Bovine Viral Diarrhea Virus NS3 Serine Proteinase: Polyprotein Cleavage Sites, Cofactor Requirements, and Molecular Model of an Enzyme Essential for Pestivirus Replication

JIAN XU,¹ ERNESTO MENDEZ,^{1†} PAUL R. CARON,² CHAO LIN,^{1,2} MARK A. MURCKO,²
MARC S. COLLETT,³ AND CHARLES M. RICE^{1*}

*Department of Molecular Microbiology, Washington University School of Medicine,
St. Louis, Missouri 63110-1093¹; Vertex Pharmaceuticals, Inc., Cambridge,
Massachusetts 02139-4242²; and ViroPharma, Incorporated,
Malvern, Pennsylvania 19355³*

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Members of the *Flaviviridae* encode a serine proteinase termed NS3 that is responsible for processing at several sites in the viral polyproteins. In this report, we show that the NS3 proteinase of the pestivirus bovine viral diarrhea virus (BVDV) (NADL strain) is required for processing at nonstructural (NS) protein sites 3/4A, 4A/4B, 4B/5A, and 5A/5B but not for cleavage at the junction between NS2 and NS3. Cleavage sites of the proteinase were determined by amino-terminal sequence analysis of the NS4A, NS4B, NS5A, and NS5B proteins. A conserved leucine residue is found at the P1 position of all four cleavage sites, followed by either serine (3/4A, 4B/5A, and 5A/5B sites) or alanine (4A/4B site) at the P1' position. Consistent with this cleavage site preference, a structural model of the pestivirus NS3 proteinase predicts a highly hydrophobic P1 specificity pocket. *trans*-Processing experiments implicate the 64-residue NS4A protein as an NS3 proteinase cofactor required for cleavage at the 4B/5A and 5A/5B sites. Finally, using a full-length functional BVDV cDNA clone, we demonstrate that a catalytically active NS3 serine proteinase is essential for pestivirus replication.

Pestiviruses such as bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and border disease virus (BDV) comprise a group of economically important animal pathogens affecting cattle, pigs, and sheep (for reviews, see references 61 and 92). These enveloped positive-sense RNA viruses are classified as a separate genus in the family *Flaviviridae*. Two additional genera of *Flaviviridae* are the classical flaviviruses, such as yellow fever virus (YFV) and dengue virus, and the hepaciviruses (hepatitis C virus [HCV]) (73).

Pestivirus genome RNAs are typically ~12.5 kb in size and consist of 5' and 3' noncoding regions that flank a long open reading frame (ORF) encoding a single viral polyprotein (see reference 58 for a review). Like those of HCV and the picornaviruses, genome-length pestivirus mRNAs are believed to be uncapped (22), and capping is not necessary for infectivity of transcribed RNAs (56, 59, 62, 79, 94). Translation initiation is mediated by the highly conserved 5' noncoding region that functions as an internal ribosome entry site (70, 78). The ensuing polyprotein is approximately 3,900 amino acids in length and is processed co- and posttranslationally by host and viral proteinases to produce 11 or 12 discrete products (see reference 58 for a review). With the exception of one nonviral protein, viral structural proteins are located in the N-terminal portion of the polyprotein; nonstructural (NS) or RNA replicase components are located in the remainder of the polyprotein. The order and nomenclature of cleavage products in the pestivirus polyprotein are as follows: NH₂-N^{pro}-C-E^{ns}-E1-E2-

p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. N^{pro} is an autoprotease unique to pestiviruses. C, a basic nucleocapsid protein, is followed by three virion glycoproteins, E^{ns}, E1, and E2. The remaining proteins are believed to be enzymatic or structural components of the membrane-associated viral RNA replication complex. NS3 possesses RNA binding, RNA-stimulated nucleoside triphosphatase, and RNA helicase activities (87, 95). The C-terminal polyprotein product, NS5B, contains the conserved GDD motif characteristic of RNA-dependent RNA polymerases.

Pestivirus polyprotein processing begins with *cis* cleavage at the N^{pro}/C junction by the N^{pro} autoprotease (85, 96). Cleavages at the C/E^{ns}, E1/E2, and E2/p7 sites are probably mediated in the endoplasmic reticulum lumen by host signal peptidase (80). The enzymes responsible for cleavage at the E^{ns}/E1 and p7/NS2 sites have not been defined, although the latter cleavage may also be mediated by signal peptidase (24). Similar to the case for HCV (48, 60, 83), processing at the pestivirus E2/p7 site is incomplete, leading to the production of two stable E2 forms: E2 and E2-p7. E2 but not E2-p7 is found in mature CSFV particles (24).

Processing at the 2/3 cleavage site and production of a discrete NS3 protein vary among pestivirus isolates (see reference 58 for a review). In the case of CSFV, incomplete processing at the 2/3 site leads to the production of both NS2-3 and NS3. For BVDV, the situation is more complex. BVDV isolates can be either cytopathic (CP) or non-CP in cell culture. Remarkably, production of NS3 generally correlates with cytopathogenicity. In the case of non-CP BVDV, cleavage at the 2/3 site does not occur and only uncleaved NS2-3 is observed. In CP BVDV biotypes, both NS3 and NS2-3 products are found in virus-infected cells. Several molecular events can lead to production of NS3, including duplication and rearrangement of pestivirus sequences, insertion of cellular sequences, and large in-frame deletions resulting in subgenomic defective interfering RNAs

* Corresponding author. Mailing address: Department of Molecular Microbiology, Box 8230, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110-1093. Phone: (314) 362-2842. Fax: (314) 362-1232.

† Permanent address: Departamento de Genética y Fisiología Celular, Instituto de Biotecnología/UNAM, Cuernavaca, Mor. 52271, Mexico.

(see reference 58 for a comprehensive review of this subject). In the case of the American prototype CP BVDV strain, NADL, a 270-base portion of a bovine mRNA is inserted in the NS2 region (called cIns), resulting in an in-frame insertion of 90 amino acid residues. By mechanisms that are not understood, this insertion leads to partial processing at the 2/3 site, production of both NS2-3 and NS3, and cytopathogenicity in cell culture (54).

Downstream cleavages in the pestivirus NS region (at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites) appear to be mediated by a serine proteinase activity present in the N-terminal one-third of the NS3 coding region (97). This chymotrypsin-like serine proteinase domain consists of approximately 180 residues and is present in all members of the *Flaviviridae* (73). In the flaviviruses, the upstream NS2B protein forms a complex with NS3 and is absolutely required as a cofactor for processing (73). The flavivirus enzyme has a preference for basic amino acids (Arg or Lys [13]) at the P1 cleavage site position (following the nomenclature proposed by Schechter and Berger [82]) and is required for virus replication (17). For the HCV NS3 proteinase, whose crystal structure has recently been described (41, 52), the downstream 54-residue NS4A polypeptide is an integral component of the serine proteinase (41) and dramatically stimulates cleavage at some sites (73). The HCV enzyme prefers cysteine at the P1 position, although threonine is found at the 3/4A processing site (73). For the pestivirus NS3 serine proteinase, neither the cleavage site specificity nor the cofactor requirements have been clearly defined. Transient expression of truncated BVDV polyproteins has shown that an alanine substitution for the putative serine nucleophile (Ser-1842) abolishes cleavage at all four downstream sites (97). Evidence for a pestivirus serine proteinase cofactor stems from the observation that polypeptide sequences downstream of NS3 are required for cleavage at the 5A/5B site (97).

In this study, we have examined several aspects of pestivirus NS3 serine proteinase function, using cDNA clones of the CP BVDV NADL strain. Expression of full-length NADL polyproteins with active or inactive NS3 proteinases was used to clarify the cleavages mediated by this viral proteinase. NS proteins produced by serine proteinase-dependent cleavage were isolated and their N-terminal sequences were determined, allowing us to define the cleavage site specificity of the pestiviral enzyme and compare this with those of other members of the *Flaviviridae*. Besides determining precise boundaries for the pestivirus NS proteins for future functional studies, definition of these cleavage sites allowed us to investigate the role of downstream polypeptides, in particular NS4A, in NS3 serine proteinase-mediated processing. Finally, using a functional NADL cDNA clone capable of producing infectious RNA transcripts (54), we analyzed the effect of the Ser-1842-to-alanine mutation, which inactivates the NS3 serine proteinase, on BVDV replication.

MATERIALS AND METHODS

Cell cultures, virus stocks, and plaque assays. The BHK-21 and BSC-40 cell lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 2 mM L-glutamine, nonessential amino acids, penicillin, streptomycin, and 10% fetal bovine serum (FBS). The Sf9 cell line, obtained from the American Type Culture Collection, was maintained in complete TNM-FH medium (Invitrogen) supplemented with amphotericin B and 10% heat-inactivated FBS.

vTF7-3, a vaccinia virus recombinant expressing the T7 DNA-dependent RNA polymerase (30), was amplified in BSC-40 monolayers and partially purified, and titers were determined by plaque assay on BSC-40 monolayers (38). The baculovirus recombinant bacD2-133, which expresses residues 1591 to 3988 of the NADL polyprotein (68), was amplified and its titer was determined by using Sf9 cells.

BVDV RNA infectivity assays (described below) were conducted by using

monolayers of BVDV-free MDBK cells propagated in Dulbecco's MEM (DMEM) containing sodium pyruvate and 10% horse serum (HS).

Plasmid constructs. Standard methods were used for construction and purification of plasmids for transient-expression assays (4). Expression constructs are diagrammed in Fig. 1. For expression of the entire BVDV ORF (residues 1 to 3988), a full-length cDNA copy of NADL was assembled immediately downstream from the T7 promoter. The construction of this plasmid, called pACNR/NADL, is described in detail elsewhere (54). To inactivate the serine proteinase, a mutant containing an alanine substitution at position 1842 (called pACNR/NADL S1842A) was constructed by replacing the *Bgl*I (nucleotide 6263; NADL numbering [19]) to *Nsi*I (nucleotide 6303) portion of pACNR/NADL with the corresponding fragment from pTM3/BVD 1598-3035 S1842A (described below).

Other expression plasmids for the vaccinia virus-T7 system were constructed by using pTM3 (64). This vector contains (5' to 3') a T7 promoter, an internal ribosome entry site derived from encephalomyocarditis virus, and a polylinker followed by a T7 termination sequence. Where indicated, protruding ends were filled in or removed by treatment with T4 DNA polymerase in the presence of deoxynucleoside triphosphates prior to ligation. pTM3/BVD 1598-3035 was created by cloning the *Eco*RV fragment of pBV-SD2-3' (97) into pTM3 which had been digested with *Nco*I (filled in) and *Sna*I. pTM3/BVD 1598-3035 S1842A was constructed in the same manner by using the *Eco*RV fragment from pBV-S1842A-3' (97). pTM3/BVD 1598-2360 and pTM3/BVD 1598-2360 S1842A were constructed by deleting the region between the *Dra*I and *Bln*I sites of pTM3/BVD 1598-3035 and pTM3/BVD 1598-3035 S1842A, respectively. Blunt ends were produced prior to ligation. The predicted translation products of these plasmids lack two C-terminal residues of NS3 and contain an additional four amino acids (Val-Ser-Asn-Asn) derived from the vector. pTM3/BVD 2363-2426, which encodes NS4A preceded by two additional N-terminal residues (Met-Ala) and followed by a termination codon, was constructed by PCR using the following two synthetic oligonucleotides: 5'-CATGCCATGGCTCTCGCTGAAA-3' (oligonucleotide 329; 5' positive-sense primer) and 5'-CGCGGATCTTCACAGTCTTTCAGTTCAG-3' (oligonucleotide 330; 3' negative-sense primer). The resulting product was digested with *Nco*I and *Bam*HI (shown in boldface in the oligonucleotide sequences) and cloned into pTM3 which had been digested with the same two enzymes. The structure of the NS4A insert was verified by sequence analysis. pTM3/BVD 2463-3988 was constructed by ligating the *Dra*I-*Pac*I fragment from pACNR/BVD5'3'ext. (73a) into pTM3 which had been digested with *Nco*I (filled in) and *Pac*I.

Vaccinia virus-T7 transient-expression assays. Expression assays of transfected plasmid constructs utilized subconfluent monolayers of BHK-21 cells in 35-mm-diameter dishes (approximately 10^6 cells) which had been previously infected with vTF7-3 (5 PFU per cell in 200 μ l of phosphate-buffered saline [PBS] containing 1% FBS and 1 mM $MgCl_2$) for 60 min at room temperature. After removal of the inoculum, the cells were transfected with a mixture consisting of 1 μ g of plasmid DNA and 8 μ g of Lipofectamine (Gibco BRL) in 0.5 ml of MEM. After 2.5 h at 37°C, the transfection mixture was replaced with 0.5 ml of MEM containing 1/40 the normal concentration of methionine, 2% FBS, and 50 μ Ci of Expre- ^{35}S label (NEN) per ml and incubated for 4 h at 37°C.

Cell lysis, immunoprecipitation, and protein analyses. After being labeled, monolayers were lysed in 0.5% sodium dodecyl sulfate (SDS) containing 20 μ g of phenylmethylsulfonyl fluoride (PMSF), and cellular DNA was sheared by repeated passage through a 27-gauge needle. Immunoprecipitations using BVDV-specific sera and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were conducted essentially as previously described (18, 76). Region-specific rabbit antisera specific for BVDV and CSFV proteins have been described elsewhere (18, 57, 97) and are summarized in the legend to Fig. 1.

N-terminal sequence analysis. For preparation of metabolically labeled NS4A and NS4B BVDV proteins, BHK-21 cells were infected, as described above, with vTF7-3. After adsorption for 60 min at room temperature, the inoculum was removed and the cells were transfected for 2.5 h at 37°C with a mixture consisting of 1 μ g of pTM3/BVD1598-3035 and 8 μ g of Lipofectamine (Gibco BRL) in 0.5 ml of MEM. After transfection, the monolayers were washed twice with prewarmed medium lacking the amino acid used for radiolabeling and labeled for 4 h in the same medium containing 2% FBS and 200 μ Ci of the indicated 3H -labeled amino acid per ml. The 3H -amino acids used were valine (29 Ci/mmol), leucine (143 Ci/mmol), and isoleucine (93 Ci/mmol) (Amersham). For preparation of [^{35}S]methionine-labeled NS4B, the monolayers were labeled for 4 h in MEM containing 1/80 the normal concentration of methionine and 100 μ Ci of Expre- ^{35}S label (NEN) per ml. After labeling, the monolayers were washed twice with cold PBS and lysed in 0.5% SDS containing 20 μ g of PMSF per ml. In most cases, material from $\sim 10^7$ cells was used for isolation of radiolabeled proteins for sequence analysis. NS4A and NS4B were immunoprecipitated by using 20 μ l BVDV-specific polyclonal rabbit antisera α 2396 and K1, respectively (57, 97). Immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immobilon polyvinylidene difluoride (Immobilon P) membranes, and localized by autoradiography.

For NS5A and NS5B, baculovirus expression was used to prepare sufficient quantities for direct N-terminal sequence analysis. Sf9 cells in a 150-mm-diameter dish (approximately 2×10^7 cells) were infected with baculovirus recombinant bacD2-133 (68) at a multiplicity of infection of ~ 5 PFU per cell. After adsorption for 60 min at room temperature, the inoculum was removed and replaced with fresh medium. At 48 h postinfection, the infected monolayers were

FIG. 1. The BVDV polyprotein, region-specific antisera, and expression constructs. The diagram at the top represents the BVDV strain NADL polyprotein, with the locations of the cleavage sites, the structural proteins (shaded boxes), and the NS proteins (open boxes) indicated. Enzymes responsible for proteolytic processing include host signal peptidase (◆), the N^{pro} autoprotease, the NS3 serine proteinase (↘), and as yet unidentified activities (?). Region-specific antisera used in this study are indicated by the subregions (horizontal gray bars) used for immunization. These regions include the following amino acids: 873 to 1015 (D31; E2 specific), 1335 to 1351 (α1335; NS2 specific), 1807 to 2093 (G40; NS3 specific), 2133 to 2480 (B10; reacts with NS3, helicase region), 2396 to 2414 (α2396; NS4A specific), 2890 to 3192 (62D; NS5A specific), and 3409 to 3692 (B3B; NS5B specific). K1 antiserum (57) (kindly provided by R. Stark and H.-J. Thiel) was produced against the amino acid 2555 to 2766 subregion of the CSFV polyprotein (homologous to residues 2645 to 2856 of the NADL polyprotein) and reacts with BVDV NS4B (57). Processing sites for the NS3 serine proteinase, as determined in this study by the N-terminal sequences of NS4A (N-terminal residue 2363), NS4B (residue 2427), NS5A (residue 2774), and NS5B (residue 3270), are indicated (vertical shaded lines). Although the precise location of the NADL 2/3 cleavage site has not been determined, Gly-1680 corresponds to the NS3 N terminus determined for a number of other CP pestiviruses (58). Expression constructs, detailed in Materials and Methods, are shown below and drawn to scale to indicate the region of the NADL polyprotein expressed. T7-driven mammalian expression constructs were either pACNR (full-length) (54) or pTM3 (all others) derivatives. The baculovirus recombinant (bacD2-133) (68) used to produce NADL NS5A and NS5B is also shown. Regions of the BVDV polyprotein expressed by these constructs are given at the left, as are short names used in the text and subsequent figures. Asterisks denote N- or C-terminally truncated proteins. Mutant derivatives in which the NS3 proteinase serine nucleophile (Ser-1842) was inactivated by replacement with alanine are indicated (S1842A).

washed twice with cold PBS, harvested by being scraped into PBS, and collected by centrifugation at $100 \times g$ for 10 min. The cell pellet was resuspended in ice-cold hypotonic buffer (25 mM Tris-Cl [pH 8.0], 1 mM MgCl₂, 10 mM KCl, 20 μg of PMSF per ml, 5 μg of leupeptin per ml, 50 μg of antipain per ml, 1 μg of pepstatin per ml, 0.02% 2-mercaptoethanol) and lysed by 20 strokes with a tight-fitting Dounce homogenizer. The disrupted cells were pelleted by centrifugation at $8,000 \times g$ for 10 min, and the pellet was resuspended in hypotonic buffer containing 1% Triton X-100. After shaking at 4°C for 30 min, the detergent-insoluble fraction was pelleted at $8,000 \times g$ for 10 min. The resulting pellet containing the majority of the BVDV NS proteins was solubilized in SDS sample buffer and separated by SDS-PAGE. Proteins were transferred to Immobilon polyvinylidene difluoride membranes and localized by Coomassie blue staining.

Partial amino acid sequence analyses of radiolabeled and unlabeled protein samples were performed as described previously (14, 53).

Infectivity analyses of transcribed full-length NADL RNA. Template DNAs were prepared by linearization of pACNR/NADL and pACNR/NADL S1842A DNAs with Sse8387I. Full-length uncapped RNAs were transcribed by using the T7 RNA polymerase MEGAscript system (Ambion). [³H]UTP was included during transcription to monitor the yield and integrity of the RNA products (75). After transcription, template DNA was degraded by digestion with DNase I (Ambion), and RNA products were extracted with phenol and then chloroform, concentrated by ethanol precipitation, and resuspended at 2 μg/μl in RNase-free water.

For transfection, 1 μg or 100 ng of transcript RNA was added to 200 μl of RNase-free PBS containing 5 μg of Lipofectin (Gibco-BRL). Samples were incubated for 10 min at 4°C. During this time, MDBK monolayers (50 to 70% confluent) grown in six-well plates (35-mm diameter) were washed twice with RNase-free PBS. After the final wash was removed, the transfection mixtures were added to the cell monolayers, which were then incubated for 10 min at room temperature with occasional rocking. A 2-ml volume of DMEM containing 10% HS was added to each well, the contents were mixed, and the supernatant was removed. The cells were washed once more with the same medium and overlaid with 1.5% low-melting-point agarose in MEM containing 5% HS. Alternatively, MDBK cells were transfected by electroporation and dilutions of transfected cells were plated on MDBK monolayers to determine infectious centers (54). Following incubation for 3 to 4 days at 37°C, the cells were fixed with 3.7% formaldehyde, the agarose plugs were removed, and BVDV plaques were visualized by staining with crystal violet (74).

Sequence alignments and molecular modeling. Sequence alignments were generated by using the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, Wis.) and were used to map the side chains lining the S1 site onto the framework of known chymotrypsin-like serine proteases. Models were constructed by using structures with similarities in the residues lining the S1

site. The BVDV model was constructed by using elastase (protein database [PDB] entry 1HNE) as a framework, while the YFV model was based on trypsin (PDB entry 1NTP). Side chains surrounding the S1 sites were generated by using Quanta96 (Molecular Simulations Inc., San Diego, Calif.). Graphic representations of the pockets were generated by using the program GRASP (66).

RESULTS

Mutation of Ser-1842 blocks processing at downstream sites. Previous experiments examining expression of various truncated BVDV polyproteins in mammalian and insect cell systems demonstrated that an active NS3 serine proteinase was required for processing at downstream sites (97). However, discordant results were obtained regarding processing at the 2/3 junction: apparent NS3-dependent cleavage was observed in mammalian cells (97) but not in the insect cell system (68). We reexamined this issue, using the vaccinia virus-T7 system to drive expression of the entire BVDV polyprotein in mammalian cells. Expression constructs consisted of the T7 promoter followed by the full-length BVDV (strain NADL) cDNA, which had been assembled from sequenced clones described elsewhere (54). At position 1842, the parental construct encodes the serine residue believed to be the nucleophile of the pestivirus NS3 serine proteinase catalytic triad (97). A mutant derivative, S1842A, contained an alanine substitution at this position. Polyprotein processing phenotypes were examined by metabolic labeling followed by immunoprecipitation with rabbit polyclonal antiserum specific for E2, NS2, NS3, or NS5A. Extracts from BVDV-infected MDBK cells were radiolabeled and processed in parallel to provide markers for authentic viral cleavage products.

As shown in Fig. 2, identical BVDV-specific proteins of the expected sizes for E2, NS2, NS3, and NS5A (18, 20) were observed in BVDV-infected cells and for the transfected parental construct. For the S1842A mutant, proteins comigrating

FIG. 2. Processing phenotypes of full-length BVDV NADL polyproteins containing Ser-1842 or Ala-1842. BHK-21 monolayers were infected with vTF7-3 alone or infected with vTF7-3 and transfected with either pACNR/NADL or pACNR/NADL S1842A and labeled from 3 to 7 h postinfection as described in Materials and Methods. For production of authentic BVDV protein standards, MDBK cells were mock infected or infected with BVDV NADL rescued from cDNA (ACNR/NADL; 1 PFU/cell) and labeled from 20 to 24 h postinfection with 40 μ Ci of Expre³⁵S³⁵S label per ml. Cell lysates were prepared and immunoprecipitated by using the indicated BVDV region-specific antisera as described in the legend to Fig. 1. Immunoprecipitated proteins were separated by SDS-8%PAGE. Only the relevant portions of the gels are shown; BVDV-specific proteins are indicated at the right. Note that the cleavage efficiency at the 2/3 site is lower and that NS2-3 appears as a doublet when the BVDV polyprotein is expressed in BHK cells by using the vaccinia virus-T7 system. The basis for these differences from BVDV-infected MDBK cells is not known.

with E2 and NS2 were observed; however, NS3 and NS5A were not detected. Rather, large uncleaved polyproteins immunoprecipitating with NS3- and NS5A-specific antisera were observed. Other immunoprecipitations demonstrated that NS4B and NS5B were produced in BVDV-infected cells and for the parental construct but not for the S1842A mutant (data not shown). These observations are consistent with previous results (97) and demonstrate that the NS3 serine proteinase is re-

quired for processing at the 3/4A, 4A/4B, 4B/5A, and 5A/5B sites but not for processing in the E2-NS2 region or at the 2/3 site (see Discussion).

Definition of NS3 serine proteinase-dependent cleavage sites. Although the polyprotein cleavage sites for the flavivirus and HCV serine proteinases have been determined (73), the sites cleaved by the pestivirus NS3 serine proteinase have not been previously identified. To determine the sequences at the four NS3 proteinase cleavage sites, the N-terminal sequences of NS4A, NS4B, NS5A, and NS5B were determined. Sufficient quantities of BVDV-specific proteins for N-terminal sequence analysis were not readily obtained from virus-infected MDBK cells. Since the vaccinia virus-T7 and baculovirus expression systems appear to faithfully reproduce authentic BVDV polyprotein processing at serine proteinase-dependent sites (68, 97) (Fig. 2), these systems were used as sources of BVDV-specific cleavage products.

By using the vaccinia virus-T7 system to drive expression of pTM3/BVDV1598-3035 (2*-5A*), NS4A and NS4B were metabolically labeled with either [³H]leucine, [³H]valine, [³⁵S]methionine, or [³H]isoleucine, immunoprecipitated with region-specific antisera, separated by electrophoresis on SDS-polyacrylamide gels, and electroblotted onto Immobilon P membranes. The appropriate bands were localized and sequenced for 14 or 15 cycles of Edman degradation. Representative results are shown in Fig. 3. For each protein, the deduced sequence was sufficient to unambiguously define its N terminus within the BVDV polyprotein. For NS4A, leucine residues were recovered at positions 7, 8, and 11 and valine was recovered at position 9. These data establish the N terminus of NS4A as residue 2363. For NS4B, valine was recovered at position 5, isoleucine residues were recovered at positions 8 and 12, and a methionine residue was recovered at position 9. These data establish the N terminus of NS4B as residue 2427.

For NS5A and NS5B, sufficient quantities of unlabeled proteins could be isolated by SDS-PAGE of a detergent-washed membrane fraction derived from recombinant baculovirus bacD2-133-infected cells (Fig. 1). N-terminal sequences obtained from these samples establish the N termini of NS5A and NS5B as residues 2774 and 3270 of the NADL polyprotein, respectively (Fig. 3).

Assuming that C-terminal trimming or additional cleavages do not occur, BVDV NS4A is 64 amino acid residues in length (predicted molecular mass, 7,179 Da), NS4B is 347 residues (predicted molecular mass, 38,332 Da), NS5A is 496 residues (predicted molecular mass, 55,716 Da), and NS5B is 719 residues (predicted molecular mass, 81,918 Da). These predicted sizes are in rough agreement with the apparent molecular weights of the BVDV strain NADL NS proteins estimated by SDS-PAGE (18, 20, 95).

Inspection of the sequences surrounding the NADL cleavage sites and comparison with the homologous regions deduced from the nucleotide sequences of other pestiviruses reveal an invariant leucine residue at the P1 position of NS3 serine proteinase-dependent cleavage sites (Fig. 4). The apparent preference for a leucine residue at the P1 position differs from that of the classical flaviviruses and HCV (see Discussion). At the P1' position, a serine residue is found at the 3/4A, 4B/5A, and 5A/5B sites, whereas an alanine is present at the 4A/4B site. Other than these conserved residues at the P1 and P1' positions, the aligned sequences surrounding the cleavage sites did not reveal any additional common features that might be important for determining cleavage site specificity of the NS3 serine proteinase. Clearly, other determinants must be important for site-specific cleavage, since 17 additional Leu-Ser dipeptide sequences are present in the

FIG. 3. N-terminal sequences of BVDV NS4A, NS4B, NS5A, and NS5B. The graphs for NS4A and NS4B represent uncorrected counts per minute released per sequencing cycle. In these sequences, the amino acid residues determined by N-terminal sequence analysis of radiolabeled BVDV-specific proteins are indicated (underlined); the remaining residues are deduced from BVDV strain NADL nucleotide sequence data (19). For NS5A and NS5B, residues determined by direct N-terminal sequencing of baculovirus-expressed cleavage products are shown in boldface and aligned with the nucleotide (19) and deduced polypeptide sequences. Positions at which no specific residue could be identified are indicated by a question mark. Sequences for NS4A, NS4B, NS5A, and NS5B begin at NADL polypeptide residues 2363, 2427, 2774, and 3270, respectively. The single-letter code for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

NADL NS polypeptide (data not shown), yet cleavages at these sites are not apparent.

BVDV NS4A functions as a serine proteinase cofactor. Previous studies demonstrated that the BVDV NS3 serine proteinase expressed alone was not sufficient for cleavage at the 5A/5B site (97). Specifically, a proteinase construct spanning polypeptide residues 1591 to 2191 was active for *trans* cleavage at the 4A/4B, 4B/5A, and 5A/5B sites of a substrate beginning in the middle of the proteinase domain and extending to the C terminus of the BVDV polypeptide (residues 1761 to 3988). This proteinase was not, however, capable of cleaving at the 5A/5B site of a substrate which began just downstream of the NS5A N terminus (from residues 2796 to 3988). By using a proteinase construct extending into the NS4B region (residues 1591 to 2645), the same 5A-5B substrate could be cleaved efficiently in *trans*. These results suggested that sequences between residues 2191 and 2645 were required for serine proteinase-dependent processing at the 5A/5B site. This region of the polypeptide encompasses the C-terminal 25% of the NS3 RNA helicase domain, the complete NS4A protein, and the first 219 residues (63%) of NS4B.

This observation is strikingly reminiscent of HCV polypeptide processing, in which NS4A functions as an integral component of the NS3 serine proteinase, leading to a dramatic stimulation in cleavage at certain sites (see Discussion). Having defined the boundaries of BVDV NS4A, we tested the ability of this 64-residue protein to function as a proteinase cofactor. The vaccinia virus-T7 system was used for expression of various BVDV polypeptides (Fig. 1), and the products were

analyzed by metabolic labeling, immunoprecipitation, and SDS-PAGE (Fig. 5). The proteinase substrate (4B*-5B) initiated 37 residues downstream from the NS4B N terminus and terminated at the end of the BVDV ORF. The proteinase construct (2*-3) contained 82 C-terminal residues of NS2 (assuming that NS3 begins at residue 1680 [58]) and the entire NS3 coding region except for two C-terminal amino acids. Transfection of the substrate construct alone yielded a major band of ~170 kDa that reacted with antisera specific for NS4B, NS5A, and NS5B. A series of smaller, less prominent products was also observed, presumably as a result of premature termination and/or degradation. This pattern was unchanged when the substrate was coexpressed with the 2*-3 proteinase or an inactive derivative (2*-3 S1842A; Fig. 5, α 3hel panel). Only when both the 2*-3 proteinase and NS4A were coexpressed with the substrate were cleavage products observed. The NS4B-specific antiserum recognized a product which migrated slightly faster than the full-length NS4B marker produced by the 2*-5A* polypeptide (Fig. 5, top panel). This is consistent with cleavage at the 4B/5A site of the 4B*-5B substrate to produce a form of NS4B truncated by 37 N-terminal residues (~4 kDa). NS5A-specific antiserum recognized a doublet migrating at ~58 kDa, and the NS5B-specific antiserum recognized a protein of ~75 kDa. Both of these antisera recognized a 130-kDa product that was not immunoprecipitated by anti-NS4B and is therefore likely to be 5A-5B. Taken together, these data suggest that NS4A functions as an NS3 serine proteinase cofactor that is required for efficient cleavage at both the 4B/5A and the 5A/5B sites. Interestingly, the level of NS4A

BVDV-NADL	GKALKQVTGL	SSAENALLVA	2363	3/4A
BVDV-SD1	A.....		2273	
BVDV-Osloss	V.....	I.....	2350	
BVDV-Type 2	..R.....T.....	I.....	2347	
CSFV-Alfort	..R.....V.....	..T.....	2273	
CSFV-Brescia	..R.....V.....	..T.....	2273	
BDV-BD31	..R.....T.....		2271	
BVDV-NADL	DGTETELKEL	ASGDVEKIMG	2427	4A/4B
BVDV-SD1	E.....		2337	
BVDV-Osloss	E.K.....	..V...MDR..E	2414	
BVDV-Type 2	..KDS.....	..V...LD.YLD	2411	
CSFV-Alfort	E.K.....	..Q...QRCVE	2337	
CSFV-Brescia	E.K.....	..Q...QRC.E	2337	
BDV-BD31	..K.....	..Q...IQRCTE	2335	
BVDV-NADL	MDSQGKIRNL	SGNYILDLIY	2774	4B/5A
BVDV-SD1	E.....	H	2684	
BVDV-Osloss	..E.....		2761	
BVDV-Type 2	..E.....	F	2758	
CSFV-Alfort	V..E.....Q..	..S...E.L.	2684	
CSFV-Brescia	V..E.....Q..	..S...E.L.	2684	
BDV-BD31	V..E...M.S.	LIE.LQ	2682	
BVDV-NADL	VGSRTYAMKL	SSWFLKASNK	3270	5A/5B
BVDV-SD1	T...	Q..S.	3180	
BVDV-Osloss	..A...T...	Q..S.	3257	
BVDV-Type 2	..A...T...	...STQVTK.	3255	
CSFV-Alfort	..AKV.S...	..N.VIQEE..	3181	
CSFV-Brescia	..AKV.S...	..N.VMQEE..	3181	
BDV-BD31	..A.V.T...	..N.V.Q.ESG	3179	
CONSENSUS	P1↓P1'	L S A N		

FIG. 4. Alignment of sequences flanking the serine proteinase-dependent cleavage sites. As determined from the N-terminal sequence data shown in Fig. 3, the 10 amino acid residues on either side of the 3/4A, 4A/4B, 4B/5A, and 5A/5B cleavage sites are aligned for BVDV strain NADL and selected pestivirus sequences. As shown at the right, NS4A, NS4B, NS5A, and NS5B begin at NADL polyprotein residues 2363, 2427, 2774, and 3270, respectively. The corresponding polyprotein residues for the other aligned pestivirus sequences are also given. The P1 and P1' positions are shown (boldface). The pestivirus sequences are from the following sources: NADL (19); BVDV SD-1 (23); BVDV Osloss (21); BVDV type 2 (77); CSFV Alfort-Tübingen (55); CSFV Brescia (63); and BDV BD31 (accession no. U70263).

accumulation when the protein was expressed independently was much lower than when the protein was expressed in the context of the 2*-5A* polypeptide (Fig. 5, bottom panel, lane 3). Slightly increased accumulation of NS4A was observed when it was coexpressed with substrate and active or inactive NS3 proteinase (Fig. 5, bottom panel, lanes 9 and 10). Despite this low level of NS4A, a majority of the substrate was processed in the presence of active proteinase and NS4A.

A catalytically active serine proteinase is essential for BVDV replication. Cleavages mediated by the NS3 serine proteinases of viruses from all three genera of *Flaviviridae* are believed to be important for virus replication. For flavivirus YFV, this was formally demonstrated by examining the effects of mutations that inactivate the serine proteinase or block cleavage at specific sites in the context of an infectious molecular clone (16, 17, 65). Recently, we constructed a full-length cDNA clone of the NADL strain of BVDV from which infectious RNA could be transcribed (54). The transcribed pestivirus RNA was of high specific infectivity, and recovered virus was similar to the parental virus in terms of plaque phenotype, growth properties, and the pattern of virus-specific proteins. We used this system to test the infectivity of full-length RNA transcripts containing the S1842A mutation. The yields of full-length tran-

scripts produced in the transcription reaction from the wild-type (pACNR/NADL) and the S1842A mutant templates were similar (data not shown). Transcribed RNAs were transfected into MDBK monolayers, which were then either overlaid with agarose to assay directly for plaque formation or incubated in culture medium for 2 days before being assayed for release of infectious virus. Both Lipofectin ($\sim 10^3$ to 10^4 PFU/ μ g of RNA) and electroporation (10^4 to $>10^5$ PFU/ μ g of RNA) transfection methods were used in different experiments. Figure 6 shows the results of a representative Lipofectin-mediated RNA transfection/plaque assay. More than 10^3 plaques were observed for the wild-type parent; none were observed for the S1842A mutant. In a second experiment, electroporation with the wild-type transcripts resulted in the appearance of complete CP effect within 2 days and yielded 2.9×10^4 PFU/ μ g of RNA and virus titers in the culture medium of $>2 \times 10^5$ PFU/ml. In contrast, monolayers transfected with the S1842A

FIG. 5. NS4A functions as an NS3 serine proteinase cofactor. BHK-21 cell monolayers were infected with vTF7-3 alone (lane 5) or infected with vTF7-3 and transfected with the indicated plasmids (plus sign over the respective lanes) (see Fig. 1). Between 3 and 7 h postinfection, the cells were metabolically labeled as described in Materials and Methods. Cell lysates were immunoprecipitated with the following BVDV-specific rabbit antisera: NS4B-specific K1 (α 4B), NS5A-specific 62D (α 5A), NS5B-specific B3B (α 5B), NS3-specific B10 (α 3hel), and NS4A-specific α 2396 (α 4A). Immunoprecipitated proteins were solubilized and separated by electrophoresis on 8% (α 5A, α 5B, and α 3hel) or 14% (α 4B and α 4A) polyacrylamide-SDS gels. Only the relevant portions of the gels are shown; BVDV-specific proteins are indicated at the right.

FIG. 6. Full-length transcripts containing Ala-1842 are not infectious. As described in Materials and Methods, MDBK monolayers were transfected with a mixture of Lipofectin and 1 μ g of RNA transcribed from either pACNR/NADL (clone 5) or pACNR/NADL S1842A. To assay for plaque formation, transfected monolayers were overlaid with agarose, incubated for 3 days at 37°C, fixed, and stained with crystal violet. Identical results were obtained in several independent experiments.

mutant RNA failed to exhibit any CP effect and yielded no plaque-forming virus in the medium, even after a prolonged culture period (7 days). To rule out the possibility that the S1842A mutation led to a replication-competent but non-CP virus, transfected cells were analyzed for production of BVDV antigens by immunostaining or by metabolic labeling and immunoprecipitation. BVDV-specific antigens were readily detected for both CP BVDV and non-CP BVDV control RNAs. In contrast, no BVDV-specific proteins were detected for the S1842A mutant by either method (data not shown). These results indicate that the NS3 serine proteinase activity is essential for efficient pestivirus replication.

DISCUSSION

Results presented here and elsewhere (see references 58 and 73 for reviews) reveal both striking similarities and distinct differences in NS3 serine proteinase-mediated processing in the *Flaviviridae*. For all three genera, inactivation of this enzyme blocks polyprotein cleavage at multiple sites. The pestivirus and HCV serine proteinases cleave at four downstream sites in the NS region (3/4A, 4A/4B, 4B/5A, and 5A/5B). The flavivirus enzyme, in addition to cleaving at three downstream sites (3/4A, 4A/2K, and 4B/5), also mediates additional upstream cleavages in the structural and NS2 regions, including autocatalytic cleavage at the 2B/3 site. Other aspects of flavivirus NS region processing are also distinct from those of pestiviruses and HCV, including a signal peptidase cleavage that generates the N terminus of NS4B (47) and a lack of further processing of the 100-kDa NS5 protein.

N-terminal sequence analysis of the BVDV NS3 serine proteinase-dependent cleavage products shows that the specificity of the pestiviral enzyme differs from that of the flaviviruses and HCV. At all four sites, leucine was found at the P1 position, and either serine, alanine, or asparagine was found at the P1' position. Other than the asparagine at the P1' position of the BDV 5A/5B site, these P1-P1' residues are conserved among pestivirus isolates (Fig. 4). Inspection of residues immediately flanking these sites has not revealed any other common features which might contribute to substrate specificity of the enzyme. For viruses from all three genera of the *Flaviviridae*, amino acids with short side chains seem to be preferred at the P1' position. In contrast, while the BVDV NS3 proteinase prefers leucine at the P1 site, the HCV proteinase favors cys-

teine or threonine (6, 33, 42, 43, 45) and the flavivirus enzyme prefers basic residues (reviewed in reference 13) (Table 1).

Structural comparisons of the P1 binding sites of a number of chymotrypsin-fold serine proteases indicate that substrate specificity is determined mainly by a small number of residues (67). Mutations of these residues can change the specificity of an enzyme, as demonstrated for trypsin (36), α -lytic protease (46), and other members of this enzyme family, including the HCV NS3 proteinase (27). These residues are located near the catalytic serine residue in both the tertiary structure and primary polypeptide sequence (Table 1). The high degree of sequence conservation surrounding this serine allows sequence alignments in this region to be constructed with a high level of confidence. This in turn allows construction of homology models which can be used to predict or rationalize cleavage specificity (35).

Models for the P1 specificity pockets of the NS3 serine proteinases of the *Flaviviridae* compared to those of chymotrypsin, elastase, and trypsin are shown in Fig. 7. Residues known or predicted to contribute to P1 specificity include positions 189, 190, 213, 216, and 228 (chymotrypsin numbering system [35]; Table 1). In particular, the residue at position 216 defines the depth of the P1 specificity pocket, whereas the residues at positions 190 and 213 help to determine its width. The specificity site in the BVDV proteinase model resembles those of elastase and chymotrypsin: glycine at position 216 results in a deeper binding pocket, but asparagine at position 190 limits its width (Fig. 7). This is consistent with the leucine substrate specificity that we observe for BVDV proteinase and is also observed for *Streptomyces griseus* protease B, which has a glycine at position 216 and valine at 190 (39). Chymotrypsin can accommodate larger hydrophobic residues due to the presence of Gly-216 and Ser-190. For the HCV proteinase, the crystal structure (41, 52) confirmed the prediction that its P1 specificity pocket would resemble that of elastase (69). In both enzymes, residues other than glycine are located at position 216 (Table 1). The combination of this and relatively large hydrophobic residues at positions 190 (leucine) and 213 (phenylalanine) effectively seals off a shallow hydrophobic P1 specificity pocket, consistent with the observed preference for cysteine. The flavivirus NS3 proteinase has specificity for basic residues, as does trypsin (32). In trypsin, this specificity has been shown to be due to Asp-189 (10). Sequence alignments suggest that the flavivirus NS3 proteinase also has an aspartate at this location (8, 31). Changing this aspartate to lysine in trypsin, which is the residue found at this position in the BVDV alignment, does not enhance the specificity of trypsin

TABLE 1. Sequence alignment of residues surrounding the P1 specificity pocket of chymotrypsin-like serine proteinases

Enzyme and source	P1 specificity ^a	P1 specificity pocket ^b (residue)				
		189	190	213	216	228
NS3, pestivirus	L	K	N	V	G	S
Protease B, <i>S. griseus</i>	L	C	V	T	G	F
Chymotrypsin	F, Y, W	S	S	V	G	Y
NS3, HCV	C, T	L	L	F	A	F
Elastase	A, V	G	V	A	V	F
NS3, flavivirus	K, R	D	Y	Y	G	I
Trypsin	K, R	D	A	V	G	Y

^a For the listed serine proteinases, residues preferred at the P1 position are indicated. The single-letter amino acid code is used (see Fig. 3 legend).

^b Residue numbers refer to the standard chymotrypsin numbering system as described by Greer (35).

FIG. 7. Comparison of the shape of the BVDV NS3 serine protease P1 specificity pocket with those of other serine proteases. A structural model of the residues surrounding the P1 specificity pocket of BVDV was constructed as described in Materials and Methods. GRASP surface representations (66) of the P1 specificity pockets of structurally aligned proteases are colored by charge, with acid residues colored red and basic residues colored blue. (A) BVDV model; (B) HCV NS3 protease (41); (C) YFV model; (D) chymotrypsin (PDB 6CHA); (E) elastase (PDB 1HNE); (F) trypsin (PDB 1NTP). The numbered residues correspond to those highlighted in Table 1 and the text.

towards acidic residues, possibly because the lysine side chain is reoriented away from the P1 specificity site (32).

Previous work suggested that sequences downstream of the BVDV NS3 serine protease domain were required for processing at the 5A/5B site (97). *trans*-Processing experiments reported here show that BVDV NS4A is essential for cleavage at both the 4B/5A and the 5A/5B sites. Similar observations hold for HCV (73), in which the 54-residue NS4A protein forms a complex with the NS3 serine protease domain (7, 25, 51, 81) and enhances processing at downstream sites (5, 26, 49, 89). HCV NS4A is required for cleavage at the 4B/5A site, but unlike in BVDV, processing at the HCV 5A/5B site occurs with reasonable efficiency in the absence of the cofactor (see reference 86 and citations therein). Structural analysis of an HCV proteinase-NS4A peptide complex (41) has shown that a central hydrophobic region of NS4A, which is essential for cofactor activity (7, 11, 50, 84, 93), forms an integral component of the N-terminal domain of the proteinase (41). For the flaviviruses, the upstream NS2B protein forms a stable complex with the NS3 serine protease domain after autocatalytic cleavage at the 2/3 site (3, 15). NS2B is required for cleavage at all NS3 proteinase-dependent sites (1, 3, 12, 29). A central region in flavivirus NS2B, which is highly conserved and rich in charged residues, has been implicated in cofactor function (15, 28). In future experiments with the pestivirus NS4A cofactor, it will be interesting to examine requirements for cleavage at the 3/4A and 4A/4B sites, to define residues in NS4A important for cofactor function, and to elucidate its mechanism of action, whether similar to or distinct from those of the other genera.

Production of NS3 is a hallmark of CP BVDV strains and occurs by varied mechanisms (58). In several cases, the pro-

teinases responsible for the N-terminal (2/3) cleavage of the NS3 polypeptide have been defined. In CP BVDV strains Osloss, CP14, and CP1, insertion of cellular ubiquitin sequences adjacent to NS3 creates a processing site for the cellular enzyme ubiquitin carboxyterminal hydrolase. In other CP BVDV strains (Pe515, CP6, CP4), duplications and rearrangements of BVDV RNA sequences have led to the juxtaposition of N^{pro} immediately upstream of an additional copy of the NS3 coding sequence. Similarly, in the defective CP BVDV isolate CP9, N^{pro} and NS3 are found adjacent to one another as a result of a large in-frame deletion. For these various gene fusions, autocatalytic cleavage by N^{pro} is believed to produce the proper NS3 N terminus, corresponding to Gly-1590 (using the numbering for the non-CP strain SD-1 polyprotein sequence [23]).

For two CP strains, NADL and CP7, short in-frame insertions of either cellular (NADL) or duplicated viral (CP7) sequences in the NS2 region upstream of the putative NS3 cleavage site lead to NS3 production (54, 56, 91). Recent work on CP7 has clearly shown that cleavage at the 2/3 site is independent of the NS3 serine protease activity (91). Previous studies of NADL gave discordant results regarding the involvement of the NS3 serine protease in this cleavage (68, 97). Data reported here, where NS2 was produced normally after inactivation of the NS3 serine protease, suggest the involvement of a distinct proteolytic activity. This discrepancy may be related to aberrant processing in the NS2 region when truncated (versus full-length) polyproteins are used for expression (98). Although additional work is needed to definitively establish the NS3 N terminus, preliminary microsequence analysis of NADL NS3 has yielded cysteine residues at positions 5 and 13, which is consistent with Gly-1680 as the N-terminal residue (98). This

TABLE 2. Properties of pestivirus NS proteins generated by NS3 serine proteinase-mediated cleavages

Protein	No. of amino acid residues ^a	Predicted polypeptide molecular mass (kDa) ^b	pI ^c	Hydropathy ^d	Amino acid identity ^{b,e} vs BVDV-1 (%)				Function(s) ^f
					BVDV-1	BVDV-2	CSFV	BDV	
NS3	683	75–76	7.3–8.1	Hydrophilic	97–100	91–92	89–90	91–92	Ser proteinase, RNA helicase
NS4A	64	7.2	4.6–5.2	Hydrophobic/hydrophilic	93–100	78–83	86–89	86–89	Ser proteinase cofactor
NS4B	347	38–39	7.5–8.8	Hydrophobic	92–97	84–86	76–78	76–77	Replicase component?
NS5A	496–497	55–56	6.8–8.9 ^c	Hydrophilic	83–91	67–69	57–59	60–61	Replicase component?
NS5B	718–719	81–82	9.1–9.4	Hydrophilic	89–94	75–76	70–71	72–74	RNA-dependent RNA polymerase?

^a Protein boundaries and lengths are based on the cleavage sites determined here for the NADL strain. NS3, whose N terminus is not generated by NS3 serine proteinase-mediated cleavage (this report), is assumed to begin with NADL polypeptide residue 1680 (58) (see Discussion) or SD-1 residue 1590 (23).

^b Full-length or partial polypeptide sequences aligned and analyzed included: BVDV strains NADL (P19711), Osloss (M96687), SD-1 (Q01499), CP-7 (U63479), CP13 (Z73248), NCP9 (U03912), NCP1 and CP1 (M62430 and M62431), Pe515 NCP and CP (M96640 and M96641), NY1 (L35850), Oregon (L35851), and Singer (L35852), and accession no. U18059 (type 2) (77); CSFV strains Alfort (P19712), Alfort/187 (X87939), Brescia (P21530), C (Z46258), Glentorf (U45478), and Riems (U45477) and accession no. D49532, D49533, U21328, U21329, and X96550; and BDV strains BD31 (U70263) and Clover lane (U00892). Database searches were conducted by using BLAST; DNA Star was used to align protein sequences and calculate the predicted molecular mass net charge, pI, hydropathy, and amino acid identity. On the basis of these analyses, ranges for the values are presented.

^c Predicted isoelectric points for BVDV NS5A proteins range from 6.8 to 7.7. For CSFV, NS5A pIs are more basic, ranging from 8.0 to 8.9.

^d Hydropathy analyzed by using the program of Kyte and Doolittle (44).

^e Range of percent identical amino acids in the aligned pestivirus NS protein sequences. In the first column, the numbers give the range of sequence conservation among BVDV type 1 isolates (see footnote *b*). In the BVDV-2, CSFV, and BDV columns, the ranges of percent sequence identity are given for comparisons of BVDV type 1 sequences with BVDV type 2, CSFV, or BDV strains, respectively. See references 58 and 77 for a discussion of pestivirus classification and BVDV types.

^f Possible or predicted functions are indicated by a question mark. See Discussion for further details.

polyprotein position is identical to that predicted for the CP strains described above (e.g., Gly-1590 in the SD-1 sequence). If this result is confirmed, then the 90-amino-acid cellular insert in NS2 of the NADL virus somehow promotes cleavage at a site 53 residues downstream. Whether the NADL and CP7 inserts target the BVDV polyprotein for site-specific cleavage at the 2/3 site by a cellular enzyme or activate a cryptic activity residing in the NS2-3 region remains to be determined. It is interesting that NS3 is still produced, albeit inefficiently, by CSFV strains that do not contain insertions or rearrangements such as those described for CP BVDV (58). It is also noteworthy that in the case of HCV, the NS2-3 region encodes a Zn²⁺-stimulated proteolytic activity, distinct from the NS3 serine proteinase, which is responsible for efficient autocatalytic cleavage at the 2/3 site (34, 37, 71).

On the basis of the cleavage site determinations reported here, features of the pestivirus NS proteins (except NS2, for which the N terminus remains to be determined) are summarized in Table 2. The NS3 proteinase/RNA helicase is the most highly conserved NS protein and predicted to be hydrophilic with a net positive charge. The NS4A cofactor is 64 residues long, quite acidic, and also highly conserved among pestiviruses. Similar to the 54-residue HCV NS4A, the N-terminal third of the pestivirus NS4A protein is hydrophobic and the C-terminal portion is highly charged and acidic. The N-terminal hydrophobic region of HCV NS4A is believed to be responsible for membrane association and stabilization of the NS3-4A proteinase complex (89). The central region of BVDV NS4A contains a number of conserved charged residues and in this respect differs from HCV NS4A, which contains mainly uncharged hydrophobic residues that also stabilize interactions with NS3 required for cofactor function (see discussion in reference 41). Little is known about the function of NS4B for any member of the *Flaviviridae*. Pestivirus NS4B is basic with a number of hydrophobic regions, perhaps indicative of membrane association. Although the predicted molecular mass of NS4B is 38 to 39 kDa, the protein migrates at ~30 kDa as determined by SDS-PAGE. Whether this reflects additional processing at the C terminus, other posttranslational modifications, or simply aberrant migration is unknown. NS5A is a hydrophilic protein, a presumed replicase component, and

shows considerable divergence between BVDV and CSFV isolates. Recent work has shown that HCV NS5A and flavivirus NS5 proteins are serine phosphoproteins (40, 72, 90). The highly basic NS5B protein is predicted to encode the pestivirus RNA-dependent RNA polymerase. This activity was recently demonstrated for partially purified preparations of HCV NS5B (9) and dengue virus NS5 (88) but has not yet been reported for pestivirus NS5B.

Processing of the polyproteins of positive-strand animal RNA viruses regulates important steps in virion assembly and RNA replicase function. This is also believed to be the case for NS region cleavages in the polyproteins of the *Flaviviridae*. Using a functional BVDV cDNA clone, we have now shown that the NS3 serine proteinase is essential for pestivirus replication. Previous studies with YFV demonstrated that mutations in NS3 serine proteinase catalytic residues (17) or in the NS2B cofactor (15) or substitutions affecting cleavage at specific sites (2, 16, 65) impaired or blocked flavivirus replication. Given the numerous similarities among the NS3 proteinases and polyprotein-processing schemes of members of the *Flaviviridae*, it seems likely that the HCV proteinase is also necessary for replication of hepaciviruses, further validating its choice as a target for antiviral drug development. Future studies will focus on determining the steps in viral RNA replication which are regulated by NS3-mediated processing events.

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ADDENDUM IN PROOF

Tantz et al. (J. Virol. 71:5415–5422, 1997) have independently determined the serine proteinase-dependent processing

sites for BVDV strain CP7. Their results (CP7) and ours (NADL) are in complete agreement.

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