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Mapping of two antigenic domains on the NS3 protein of the pestivirus bovine viral diarrhea virus

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Abstract

The immunodominant NS3 (p80) protein of the pestivirus bovine viral diarrhea virus (BVDV) functions as a serine protease and a RNA helicase. To identify antigenic domains of the BVDV NS3, a panel of monoclonal antibodies (mAbs) was tested against fragments of the protein expressed in *E. coli*. Two large overlapping NS3 fragments, A (amino acids [aa] 1–434) and B (aa 368–683) which together contain all NS3 sequences, were used to screen mAbs for reactivity. Two mAbs, 21.5.8 and 1.11.3, were reactive to fragment A (in ELISA only) and one mAb, 20.10.6, was reactive to fragment B (in ELISA and Western blotting). Further mapping demonstrated that the smallest fragment mAbs 21.5.8 and 1.11.3 bound to was comprised of aa 205–369 (domain A). In Western blotting, the smallest fragment reactive with mAb 20.10.6 was comprised of aa 368–549 (domain B). However, in indirect ELISA, mAb 20.10.6 also demonstrated high reactivity to a smaller fragment comprising aa 368–512 (domain B′). This indicated that the epitope of mAb 20.10.6 was conformational and not linear. Blocking ELISAs using these mAbs and type 1 and type 2 BVDV antisera demonstrated that an immunodominant region of the NS3 protein in cattle is defined by aa 205–549.

Keywords: Bovine viral diarrhea virus; Pestivirus; NS3 protein; Epitopes; Monoclonal antibodies

1. Introduction

Bovine viral diarrhea virus (BVDV) causes a variety of economically important diseases in cattle including reproductive and respiratory disorders, hemorrhagic syndrome, persistent infections and mucosal disease, a usually fatal condition of persistently infected (PI)

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cattle (Baker, 1995). The virus' importance to the cattle industry is highlighted by the announcement in recent years of eradication programs in several European countries (Bitsch and Rønsholt, 1995) and the recently announced position statement from the Academy of Veterinary Consultants calling for adoption of measures to control and target eventual eradication of BVDV from North America (Grotelueschen, 2002). A key element in the control and eradication of BVDV is the identification and removal of PI cattle (Lindberg and Alenius, 1999) because these animals shed virus continuously and are the major source of new herd infections.

BVDV is a member of the genus pestivirus, a group of small RNA viruses in the family *Flaviviridae* (HCV) (Wengler, 1991). Two species of BVDV exist: type 1 (BVDV1) and type 2 (BVDV2) (Pellerin et al., 1994; Ridpath et al., 1994). Each species consists of cytopathic and noncytopathic strains (biotypes), which propagate with or without cytopathic effect in cell culture, respectively.

The approximately 12.5 kb BVDV genome encodes four structural proteins, including the major neutralizing E2 protein (Donis et al., 1988) for which epitopes have previously been mapped (Paton et al., 1992; Deregt et al., 1998a,b), and several non-structural proteins (Collett et al., 1988; Meyers and Thiel, 1996). Among the non-structural proteins, the NS3 (80 kDa)/NS2-3 (125 kDa) proteins are highly immunogenic. The NS3 protein is highly conserved among pestiviruses (about 90% amino acid identity for BVDV1 and BVDV2) and is derived from coding sequences of the larger NS2-3 protein (Ridpath and Bolin, 1995; Meyers and Thiel, 1996). It is observed as a separate entity only in cells infected with cytopathic BVDV strains and is the marker protein of these strains (Donis and Dubovi, 1987).

The NS3 protein of BVDV is bi-functional with protease and NTPase/RNA helicase activity (Wiskerchen and Collett, 1991; Tamura et al., 1993; Warrener and Collett, 1995; Tautz et al., 2000). These functions are expressed in two domains: the serine protease domain in the N-terminal one-third of the protein involved in polyprotein cleavage of viral non-structural proteins (Wiskerchen and Collett, 1991) and the helicase domain comprising conserved motifs in the C-terminal two-thirds of the protein involved in unwinding duplex RNAs during virus replication (Tamura et al., 1993; Warrener and Collett, 1995; Kadaré and Haenni, 1997; Gu et al., 2000).

The high immunogenicity of the NS3 protein is evidenced by the observation that monoclonal antibodies are readily produced to the viral protein (Corapi et al., 1990; Deregt et al., 1990, 1991; Shannon et al., 1991). In addition, the NS3 protein is used as an antigen in ELISA for detection of BVDV antibodies in cattle (Lecomte et al., 1991; Paton et al., 1991). Although it has important immunological and diagnostic significance, with the exception of the recent identification of a critical amino acid for the epitope of a rare BVDV1-specific monoclonal antibody (Brown et al., 2002) and a report of an immunoreactive 70 amino acid region (Lecomte et al., 1991), the antigenic sites of the protein are not well defined. In this study, in an effort to identify an immunodominant $\leq$25 kDa NS3 fragment for possible use in a diagnostic fluorescent polarization assay (Lin and Nielsen, 1997), we examined the reactivity of NS3 fragments with murine monoclonal antibodies and bovine sera and identified two immunodominant antigenic domains on the NS3 protein.

2. Materials and methods

2.1. Production of NS3 constructs

Regions of the NS3 gene of the type 1a Singer strain were reverse transcribed and amplified by PCR using methods previously described (Gilbert et al., 1997). Primers for PCR were designed from the published sequence (Pellerin et al., 1995). PCR products were cloned into the pET-30a-c(+) vector (Novagen, Madison, WI). *E. coli* BL21 (DE3) cells (Novagen) were transformed with the recombinant plasmid for expression of N-terminal six histidine-tagged constructs (Fig. 1). Nucleic acid sequencing of the NS3 constructs ($\sim$500–900 nucleotides of the 5′ end, for fragments A, B, and D, or the entire coding region for other fragments) was performed as previously described (Gilbert et al., 1997).

2.2. Purification of expressed NS3 constructs

Expressed NS3 fragments were purified under denaturing conditions as per the manufacturer's

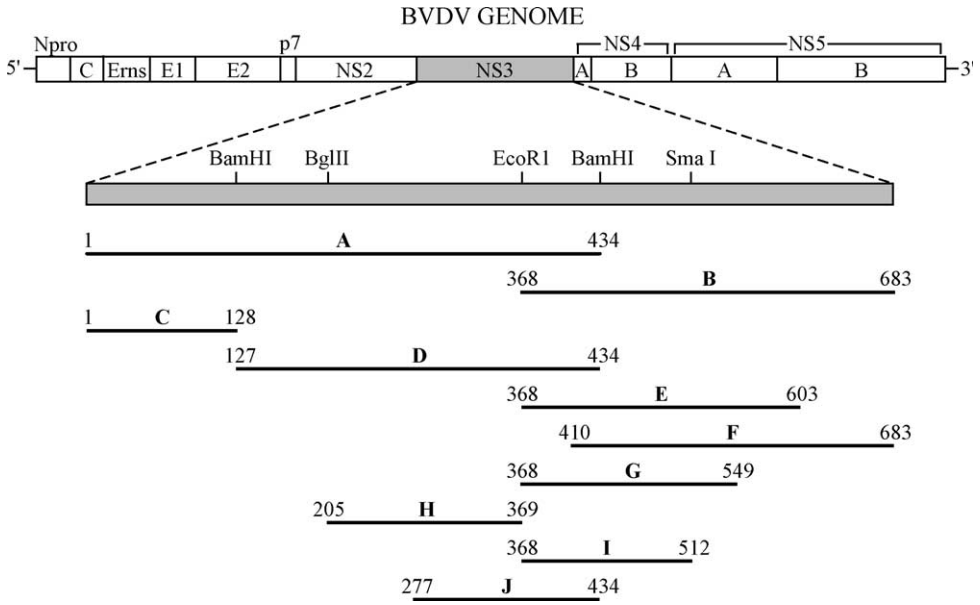

Fig. 1. Cloning strategy for the NS3 fragment-fusion proteins. Regions of the NS3 gene (Singer strain) were cloned into the pET-30a-c(+) vector for expression of N-terminal six histidine-tagged constructs. The location of the NS3 gene, which codes for the 683-amino acid, NS3 protein, and the positions (N- and C-terminal amino acids) of cloned NS3 fragments A–J are shown.

instructions (Novagen) as the majority of protein occurred in the insoluble fraction. Binding, wash, and elution buffers used were those formulated by Novagen. Proteins were first solubilized in binding buffer containing 8 M urea which was adjusted to 6 M urea prior to Ni-NTA agarose (QIAGEN, Mississauga, Ontario, Canada) column chromatography. After the protein was added to the column, the slow removal of urea was accomplished by washing with wash buffer containing decreasing amounts of urea (6 M [high end] to 0 M [low end] in 1 M steps) to encourage protein re-folding. NS3 fragments were eluted from the column in elution buffer containing 250 mM imidazole, and stored in this buffer at 4 °C for ELISAs and at −70 °C for Western blot analysis.

2.3. Monoclonal antibodies

The production and characterization of NS3 monoclonal antibodies (mAbs), 20.10.6, 5.2.1, 9.10.4, 4.11.4, 21.5.8, 12.7.3, 15.14.5, 16.1.5, 1.11.3, 115, and 184, used in this study, were previously described (Corapi et al., 1990; Deregt et al., 1990, 1991). All of the mAbs were raised against native (non-denatured) antigen.

2.4. Bovine sera

All calves used to produce BVDV antiserum were from the BVDV-free herd located at the Lethbridge Laboratory. Paired pre-inoculation and day 21 post-inoculation sera from eight calves inoculated once intranasally with BVDV1 strains Singer (cytopathic) or Hastings (noncytopathic), or BVDV2 strains 125c (cytopathic) or 24515 (noncytopathic) (two calves for each virus) were used in this study. The post-inoculation sera had virus neutralizing antibody titers than ranged from 1/128 to 1/65,536 against the homologous virus. In addition, post-inoculation sera were derived from another 12 cattle for which pre-inoculation sera were unavailable. These cattle were inoculated once intranasally with Singer ($n = 1$), 24515 ($n = 8$), or V1127, a noncytopathic BVDV1 isolate obtained from a bison ($n = 3$). These sera were collected at 14–70 days post-inoculation and had virus neutralizing antibody titers which ranged from 1/512 to 1/16,384 against the Singer strain or the homologous virus (for 24515-inoculated cattle). All procedures complied with the guidelines of the Canadian Council on Animal Care.

2.5. Western blotting

NS3 fragments were electrophoresed in 12% SDS–polyacrylamide gels at 200 V for 45 min. Polyvinylidene fluoride (PVDF) membranes (Immobilin-P, Millipore Corporation, Bedford, MA) (pre-wetted in methanol) and gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, 1.3 mM SDS) for 30 min. Transfer of NS3 fragments onto PVDF membranes occurred in a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, Mississauga, Ontario, Canada) at 15 V for 30 min. After blocking with PBST buffer (0.01 M PBS, pH 7.4, 0.05% Tween-20) containing 5% skim milk powder for 1 h, individual PVDF membranes were rinsed with PBST and incubated overnight with 1/250 dilutions in PBST of NS3-specific mAbs (mouse ascites fluids) or a 1/2000 dilution of anti-histidine (RGS) mAb (QIAGEN). All incubations occurred at room temperature. After washing the membrane in PBST, membranes were incubated with a 1/1000 dilution of goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (KPL, Canadian Life Technologies, Burlington, Ontario, Canada) for 1 h. Finally, after washing in PBST and PBS, reactions were detected with 4-chloro-1-naphthol (Bio-Rad Laboratories) as per manufacturer's instructions.

2.6. Indirect and blocking ELISAs

For indirect ELISA, Ni-NTA agarose-purified NS3 fragments in coating buffer (0.5 M $NaHCO_3/Na_2CO_3$, pH 9.3) were applied at 1 μg/well onto 96-well polystyrene microtiter plates (Nunc, VWR Canlab, Mississauga, Ontario, Canada) and incubated overnight at 4 °C. All subsequent incubations occurred at room temperature and plates were washed five times with PBST between each step. To block unreacted sites, PBST containing 5% horse serum (HS) was applied and incubated for 90 min. After washing the plate, a 1/100 dilution of mouse ascites fluids or a 1/200 dilution of bovine serum, in PBST containing 1% HS (diluent), were added to duplicate wells and incubated for 60 min. Following the incubation of the primary antibody, a 1/2000 dilution of goat anti-mouse IgG-HRP or goat anti-bovine IgG-HRP in diluent was added and incubated for 60 min. After washing with PBST and a final rinse with PBS, the substrate 1.9 mM 2,2′-azino-bis(3-ethylbenzthiazoline-sulfonic acid) (ABTS) (Sigma, Oakville, Ontario, Canada)–4.3 mM H_2O_2 in 50 mM sodium citrate (pH 4.5) was added to the plates and incubated for 20 min. Optical density (OD) values were measured at 414 nm with a Labsystems Multiskan RC plate reader. To determine reactivity for bovine sera, positive/negative (P/N) values were utilized, which is the mean OD of the post-inoculation (positive) serum divided by the mean OD of the pre-inoculation (negative) serum for individual animals. If the P/N value was $\geq$1.5, the post-inoculation serum was considered to demonstrate reactivity with the NS3 fragment.

For blocking ELISA, NS3 fragments were coated at 0.5–1 μg/well and the procedures were as above with the following modifications. Undiluted BVDV antibody-positive and negative bovine sera were first incubated on the plates for 90 min. After washing, a 1/100–1/500 dilution of monoclonal antibody was added and incubated for 1 h. Finally, a 1/2000 dilution of goat anti-mouse IgG-HRP in diluent was added and incubated for 60 min for detection of bound monoclonal antibody. The percentage blocking by bovine serum was calculated by the formula: $(A - B)/A \times 100\%$ where A is the absorbence in the presence of reference negative serum and B is the absorbence in the presence of the test sample. Samples were considered positive when the level of blocking was $\geq$50%.

3. Results

3.1. ELISA reactivity of monoclonal antibodies with NS3 fragments

Ten NS3 fragment-fusion proteins were produced for mapping of epitopes (Figs. 1 and 2). For unknown reasons, the histidine-tagged fusion proteins migrated on gels with higher apparent molecular weights (MWs) than expected from their predicted MW (Fig. 2). This was consistently observed for all fusion proteins but was more noticeable with the smaller proteins. Purification with Ni-NTA agarose gave only a single product for each construct and the identity of all cloned fragments were confirmed by nucleotide sequencing, therefore we were confident of their authenticity. Except for one mis-incorporation noted

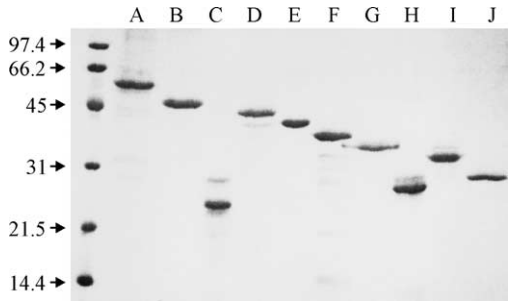

Fig. 2. Expression of NS3 fragments in *E. coli*. Bacteria containing the recombinant pET-30a-c(+) expression vectors were induced for 3 h with 1 mM IPTG. The resultant proteins were solubilized, purified by Ni-NTA agarose column chromatography, boiled for 5 min with loading buffer, and resolved in a 13.5% SDS–polyacrylamide gel. The lane letters at the top indicate the NS3 fragment-fusion proteins (A–J) shown for each lane. Molecular weight markers (in kDa) occur in the first lane. Predicted molecular weights of the fusion proteins occur in Table 1.

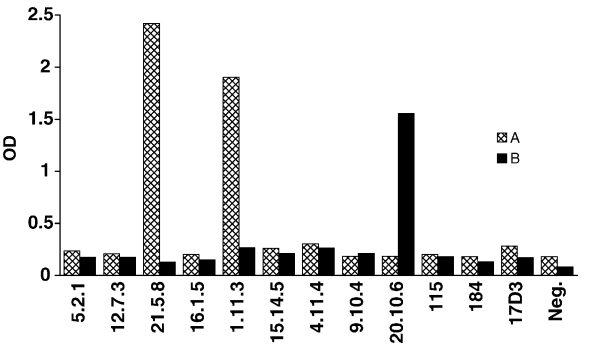

Fig. 3. Reactivity of mAbs with overlapping NS3 fragments A and B in indirect ELISA. A panel of eleven NS3 mAbs and an equine arteritis virus (negative control) mAb (17D3) were incubated with fragments A and B to screen mAbs for reactivity. Neg. = no mAb (negative) control.

in fragment A (below), all sequences obtained for the cloned fragments were in agreement with the published Singer strain sequence (Pellerin et al., 1995). Since difficulties were encountered in the expression of a full-length NS3 protein, mAbs were first screened against two large overlapping fragments, A (amino acid [aa] positions 1–434) and B (aa 368–683), which cover the entire NS3 sequence. In ELISA, from 11 mAbs screened, two mAbs (21.5.8 and 1.11.3) were reactive to fragment A and one mAb (20.10.6) was reactive to fragment B (Fig. 3).

The reactivities of mAbs 21.5.8, 1.11.3, and 20.10.6 with NS3 fragments are summarized in Table 1. Because of a known mis-incorporation of a base in the sequence of fragment A resulting in an amino acid change at position 91 (Ser to Arg), fragment C (aa 1–128) was produced, identical to the published sequence. None of the 11 mAbs were reactive to this fragment. This indicated that mAbs 21.5.8 and 1.11.3 bound downstream of sequences in fragment C and, as expected, reactivity of these two mAbs was demonstrated with fragment D (aa 127–434). Knowledge that mAb 21.5.8 inhibited helicase activity (Warrener and Collett, 1995) suggested that

Table 1
Summary of NS3 fragments and reactivity of BVDV-specific antibodies in indirect ELISA

Fragment	Amino acids	MW[a] (kDa)	Monoclonal antibody			Bovine sera ($n = 8$)
			21.5.8	1.11.3	20.10.6	
A	1–434	47.4 (55.3)	+	+	–	6 (75)[b]
B	368–683	35.4 (40.9)	–	–	+[c]	8 (100)
C	1–128	13.9 (20.9)	–	–	–	1 (12)
D	127–434	33.7 (41.1)	+	+	–	6 (75)
E	368–603	26.4 (36.0)	–	–	+[c]	8 (100)
F	410–683	30.7 (35.7)	–	–	–	7 (88)
G	368–549	20.1 (28.9)	–	–	+[c]	7 (88)
H	205–369	18.5 (24.7)	+	+	–	8 (100)
I	368–512	15.8 (25.2)	–	–	+	5 (62)
J	277–434	17.5 (24.5)	–	–	–	3 (38)

[a] Predicted molecular weight (MW) in kDa of the NS3 sequence followed by the MW of the corresponding fusion protein in parenthesis.
[b] Number and percentage (in parenthesis) of post-inoculation bovine sera demonstrating reactivity (P/N value ≥ 1.5).
[c] NS3 fragments which were also reactive in Western blots with mAb 20.10.6.

this mAb likely bound at or near conserved helicase motifs. Therefore, a smaller fragment, H (aa 205–369), was produced which contained motifs I "G^{229}AGKT" and motif II "D^{321}EYH", also known as Walker motifs A and B, respectively (Walker et al., 1982). Both mAbs, 21.5.8 and 1.11.3, were reactive to this fragment. For further mapping, fragment J (aa 277–434) which eliminated motif I was produced, however, neither mAb was reactive to fragment J. Thus, the antigenic domain of these mAbs, designated domain A, was contained within aa 205–369.

To further map the antigenic domain of mAb 20.10.6, three fragments were produced: E (aa 368–603), F (aa 410–683), and G (aa 368–549) (Fig. 1 and Table 1). Fragment F, for which N-terminal sequences of fragment B were deleted, was non-reactive, whereas the C-terminally truncated fragments E and G were both reactive. A further C-terminal truncation lead to production of fragment I (aa 368–512) which was also reactive giving a similar OD value as the larger reactive fragments. The fragment J (aa 277–434) was unreactive with this mAb which confirmed findings with fragments A and D. Thus, the antigen domain of this mAb, designated antigenic domain B′, was contained within aa 368–512.

3.2. Reactivity of the mAbs to NS3 fragments by Western blotting

All 11 mAbs were screened for reactivity with fragments A and B in Western blotting. Only a single mAb, 20.10.6, demonstrated reactivity (with fragment B) (data not shown). This suggested that the epitope for 20.10.6 was linear. However, Western blotting demonstrated that whereas the mAb reacted with fragments E (aa 368–603) (not shown) and G (aa 368–549), it did not react with fragment I (aa 368–512) (Fig. 4A) which was reactive in ELISA. To ensure transfer of all fragments, blots were also reacted with an anti-histidine mAb (Fig. 4B). Because of the ELISA positivity/Western blot negativity of fragment I, it was concluded that the epitope of 20.10.6 is conformational and not linear. The region represented by aa 368–549 (fragment G), was designated as antigenic domain B (to distinguish it from domain B′ [aa 368–512]) since it was apparent that C-terminal sequences in fragment G were required for Western blot reactivity as well as mAb avidity in a blocking ELISA (Section 3.4).

3.3. ELISA reactivity of bovine sera to NS3 fragments

To determine reactivity with bovine sera, NS3 fragments were tested in indirect ELISA with paired post-inoculation (positive) sera and pre-inoculation (negative) sera from eight calves inoculated with either BVDV1 or BVDV2. Positive/negative values were calculated from the average OD value for each sera and those post-inoculation sera giving P/N values of $\geq$1.5 were scored as reactive. Actual positive P/N values ranged from 1.5 to 8.9.

As shown in Table 1, NS3 fragments bearing mAb epitopes were reactive with the majority of positive bovine sera. All eight positive sera reacted with fragment H bearing epitopes of mAbs 21.5.8 and 1.11.3 and seven of eight positive sera (88%) reacted with fragment G, the smallest fragment reactive with mAb 20.10.6 in Western blots. Sera from calves inoculated with BVDV2 demonstrated reactivity with NS3 fragments more often and generally gave higher P/N values than sera of calves inoculated with BVDV1 (data not shown).

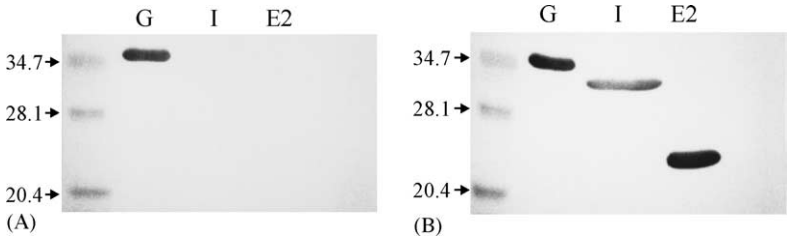

Fig. 4. Western blotting reactivity of mAb 20.10.6. Panel A, mAb 20.10.6 was incubated with NS3 fragments G (aa 368–549) and I (aa 368–512) and a BVDV E2 fragment-fusion protein (negative control). Panel B, an anti-histidine mAb was incubated with the same proteins as in panel A to demonstrate blot transfer of all histidine-tagged proteins. Molecular weight markers (in kDa) occur in the first lane in each panel.

Table 2
Reactivity of antibodies to NS3 fragments H and G in BVDV-inoculated calves as demonstrated in a blocking ELISA

Fragment	Monoclonal antibody[a]	Pre-inoculation sera[b]	Post-inoculation sera[b]
H (aa 205–369)	21.5.8	0/8	14/20 (70%)
H (aa 205–369)	1.11.3	0/8	20/20 (100%)
G (aa 368–549)	20.10.6	0/8	17/20 (85%)

[a] Sera were tested for their ability to compete with the indicated monoclonal antibody in ELISA.

[b] Number of pre-inoculation and post-inoculation sera which gave a positive reaction (greater than 50% blocking of the monoclonal antibody)/total of each sera tested.

3.4. Blocking of monoclonal antibodies by bovine sera in blocking ELISAs

To determine whether bovine antisera recognized the same antigenic domains as the mAbs, 20 BVDV positive bovine sera were tested in blocking ELISAs. As shown in Table 2, 14 (70%) and 20 (100%) of the bovine sera blocked the binding of mAbs 21.5.8 and 1.11.3, respectively, to fragment H, and 17 (85%) blocked the binding of mAb 20.10.6 to fragment G. All eight pre-inoculation negative sera gave negative responses as expected. Fragment I was also tested in the blocking ELISA with mAb 20.10.6. In addition to observed blocking with positive sera, some pre-inoculation negative sera also blocked the mAb (data not shown). This indicated that the avidity of mAb 20.10.6 to fragment I was weak.

3.5. Schematic summary

As shown in Fig. 5, the two antigenic domains are located in the N-terminal helicase domain (the minimal protease domain comprises approximately the first 209 amino acids) (Tautz et al., 2000).

4. Discussion

In this study, we mapped the epitopes of three mAbs to two antigenic domains, both of which were determined to be immunodominant in cattle. All three mAbs recognize highly conserved epitopes as they reacted with 94–100% of BVDV isolates tested ($n = 70$) (Corapi et al., 1990). Two mAbs, 21.5.8 and 1.11.3, recognize epitopes in domain A (aa 205–369), whereas mAb 20.10.6 recognizes an epitope in domain B (aa 368–549). Other mAbs could not be mapped to NS3 fragments possibly because they react to epitopes whose conformation could not be duplicated or because they involve very widely spaced residues. Lack of expression of a full length NS3 protein did not allow investigation of the latter. Antigenicity may also have been better preserved had the fragments been expressed in a eukaryotic system.

The epitopes of mAbs 21.5.8 and 1.11.3 are conformational as determined by their lack of Western blot reactivity. These mAbs mapped to a region containing Walker helicase motifs I (G^{229}AGKT) and II (D^{321}EYH). It is not known whether these mAbs recognize overlapping epitopes as their previous characterization (Corapi et al., 1990) did not involve competitive binding assays. However, mAb 21.5.8 was reactive with more BVDV isolates than 1.11.3 (Corapi et al., 1990) indicating that they do not share an identical epitope. The mAb 21.5.8 was previously found to inhibit helicase activity (Warrener and Collett, 1995) and the mapping of its epitope is consistent with this observation.

The epitope of mAb 20.10.6 was also found to be conformational because of the lack of reactivity in Western blots of fragment I (aa 368–512). In ELISA, fragment I gave similar OD values to those observed for the larger fragment G (aa 368–549). The binding of

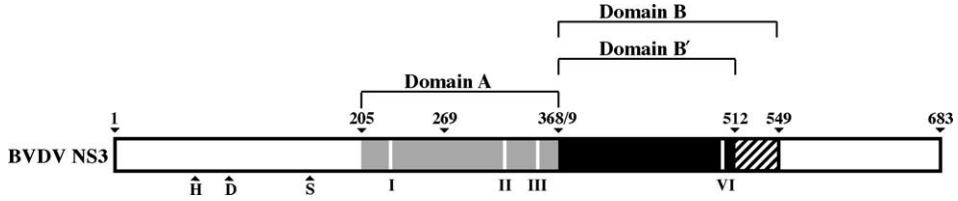

Fig. 5. Location of the antigenic domains of the BVDV NS3 protein mapped in this study. The additional 37 amino acids (513–549) in domain B, not occurring in B′, confers Western blotting reactivity with the mAb 20.10.6. Numbering begins at the first amino acid of the NS3 protein. The positions of the protease catalytic triad of residues H (His), D (Asp), and S (Ser) and the highly conserved helicase motifs I (G^{229}AGKT), II (D^{321}EYH), III (T^{350}ATPA), and VI (Q^{502}RRGRVGR) are indicated.

this mAb to fragment I appears weak, however, since some negative sera blocked its binding in the blocking ELISA. If the epitope of mAb 20.10.6 was linear as suggested by the positive Western blotting results with larger fragments, fragment F (aa 410–683) or fragment I (or both) should have reacted in Western blots, but neither was reactive. Furthermore, these fragments did not contain mis-incorporated sequences. We have previously reported on a mAb reactive to a conformational epitope in Western blot (Deregt et al., 1998a) and conclude that some of these epitopes may re-fold and re-attain conformation during blotting procedures.

To summarize, the region of aa 368–410 contains a critical part of the epitope of mAb 20.10.6 since fragment F lacks the N-terminal sequences of fragment B and was non-reactive. This region is not sufficient in itself for expression of the epitope as shown by the lack of reactivity of fragment J (aa 277–434). Fragment I, representing domain B′ (aa 368–512), contains the essential amino acids for mAb 20.10.6 reactivity in indirect ELISA but its binding is weak as indicated by the blocking ELISA results. Downstream sequences contained in fragment G (specifically within aa 513–549) are apparently required to allow epitope re-folding, and thus Western blot reactivity, and are also required to increase the strength of mAb binding in ELISA, probably by allowing adoption of an optimal epitope conformation. Thus, the epitope of mAb 20.10.6 appears to be more functional/stable in the larger domain (B) than in the smaller domain (B′).

The number of positive bovine sera reacting with the NS3 fragments in the indirect ELISA ranged from only one (12%) for a fragment comprising the first 128 aa of the protease domain to eight (100%). The fragments showing reactivity with the majority of bovine sera in indirect ELISA were those bearing the epitopes of the mAbs (62–100% of bovine sera) and fragment F (aa 410–683) (88%).

Fragment H (aa 205–369), bearing the epitopes of mAbs 21.5.8 and 1.11.3, was reactive with all eight positive bovine sera in indirect ELISA showing immunodominance for this region of the NS3 protein (domain A) in cattle. This small fragment was reactive with two more bovine sera than the larger fragments A (aa 1–434) and D (aa 127–434) containing domain A. The two positive sera which failed to react with the larger fragments were both from calves infected with the type 1b Hastings strain. It is suspected that these calves responded to a different domain A epitope than the other calves and that this epitope was presented sub-optimally on the larger fragments. When fragment H was used in the blocking ELISA, all 20 positive bovine sera blocked the binding of mAb 1.11.3, again demonstrating the immunodominance of domain A. Previously, Kwang et al. (1991) expressed a NS3 fragment of the BVDV NADL strain (aa 127–432) as a fusion protein containing glutathione-*S*-transferase which was immunodominant in cattle. Our results are in agreement with this study and we have shown that the limits of this domain can be further reduced to aa 203–369.

In indirect ELISA, fragments bearing the mAb 20.10.6 epitope were reactive with five (62%) to eight (100%) of the post-inoculation bovine sera, for the smallest fragment I (aa 368–512) to the two largest fragments, B (aa 368–683) and E (aa 368–603), respectively. The slightly larger fragment G (aa 368–549) also demonstrated more reactivity (88%) than fragment I. It is apparent that fragment G contains (an) additional bovine epitope(s) not contained in fragment I. A high percentage (85%) of the BVDV antibody-positive bovine sera blocked mAb 20.10.6 binding to fragment G in the blocking ELISA, further demonstrating that domain B (aa 368–549) is immunodominant in cattle. This immunodominant domain has not been previously described.

Recently, Brown et al. (2002) mapped four mAbs to a large NS3 N-terminally truncated fragment of approximately 500 amino acids beginning at position 189 (corresponding to the Singer NS3 sequence). They identified a critical amino acid at position 269 as part of the epitope of a rare BVDV-1 specific NS3 mAb and found that C-terminal sequences of NS3 were important for expression of their mAb epitopes. The position 269 (as indicated in Fig. 5) occurs in domain A which is recognized by two mAbs in our study. However, in contrast to their study, mAb epitopes in our study were found to occur in two discrete antigenic domains in which C-terminal NS3 sequences played no part.

Both the BVDV and the hepatitis C virus (HCV) NS3 proteins belong to the superfamily II of RNA helicases (Kadaré and Haenni, 1997). The immunodominant region (aa 116–431) of the HCV NS3 occurs

in the N-terminal helicase region (Chien et al., 1999) as does an immunodominant region of the BVDV NS3 (Fig. 5). The physical structure of the BVDV NS3 helicase domain is unknown, however the structure of the HCV NS3 helicase domain has been determined by X-ray crystallography and is ''Y''-shaped and composed of three nearly equal-sized structural domains 1 (left arm), 2 (right arm), and 3 (stem) (Cho et al., 1998; Kim et al., 1998; Yao et al., 1997). Helicase motifs (I and II) occur in domain 1, motif VI is contained in domain 2, and motif III is contained in a loop that connects domains 1 and 2 (Cho et al., 1998). These four helicase motifs are highly conserved between the HCV and BVDV (Kwong et al., 2000) and because of functional and sequence homology (Miller and Purcell, 1990; Ohba et al., 1996), it is possible that the overall structure of the BVDV helicase domain is similar to that of HCV helicase domain. In fact, Brown et al. (2002), produced a homology model of the BVDV NS3 helicase region that resembles the triangular structure of the HCV helicase. Thus, it is possible that antigenic domains A and B are contained in two separate structural domains of the BVDV helicase.

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