Sequence Specificity in the Interaction of Bluetongue Virus Non-structural Protein 2 (NS2) with Viral RNA*

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The non-structural protein NS2 of Bluetongue virus (BTV) is synthesized abundantly in virus-infected cells and has been suggested to be involved in virus replication. The protein, with a high content of charged residues, possesses a strong affinity for single-stranded RNA species but, to date, all studies have failed to identify any specificity in the NS2-RNA interaction. In this report, we have examined, through RNA binding assays using highly purified NS2, the specificity of interaction with different single-stranded RNA (ssRNA) species in the presence of appropriate competitors. The data obtained show that NS2 indeed has a preference for BTV ssRNA over nonspecific RNA species and that NS2 recognizes a specific region within the BTV10 segment S10. The secondary structure of this region was determined and found to be a hairpin-loop with substructures within the loop. Modification-inhibition experiments highlighted two regions within this structure that were protected from ribonuclease cleavage in the presence of NS2. Overall, these data imply that a function of NS2 may be to recruit virus messenger RNAs (that also act as templates for synthesis of genomic RNAs) selectively from other RNA species within the infected cytosol of the cell during virus replication.

Viruses that have a segmented RNA genome face a challenging task in the recruitment and assortment of specific viral-coded RNA species in the infected cells. It is commonly believed that each of the viral segments must possess some specific sequence or structures, which is recognized by one or more virally encoded proteins to facilitate these processes. Generally, viral RNA-binding proteins are distinguished by being in one of two categories. The first includes proteins that are associated with the nucleocapsid (nucleoproteins) (1) and are involved in the replication process (transcription and packaging) of the viral genome (2, 3). The second includes proteins that play an essential role in recruiting, transporting (4), modifying (5), and translating (6) viral RNA. These proteins can also interact with cellular RNA to suppress the expression of regulatory genes (7), so protecting the viral RNA from cellular recognition mechanisms to use the cellular machinery for virus propagation (6). Members of the Reoviridae have segmented double-stranded RNA genomes enclosed within the double capsids of the virions. During virus entry into the host cells the outer capsid proteins are lost, allowing the viral core to initiate the transcription of genomic RNAs. The newly synthesized single-stranded RNA species are subsequently released into the cytosol and in turn serve both as templates for viral double-stranded RNA genome synthesis and also act as messengers for the synthesis of viral proteins within the cytoplasm (see review, see Ref. 8). However, to date it is not known how the 10–12 RNA segments are specifically recruited and transported to the virus replication and assembly sites within the cytoplasm and if any specific sequence is involved. Bluetongue virus is an orbivirus within the Reoviridae family and has a genome of 10 double-stranded RNA segments, each of which encodes a single major protein. Whereas seven of these proteins (VP1–VP7) are assembled into the viral core and outer capsid, the remaining three or four are non-structural proteins (NS1, NS2, and NS3/NS3A) that are believed to be involved in virus replication, assembly, and morphogenesis. Of these, only NS2 is an RNA-binding protein, all others are structural, the three core proteins (VP1, VP4, and VP6) that form the particle complex and are closely associated with the genomic RNA within the core. Whereas considerable information is available on the structure-function relationship of each of the 7 capsid proteins and their assembly pathway, relatively little is known about the virus replication process and RNA recruitment and packaging.

For a number of animal and plant viruses, replication complexes, transcription complexes, replication and assembly intermediates, as well as nucleocapsids and virions accumulate in specific locations within the host cell in structures described as “virus assembly factories” or “virus inclusion bodies.” Virus inclusion bodies (VIBs)1 are also seen in the cytoplasm of BTV as well as in Reo- and rotavirus-infected cells. Early in infection the VIBs appear as granular material scattered throughout the cell but they later coalesce to form a prominent inclusion with a perinuclear location (9–11).

The 41-kDa NS2 protein, which is synthesized at high levels early in BTV-infected cells, is predominantly associated with VIBs (9–11). When NS2 is expressed by baculovirus vectors, VIBs are formed readily in insect cells. The morphology is

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1 The abbreviations used are: VIB, virus inclusion bodies; BTV, Bluetongue virus; ssRNA, single-stranded RNA; EMSA, electrophoretic mobility shift assay; nt, nucleotide(s); UTR, untranslated region; GFP, green fluorescent protein; NS2, non-structural protein 2.
similar to that found in BTV-infected cells and suggests that neither other viral proteins nor a full complement of viral RNA is necessary for VIB formation (12). VIBs are believed to be the sites at which virus assembly occurs as both viral structural proteins and capsid structures have been shown to localize within VIBs (13). NS2 is the only phosphorylated BTV protein that possesses both NTP binding and nucleotidylphosphohydrolase activity although neither the significance of this modification nor the activity is clear (14–16). The protein is generally hydrophilic, being rich in charged residues, has a strong affinity for ssRNA but not for double-stranded RNA suggesting it may have a role in the recruitment of RNA for replication (17). However, data obtained from previous studies demonstrates that the ssRNA-binding activity of NS2 is nonspecific (15, 18, 19). NS2 contains a number of RNA-binding sites offering the possibility that certain site(s) may have a higher affinity for BTV transcripts than other ssRNA species.

Many steps in the replication cycle of BTV may require specific recognition of viral RNA sequences and such specific recognition must be accomplished by one or more of the proteins encoded by the BTV genome. The activities of NS2 (formation of VIBs, phosphorylation, and RNA-binding) suggest that it might play an important role in the viral replication cycle, particularly with respect to the recruiting and packaging of BTV ssRNA prior to encapsidation. To define the interactions between NS2 and RNA in further detail we have expressed NS2 in the baculovirus expression system and purified it to homogeneity. The purified NS2 was used in electrophoretic mobility shift assays (EMSA) with complete or incomplete fragments of BTV RNAs as well as with non-BTV ssRNAs. The establishment of a defined assay for NS2 competitive RNA binding has allowed us to demonstrate a specificity for BTV RNAs over any other and offers compelling support for its role in the BTV life cycle.

**EXPERIMENTAL PROCEDURES**

**Virus, Cells, and Purification of Recombinant NS2—**Trichoplusia ni cells (Tn5) were grown in suspension at 28 °C in TC100 medium supplemented with 10% fetal calf serum (Invitrogen). Recombinant baculovirus (AcBTV10-NS2) containing the BTV10 NS2 gene was propagated as described previously (12). The recombinant NS2 protein from infected Tn5 cell extracts was purified essentially as described previously (16). The purified NS2 was concentrated using a Centricon-10 (Amicon) centrifugal concentrator with a molecular cut-off of 10 kDa. Aliquots of NS2 were stored either at 4 °C (short term storage) or at −70 °C (long term storage).

**Western Blotting—**After resolution by SDS-PAGE, proteins were transferred to a nitrocellulose membrane by electroblotting and NS2 was identified using a guinea pig anti-NS2 polyclonal antibody by standard procedures (20). Proteins were visualized by chemiluminescence (Amersham Biosciences).

**DNA Templates for in Vitro Transcription—**Templates for in vitro transcription were prepared by PCR from existing full-length cDNA clones of BTV10. The sequence of the T7 promoter was incorporated in the sense primers and restriction sites were placed at the 5′-ends of sense and antisense primers to facilitate cloning of the PCR products where needed. The cDNA plasmid pCW219 containing the 5′ and 3′ noncoding regions of genome segment S10 fused to the hepatitis δ virus ribosome binding sequence was constructed from the full-length cDNA clone pEC-NS3 by digestion with EcoRI and Bsu36I, which removes the coding and 5′ noncoding region of S10 and ligates with oligonucleotides that encode the T7 promoter, the 5′-noncoding region of S10, and a NotI linker. The plasmid pCW219 was then cut with EcoI, blunted with Klenow polymerase, and ligated with the blunted Ncol-EcoRI fragment of pEGFP (Clontech) to generate plasmid pCW353, which contains the epidermal growth factor protein coding sequence flanked by the 5′ and 3′ noncoding regions of the S10 genome segment (21). For the generation of non-BTV transcripts pTrEx4 (Novagen) was linearized with SnaBI and used as a template to generate an in vitro RNA transcript of 850 nt length.

**In Vitro Transcription and RNA Labeling—**Transcripts were produced and labeled using a T7 RiboMAX large scale RNA production system (Promega), with the following modifications to the manufacturer’s instructions. Each transcription reaction was set up to include supplied buffer, 4 mM each of ATP, CTP, GTP, 0.5 mM UTP, 1–2 μg of template DNA, 10 μCi of [α-32P]UTP, and 2 μl supplied enzyme mixture in a 20-μl volume. For preparation of unlabeled transcripts 4 μl each of ATP, CTP, GTP, and UTP were used for each reaction. The reactions were carried out for 3–4 h at 37 °C. Two units of BQI RNase-free DNase were then added and incubated for a further 15 min. Following extraction with phenol/chloroform and purification on a 25-gp spin column (Pharmacia), the transcripts were precipitated with isopropon alcohol in the presence of 0.3 M sodium acetate (pH 5.2). The radiolabeled transcripts were re-suspended in water, typically 50 μl. For measuring nonspecific RNA and RNA activity concentration 1 μl was precipitated with 5% trichloroacetic acid and radioactivity was counted in a scintillation counter. The concentration of unlabeled RNA transcript was measured by optical density (OD) at 260 nm.

**RNA-NS2 Binding and EMSA—**In a typical binding assay (20 μl), 3 μg of NS2 was added to RNA binding buffer (final concentration 2 mM MgCl<sub>2</sub>, 60 mM KCl, 150 mM NaCl, 20 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 unit of RNasin (Promega) and incubated for 10 min at room temperature. Radiolabeled RNAs were heated to 65 °C for 2 min, the mixture of NS2 in binding buffer was added and incubated for 30 min at 4 °C. In competition reactions, unlabeled RNAs were added to the 32P-labeled transcripts prior to the 65 °C heating step. Reactions were terminated by addition of bromphenol blue dye and analyzed on 1% (for long transcripts with >800 nt) or 2% (for shorter transcripts with <300 nt long) agarose gels in TBE (Tris borate-EDTA) (89 mM Tris, pH 7.4, 89 mM boric acid, 2.5 mM EDTA). The gels were dried and analyzed by autoradiography or using a Amersham Biosciences PhosphorImager. Signals were quantified using ImageQuant version 5.0 (Amersham Biosciences) and data were plotted using Sigma Plot 2000 program. In competition binding experiments, the level of competition was assessed from the reduction in the formation of low-mobility radiolabeled RNA-protein complexes.

**Comprehensive Analysis of RNA Structures—**The prediction of the secondary structure of RNA transcripts were performed by using mfold version 3.1 (21) on the mfold RNA server of the Rensselaer Polytechnic Institute.

**RNA Structure Mapping—**RNAs for secondary structure mapping were prepared by in vitro transcription as described above. RNA secondary structure probing followed the protocol described in Ref. 22. In brief, transcripts (10 μg) were 5′-end labeled with γ-32P-ATP using a dephosphorylation-rephosphorylation strategy (23), purified from 6 or 10% acrylamide, 7 μm urea gels, and dissolved in water. All reactions contained 10,000–50,000 cpm of 5′-labeled transcripts and 10 μg of yeast carrier RNA. Enzymatic probing reactions were performed using RNase T<sub>1</sub> and U<sub>0</sub>, whereas for chemical probing, lead acetate and imidazole were used. An RNA size marker was provided by alkaline hydrolysis. Samples were mixed with an equal volume of formamide gel loading buffer, heated at 80 °C, and loaded immediately onto 6, 10, or 17.5% acrylamide, 7 μm urea gels.

**RESULTS**

**NS2 Has Preference for BTV Transcripts Over Nonspecific ssRNA—**BTV NS2 protein, which contains a large number of arginine and lysine residues (16% of total residues) with net charge of −3, has inherent single-stranded RNA binding affinity. To investigate whether the protein has any specificity for BTV sequences, several competitive RNA binding assays were performed using specific <sup>32</sup>P-labeled RNA probes, appropriate competitors, and purified NS2. NS2 was purified to homogeneity from insect cell cultures infected with a recombinant baculovirus expressing NS2 as described under “Experimental Procedures” and confirmed by SDS-PAGE and Western analysis (Fig. 1A). Following preliminary ranging experiments ~75 pmol of NS2 were routinely used for each binding assay. As poly(U) had been shown previously to bind NS2 (19), initial RNA binding studies, used poly(U) (i.e. 220–240 nt) as nonspecific competitor RNA. To provide a suitable probe of similar length, BTV RNA transcripts were synthesized in vitro from a DNA fragment of 232 nt that was generated from the 5′ terminal region (including the short 8 nt of the 5′ terminal sequences) of the DNA clone of BTV RNA segment 4. For each...
RNA binding assay. 0.7 fmol (0.7 × 10^{-9} \text{ mol}) of BTV transcripts were used in the presence of increasing amounts (7 × 10^{-9} to 7 × 10^{-4} \text{ mol}) of poly(U) competitor RNA binding was performed in the presence of competitors representing different subregions of the S10 RNA. Initially full-length S10 transcripts (822 nt) were self-competed to verify the results obtained with segment 9 above were also apparent with segment 10 RNA. As before, effective competition was apparent at a labeled:unlabeled probe ratios of 1:10 (Fig 2A, lane 4) and significant amounts of the complex were lost in the presence of 100-fold excess (1 × 10^{-5} \text{ mol}) of unlabeled RNA transcripts (Fig. 2A, lane 5). Competition patterns for segment 10 RNA and a partial S9 sequence were the same and substantially different than the competition pattern obtained with the two nonspecific sequences used. Both full-length S10 and the S9 fragment competed efficiently when compared with the nonspecific competitors. Thus, sequence specificity was also apparent for segment 10 RNA.

The 5' and 3' terminal sequences of 10 segments of BTV genome, like other segmented RNA genome viruses, are highly conserved and could possess the common recognition signals for binding NS2. Moreover, the two terminal sequences of each mRNA are complimentary and have the potential to form partial duplex (or panhandle) structures (24). The non-coding regions of S10 RNA, in particular, have the potential to form a strong secondary structure as it has particularly long untranslated regions (UTRs) in comparison with other BTV transcripts. To investigate if the specific recognition sequences for NS2 binding are localized within the non-coding regions of BTV RNA, a DNA fragment of 125 nt that includes only the 5' and 3' UTR of the S10 genome segment was constructed and used as template for in vitro transcription. When the labeled full-length S10 RNA transcripts (1 × 10^{-8} \text{ mol}) were incubated with NS2 in the presence of increasing amounts (1 × 10^{-8} to 1 × 10^{-10} \text{ mol}) of unlabeled UTR transcripts and the products analyzed by EMSA, competition was substantially different when compared with full-length S10 or its coding region (Fig. 2C).

**NS2 Recognizes Particular Regions within BTV Transcripts, but Not the Non-coding Sequences**—To investigate further the sequence specificity of the NS2-BTV RNA interaction, full-length S10 RNA transcripts (822 nt) were used as labeled probe and RNA binding was performed in the presence of competitors arabidopsis thaliana.

**NS2 and competitor in 10-fold increments (probe:competitor, 1:10 to 1:10,000).** When RNA-protein complexes were analyzed in EMSA as described under “Experimental Procedures,” poly(U) competed with BTV RNA binding only when present at vast excess (Fig. 1B, lane 8). No competition was apparent at up to 100-fold molar excess of poly(U) over BTV RNA (Fig. 1B, lane 4). Some competition (−30%) was apparent at a poly(U):BTV ratio of 10,000 (Fig. 1B, lane 6) but complete prevention of NS2 binding to BTV RNA was only shown at a poly(U):BTV ratio of >100,000, (Fig. 1B, lane 7). To confirm that poly(U) was acting as a typical nonspecific RNA competitor, an alternate non-homopolymeric and non-viral sequence was also used as competitor for the BTV probe as above. A nonspecific competitor RNA transcript of 850 nt length (generated from linearized pTriEx4), equivalent to the length of the S10 radiolabeled probe (822 nt) began to compete with the specific BTV probe at a 1:10,000 molar excess (Fig. 1C, lane 6), similar to the competition assay in which poly(U) was used as a nonspecific competitor (Fig. 1B, lanes 6). To further investigate the NS2 specificity for BTV sequences over the nonspecific sequences, a second set of competitive RNA binding assays were performed with specific competitor in which both the probe and the competitor were derived from BTV RNA. For this, both the probe and the competitor ssRNA were generated from the 5' terminal fragment of RNA segment 9, a 273-nt fragment similar in length to the poly(U). In contrast to competition with nonspecific competitors (panels B and C), NS2 binding to the labeled BTV probe RNA was effective at much lower unlabeled:labeled molar ratios of RNA transcripts. Competition was apparent by EMSA at only 100-fold excess (cf. >10,000-fold for poly(U), and pTriEx4 transcripts) of the unlabeled RNA competitor confirming specificity in the binding BTV RNA by NS2 (Fig. 1D, lane 4). When the radioactivity associated with the low-mobility complexes in panels B–D was quantitated by phosphorimaging and compared, a difference between viral and non-viral transcripts in their ability to compete with labeled viral RNA was apparent. For example, in the presence of 100-fold excess of specific competitor there was ~50% reduction in the formation of radiolabeled complex (Fig. 1D, lane 4), while at the same molar ratio of the nonspecific competitor only ~25% reduction was achieved (Fig. 1C, lane 4).

Differences between specific and nonspecific competitor were enhanced at higher concentrations of competitor. At 1,000 molar excess the nonspecific competitor retained 65–70% of the low-mobility RNA-NS2 complex, whereas the same concentration of specific competitor essentially prevented labeled complex formation (~1% signal). Similar differences were apparent when poly(U) was used as nonspecific competitor (Fig. 1B, lanes 4 and 5). These results provide convincing evidence that NS2 is able to distinguish between viral and non-viral RNA sequences.
Quantitation of radiolabeled RNA-NS2 complexes showed only a 40% reduction in the presence of 10,000 molar excess of the 5’ and 3’ UTR competitor transcripts similar to that obtained by a nonspecific poly(U) competitor.

As it was possible that the transcripts generated from only UTR constructs might not be able to form the correct secondary structure to be recognized by NS2 because of lack of an intervening sequence, a construct consisting of nonspecific sequences from the coding region of the GFP protein flanked by the 5’ and 3’ UTRs of the S10 genome segment was also prepared. The complete UTR-GFP transcript comprises 846 nt, equivalent to the size of full-length S10 transcripts. As for the non-coding region alone, the insertion of the GFP sequence between the two termini of S10 RNA also failed to show effective competition on NS2 binding (data not shown). These data show that the terminal non-coding regions of S10 do not possess the specific recognition signal for NS2 and suggest that the specificity of interaction resides within the coding region of BTV transcripts. To confirm this, an additional competition assay was undertaken using BTV transcripts, which represent only the coding region (total length of 697 nt) of the S10 genome segment as competitor. The coding region transcripts were found to compete efficiently with full-length labeled transcripts for NS2 binding (Fig. 2B), similar to the efficiency evident with full-length unlabeled transcripts (cf. Fig. 2A). These data confirm that a specific region within the coding sequences of BTV RNAs possesses a recognition signal for NS2.

Identification of Specific Sequences of BTV Transcripts Recognized by NS2—To identify the NS2 binding recognition signal within BTV RNAs, transcripts representing various regions of the coding sequences of the BTV RNA segment were generated (Fig. 3). Initial data showed that the first half of the coding region, representing 20–392 nt of the S10, was as effective a competitor for the full-length labeled S10 transcripts for binding to NS2 (Fig. 4A) as transcripts of the complete S10 coding region (i.e. 697 nt transcripts, see Fig. 2B). This data localized the NS2 binding signal to the first 372 nt of the coding region of S10 segment.

To further map the precise binding region within the 372-nt fragment of S10 RNA, initially 2 different DNA fragments, 20–206(a) and 113–299(b), were generated (see Fig. 3) and specific transcripts were produced from each DNA template. Each type of transcript was the same length, 186 nt, and when each was tested for their ability to compete with full-length labeled transcripts, “a” transcripts provided effective competition, whereas “b” transcripts failed to compete as efficiently (Fig. 4, B and C). To confirm the specificity of the transcripts further competition assays were undertaken in which radiolabeled a or b transcripts were competed for NS2 by excess amounts of unlabeled b or a transcripts. When the radioactivity bound to NS2 was measured, b transcripts failed to displace a transcripts as efficiently as a competitor versus a b probe. Even at 10,000-fold molar excess of b competitor there was only 58% reduction in the formation of RNA-NS2 complexes. However, when a transcripts were used as competitors for the b probe in the same concentration as above, the results showed a 92% reduction in radioactive complex formation. Similarly at 100-fold molar excess the a competitor reduced the radiolabeled b RNA-NS2 complex by 67%, whereas b competitor reduced it by only 33% (Fig. 4D). Therefore the recognition signal for NS2 binding must lie within nucleotides 20 and 206 of the S10 RNA. The finer mapping of the NS2 binding region within this region was undertaken subsequently. The a fragment of 186 nt (20–206 nt) of the S10 segment was subdivided into four regions (a1, 20–100 nt, a2, 79–151 nt, a3, 99–170 nt, and a4, 160–199 nt, respectively) and in vitro transcripts were tested for relative competitiveness by EMSA. The competition efficiency was assayed by their relative ability to displace the RNA-protein complexes as described above. This analysis showed that only a3 (99–170 nt) transcripts effectively competed for the formation of S10 RNA-NS2 complexes (Fig. 5C). The displacement of the lower mobility complexes started to occur at 1:100 molar excess of the cold a3 competitor (Fig. 5C, lane 5), consistent with previous results using a specific competitor. All three other transcripts exhibited nonspecific binding with NS2 in the absence of unlabeled RNA (Fig. 5, A, B, and D). Secondary structure analysis of these four RNA fragments showed that a3, but not the other 3 RNAs, could adopt the predicted secondary structure (Fig. 6C). Because the first 372 nt, a and a3, subgenomic transcripts could compete with the full-length transcripts for NS2 it was possible that there may be a particular structure(s) within these subgenomic transcripts that has affinity for NS2. To obtain some evidence for NS2 binding to particular secondary structures within BTV transcripts, potential secondary structures of these ssRNA species of S10 RNA were generated using mfold (22–25). This revealed that fragments a and a3 could potentially form struc-
and lane 2, excess of the competitor, the percentage of bound B in 10-fold increments. – of the competitor (molar ratio of competitor:labeled probe 1:1 open circles).

C

Secondary structure (Fig. 6 competitive subfragment of S10, contained the predicted secondary is shown in Fig. 7. Nucleotide numbering is as for the nter with a stem, a loop at the upper part of the stem, and two smaller loops forming beyond the main loop (Fig. 6, B and C), whereas fragments a1, a2, and a4 could not form this structure (data not shown). A similar structure was also present both in the predicted secondary structure of the first 372 nucleotides of S10 (Fig. 6A). Secondary structure analysis of a1, a2, a3, and a4 was consistent with this hypothesis as only a3 RNA, the competitive subfragment of S10, contained the predicted secondary structure (Fig. 6C).

RNA Structure Mapping of Fragments 1–206 and a3—Whether indeed the predicted RNA secondary structure exists within the S10 structure binding region was further verified experimentally. Two different RNAs, fragments 1–206 and a3 (206 and 71 nt long, respectively), were end-labeled and subjected to chemical and enzymatic structure probing (26). For the enzymatic structure probing, the single-stranded specific RNase T1 was used, which cleaves at the 3' of unpaired G residues, and RNase U2, which cleaves at the 3' of unpaired A or G with a preference for A's. For the chemical probing we used imidazole (27) and lead acetate (28), which are both single stranded-specific. Reactions were analyzed on denaturing gels of various acrylamide concentrations to maximize the information from each probing reaction. Sites of cleavage were scored only from those reactions where 80–90% of the probes remained intact. A selection of the biochemical analyses and a diagrammatic summary is shown in Fig. 7. Nucleotide numbering is as for the BTV S10 genome segment.

Fig. 4. Competition binding experiment with fragments 20–391, a and b of S10. Reactions were performed as described previously. A–C, full-length S10 as radiolabeled probe. A, S10 nt 30–391 as competitor; B, S10 a fragment (nt 20–206) as competitor; C, S10 b fragment (nt 113–298) as competitor. For all panels lane 1 is labeled probe only and lane 2, 32P-labeled probe and NS2, A, lanes 3–7 increasing amounts of the competitor (molar ratio of competitor:labeled probe 1:1–10,000:1) in 10-fold increments. B and C, lane 3, molar ratio of competitor:labeled transcript, 10:1; lane 4, 100:1; lane 5, 300:1; lane 6, 600:1; lane 7, 1,000:1; and lane 8, 10,000:1. D, unlabeled fragment was used to compete with labeled a fragment, and vice versa. The percentage of a fragment probe bound to NS2 in increasing concentrations of competitor b is indicated by solid circles. At 1 \times 10^{-7} M (100-fold molar excess) the percentage of bound a fragment is 67.08%, at 5 \times 10^{-7} M (500-fold) is 47.75%, at 1 \times 10^{-6} M (1,000-fold) is 47.24%, and at 1 \times 10^{-5} M (10,000-fold) is 42.28%. The percentage of b fragment probe bound to NS2 is indicated by the open circles. At 1 \times 10^{-7} M (100-fold molar excess) the percentage of bound b fragment is 33.24%, at 500-fold molar excess of the competitor, the percentage of bound b fragment is 26.54%, at 1,000-fold excess 11.51% and at the maximum concentration of the competitor RNA a (10,000-fold excess) is 7.87%.

The data from the structure probing of fragments 1–206 and a3 clearly indicate that nucleotides 100–112 and 157–168 form a stem region, which we refer to as the main stem. This was most evident in the chemical probing reactions with imidazole and lead acetate. Structure probing with either reagent generated a similar pattern of bands, which were in effect footprints highlighting the major regions of single- and double-stranded RNA. Although resistant to chemical reagents, the main stem was reactive to some extent to RNases T1 and U2, probably indicating occasional breathing by this stem. This was more evident in the a3 fragment, which lacks the flanking sequences of 1–206 and may be more likely to breath. In either RNA, the loop bounded by the main stem, corresponding to nucleotides 113–156, gave predominantly single-stranded reactivities. Thus the RNA structure present in the S10 mRNA that is specifically recognized by NS2 is a hairpin-loop. Other reactivities were present, however, which suggest that additional substructures are present within the loop region. We propose that the loop contains two additional small stem-loops (Fig. 7C, "ears" 1 and 2). This proposal is based on the reduced accessibility of nucleotides 123–127, 131–134, 136–138, and 146–149 to imidazole and lead cleavage and reduced cleavage by RNases in this region. The remainder of the main loop remained highly accessible to the chemical probes and had, overall, more evident RNase cleavages. The experimentally determined structure is therefore very similar to that predicted by mfold. The main difference is that the region of base pairing predicted by mfold between residues 115–118 and 151–154 is not present; these residues are clearly single-stranded (Fig. 7, panels A and C).

To investigate further interactions between NS2 and the hairpin-loop, the a3 transcript was subjected to similar structure probing, but in the presence of increasing concentrations...
of purified NS2 (Fig. 7B). With the enzymatic probes, a number of bases within the structure were found to have reduced reactivity in the presence of NS2. These were clustered in two areas, the main loop (G151, A152) and the 5' arm of the main stem (G104, G106, G110). With the chemical probes, the presence of NS2 had little impact on the cleavage pattern (data not shown). Although the presence of NS2 protected certain residues, we did not see any gross rearrangements of the RNA, nor large stretches of protected bases, at least with this methodology.

**DISCUSSION**

Our studies demonstrate for the first time that BTV protein NS2 previously shown to bind any ssRNA species has specificity for BTV RNA sequences. Previous studies on the RNA-binding activity of NS2 failed to identify sequence or structural specificity for NS2, (12, 15, 19, 29–31), although Theron and Nel (32) indicated that NS2 may have a weak preference for the 3' terminal half of the S8 RNA segment (621–1124 nt). Recently, NS2 was shown to have three RNA-binding domains each with a different affinity for ssRNA but with only nonspecific ssRNA-binding activity (15, 33). These results were obtained by using radiolabeled non-BTV probes, transcripts of the rotavirus S8 gene, or luciferase. Furthermore, the recombinant NS2 used was expressed in *E. coli*, which, as the natural hosts of BTV are eukaryotic (e.g. gnat and animals), may lack significant post-translational modifications. For example, although the role of the phosphorylation of NS2 in RNA binding is unclear, NS2 expressed in *E. coli* would not be phosphorylated and may have a compromised activity.

We used purified recombinant NS2 expressed by a eukaryotic expression system to allow the appropriate modification of the expressed protein such as phosphorylation (12), and the optimum concentration of purified NS2 necessary for ssRNA-NS2 complex formation was assessed prior to an investigation of specificity of the RNA-binding activity. Based on preliminary studies ~75 pmol of NS2 was used for RNA binding experiments but, as NS2 forms multimers in solution, the active binding molecule for BTV RNA cannot be deduced. Similarly, the functional proportion of the purified protein is unclear making specific activity measurements impossible. Nevertheless, the assay allowed the relative binding efficiency of various RNA substrates to be assessed.

Assays of purified NS2 in combination with BTV-derived ssRNA probes and various BTV-specific ssRNA competitors, as well as non-BTV ssRNA competitors, were used to determine the NS2 specificity for BTV sequences versus non-BTV sequences. Using gel retardation analysis, we measured the relative ability of a competitor to compete with BTV RNA and demonstrated that NS2 has a higher affinity for BTV RNA than for nonspecific ssRNA. The data implied that there are particular site(s) within NS2 solely responsible for binding BTV RNA. Prior studies have indicated that NS2 is likely to have multiple RNA-binding domains (33, 34) consistent with our findings that NS2 possesses both specific and nonspecific ssRNA binding activity.

In other segmented RNA genome viruses such as influenza virus, replication and packaging are mediated by cis-acting signals, which are located at the 3' and 5' ends of the viral segments. The 3' and 5' terminal non-coding regions of each RNA segment have short highly conserved sequences that form partial duplex structure (the panhandle stem-loop structure) and longer non-conserved sequences. The packaging signals in all RNA segments are identical and include the terminal stem-loop structure (35, 36). However, reverse genetics of influenza A has recently shown that sequences within the coding region of the neuraminidase vRNA possess a signal that drives incorporation of this segment into virions (37). This salient study, in contrast to previous reports, suggests that unique sequences within the coding regions of individual vRNA segments are responsible for the selective mechanism of vRNA recruitment into virions. Unlike influenza virus, in members of Reoviridae, neither the RNA packaging mechanism nor the role of specific signals is clearly understood. However, recent study has provided some understanding of the RNA packaging and assembly mechanism for a much simpler virus, bacteriophage φ6, which has a genome of only 3 double-stranded RNA segments (38–40). The packaging signals of φ6 RNA segments are located within the 5' non-coding regions and although packaging of all segments share similar elements, each folds into a different secondary structure (40).

Analogous to the RNA genome segments of influenza virus, all RNA segments of BTV and other orbiviruses have short conserved 5' and 3' termini that are shared by each of the RNA segments. In addition, each non-coding sequence also includes non-conserved sequences of variable lengths. The results from our studies demonstrate that neither the short conserved non-coding sequences, the potential panhandle structures (formed by the partial complimentary sequences of the 5' and 3' termini), nor the variable non-coding sequences had any specificity for NS2 binding. In contrast, sequences within the coding region of each RNA segment were responsible for a specific interaction between NS2 and BTV RNA. Furthermore, the data also suggest the likelihood that the secondary structure rather than primary sequence are critical for the recognition of BTV RNA by NS2.

Chemical and enzymatic structure probing confirmed that the NS2-binding region of the S10 RNA transcript folds into a...
**FIG. 7. Structure probing of RNAs 1–206 and α3.** Base numbers correspond to those of the S10 mRNA. Products were analyzed on denaturing acrylamide, 7 M urea gels and data were collected from 6, 10, and 17.5% gels. Enzyme units per reaction (U2, T1), hours of reaction (I, imidazole), and millimolar concentration in reaction (Pb, lead) are shown. OH-, alkaline hydrolysis ladder; R, RNA only. In panel A, the gels shown are 6% (RNA 1–206) and 10% (RNA α3) gels.
secondary structure very similar to that predicted by mfold. This hairpin-loop structure possesses two minor stem-loops (ears) within the main loop region and binds NS2 in two regions, the main stem and part of the main loop. The identification of protected regions was only possible using ribonucleases as smaller chemical probes, which were expected to offer a more refined footprint, gave no protection. This may be the consequence of inactivation of NS2 activity by the chemical probes or that the bound NS2 did not restrict access of the chemicals to the bases. It will be of interest to probe the NS2-hairpin interaction using hairpin variants with modified primary and secondary structure. Similarly, it will be important to determine whether the protection afforded by bound NS2 is a consequence of the binding of a single NS2 molecule, with two contact sites, or multiple NS2 proteins.

A key question is whether the RNA motif recognized by NS2 in S10 is present in all other BTV segments, because this would represent a common element for the recruitment of viral RNAs. Using computational analysis, very similar structures can be identified in all BTV RNA segments (data not shown). Interestingly, these structures are found within the coding regions of the different BTV segments, although at different sites. It will be possible to confirm that these motifs bind NS2 specifically using the methodologies developed in the present work.

A number of RNA-binding proteins recognize specifically the structure of the RNA and include the Gag polyproteins of the spleen necrosis virus and Murine leukemia virus. These proteins recognize packaging signals that are contained in hairpin structures with no apparent sequence homology (41). Also, the RNA-dependent RNA polymerase of influenza A virus exhibits structural specificity for an internal RNA loop of the viral promoter (42). However, RNA-binding proteins of none of the other members of Reoviridae family, such as reovirus or rotavirus have been shown to have RNA structural specificity, although rotavirus NSP3 appears to bind a linear sequence found at the 3′ end of all rotavirus RNA segments (43).

It is unclear how the packaging of the segmented RNA genome of members of the Reoviridae is achieved. That the fact that mutants in the rotavirus NSP2 protein (ssRNA-binding protein and a major component of IBs) produces virions that are “empty” of viral RNA at non-permissive temperatures (44) suggests that non-structural proteins may play a role in this process. It is tempting to speculate that NS2 may be responsible for the sequestration and assortment of viral ssRNA molecules that are subsequently packaged by a separate viral protein, or protein complex, e.g. the polymerase complex. Recent data on influenza packaging showing that packaging occurs by a sequence in the coding region of influenza genes supports the mechanism we hypothesize for BTV.

Our model would also account for the nonspecific substrate specificity shown by the polymerase protein of ds6 (45) as template selectivity would have been achieved by selective encapsidation prior to polymerase action. Our recent emerging data suggests a similar lack of sequence specificity by the BTV polymerase protein VP1.2 In BTV-infected cells, NS2 occurs in VIBs, which are the sites of viral assembly where viral proteins and capsids accumulate. We speculate that VIBs are formed by the multimerization of NS2 and that BTV ssRNA is specifically recruited to such assemblies to interact with VP1, VP4, and VP6, the transcriptase complex, and subsequently be encapsulated by a single shell of VP3 to form the initial core of the assembling virus. Second-strand synthesis to form the mature genome would then occur within the cores. NS2 binding to ssRNA and multimerization into a VIB may make an RNA molecule unavailable for translation. It would therefore act as a control mechanism determining whether an RNA molecule is translated or packaged. VIBs are also formed in insect cells by baculovirus-expressed NS2 suggesting that neither BTV RNA nor any other viral proteins are necessary for multimerization of NS2. The effects of the presence of BTV RNA on the assembly efficiency or stability of VIBs are not known.

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1–206) and 17.5% (RNAo3). In panel B, RNA o3 was subjected to structure probing in the absence (0) or presence of increasing amounts of purified NS2 protein (0.1, 0.2, and 1 μg, indicated by a triangle). Here, RNases T1 and U2 were used at 0.1 and 0.2 units, respectively, per reaction. Panel C, summary of the probing results for RNA o3. The sensitivity of bases to the probes is indicated by a bold arrow, a light arrow, and an asterisk (RNase T1), or a slender arrow (imidazole). The lead acetate probing data, similar to that seen with imidazole, is omitted for clarity. Bases protected from RNase cleavage in the presence of NS2 are bold and italicized. RNA 1–206 probing results are not summarized; they were similar to those of RNA o3 in the appropriate region (see text).
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Sequence Specificity in the Interaction of Bluetongue Virus Non-structural Protein 2 (NS2) with Viral RNA
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