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Serine Protease of Pestiviruses: Determination of Cleavage Sites†

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The single-stranded genomic RNA of pestiviruses is of positive polarity and encompasses one large open reading frame of about 4,000 codons. The resulting polyprotein is processed co- and posttranslationally by virus-encoded and host cell proteases to give rise to the mature viral proteins. A serine protease residing in the nonstructural (NS) protein NS3 (p80) has been shown to be essential for the release of the NS proteins located downstream of NS3. In this report the NS3 serine protease-dependent cleavage sites for bovine viral diarrhea virus (BVDV) strain CP7 are described. Proteins used for analysis were generated in *Escherichia coli* or in eukaryotic cells by the use of the T7 vaccinia virus system. The N termini of NS4A, NS4B, NS5A, and NS5B were determined by protein sequencing. Analysis of the data obtained showed that leucine at P1 is the only position conserved for all cleavage sites. At P1′ alanine is found at the NS4A-NS4B site, whereas serine resides at this position at the NS3-NS4A, NS4B-NS5A, and NS5A-NS5B cleavage sites. For all cleavage sites the amino acids found at P1 and P1′ are conserved for different genotypes of pestiviruses, despite the high degree of sequence variation found between these viruses. It is therefore assumed that the cleavage sites determined for BVDV CP7 are representative of those for all pestiviruses.

Bovine viral diarrhea virus (BVDV), classical swine fever virus (CSFV), and ovine border disease virus (BDV) are members of the genus *Pestivirus* in the family *Flaviviridae* (53). This virus family also comprises the genus *Flavivirus* and the hepatitis C virus (HCV) group (41). The pestivirus genome is a single-stranded RNA molecule of positive polarity with a length of usually 12.3 kb (4, 6, 12, 14, 30, 37, 40, 42, 43). The genome encodes a single polyprotein of about 4,000 amino acids. The polyprotein of noncytopathogenic pestiviruses is cleaved co- and posttranslationally into at least 11 proteins, namely, N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, and NS5B (5, 7, 17, 33, 46, 54). The genomes of cytopathogenic pestivirus strains express at least one additional protein, called NS3 (p80); this protein can be generated in different ways (33, 34, 50–52).

Studies on the processing of the N-terminal third of the pestivirus polyprotein included determination of the N termini of the respective cleavage products by protein sequencing. The first cleavage product of the polyprotein, the autoprotease N^{pro}, generates its own C terminus and thereby the N terminus of the capsid protein (C) (46). Cleavages between the structural proteins are thought to be catalyzed by cellular enzymes. The C-E^{rns}, E1-E2, and E2-p7 cleavage sites are most likely processed by the cellular enzyme signal peptidase (17, 44). The E^{rns}-E1 cleavage is probably also mediated by a cellular enzyme; however, signal peptidase cleavage is considered unlikely (44). Processing at the p7-NS2 cleavage site is the subject of several ongoing investigations; preliminary results also suggest cleavage by cellular signal peptidase (49a).

It has been shown previously that BVDV nonstructural (NS) proteins are processed authentically when expressed in eukaryotic cells by the T7 vaccinia virus system (58). The same study demonstrated that a serine protease residing in the N-terminal region of NS3 is essential for generation of its own C terminus and for processing of the downstream cleavage sites, leading to the release of NS4A, NS4B, NS5A, and NS5B. In contrast to the data available for the pestivirus structural proteins, the knowledge about the localization of the NS proteins is so far limited to estimations based on the apparent molecular weights of the mature proteins. In particular, none of the cleavage sites has been determined. Moreover, prediction of putative cleavage sites was hampered by the fact that the sequence motifs at the cleavage sites are not conserved between the different genera of the family *Flaviviridae.*

In this report we describe the expression of the pestivirus NS proteins in heterologous systems. After partial purification, the N termini of NS4A, NS4B, NS5A, and NS5B were determined by protein sequencing.

MATERIALS AND METHODS

Cells and viruses. MDBK cells were obtained from the American Type Culture Collection (Rockville, Md.). BHK-21 cells (BSR clone) were kindly provided by J. Cox (Federal Research Centre for Virus Diseases of Animals, Tübingen, Federal Republic of Germany [FRG]). Virus strain CP7 has been described previously (11). Cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (FCS). The T7 vaccinia virus (vTF7-3) (19) was generously provided by B. Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.).

Infection of cells. Freeze-thaw lysates of BVDV-infected cells were used for infection of culture cells. The multiplicity of infection was usually 0.1. Cell lysates were prepared at 48 h postinfection and stored at −70°C.

Nucleotide sequencing. For analysis of double-stranded DNA, the T7 Sequencing Kit (Pharmacia Biotech, Freiburg, FRG) was used. Computer-aided sequence analysis was carried out on a Digital Microvax II, using the University of Wisconsin Genetics Computer Group software (15).

PCR. For PCR, Appligene *Taq* DNA polymerase (Oncor, Gaithersburg, Md.) together with the buffer supplied by the manufacturer was used. Amplifications were carried out with a Perkin-Elmer 9600 Thermocycler. The reaction volume was 50 μl and contained 250 μM deoxynucleoside triphosphates and 30 pmol of each primer. After 30 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 30 s, an incubation at 72°C for 3 min was carried out. PCR products were separated by

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agarose gel electrophoresis; for purification of DNA fragments, a QIAEX II gel extraction kit (Qiagen, Hilden, FRG) was used.

Radioimmunoprecipitation (RIP) and SDS-PAGE. Either 1.5×10^6 MDBK cells or 5×10^5 BSR cells (3.5-cm-diameter dish) were labeled in medium lacking the amino acid(s) used for labeling for 5 to 6 h with 500 μCi of [^{35}S]methionine-cysteine or 300 μCi of [^{3}H]leucine. 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 crosslinked *Staphylococcus aureus* was used (25). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins with molecular masses of up to 50 kDa was performed by the use of Tricine gels as described by Schägger and Jagow (45); for separation of larger polypeptides, SDS gels as described by Doucet and Trifaro (16) were used. Gels were processed for fluorography with En^{3}Hance (New England Nuclear, Boston, Mass.).

Transient expression with the T7 vaccinia virus system. A total of 5×10^5 BSR cells (3.5-cm-diameter dish) were infected with vTF7-3 (19) at a multiplicity of infection of 5 in medium lacking FCS. After 1 h of incubation at 37°C, the cells were washed twice with medium lacking FCS, followed by DNA transfection (10 μg of plasmid DNA; a Mammalian Transfection Kit [Stratagene] was used). After 4 h of incubation at 37°C, cells were washed two times with label medium lacking the amino acid(s) used for labeling and incubated in this medium for 0.5 h at 37°C. Cells were labeled in 1 ml of label medium containing the radioactive amino acid(s) for 5 to 6 h, washed with phosphate-buffered saline, and stored at −70°C.

N-terminal sequence analysis of radiolabeled proteins. Proteins used for radiosequencing were generated by transient expression in the T7 vaccinia virus system (see above). Labeling was carried out with 500 μCi of [^{35}S]methionine (about 1×10^6 cells) or 300 μCi of [^{3}H]leucine (about 2×10^6 cells) in 1 ml of labeling medium lacking methionine or leucine, respectively. Proteins were immunoprecipitated (see above), separated by SDS-PAGE, and transferred to an Immobilon polyvinylidene difluoride membrane. The protein was localized by autoradiography and subjected to automated Edman degradation.

Protein expression in *Escherichia coli*. For expression directed by pARHS3 (13), the bacterial strain BL21-DE3 expressing the DNA-dependent RNA polymerase of bacteriophage T7 (47) was used. Twenty-five milliliters of an overnight culture of transformed bacteria, grown at 37°C in Luria-Bertani (LB) medium containing 100 μg of ampicillin per ml, was added to 1 liter of LB containing 100 μg of ampicillin per ml and incubated at 37°C until the suspension reached an optical density at 660 nm of 0.6. After addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 10 mM, bacteria were incubated for 2 h at 37°C. Subsequently, cells were harvested by centrifugation (1,600 rpm at 4°C for 3 min), washed once with 100 ml of phosphate-buffered saline, and resuspended in 40 ml of 100 mM Tris, pH 8.0. After addition of 5 ml glycerol and 5 ml of Nonidet P-40 (NP-40) (10% [vol/vol] in double-distilled water), cells were sonified (Cell Disruptor B-15; Branson) and incubated on ice for 4 to 6 h. The supernatant from a final centrifugation (20,700 rpm at 4°C for 30 min) was further processed by affinity chromatography.

Partial purification of NS4A from *E. coli*. A protein A-Sepharose matrix (Pharmacia) was incubated with 10 volumes of a 1:10 dilution of anti-pep14 serum (33) in 100 mM Tris-HCl (pH 8.0) for 4 h at room temperature. The material was loaded in a column and washed with 200 volumes of 50 mM Tris-HCl pH 8.0 (solution A) and 20 volumes of 1% (vol/vol) NP-40–100 mM Tris (pH 8.0)–10% (vol/vol) glycerol (solution B). Subsequently, the bacterial extract was loaded on the column. After washing with 200 volumes of solution B and 100 volumes of solution C (0.1% [vol/vol] NP-40, 10 mM Tris [pH 8.0], 10% [vol/vol] glycerol), proteins bound to the matrix were eluted with solution D (0.1% [vol/vol] NP-40, 100 mM glycine [pH 2.7], 10% [vol/vol] glycerol). After neutralization with 0.1 volume of 1 M Tris-HCl (pH 9), the eluate was separated by SDS-PAGE, and NS4A was eluted from the polyacrylamide gel. After another SDS-PAGE, the proteins were transferred to an Immobilon polyvinylidene difluoride membrane, localized by Coomassie blue staining, and subjected to automated Edman degradation.

Construction of T7 expression plasmids. All T7 expression constructs were based on pSKT7T (9), a pBluescript SK− derivative kindly provided by K. K. Conzelmann (Federal Research Centre for Virus Diseases of Animals, Tübingen, FRG). All nucleotide or amino acid positions given in this paper refer to the sequence of BVDV strain SD-1 (14). To create blunt ends, the Klenow fragment of DNA polymerase I was used.

(i) pC7.3898. A *Bam*HI-*Xba*I fragment (nucleotides [nt] 5143 to 7724) of pC7.1 was cloned into pRN653B (51) restricted with the same enzymes. The resulting plasmid, pRN7.7, encodes an open reading frame consisting of nine vector-derived amino acids (including an in-frame AUG codon) and amino acids [aa] 1587 to 2247 of the CP7 polyprotein. Into pRN7.7, restricted with *Pst*I (blunt) and *Nsi*I, an *Nsi*I-*Sac*II (blunt) fragment (nt 7438 to polylinker) of cDNA clone pCP7.21 (nt 6725 to 10803) was ligated, leading to pRN10.8. A *Bam*HI fragment (nt 10219 to polylinker) of cDNA clone pCP7.31 (nt 8968 to 12161) was inserted into pRN10.8 previously treated with *Bam*HI (partial cut) (nt 10219 to polylinker) and calf intestine alkaline phosphatase. From this plasmid, pRN12.2, an *Nco*I (blunt)-*Sma*I fragment was isolated and cloned into pSKT7T digested with *Sma*I and treated with calf intestine alkaline phosphatase, leading to pC7.3898. This plasmid encompasses the open reading frame of CP7 from aa 1587 downstream to the authentic stop codon (downstream of aa 3898). All further plasmids used to direct transient expression in the T7 vaccinia virus system in this study are based on pC7.3898; these plasmids encode polyproteins which are C-terminally truncated. The number together with the plasmid name indicates the position of the last amino acid encoded by the respective construct.

(ii) pC7.2309, -2319, -2329, and -2339. The corresponding negative-sense oligonucleotides (see below) encompassing the stop codon at the respective position and an *Sma*I site at the 5′ end were used for PCR amplification together with oligonucleotide B32 (positive orientation), with pC7.3898 as the template DNA. The resulting PCR fragments were restricted with *Sma*I and *Bgl*II and ligated into pC7.3898 from which a *Bgl*II-*Xba*I (blunt) fragment (nt 7106 to polylinker) had been deleted. The identity of the inserted sequences was checked by DNA sequencing.

(iii) pC7.2349. Following PCR with primers B32 (positive orientation) and B2349S (negative orientation) with pC7.3898 as the template DNA, the resulting product was digested with *Msc*I and *Nsi*I. This fragment was cloned into pC7.3898 carrying an *Msc*I-*Pst*I deletion (nt 7138 to polylinker). The identity of the inserted sequence was checked by DNA sequencing.

(iv) pC7.2638, -2664, -2672, and -2683. Separate amplifications with primer B32 and the respective negative-sense primer were conducted on pC7.3898 template DNA. The products were incubated with *Nsi*I and *Sma*I (originating from the 5′ end of the oligonucleotide) and were used to replace an *Nsi*I-*Xba*I (blunt) fragment (nt 7438 to polylinker) in pC7.3898. The inserted sequences were verified by DNA sequencing.

(v) pC7.3142. From pC7.3898 an *Nco*I-*Eco*RV fragment (polylinker to nt 9809) was isolated and ligated into pC7.3898 from which the entire cDNA insert was previously removed by restriction with *Nco*I and *Bam*HI (blunt). In the resulting plasmid, pC7.3142, aa 3142 is followed by a UGA stop codon.

pC7.3179 and -3193. Based on pC7.3898 template DNA, sense oligonucleotide B60, and the respective antisense oligonucleotide, PCR products containing the translational stop signals and a primer-derived *Sma*I site at the 3′ end were generated. These DNAs were digested with *Eco*RV and *Sma*I and used to replace an *Eco*RV-*Xba*I (blunt) fragment (nt 9809 to polylinker) in pC7.3898. The sequence of the inserted DNA was checked by DNA sequencing.

Oligonucleotides used in PCR. The following oligonucleotides were used (the BVDV sequence is given in capital letters; all sequences correspond to the minus strand): B2309S, 5′-ggcccgggctaTTGATCTTCTATAGTGTATA-3′ (nt 7293 to 7312); B2319S, 5′-ggcccgggctaATATTGAAGGTGGGTTGTGT-3′ (nt 7323 to 7342); B2329S, 5′-ggcccgggctaCTTCCCCTCAGTTCTTATGG-3′ (nt 7353 to 7372); B2339S, 5′-cggcccgggctaACCCACTGCTAATTCCTTT-3′ (nt 7384 to 7402); B2349S, 5′-CCTCTGATGCATAcTaCGAGATGGAACCC-3′ (nt 7420 to 7448); B2638S, 5′-ggcccgggctaCAGGTGATATATGAGCCTC-3′ (nt 8281 to 8299); B2664S, 5′-CGAAGGCTTCGAACtaTATCAAGGTG-3′ (nt 8368 to 8393); B2672S, 5′-cggcccgggctaTAGTTCGAAGGCTTCGA-3′ (nt 8382 to 8398); B2683S: 5′-cggcccgggctaCAGGTTCCTTATCTTCCC-3′ (nt 8417 to 8434); B3179S, 5′-cggc ccgggctaCAGCTTCATGGTATAGGT-3′ (nt 9905 to 9922); and B3193S, 5′-cggcc cgggctaCAAGCTCATCTGTTTATTTG-3′ (nt 9945 to 9964). The following oligonucleotides have the positive orientation: B32, 5′-TAAGGAATGGGGAGGTCA -3′ (nt 7002 to 7019), and B60, 5′-GGGAGTTATTAGAGCAGG-3′ (nt 9714 to 9731).

Construction of the bacterial expression plasmids. The plasmids are based on pAHRS3 (13), which was kindly provided by L. de Moerlooze. To create blunt ends, the Klenow fragment of DNA polymerase I was used.

(i) pARC7. For construction of pARC7, a *Bam*HI-*Nsi*I (blunt) fragment (nt 5143 to 7438) of cDNA clone pC7.1 (50) was ligated into pCITE-2C restricted with *Bam*HI and *Xho*I (blunt). In the resulting plasmid an AUG codon and six vector-derived amino acids are followed by aa 1587 to 2351 of BVDV CP7; in addition, six histidine codons and a translational stop codon were fused to the C terminus of the BVDV open reading frame. From the resulting plasmid an *Nco*I-*Xba*I fragment, including the complete open reading frame together with the stop codon, was cloned into pARHS3 previously restricted with *Nco*I-*Xba*I, resulting in pARC7.

(ii) pARC7PM. pC7PM1 (50) encodes a substitution of serine 1752 by alanine, rendering the NS3 serine protease inactive. pARC7PM, carrying this protease mutation, was constructed by replacing a *Bam*HI-*Sac*I fragment (nt 5143 to 5859) of pARHC7 with the respective segment of pC7PM1.

RESULTS

Previous studies have shown that a serine protease residing in the N-terminal region of NS3 is essential for generation of pestivirus NS proteins (58). Thus far, the exact positions of the cleavage sites in the NS region of the viral polyprotein have not been determined. This is in part due to the relatively low abundance of pestivirus proteins in infected tissue culture cells. In order to obtain larger amounts of the pestivirus NS proteins for further analyses, we started to express relevant parts of the polyprotein in heterologous systems.

Expression in *E. coli* is often suitable for obtaining large amounts of recombinant proteins. For pestiviruses, however,

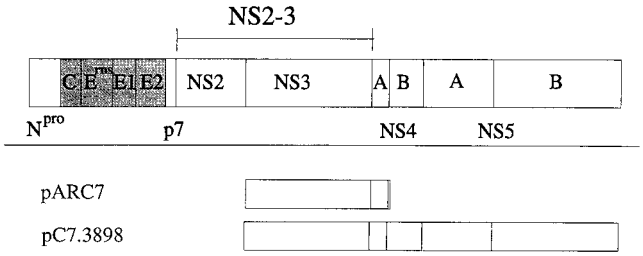

FIG. 1. Schematic drawing of the BVDV polyprotein. The structural proteins are marked by shaded boxes. Below the polyprotein, the genomic regions encoded by the bacterial expression plasmid (pARC7) and the basic construct for transient expression in eukaryotic cells (pC7.3898) are indicated. pARC7 encodes NS3, NS4A, and a small part of NS4B (aa 1587 to 2351 of the polyprotein of CP7). The open reading frame of pC7.3898 encompasses aa 1587 to 3898 of the CP7 polyprotein. Additional constructs for transient-expression experiments were derived from pC7.3898 by C-terminal truncations of the encoded polyprotein.

this approach was hampered by the fact that expression of NS3 is highly toxic to bacteria (reference 55 and unpublished data). Interestingly, it was possible to express BVDV NS3 in the bacterial expression vector pARHS3 (13, 55). By using this vector, a cDNA fragment encompassing the genomic region encoding NS3, NS4A, and a small part of NS4B of BVDV CP7 (11, 50) (aa 1587 to 2351, with numbering according to the BVDV SD-1 sequence [14]) was cloned into pARHS3, resulting in plasmid pARC7 (Fig. 1). The release of NS4A from the polyprotein fragment encoded by pARC7 was monitored by Western blotting with anti-pep14 serum (33), specific for NS4A (Fig. 2A). A 7-kDa protein was specifically detected after expression of pARC7 in bacteria; this polypeptide comigrated with NS4A derived from CP7-infected MDBK cells, indicating authentic processing at the NS3-NS4A and NS4A-NS4B sites in *E. coli*. NS4A is not generated after expression of pARC7PM (Fig. 2A); the latter encodes a polyprotein fragment in which the NS3 serine protease was inactivated by a point mutation (50). Due to the gel system used, the expected 85-kDa protein consisting of uncleaved NS3-NS4A* could not be demonstrated in Fig. 2A; however, the 85-kDa protein was recognized by anti-pep14 serum (data not shown). Accordingly, NS3 and the 85-kDa NS3-4A* protein (Fig. 2B)

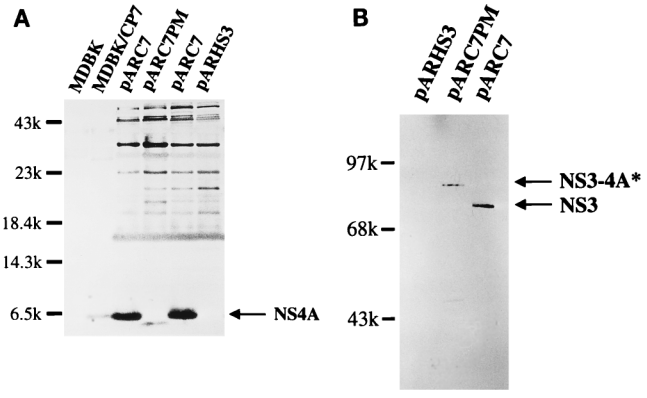

FIG. 2. Western blot of proteins from *E. coli* and MDBK cells. pARC7PM is based on pARC7 but carries a point mutation which renders the NS3 serine protease inactive (50). Bacteria transformed with pARC7PM or the parental plasmid pARHS3 served as controls. (A) Western blot with an antiserum specific for NS4A. MDBK cells infected with BVDV CP7 served as a positive control. The NS3-NS4A precursor was not detected in this gel system. (B) Western blot with an anti-NS3 monoclonal antibody.

Pestivirus strain:		Position of cleavage site:
BVDV CP7	GKALKQVVG**L** **S**SAENALLIA	NS3/NS4A
Osloss		(aa 2272/2273)
NADL	T V	
CSFV Alfort	R T V	
Brescia	R T V	
BVDV2-890	R T T	
BDV X818	R T A V	
BVDV CP7	EGKETELKE**L** **A**VGDLDKIMG	NS4A/NS4B
Osloss	M R E	(aa 2336/2337)
NADL	D T S VE	
CSFV Alfort	Q VQRCVE	
Brescia	Q VQRC E	
BVDV2-890	D DS YLD	
BVDV CP7	MDSEGKIRN**L** **S**GNYVLDLIY	NS4B/NS5A
Osloss	I	(aa 2683/2684)
NADL	Q I	
CSFV Alfort	V Q S I E L	
Brescia	V Q S I E L	
BVDV2-890	I F	
BVDV CP7	VGARTYTMK**L** **S**SWFLQATNK	NS5A/NS5B
Osloss	SS	(aa 3179/3180)
NADL	S A K S	
CSFV Alfort	KV S N VI EE	
Brescia	KV S N VM EE	
BVDV2-890	ST V K	
CONSENSUS	**L** **S**	
	A	

FIG. 3. Amino acid sequences flanking the cleavage sites in the NS protein regions of different pestiviruses. Only amino acids which differ from the sequence of BVDV CP7 are listed. The amino acid sequences were derived from the published nucleotide sequences for BVDV CP7 (31), BVDV Osloss (12), BVDV NADL (6), CSFV Alfort (Tübingen) (30), CSFV Brescia (37), BVDV2-890 (42), and BDV X818 (partial sequence) (4). The numbering of the amino acids is derived from the BVDV SD-1 sequence (14).

were detected by Western blotting with an anti-NS3 monoclonal antibody (8.12.7 [10]) after expression of pARC7 or pARC7PM in bacteria (Fig. 2B). NS4A expressed by pARC7 was partially purified from the bacterial extracts by immunoaffinity chromatography with anti-pep14 serum (data not shown); the eluate was separated by SDS-PAGE and blotted onto a membrane. NS4A was localized by Coomassie blue staining and subjected to Edman degradation. Seven of eight N-terminal amino acids were identified, resulting in the sequence SSAEXALL. An alignment of the obtained amino acid sequence with the CP7 polyprotein sequence revealed that NS4A starts with serine 2273. Thus, the cleavage site at the C terminus of NS3 is located between leucine 2272 (P1 position) and serine 2273 (P1′ position) in a short region of the polyprotein which is conserved among all pestiviruses analyzed so far (Fig. 3) (see Discussion).

Expression of polyprotein fragments larger than the one described above turned out to be very inefficient in the bacterial system. Therefore, the cleavage sites downstream of NS4A in the polyprotein were determined after expression in the T7 vaccinia virus system (19). A series of plasmids, all based on C7.3898 (Fig. 1), was constructed and expressed in eukaryotic cells. The open reading frames contained in these plasmids were under control of a T7 RNA polymerase promoter. pC7.3898 encompasses the coding region of the BVDV CP7 genome from NS3 downstream to the translational stop codon after codon 3898. In addition, several plasmids encoding C-terminally truncated open reading frames were derived from pC7.3898 by the introduction of stop codons. Detection of

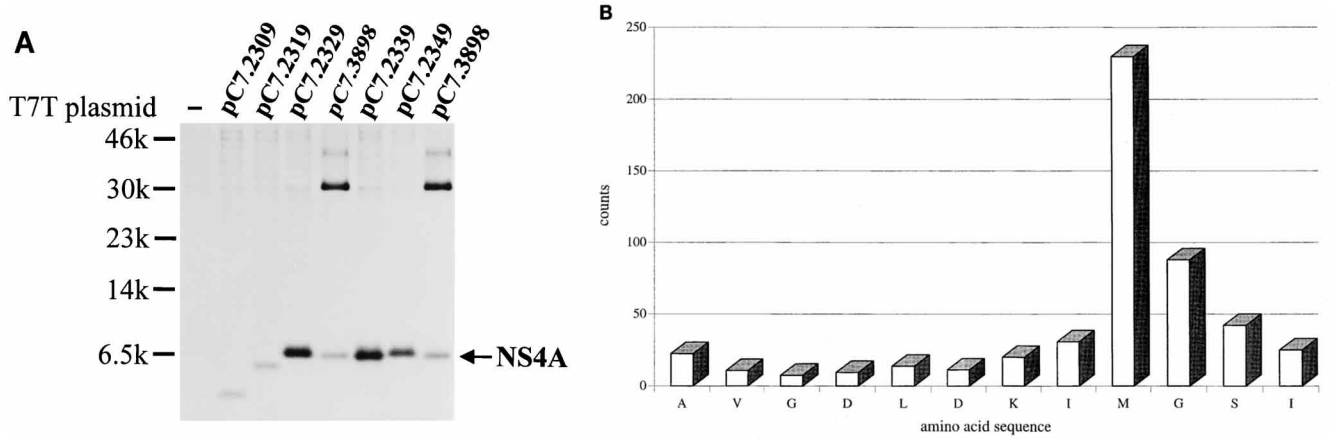

FIG. 4. (A) RIP with antiserum P1, which reacts with NS4A and NS4B (33). Metabolically labeled proteins were generated by transient expression in the T7 vaccinia virus system. The expression plasmids are all based on pC7.3898 (Fig. 1). Transfection of this plasmid into BSR cells preinfected with vaccinia virus vTF7-3 gave rise to authentically processed BVDV NS proteins, which served as positive controls. The numbers together with the plasmid names indicate the position of the C-terminal amino acid encoded by the construct. The position of authentic NS4A is indicated. (B) Radiosequencing diagram for determination of the N terminus of NS4B. NS4B was generated by transient expression of pC7.3898 in cells metabolically labeled with [^{35}S]methionine. The graph shows the counts per minute released by each Edman degradation step corrected by subtraction of the background level. The amino acid sequence of BVDV CP7 beginning with alanine 2337 is aligned with the degradation steps.

truncated processing products expressed from these plasmids should indicate the locations of the cleavage sites.

Viral proteases often recognize short amino acid motifs surrounding the cleavage sites of their substrates. For HCV, which shows a high degree of similarity to pestiviruses, especially with regard to the NS protein region, it was recently demonstrated that the amino acids at the P1 and P1′ positions are highly conserved (22); it turned out that the amino acid residue at the P1 position is especially critical for cleavage (2, 27). Since the NS3-NS4A cleavage site of BVDV CP7 is flanked by leucine and serine residues, the stop codons were introduced preferentially downstream of leucine residues which were followed either by serine or amino acids with small hydrophobic side chains. After transient expression of these constructs in BHK cells (BSR clone), the pestivirus proteins were analyzed by RIP and SDS-PAGE. Comparison of the molecular weights of NS4A derivatives expressed from pC7.2309, pC7.2319, pC7.2329, pC7.2339, pC7.2349, or pC7.3898 indicated that proteins with a C terminus located at aa 2309 or 2319 migrated faster than authentic NS4A (Fig. 4A). The NS4A derivative expressed from pC7.2349 showed a slightly lower mobility by SDS-PAGE than the wild-type protein. Therefore, the NS4A-NS4B cleavage site was assumed to be located in the region between aa 2325 and 2345. A leucine-serine dipeptide, as determined for the NS3-NS4A cleavage site, is not present in the region estimated to encompass the NS4A-NS4B cleavage site. However, this region of the polyprotein contains two leucine residues; leucine 2333 is followed by lysine, and an alanine residue is present downstream of leucine 2336. Accordingly, the leucine-alanine dipeptide at positions 2336 and 2337 showed the highest degree of similarity to the NS3-NS4A cleavage site. In order to determine the N terminus of NS4B, pC7.3898 was transiently expressed in cells labeled with [^{35}S]methionine. NS4B was enriched by RIP with antiserum P1 (33), and the precipitates were separated by SDS-PAGE, blotted onto a membrane, and subjected to Edman degradation. The samples derived from each degradation step were quantitated in a beta counter. The amount of radioactivity released by each degradation step is given in Fig. 4B. The peak of radioactivity is contained in the sample originating from degradation step 9. This result is in accordance with NS4A-NS4B cleavage between leucine 2336 and alanine 2337, since there is only one methionine residue (aa 2345) in the CP7 polyprotein between aa 2300 and 2360, encompassing the region previously mapped for the NS4A-NS4B cleavage site.

In order to roughly define the region of the polyprotein where cleavage between NS4B and NS5A takes place, the apparent molecular weights of NS4B derivatives expressed from pC7.2638, pC7.2664, pC7.2672, pC7.2683, and pC7.3898 were compared by SDS-PAGE (Fig. 5A). NS4B encoded by pC7.2683 comigrated with the native NS4B molecule expressed from pC7.3898. The additional bands with molecular masses of about 36 and 37 kDa after expression of pC7.2672 and pC7.2683, respectively, most likely represent uncleaved NS4A-NS4B precursor molecules. The reason for the inefficient processing of NS4A-NS4B expressed from pC7.2683 is not known, but it may be due to conformational changes induced by the C-terminal truncation. Interestingly, the NS4A-NS4B precursor was found to be relatively stable in BVDV-infected cells (8).

NS4B derivatives generated from constructs pC7.2638, pC7.2664, and pC7.2672 migrated faster than authentic NS4B expressed from pC7.3898. In the polyprotein of BVDV CP7, the amino acid leucine 2683 is followed by a serine residue. This dipeptide is identical to the NS3-NS4A cleavage site and is conserved among all pestiviruses analysed so far (see below). For direct protein sequencing of NS5A, construct pC7.3179 was expressed transiently in cells labeled with [^{3}H]leucine. Figure 5B shows the radioactivity released by each degradation step. Peaks of radioactivity were located at positions 6, 8, and 12. As depicted in Fig. 5B, this result is in accordance with cleavage of NS4B-NS5A between leucine 2683 and serine 2684.

The position of the NS5A-NS5B cleavage site was determined by a series of experiments similar to those described above. NS5A derivatives generated from plasmids pC7.3142, pC7.3179, and pC7.3193 were compared by SDS-PAGE with NS5A derived from pC7.3898. The NS5A proteins expressed from pC7.3179 and pC7.3898 comigrated (Fig. 6A). In comparison to these molecules, NS5A originating from pC7.3193

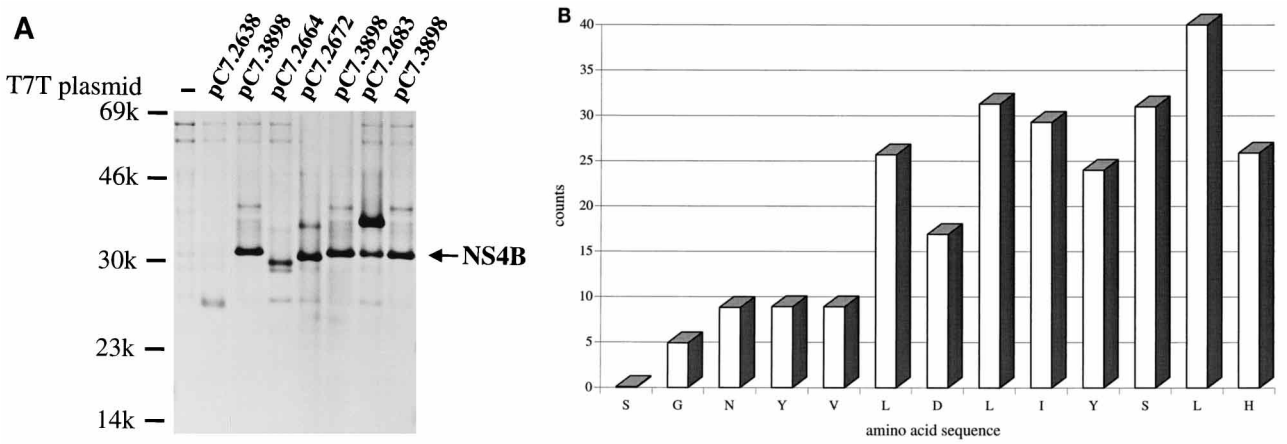

FIG. 5. (A) RIP with antiserum P1, which is specific for NS4A and NS4B. Metabolically labeled proteins were generated by transient expression in the T7 vaccinia virus system. The expression plasmids are all based on pC7.3898 (Fig. 1). The position of authentic NS4B is indicated. (B) Radiosequencing diagram for determination of the N terminus of NS5A. NS5A was generated by transient expression of pC7.3179 in cells metabolically labeled with [^{3}H]leucine. The graph shows the counts per minute released in each Edman degradation step corrected by subtraction of the background level. The experiment was repeated once; in the second experiment the peaks of radioactivity were located at the same positions. The amino acid sequence of BVDV CP7 starting with serine 2684 is aligned with the degradation steps.

showed slightly slower migration; in addition to this larger protein, a minor protein comigrated with authentic NS5A. This protein may represent a product of partial processing at the authentic cleavage site. Expression of pC7.3142 led to a C-terminally truncated NS5A molecule. These experiments indicated that the NS5A-NS5B cleavage site is probably located around aa 3179. At this position a leucine (position 3179)-serine (position 3180) dipeptide is conserved in the polyproteins of all published pestivirus sequences (see Discussion). Twelve amino acids downstream of this putative cleavage site, a methionine residue (position 3191) is located in the CP7 polyprotein. NS5B generated by transient expression of pC7.3898 was then labeled metabolically with [^{35}S]methionine and used for N-terminal protein sequencing. The distribution of the radioactivity in the samples from the Edman degradation is shown in Fig. 6B; only amino acid position 12 showed a pronounced peak, confirming serine 3180 as the N-terminal amino acid of NS5B.

DISCUSSION

The polyprotein of noncytopathogenic BVDV is cleaved into 11 polypeptides: N^{pro}, C, E^{rns}, E1, E2, p7, NS2-3, NS4A, NS4B, NS5A, NS5B (5, 17, 33, 46, 54). Whereas the structural proteins are released from the polyprotein mostly by cellular enzymes (17, 44), generation of the NS proteins downstream of NS3 is dependent on a serine protease residing in the N-terminal domain of NS3 (58). The exact cleavage sites, however, have so far not been identified. This was in part due to the relatively low levels of viral protein synthesis in pestivirus-infected cells. In addition, the N termini of BVDV NS proteins purified from infected cells could not be analyzed by Edman degradation (unpublished data). The reason for this failure is not known; it is possible that chemical alteration of the N-terminal amino acid(s) occurred during the purification procedure. Alternatively, the N-terminal amino acid(s) may be modified in the infected cell. In this study the N termini of the

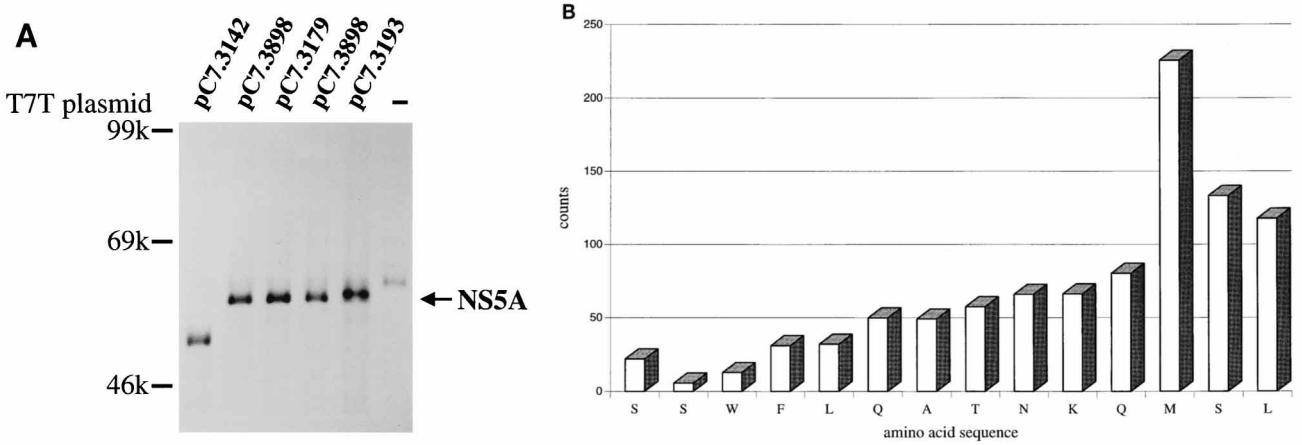

FIG. 6. (A) RIP with antiserum 62D specific for NS5A (5). Metabolically labeled proteins were generated by transient expression in the T7 vaccinia virus system. The expression plasmids are all based on pC7.3898 (Fig. 1). The position of authentic NS5A is indicated. (B) Radiosequencing diagram for determination of the N terminus of NS5B. NS5B was generated by transient expression of pC7.3898 in cells metabolically labeled with [^{35}S]methionine. The graph shows counts per minute released in each Edman degradation step corrected by subtraction of the background level. The amino acid sequence of BVDV CP7 starting with serine 3180 is aligned with the degradation steps.

BVDV proteins NS4A, NS4B, NS5A, and NS5B were determined by using proteins generated either in bacteria or by transient expression in eukaryotic cells with the T7 vaccinia virus system. These systems allowed us to obtain viral proteins suitable for protein sequencing.

A comparison of the amino acid sequences flanking the cleavage sites affected by the NS3 serine protease in the polyprotein of BVDV demonstrates that P1 and P1′ are the only conserved positions (Fig. 3). The leucine residue at P1 is conserved for all cleavage sites. At three of four cleavage sites, serine is found at the P1′ position. At the NS4A-NS4B cleavage site only, serine is replaced at P1′ by alanine. Thus, at all cleavage sites an amino acid with a small uncharged side chain resides at P1′. It has been shown by pulse-chase experiments that the NS3-NS4A and NS4B-NS5A cleavages occur very rapidly. In contrast, the NS4A-NS4B cleavage is delayed (8). Future experiments will show whether the replacement of serine at the P1′ position by alanine is involved in retardation of processing between NS4A and NS4B. In BVDV-infected cells, processing at the NS5A-NS5B site, composed of a leucine-serine dipeptide, also occurs in a delayed fashion (8), indicating that the processing kinetics at a specific cleavage site is probably regulated not only by the amino acids found at the P1 and P1′ positions.

Despite a high degree of sequence variation between the different genotypes of pestiviruses, the amino acids at the P1 and P1′ positions of the cleavage sites in the NS protein region are conserved (Fig. 3). It appears justified to assume that the N termini of the NS proteins determined in this study for BVDV CP7 are representative of those for all pestiviruses.

The members of the HCV group show a number of interesting similarities to pestiviruses. This is especially true with respect to the NS region and its individual proteins. While the generation of uncleaved NS2-3 is unique for pestiviruses, HCV and BVDV otherwise have the same number of NS proteins. The molecular weights, hydrophobicity patterns, and enzymatic activities of the respective proteins are quite similar (48, 49, 57). The serine proteases residing in the NS3 molecules of both viruses are essential for processing of the NS proteins downstream of NS3 (1, 22, 24, 58). With the HCV system it was demonstrated that NS4A is an essential cofactor of the NS3 serine protease (18, 28). A similar function was postulated for the NS4A-NS4B region of pestiviruses (58). A comparison of the cleavage sites downstream of NS3 also shows some interesting parallels between HCV and pestiviruses. For HCV the consensus sequence at these cleavage sites is P6 (D or E), P1 (C or T), and P1′ (S or A) (22). The importance of P6, however, is unclear, since amino acid changes at this position had no effect on cleavage efficiency (2, 27). A corresponding conservation of P6 was not found for the pestivirus polyprotein. For HCV a cysteine residue is usually found at the P1 position; only at the NS3-NS4A site, which is processed exclusively in *cis*, threonine is found at P1. Interestingly, replacement of cysteine at P1 by leucine, thereby mimicking the cleavage site of pestiviruses, led in vitro to an enhanced cleavage efficiency (27). For HCV and pestiviruses serine is conserved at position P1′; the change of serine to alanine, which is found at P1′ at the NS4A-NS4B site of pestiviruses, is present in some HCV strains (cited in reference 22). These striking similarities concerning the NS3-dependent cleavage sites underline the phylogenetic relationship between HCV and pestiviruses.

According to sequence pattern analyses, the NS3 serine proteases of all members of the *Flaviviridae* and the capsid protease of Sindbis virus belong to the family of trypsin-like proteases (3, 20). The amino acids preceding the catalytic serine residue in the active centers of trypsin or chymotrypsin are involved in formation of the S1 substrate binding pocket (23, 56). The side chain of the amino acid which is located 6 aa upstream of the catalytically active serine (aa 189 according to chymotrypsin numbering) is located at the bottom of the substrate binding pocket and thus is believed to be especially critical for the substrate specificity of these enzymes (21). At the corresponding position an aspartic acid is found in trypsin as well as in NS3 of flaviviruses, which is in line with the preference of both enzymes for cleavage after positively charged amino acids.

Interestingly, the recently resolved three-dimensional structure of the HCV NS3 protease domain showed that the amino acids which comprise the S1 specificity pocket of this enzyme are Leu-135, Phe-154, and Ala-157 (26, 29). Phe-154, which is localized at the bottom of the S1 pocket, is believed to interact with the amino acid at P1 (Thr or Cys). Thus, the amino acid which interacts with P1 is not located 6 aa upstream of the active-site serine (Ser-139). Because of the already-mentioned similarities between pestiviruses and HCV, it seems reasonable to assume that HCV and pestivirus NS3 proteases show more resemblance to each other than to flavivirus and cellular enzymes.

So far, noncytopathogenic pestivirus strains are the only members of the *Flaviviridae* which show no processing of NS2-3. Cytopathogenic pestivirus strains have evolved different mechanisms for the expression of an additional protein, representing the C-terminal part of NS2-3, namely, NS3 (p80) (11, 38, 39). Recent studies have shown that generation of the N terminus of NS3 in cytopathogenic-BVDV-infected cells can be mediated by a viral or a host cell protease (32, 33, 51, 52). Thus, in cells infected with cytopathogenic pestiviruses, two proteases, namely, NS2-3 and NS3, are present and probably capable of processing the viral NS protein region, whereas in noncytopathogenic-pestivirus-infected cells usually only the NS2-3 protease catalyzes the corresponding cleavages. Future studies will show whether the proteases differ in their activities. Differences in the fine tuning of polyprotein processing might be important for regulation of viral replication and ultimately also for cytopathogenicity of pestiviruses.

The infectious pestivirus cDNA clones which have been described recently (31, 35, 36, 43) are promising tools for future investigations particularly with regard to the mechanism of RNA replication. The knowledge of the N termini of the pestivirus NS proteins downstream of NS3 is essential for future studies concerning the functions of the individual proteins. Because of the close relationship between pestiviruses and HCV, it appears justified to assume that results obtained for pestiviruses will also lead to a better understanding of the life cycle of HCV.

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ADDENDUM

Xu et al. (J. Virol. 71:5312–5322, 1997) have independently determined the serine proteinase-dependent processing sites for BVDV strain NADL. Their results (NADL) and ours (CP7) are in complete agreement.

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