The NS5A Protein of Bovine Viral Diarrhea Virus Contains an Essential Zinc-Binding Site Similar to That of the Hepatitis C Virus NS5A Protein

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The recent demonstration that the NS5A protein of hepatitis C virus (HCV) contains an unconventional zinc-binding site with the format Cx¹⁷CxCx²⁰C and the presence of a similar sequence element in the NS5A proteins of members of the *Pestivirus* genus has led to the hypothesis that the NS5A protein of the pestivirus bovine viral diarrhea virus (BVDV) is a zinc-binding protein. A method for the expression and partial purification of BVDV NS5A was developed, and the partially purified protein was analyzed for zinc content by atomic absorption spectroscopy. BVDV NS5A was found to coordinate a single zinc atom per protein molecule. Mutation of any of the four cysteines of the predicted zinc-binding motif eliminated zinc coordination. Furthermore, analysis of mutations at these cysteine residues in the context of a BVDV replicon system indicated that these residues were absolutely essential for RNA replication. The recently determined crystal structure of the N-terminal zinc-binding domain of the HCV NS5A protein, combined with secondary structure predictions of the region surrounding the mapped BVDV zinc-binding region, indicates that the BVDV zinc-binding motif fits the general template Cx²³CxCx²⁴C and likely comprises a three-stranded antiparallel β -sheet fold. These data highlight the similarities between the *Hepacivirus* and *Pestivirus* NS5A proteins and suggest that both proteins perform a not-yet-defined function in RNA replication that requires coordination of a single zinc atom.

The *Pestivirus* genus contains numerous animal pathogens of agricultural importance, including classical swine fever virus, border disease virus, and bovine viral diarrhea virus (BVDV) (52). Pestiviruses are classified in the *Flaviviridae* family, a large family of diverse RNA viruses including, in addition to the pestiviruses, the *Flavivirus* genus, which includes the classical flaviviruses such as yellow fever and dengue viruses and the *Hepacivirus* genus, containing hepatitis C virus (HCV) (33, 52). Pestiviruses are more closely related to HCV than to the classical flaviviruses, and the pestiviruses have been used as a surrogate model for HCV (33). Although recent advances now permit study of the complete life cycle of HCV in cell culture (32, 53, 58), BVDV continues to be of high interest given its ability to cause fatal mucosal disease in cattle and widespread infection in livestock (49). BVDV represents the type virus of the *Pestivirus* genus and therefore is the best-characterized member of this group.

BVDV is an enveloped virus containing a single positive-sense RNA of approximately 12.3 kb (7, 11, 14, 16, 18, 37). This RNA contains a single large open reading frame flanked by highly structured 5' and 3' nontranslated regions that can directly serve as an mRNA in the cytoplasm of an infected cell (7, 8, 11, 14, 17, 37, 39). The 5' nontranslated region contains an internal ribosome entry site that directs translation of the

open reading frame to produce a large polyprotein (7, 8, 11, 14, 17, 37, 39). The viral proteins are organized in the following order in the polyprotein: NH₂-N^{pro}-C-Erns-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH (13, 15). The viral polyprotein is processed both co- and posttranslationally by a combination of viral and cellular protease activities (29, 42, 44, 46, 55–57). Like those of the other members of the family *Flaviviridae*, the BVDV polyprotein is organized with the structural proteins (C, E^{rn}s, E1, and E2) and proteins with putative roles in virion biogenesis (N^{pro}, p7, and NS2) in the N-terminal region and the RNA replication machinery (nonstructural [NS] proteins NS2–NS5B) in the C-terminal region (12, 13, 15). The BVDV replication machinery, or replicase, is a membrane-associated complex of viral and, most likely, cellular proteins (21, 22). The functions of at least some of the viral components of the replicase have been defined. The NS2 protein, in conjunction with the amino terminus of NS3, functions as an autoprotease that cleaves the NS2-NS3 junction of the polyprotein (28, 29). Cleavage of this bond is required for RNA replication and linked to BVDV cytopathogenicity and pathogenesis; cellular protein sequences that modulate cleavage efficiency and subsequent disease states are often inserted in this region of the polyprotein (28, 36, 48; reviewed in reference 49). NS3 is a multifunctional protein with a helicase/nucleoside triphosphatase and a serine protease activity responsible for all downstream polyprotein cleavages (5, 9, 23, 24, 28, 36, 47, 48, 54, 56, 57). The NS4A peptide is tightly associated with NS3 and serves both as a cofactor for the NS3 protease activity and as a membrane tether to localize NS3 to

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membranes (46, 57). On the basis largely of studies with HCV (27), BVDV NS4B is believed to function as an integral membrane scaffold upon which the replicase complex assembles, as well as functioning to reorganize cellular membranes in the infected cell. NS5B is the viral RNA-dependent RNA polymerase (10, 12, 14, 30, 34, 45, 59). The function(s) of NS5A in the BVDV replicase remains to be defined.

The BVDV NS5A protein is a large, hydrophilic phosphoprotein of approximately 56 to 58 kDa. Like HCV NS5A, the protein is likely associated with cellular membranes via an amino-terminal helix that inserts itself into the luminal leaflet of ER-like membranes (6, 20, 38). Recently, the nuclear magnetic resonance structure of the BVDV membrane anchor has been determined, and molecular dynamic simulations suggest that this helix interacts with membranes in a manner similar to that of the HCV NS5A anchor (43). Collectively, these data suggest that the HCV and BVDV proteins serve similar functions in the respective replicases, despite the fact that the proteins have only approximately 15% amino acid identity (HCV strain Con1 compared to BVDV strain NADL). This hypothesis is supported by several observations. First, the NS5A proteins of both viruses are the only replicase components in either system that can be complemented in *trans* (3, 25). Second, the HCV and BVDV NS5A proteins appear to be phosphorylated by the same or a similar cellular kinase(s) (41). Given our previous demonstration that HCV NS5A coordinates a single zinc atom and that this coordination is absolutely required for RNA replication, we decided to investigate if these properties are shared with BVDV NS5A (50).

In this report, we describe the development of a system for the heterologous expression and partial purification of the BVDV NS5A protein. This material was used to determine the zinc-binding properties of NS5A. On the basis of our previous sequence alignments of the HCV and BVDV NS5A proteins and our work with HCV NS5A, we identified four cysteine residues likely to be involved in zinc binding (50). Mutation of any one of these four cysteine residues yields an NS5A protein incapable of coordinating zinc as determined by atomic absorption spectroscopy of the partially purified protein. Furthermore, analysis of these mutant proteins with a BVDV subgenomic replicon indicates that zinc coordination is required for NS5A function. Combined with our previous HCV NS5A work, these data strongly suggest that the NS5A proteins of these two genera have similar atomic structures and likely perform the same or similar functions in viral RNA replication.

MATERIALS AND METHODS

Cloning and in vitro mutagenesis. The EcoRI fragment of the BVDV Jiv⁻ replicon (pACNR/NADL Jiv-S-*pac*) (31), containing the NS5A coding region, was subcloned into the pBluescriptSK⁺ vector to generate the vector pBluescriptSK⁺ EcoRI. This vector was then used as a template for in vitro mutagenesis with the QuikChange mutagenesis kit in accordance with the manufacturer's (Stratagene) instructions. All mutations were confirmed by DNA sequence analysis. Introduced amino acid changes are referred to via the single-letter amino acid code of the parental sequence, the amino acid numbering of BVDV NS5A, and the single-letter amino acid code of the mutation; for example, C34G corresponds to a mutation of the cysteine residue at amino acid 34 of BVDV NS5A to a glycine. Conversion of the BVDV NS5A numbers used in this report to the amino acid numbering scheme of the BVDV strain NADL polyprotein can be accomplished by addition of 2773 to the amino acid positions in this report. Once generated, mutations were cloned back into the BVDV Jiv⁻ replicon (pACNR/NADL Jiv-S-*pac*) and the clones were confirmed by restriction endo-

nuclease mapping and DNA sequence analysis. Clone 5'pac/NADL has been described previously (40). Generation of BVDV protein expression vector pET30ubiBVDV NS5A-Δh was performed by PCR amplification of the DNA fragment of pACNR/NADL Jiv-S-*pac* encoding amino acids 25 to 496 of the mature BVDV NS5A protein with primers that introduced SacII and KpnI restriction enzyme cleavage sites for direct cloning into previously described pET30ubiHCV NS5A-Δh, from which the HCV coding sequence had been removed (50). This expression vector generates an amino-terminal ubiquitin fusion to BVDV NS5A that is removed during expression in bacteria by coexpression of a yeast ubiquitin cleavage activity. The vector also adds a flexible carboxy-terminal linker of 32 amino acids and a hexahistidine tag (contained in the commercial pET30b vector). Mutations of the BVDV NS5A zinc-binding site residues were amplified from the pBluescriptSK⁺ EcoRI vector bearing these mutations and cloned into the expression vector by the same strategy. All expression clones were confirmed by DNA sequence analysis.

In vitro RNA transcription. Plasmid DNAs were linearized with SbfI (New England Biolabs), followed by removal of the 3' overhang with excess deoxynucleoside triphosphates, Klenow, and T4 DNA polymerase and nucleotide removal. RNA transcripts were synthesized at 37°C for 3 h in a 100-μl reaction volume containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 12 mM MgCl₂, 2 mM spermidine, 10 mM dithiothreitol, 3 mM nucleoside triphosphate, 100 U of Superscript (Roche), 100 U of T7 RNA polymerase (Epicenter Technologies), and 5 to 10 μg of linearized DNA. At the end of the reaction, 10 U of DNase I (Roche) was added and reaction mixtures were incubated at 37°C for 20 min to remove the template DNA. DNase-treated RNAs were then purified with an RNA Easy kit (QIAGEN). RNAs were eluted in 50 μl of RNase-free distilled water. The integrity of the purified RNAs was confirmed by agarose gel electrophoresis, and RNA concentrations were determined by spectrophotometric analysis.

Cell culture. Madin-Darby bovine kidney (MDBK) epithelial cells were obtained from the American Type Culture Collection. Cells were used between passages 20 and 60. Cell monolayers were propagated in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated horse serum (HS). Cells were maintained at 37°C with 5% CO₂. Cells were passaged after treatment with 0.05% trypsin–0.02% EDTA.

Electroporation of BVDV replicons and selection. MDBK cells were trypsinized, collected, and washed three times in ice-cold, RNase-free phosphate-buffered saline (PBS). Cells were resuspended in PBS at 2.0×10^7 /ml. One microgram of the in vitro-transcribed RNA was mixed with 0.4 ml of the MDBK cell suspension, transferred to a 2-mm-gap cuvette (BTX), and pulsed with a BTX ElectroSquarePorator (0.99 kV; five pulses; 99-μs pulse duration, 1.1-s intervals). After 10 min at room temperature, the cells were transferred to 3.6 ml of DMEM–10% HS. To measure the efficiency of puromycin-resistant colony formation, the transfected cells were plated at a series of densities (10^6 , 10^5 , and 10^4 cells). To maintain the total number of plated cells at 2×10^6 /100-mm-diameter dish, untransfected cells were used as a feeder layer. After 72 h, the medium was replaced with DMEM–10% HS supplemented with puromycin (5 μg/ml; Gibco Life Technologies). The medium was replaced after another 4 days. After 8 to 10 days, puromycin-resistant colonies were stained with 1% crystal violet in 50% ethanol. Colony counts from triplicate experiments were used to calculate the number of CFU per microgram of input RNA.

Immunofluorescence microscopy analysis. MDBK cells cultured on two-well culture slides (Becton Dickinson, Franklin Lakes, NJ) were washed once in PBS and fixed and permeabilized with an ice-cold methanol-acetate solution for 20 min at room temperature as described previously (1). After being washed three times in PBS, cells were incubated with anti-BVDV NS3 monoclonal antibody 184 (19) diluted 1:500 in PBS and 0.5% goat serum for 1 h at room temperature. Following three additional washes in PBS, an Alexa 594-conjugated goat anti-mouse immunoglobulin G secondary antibody (Molecular Probes, Eugene, Oreg.) diluted 1:1,000 in PBS and 0.5% goat serum was added to the cells and the mixture was incubated for 30 min at 4°C. Hoechst 33342 (1 μg/ml) was added, and cells were incubated for an additional 10 min at 4°C. Cells were washed three times in PBS, and coverslips were mounted. Slides were viewed on a Nikon Eclipse TE 300 microscope.

Blocking of BVDV replication with a polymerase inhibitor. The BVDV polymerase inhibitor VP32947 has been described previously and was kindly provided by Marc Collett (ViroPharma, Exton, PA) (13). Electroporated cells were seeded for immunofluorescence assay (see above), and cells were left untreated or treated with VP32947 at a final concentration of 250 nM at the time of seeding.

BVDV NS5A-Δh expression and purification. Expression and purification of BVDV NS5A-Δh were performed as described previously for HCV NS5A-Δh, with minor modifications (50). Briefly, 1 liter of LB medium supplemented with

FIG. 1. (A) Diagrammatic comparison of HCV and BVDV NS5A proteins. The HCV NS5A protein is divided into three domains based on the presence of two low-complexity sequence blocks (designated LCS I and LCS II, gray shading) predicted to be interdomain connecting loops. The region believed to constitute domain I (amino acids 1 to 213) contains the amino-terminal membrane-anchoring helix (designated anchor, black bar), as well as a zinc ion coordination motif (CPC tripeptide and four cysteines involved in zinc binding are shown). Domain II (amino acids 250 to 342) and domain III (amino acids 356 to 447) constitute the carboxyl-terminal half of HCV NS5A. No domain-mapping information is available for BVDV NS5A. The amino-terminal region of BVDV NS5A contains a putative membrane-anchoring helix (designated Anchor, black bar) and four conserved cysteine residues (C34, C57, C59, and C84) centered around a CPC tripeptide sequence (C57, P58, and C59). The position and spacing of these cysteine residues suggest that this region of BVDV NS5A constitutes a zinc-binding site similar to that characterized for HCV NS5A. The regions trailing this potential metal coordination site are poorly characterized for BVDV. (B) Sequence alignments of *Hepacivirus* and *Pestivirus* NS5A zinc-binding site coding regions. Residues comprising the zinc-binding site previously identified in the HCV NS5A protein are conserved across a diverse range of pestiviruses (gray shading). The numbering of these residues (top of alignment) corresponds to the amino acid sequence of the NS5A protein of the NADL isolate of BVDV. Note the difference in spacing of the outermost conserved cysteine residues between HCV and the pestiviruses and the presence of the central CPC tripeptide sequence. Virus names and respective NCBI protein identifications or GenBank accession numbers: HCV-Con 1, HCV genotype 1b sequence (AJ238799); HCV-H77, HCV infectious clone 1a H77 (AF009606); GBV-B, human GB virus B (9628102); BVDV-NADL, BVDV strain NADL (P19711); BVDV-Oregon, BVDV strain Oregon (AAC40704); BVDV-Osloss, BVDV strain Osloss (AAA02769); BVDV-SD1, BVDV strain SD1 (Q01499); BVDV-CP7, BVDV strain CP7 (Q96662); BVDV-2, BVDV strain 2 (044731); Giraffe, novel pestivirus isolated from a giraffe (620053); Reindeer, novel pestivirus isolated from a reindeer (620051); CSFV-Alfort, classical swine fever virus strain Alfort (AAB50409); CSFV-Brescia, classical swine fever virus strain Brescia (AAA43843); CSFV-Riems, classical swine fever virus strain Riems (AAA86908); CSFV KC/CS, classical swine fever virus vaccine strain KC/CS (AAC98302); BDV-1, border disease virus 1 (620062); BDV-C143 (AAB60887), border disease virus strain C143; BDV-X818, border disease virus strain X818 (AAC16444). (C) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of a representative sample of the partially purified BVDV NS5A (lane BVDV 5A) protein used in the experiments described in this report. The gel was stained with Coomassie brilliant blue R-250. The values on the left of the gel correspond to masses of the molecular size marker (lane MW) in kilodaltons.

30 μ g/liter kanamycin and 25 μ g/liter chloramphenicol was inoculated from an overnight culture of *Escherichia coli* BL21(DE3) containing pET30ubiBVDV NS5A- Δ h and the ubiquitin-specific protease pCG1 plasmids such that the cell optical density at 600 nm was approximately 0.05. Cells were grown at 37°C and

250 rpm until an optical density at 600 nm of 0.6 was reached. Cells were then chilled at 4°C for 30 min, induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and incubated for 5 h at 20°C and 250 rpm. Cells were then collected by centrifugation at 6,000 $\times$ g for 10 min and resuspended in 20 ml of

buffer A (100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 50 mM imidazole, 10 mM 2-mercaptoethanol) per liter of the original culture volume. Cells were lysed by three passes through a cold Avestin air emulsifier at 15,000 lb/in². Following lysis, cell extracts were clarified at 25,000 × *g* for 30 min at 4°C. Clarified extracts were then loaded on a 5-ml bed volume HiTrap IMAC column (Amersham/Pharmacia) charged with nickel and equilibrated with buffer A at a flow rate of 2.5 ml/min. Following extensive washing with buffer A and buffer A supplemented with 1 M NaCl, NS5A-Δh was eluted with a 25-ml linear gradient of 0 to 500 mM imidazole. Fractions containing NS5A-Δh were pooled and exchanged into buffer B (25 mM HEPES [pH 7.4], 25 mM NaCl) with a HiPrep 26/10 desalting column at a flow rate of 8 ml/min (Amersham/Pharmacia). Desalted protein was then concentrated via Amicon ultracentrifugal concentrators (Millipore). The concentration of partially purified NS5A-Δh was determined by the Bio-Rad protein assay (Bio-Rad). Protein yields were typically between 1 and 3 mg/liter of bacterial culture. Mutant proteins were of lower solubility in the initial cell lysates, and therefore more input bacterial lysate was used to produce the mutant proteins in final yields comparable to that of wild-type NS5A-Δh. Purity was estimated to be approximately 80% on the basis of visual analysis of Coomassie R-250 staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A sample of BVDV NS5A prepared by this method is shown in Fig. 1C.

Atomic absorption analysis. Atomic absorption experiments were performed by the Trace Metal Core Facility, Department of Environmental Sciences, Mailman School of Public Health, Columbia University, New York, NY. All experiments were performed according to the standard operating procedures of the core facility. Measurement of the background zinc level of the protein analysis buffer was performed, and this value was subtracted from all values determined for protein samples. All of the values reported are averages of three independent measurements of two independently prepared aliquots of each sample. Zinc values determined were based on comparison with a zinc standard calibration curve. All of the glassware and plasticware used for the preparation of BVDV NS5A-Δh was washed overnight in 50% nitric acid and then washed with copious amounts of ultrapure deionized water. All buffers and reagents were prepared with the highest-quality grade of chemicals commercially available. The concentrations of BVDV NS5A-Δh used for calculation of protein-to-zinc molar ratios were determined by Bio-Rad protein assay.

RESULTS

Identification of an HCV NS5A-like zinc-binding site in BVDV NS5A. In our previous work on HCV NS5A, we attempted to identify the most conserved and, consequently, functionally most important amino acid residues in the NS5A protein. We performed comparative sequence analysis of the NS5A proteins of a number of HCV isolates, as well as the closely related GB viruses and two members of the *Pestivirus* genus (50). This analysis showed that the sequence conservation among these diverse NS5A sequences was limited to approximately 90 amino acid residues proximal to the N terminus. A striking feature of these alignments was the presence of four conserved cysteine residues centered around a cysteine-proline-cysteine tripeptide sequence. The presence of four conserved cysteine residues in close proximity in an intracellular protein is often associated with a structural tetrahedral metal (zinc)-binding site similar to that identified for HCV NS5A. On the basis of the results of this analysis, we proposed that BVDV NS5A might contain a zinc-binding site composed of residues C34, C57, C59, and C84. The relative positions of these residues in the BVDV and HCV NS5A sequences are indicated in Fig. 1A. We extended this analysis to include a number of diverse pestivirus NS5A sequences, all of which contain conserved cysteine residues at these positions fitting the general template Cx²²CxCx²⁴C (Fig. 1B). The four cysteines previously shown to be responsible for zinc coordination in HCV NS5A are shown for comparison. Although direct interaction with zinc was only demonstrated for the Con1 1b isolate of HCV, sequences of the infectious clone 1a H77 strain

and the related virus GB virus B are also shown to highlight the conservation of this sequence element in the hepaciviruses and related viruses. In our previous analyses, the large number of cysteine residues (approximately 14, depending on the HCV isolate) in the HCV NS5A protein required an extensive mapping study to identify residues responsible for metal ion binding. In the case of BVDV NS5A, the case is much simpler, with only six cysteines present in the entire protein, four of which align well with the previously validated HCV NS5A zinc-binding site. As this comparative sequence analysis suggested that the BVDV NS5A protein coordinated a single zinc atom via contacts with four cysteine residues arranged similarly to those of the HCV NS5A protein, we decided to investigate the zinc-binding properties of BVDV NS5A and determine if metal ion coordination is also important for RNA replication in the pestiviruses.

Analysis of the zinc content of BVDV NS5A-Δh and zinc-binding site mapping. A system for the generation of milligram quantities of partially purified BVDV NS5A protein from *E. coli* was generated on the basis of our previous work with HCV NS5A and the work originally described by Huang et al. (26, 50). In this system, BVDV NS5A from the NADL isolate lacking the first 24 amino acids (NS5A-Δh), corresponding to the bulk of the hydrophobic membrane anchor, was produced as an N-terminal ubiquitin fusion with a C-terminal flexible linker and a polyhistidine tag to facilitate purification. Expression of NS5A in bacterial cells harboring an expression vector for a ubiquitin-specific protease produced soluble NS5A-Δh with the N-terminal ubiquitin tag removed in vivo. With this expression system and metal chelate chromatography, 1 to 3 mg of BVDV NS5A-Δh could be produced per liter of bacterial culture at an approximate purity of 80%. With a system to produce BVDV NS5A-Δh in hand, the zinc content of the protein and the residues responsible for zinc coordination could be determined.

By in vitro mutagenesis, the four candidate cysteine residues believed to constitute the BVDV NS5A zinc-binding site (cysteines 34, 57, 59, and 84) were mutated to glycine residues. Each mutation was generated by altering at least two nucleotide changes to limit the potential of same-site reversion of the introduced mutations in future replicon experiments (see below). The cysteine-to-glycine mutations were subcloned into the BVDV NS5A expression system, and the proteins were expressed and partially purified as described for the wild-type BVDV NS5A-Δh protein. Each of the zinc-binding site mutations generated NS5A-Δh that was of lower solubility in the bacterial lysates, suggesting that these point mutations might affect proper protein folding. Consequently, larger volumes of initial cell lysates were required to generate quantities of the mutant proteins sufficient for subsequent analyses.

The availability of relatively large quantities of BVDV NS5A-Δh protein made atomic absorption spectroscopy the method of choice for evaluating NS5A zinc content. The zinc contents of three independent preparations of wild-type NS5A-Δh and NS5A-Δh bearing each of the four cysteine mutations were determined by triplicate measurements of each sample. The zinc content of each sample (495 μg of total protein per measurement) was determined and, following subtraction of background metal contamination from a buffer-only blank, values were converted to a ratio of moles of zinc to

TABLE 1. Zinc contents of BVDV NS5A and zinc-binding site mutants

Protein prepn ^a	Avg zinc content $\pm$ SD ($\mu\text{g/liter}$) ^b	Zinc-to-protein molar ratio
Wild type	659.2 $\pm$ 3.9	0.802
C34	ND ^c	
C57	ND	
C59	ND	
C84	11.8 $\pm$ 2.5	0.014

^a 495 μg of total protein per sample measurement.^b The values shown are averages of six measurements from two preparations of protein.^c ND indicates that the value was below the level of detection.

moles of protein. Typical raw zinc content data were quite consistent, with variations of less than 1.5% between independent preparations of the same protein. As an example, for wild-type BVDV strain NADL NS5A- Δ h, the raw zinc contents of approximately 495 μg of protein were 661, 664, and 664 $\mu\text{g/liter}$ for preparation 1; 654, 658, and 657 $\mu\text{g/liter}$ for preparation 2; and 654, 657, and 664 $\mu\text{g/liter}$ for preparation 3. The ratios of moles of zinc to moles of protein determined for wild-type NS5A- Δ h, NS5A- Δ h C34G, NS5A- Δ h 57G, NS5A- Δ h C59G, and NS5A- Δ h C84G are presented in Table 1. The values shown were calculated from the average zinc content from the nine measurements collected for each protein. The ratio of moles of zinc to moles of protein determined was 0.802 for wild-type NS5A- Δ h, indicating that this protein coordinates one zinc atom per protein molecule. The value measured for this protein, 0.802, was close to the ideal value of 1 mol of zinc per mol of protein, given the purity of the NS5A- Δ h preparations (approximately 80%; Fig. 1C.). The percentage of metal-binding-incompetent protein present in our preparations is unknown, with the potential for improperly folded, aggregated, or otherwise metal-coordinating-incompetent protein potentially present and leading to a reduction in the ratio of moles of zinc to moles of protein. Nonetheless, the value determined is in good agreement with our previous results obtained with HCV NS5A and strongly suggests that BVDV coordinates a single zinc atom. The four mutant NS5A- Δ h proteins, bearing point mutations of the four putative metal-coordinating cysteine residues, had levels of zinc coordination at the level of the background buffer blank (typically undetectable levels, although the C84 mutant had levels of zinc approximately 10 $\mu\text{g/liter}$ above the background), indicating that the observed metal ion coordination of NS5A- Δ h was dependent on cysteine residues 34, 57, 59, and 84. Collectively, these data indicate that BVDV NS5A is a zinc metalloprotein coordinating a single metal ion per protein molecule.

Zinc-binding site mutations abolish BVDV RNA replication.

Our atomic absorption results demonstrated the coordination of a single zinc atom by the BVDV NS5A protein. Furthermore, mutagenesis of each the four cysteine residues of the predicted zinc-binding site residues generated mutant NS5A proteins that were incapable of zinc coordination. Although the data convincingly demonstrated zinc binding, it was not clear if this metal ion coordination was required for the activity of NS5A in BVDV RNA replication. To assess the importance of zinc binding in BVDV RNA replication, mutations of the NS5A zinc coordination residues (cysteines 34, 57, 59, and 84

TABLE 2. Analysis of zinc-binding site mutants in the BVDV replicon system

BVDV replicon ^a	Avg transduction efficiency $\pm$ SD (CFU/ μg) ^b
Wild type	$5.66 \times 10^4 \pm 8.24 \times 10^3$
Mock ^c	0
C34	0
C57	0
C59	0
C84	0

^a All replicons are based on a wild-type pACNR/NADL Jiv-S-*pac* background.^b The values shown are averages of three independent experiments.^c Mock refers to cells not receiving replicon but subjected to puromycin selection.

changed to glycine) were subcloned into noncytopathic BVDV subgenomic replicon pACNR/NADL Jiv-S-*pac*. This replicon was then introduced into MDBK cells, and following drug selection for the expression of a puromycin reporter gene, the replication efficiency of the input RNAs was determined and expressed as the number of puromycin-resistant CFU per microgram of input RNA (Table 2). The parental NADL Jiv-*pac* replicon RNA had a transduction efficiency of 5.66×10^4 CFU/ μg , a value typical for this replicon in MDBK cells. Replicon RNAs bearing mutations of any one of the four cysteines responsible for NS5A zinc-binding activity failed to produce puromycin-resistant colonies. Attempts to select revertant or pseudorevertant RNA replicons restoring replication were not successful (data not shown). Since mutations in noncytopathic BVDV RNA and replicons that render these RNAs cytopathic to cells have been described previously (29), RNA replication of these cysteine mutation bearing RNA replicons was examined in transient assays by immunofluorescent (IF) staining of transfected cells with an anti-NS3 antibody. As shown in Fig. 2, the lack of an IF signal for NS3 indicated that no RNA replication occurred in the mutant RNAs, data that were comparable to the introduction of wild-type RNAs in the presence of a BVDV polymerase inhibitor. A cytopathic BVDV subgenomic replicon (5'*pac*/NADL) that replicates efficiently in MDBK cells and produces a strong NS3 IF signal was used as a positive control in these experiments. These data indicate that the four cysteine residues of the BVDV NS5A zinc-binding site are required for RNA replication.

DISCUSSION

We have extended our previous work on the zinc-binding properties of the HCV NS5A protein to the pestiviruses by demonstrating zinc coordination by BVDV NS5A, mapping the residues responsible, and demonstrating that these interactions are required for the function(s) of BVDV NS5A in RNA replication. Previous speculation about pestivirus NS5A zinc coordination was based solely on sequence alignments with HCV, and these data were complicated by the low sequence similarity between the NS5A proteins of these genera (50). The data presented in this report provide direct evidence for the presence of a common zinc coordination site shared by the NS5A proteins of the *Pestivirus* and *Hepacivirus* genera.

The organizational similarities between the BVDV and HCV zinc-binding sites are quite striking. Both sites are orga-

FIG. 2. Confirmation of lethal phenotypes of BVDV mutants by immunofluorescence microscopy. BVDV replicons constructed in the noncytopathic Jiv backbone have the ability to spontaneously revert to a cytopathic phenotype. To be certain that the zinc-binding site mutants were indeed lethal in BVDV replication (assayed by colony formation under drug selection) and not merely a reversion to a cytopathic replicon, analysis of BVDV replication, or the lack thereof, was monitored by immunofluorescence microscopy of cells transfected with these replicons. Detection of the BVDV NS3 protein (red staining) with mouse anti-NS3 monoclonal antibody 184 (19) and an anti-mouse Alexa 594 conjugate is shown. Nuclei were counterstained for clarity with Hoechst 333421 (blue staining). In all cases, the zinc-

nized around a central CxC tripeptide sequence with amino- and carboxy-distal flanking cysteine residues. Secondary structure predictions of the BVDV NS5A protein in the regions surrounding the zinc-binding site demonstrate the presence of three β strands in an orientation similar to that observed in the HCV NS5A crystal structure. The presence of a similar secondary structure and similar zinc-binding residues suggests that, despite having very low sequence identity (approximately 15%), the NS5A proteins of two flaviviruses from two distinct genera likely have a protein fold in common. The most direct interpretation of these results is that these proteins also have similar functions in the replication of these viruses.

Although the HCV and BVDV NS5A zinc coordination sites appear similar, some differences in the spacing of the cysteine elements of the coordination site exist. The arrangement of these residues in the pestiviruses fits the general template $Cx^{22}Cx^{24}C$, whereas in the hepaciviruses these cysteines are arranged in a $Cx^{17}Cx^{20}C$ pattern. Figure 3A shows the sequences and secondary structure contents of the HCV and BVDV NS5A proteins in the region surrounding the four cysteines involved in metal ion coordination. The HCV sequence is the Con1, genotype 1b isolate, and the secondary structure assignments are from the crystal structure of the amino-terminal domain of this protein (51). The BVDV sequence is from the NADL reference strain, and secondary structures are predictions based on the PSIPRED algorithm (51). The HCV and BVDV sequences have been arranged such that their CPC tripeptide sequences are aligned. The region surrounding the HCV zinc-binding site contains three β strands designated B1, B2, and B3 (51). These elements are arranged in an antiparallel β sheet that forms a scaffold which, together with the amino-terminal 16 amino acids of the domain I structure, orients the four cysteines involved in zinc binding on the same face of the sheet. This can be clearly seen in the portion of the crystal structure of HCV NS5A shown in Fig. 3B (51). The three β strands predicted in this region of BVDV NS5A are arranged similarly to their HCV counterparts, with B1 and B2 flanking the CPC tripeptide and B3 residing in the region between B2 and the most C-terminal cysteine of the zinc-binding site. This arrangement, combined with the relatively fixed zinc-to-cysteine sulfur atom distance of 2.3 Å seen in a variety of crystal structures (2) and the structure of the HCV NS5A protein (51), suggests that the BVDV NS5A zinc-binding site is likely a three-stranded antiparallel β sheet. The four cysteines known to be involved in zinc coordination likely lie on one face of the sheet and, in conjunction with the N-terminal region of the site, constitute the zinc-binding site. A model of the arrangement of these elements relative to the position of the zinc atom in BVDV NS5A is shown in Fig. 3C. The model is very similar to the structure observed in the HCV NS5A protein crystal structure. Figure 3C presents the region

binding site mutations in replicons appeared negative for NS3 expression (C34, C57, C59, and C84). Background NS3, representing translation of the BVDV polyprotein without RNA replication, was similar in the zinc-binding site mutants and BVDV noncytopathic (JIV-) and cytopathic (NADL) replicons cured of active RNA replication by treatment with the polymerase (POL) inhibitor VP32947 (4) (right side).

FIG. 3. Comparison of HCV and BVDV NS5A zinc-binding site structures. (A) Secondary structure content of the region surrounding the zinc-binding sites of HCV and BVDV strain NADL NS5A. Sequences and secondary structures of HCV are based on the crystal structure of the Con1 isolate (Protein Data Bank accession number 1ZH1) (51). BVDV secondary structure assignments were determined by computer prediction with the PSIPRED algorithm (35). β strands are indicated by black arrows; α helices are indicated by black boxes. Strands and helices are numbered according to the nomenclature designated in the HCV NS5A crystal structure. Conserved cysteine residues involved in zinc coordination are shaded gray. (B) Crystal structure of the HCV NS5A zinc-binding site region (51). Amino acids 36 to 85 of the NS5A protein are shown, with residues following amino acid 85 removed for clarity. The three β strands comprising an antiparallel β sheet are indicated (B1, B2, and B3). The small α helix, designated H2, is also shown. The zinc atom (yellow sphere) and conserved cysteine side chains that interact with the metal ion (highlighted in red and numbered) are shown. (C) Model of the zinc-binding region of BVDV NS5A. Theoretical topology of the zinc-binding region of BVDV created by combining the secondary structure predictions in panel A with the HCV NS5A structural information in panel B. The three predicted β strands are shown (numbered B1, B2, and B3) and arranged to generate an antiparallel β sheet similar to that seen in HCV NS5A. The locations of the zinc atom (yellow) and coordinating cysteine residues (C34, C57, C59, and C84) are shown. A putative helix (light gray cylinder, labeled H2) is shown to represent a possible helix in this region of BVDV NS5A.

of BVDV corresponding to the HCV H2 helix as a semitransparent cylinder to highlight the fact that for BVDV NS5A no helix can be predicted for this region and to show that this element lies behind the amino-terminal extension, if it exists. In the HCV structure, the H2 helix seems to contribute little to the zinc-binding site and appears to stabilize the fold in this region of NS5A. Perhaps in BVDV these residues adopt another conformation that has a similar stabilizing effect. Clearly, the presence of a proline residue in this region of BVDV

NS5A suggests that this helix either does not exist in BVDV or is shorter than that observed in HCV. Other differences between the BVDV model and the HCV structure are the length and spacing of the β strands in the zinc-binding sites. BVDV NS5A has β strand 3 (B3), which is predicted to be longer than its HCV counterpart. Additionally, the loop regions between β strands in BVDV are longer than those in HCV (see the B2-to-B3 loop), but these loop regions should be more flexible to variations in size and sequence. What functional consequence these small differences have for these proteins is not clear, but they most likely do not affect zinc coordination. It is important to note that, in the case of BVDV, these are only predictions and may not accurately represent the structures in this region of the protein. Nonetheless, the conservation of several β strands and four cysteine residues between BVDV and HCV NS5A suggests that these regions likely have similar folds, as shown in our BVDV model and the structure of HCV NS5A. What the function of this zinc atom has in replication remains to be determined for both viruses.

In summary, we have presented evidence of the zinc-binding properties of the BVDV NS5A protein on the basis of sequence analysis and atomic absorption spectroscopy of partially purified protein. Additionally, this zinc-binding site has been functionally evaluated and mapped by a combination of the BVDV replicon system and atomic absorption spectroscopy. This zinc-binding site of BVDV appears to be similar to the novel four-cysteine site we previously identified for HCV NS5A. These data, combined with the body of literature for the family *Flaviviridae*, suggests that the NS5A proteins of the *Hepacivirus* and *Pestivirus* genera share important functional similarities despite low sequence conservation and therefore likely perform the same role(s) in RNA replication. Interestingly, the NS5 proteins of the classical flaviviruses do not appear to contain a zinc-binding site like the NS5A proteins of the members of the *Hepacivirus* and *Pestivirus* genera, suggesting that some functional differences in the mechanisms of RNA replication might exist among the members of the family *Flaviviridae*. This observation is particularly intriguing when it is considered that HCV and BVDV are capable of establishing persistent long-term infections whereas the classical flaviviruses lack this ability. The identification of what function or functions the NS5A protein of the pestiviruses and HCV performs still remains a pressing issue, but the identification of common, functionally important properties shared by these proteins may ultimately begin to unravel this enigma.

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