RNA Binding Domain of Jamestown Canyon Virus S Segment RNAs

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Jamestown Canyon virus (JCV) is a member of the Bunyaviridae family, Orthobunyavirus genus, California serogroup. Replication and, ultimately, assembly and packaging rely on the process of encapsidation. Therefore, the ability of viral RNAs (vRNAs) (genomic and antigenomic) to interact with the nucleocapsid protein (N protein) and the location of this binding domain on the RNAs are of interest. The questions to be addressed are the following. Where is the binding domain located on both the vRNA and cRNA strands, is this RNA bound when double or single stranded, and does this identified region have the ability to transform the binding potential of nonviral RNA? Full-length viral and complementary S segment RNA, as well as 3′ deletion mutants of both vRNA and cRNA, nonviral RNA, and hybrid viral/nonviral RNA, were analyzed for their ability to interact with bacterially expressed JCV N protein. RNA-nucleocapsid interactions were examined by UV cross-linking, filter binding assays, and the generation of hybrid RNA to help define the area responsible for RNA-protein binding. The assays identified the region responsible for binding to the nucleocapsid as being contained within the 5′ half of both the genomic and antigenomic RNAs. This region, if placed within nonviral RNA, is capable of altering the binding potential of nonviral RNA to levels seen with wild-type vRNAs.

Jamestown Canyon virus (JCV) is a member of the Bunyaviridae family, which is composed of five genera. JCV is classified in the Orthobunyavirus genus within the California serogroup. These viruses are arthropod borne and cause a neurotropic disease in humans (10, 11, 25). The genome arrangement of JCV is composed of a single-stranded, negative-sense tripartite RNA. The three segments encode for an RNA-dependent RNA polymerase (RDRP), glycoproteins Gγ and Gε, which are cleaved posttranslationally, a nonstructural M protein, nucleocapsid protein (N protein), and the nonstructural S protein (14).

Like other negative-strand RNA viruses, replication of JCV takes place in ribonucleoprotein (RNP) complexes composed of the N protein, viral RNA (vRNA) (either genomic or antigenomic sense), and the RDRP. These RNP complexes have been observed to have a circular appearance (2, 21–23, 26, 27), because the 5′ and 3′ ends of both the genomic and antigenomic RNA contain conserved complementary terminal sequences (8, 19, 28). Only genomic and antigenomic full-length RNAs are thought to be found within the RNP complex (7, 14). The mRNAs are not encapsidated, because they are truncated at the 3′ end and contain a cap derived from host cell RNAs at the 5′ end (7).

It is hypothesized that the terminal sequences on both the genomic and antigenomic RNA are necessary for encapsidation. Nucleotide additions at the 3′ or 5′ end of the Bunyamwera genome do not affect transcription; however, deletion of as little as five nucleotides from the 3′ terminus or a change in the ability to base pair at position 12 resulted in a cessation of transcription (6). These results indicate that the termini, specifically the 5′ region, contain the location of the encapsidation signal and possibly a region responsible for RDRP recognition.

Conflicting data have been reported for several members of the Bunyaviridae family regarding the location and RNA species responsible for encapsidation. Original reports regarding the Hantavirus genus described a binding preference for double-stranded RNA but with no real specificity (9). The next set of results for the Hantavirus genus suggested that while no specific sequence was identified, full-length vRNA was identified as a preferable target over RNA containing only internal RNA sequences (29). Further analysis was completed that indicated that nucleotides 1 to 39 were all that were necessary for RNA binding to occur (30). However, more recent data suggest that the panhandle conformation resulting from the base pairing of the 5′ and 3′ complementary ends is the RNA binding domain for the Sin Nombre virus (16–18). Investigations using the nucleocapsid and RNA of Bunyamwera virus, the one most closely related to JCV of all the viruses mentioned, have shown that the first 32 nucleotides at the 5′ terminus contain the RNA binding domain for vRNA. This region is thought to contain two stem-loop structures that may be mediating the specificity of viral binding (20). There is an obvious need for specific individual virus studies due to the dissimilarities found throughout this viral family.

The study reported here examines both the vRNA and cRNA of JCV S segment RNA, the specificity of RNA-nucleocapsid interaction, the location of the RNA binding region, and whether that region of the virus genome is capable of restoring RNA binding to nonviral RNA. The results coincide with those obtained with Bunyamwera virus, in that the 5′ end contains the RNA binding region but not necessarily within the first 32 nucleotides, and that this region on the cRNA also is important for binding. The 3′ end of the genome, both viral and complementary, is not involved in RNA binding. Trans-
planting 5'–end viral sequences into a nonviral background restores binding of that RNA to nearly wild-type levels.

**MATERIALS AND METHODS**

**JCV N protein purification.** The 5' segment open reading frame that encodes the JCV N protein was cloned by reverse transcription-PCR with 5' and 3' primers engineered with NeoII and XhoI (restriction sites are underlined below). The 5' forward primer was 5'-TTGCCATGGGGATGTTGTTT, and the 3' reverse primer was 5'-TTTGCGATTTGCGACTCTTTAC. The amplified fragment then was cloned into the plasmid pQE-System vector using the NeoII and XhoI sites to create the expression plasmid pQE-JCVN. The resulting expression plasmid was sequenced to ensure that there were no insertions or deletions so that the proper reading frame was maintained. Cloning into this vector created an additional 10 amino acids at the carboxyl-terminal end, including eight histidines. This cloning created a fusion protein with a predicted mass of 27 kDa. Protein expression was induced by growing a small overnight culture (100 mL), transferring it to a 1-L culture, and allowing it to grow at 29°C until the optical density at 600 nm reached 0.8. The protein was purified under native conditions using nickel-nitrioltriacetic acid column chromatography (Qiagen). The lysate, wash, and elution solutions each contained 0.5% NP-40, 10% glycerol, and the protein was eluted from the column with 100 mM imidazole (Sigma). Fractions containing purified JCV N protein were pooled and dialyzed against a buffer containing 40 mM HEPES, pH 7.4, 300 mM NaCl, and 10% glycerol. Protein concentrations were determined by using the Micro BCA protein reagent kit (Pierce). Protein was stored at –80°C.

**Preparation of RNA substrates.** The entire 5' segment of JCV N protein, both vRNA and cRNA, was amplified by reverse transcription-PCR with 5' and 3' primers engineered with a flanking AatII site and T7 promoter sequence and a BspEI restriction site. The JCVs 5' forward primer was 5'-CCGACGCTTATACGACTCTAAATTTAGTGTGCTCCACTG, and the JCVs 3' reverse primer was 5'-GCTCCGATCTAAGCTAGTACTGACTCCACTTGAGAT. The JCVc5 forward primer was 5'-ATGACGCTATACGACTCTAAATTTAGTGTGCTCCACTG, and the JCVc3 reverse primer was 5'-CCCGGA CGCTAAGCTAGTACTGACTCCACTTGAGAT. The amplified segment was cloned into the AatII and BspEI sites (underlined above) within the plasmid pBR322 vector (New England Biolabs). The placement of the T7 promoter within the primer allowed for the creation of authentic 5' viral ends. The addition of a second enzyme site, XbaI, within the reverse primers allowed for the creation of 3' ends with only one additional nucleotide. The 3' mutations were created by digesting the cloned S segment with a variety of different restriction enzymes, creating unique 3' ends. These enzymes include BglII, EcoRI, EcoNI, BglI, HpaII, EcoRV, AvaII, PsI, ScaI, and BclI. One nonviral RNA was generated from pGem7zf (+) (Promega) linearized with DraI. In order to generate a second nonviral transcript closer in length to the JCV S segment (991 nucleotides), the pGem7zf(+) vector without mutation. The hybrid transcripts were generated by the micro BCA protein reagent kit (Pierce). Protein was stored at –80°C.

**RESULTS**

Expression, purification, and UV cross-linking of recombinant JCV nucleocapsid. JCV N protein was generated with a C-terminal eight-histidine-tagged protein in Escherichia coli and was purified under native conditions using nickel affinity chromatography. The N protein was eluted with 400 mM imidazole. Eluted fractions were dialyzed against a buffer containing 40 mM HEPES, pH 7.4, 300 mM NaCl, and 10% glycerol. Protein aliquots were stored at –80°C, but when thawed they were able to retain activity and stability at 4°C for 4 weeks. Coomassie staining showed only a single band at the expected 27 kDa (data not shown). The protein preparation underwent UV cross-linking to ensure the absence of any other RNA binding proteins. The N protein was incubated with in-vitro-transcribed full-length genomic sense RNA, full-length antigenic sense RNA, or nonviral RNA. Both full-length genomic and antigenic transcripts were engineered so that they contained authentic 5' ends and a 3' end that contained only a single additional nucleotide, making it possible for a panhandle conformation to occur. Nonviral RNA was 1,256 nucleotides in length, 256 nucleotides longer than either genomic or antigenic RNA. Another nonviral RNA probe was generated that was 912 nucleotides in length. Although it was only 76 nucleotides shorter than the full-length viral genome, this probe had binding that was nearly identical to that of the larger nonviral RNA, suggesting that binding ability is not due to the number of sites available but rather to nonspecific interactions, such as electrostatic interaction and van der Waals forces.

The UV cross-linking (Fig. 1) showed the presence of monomeric, dimeric, and tetrameric forms of RNA-nucleocapsid interactions for all three forms of RNA. In the absence of N

**Filter binding assay.** Filter binding reactions were carried out in a volume of 20 μL. RNA transcripts were heated to 100°C for 10 min and then allowed to slowly cool to 25°C. The reaction buffer consisted of 40 mM HEPES (pH 7.4), 1 U Rnasin (Promega), and 23 mM [α-32P]UTP-labeled RNA, along with 20 μM KCl, 20 mM NaCl, 10 mM MgCl2, 50 μg of heparin sulfate, and 11.6 μM JCV N protein. The reaction mixtures were incubated at room temperature for 20 min and then were spotted onto a Bio-Rad (Richmond, CA) and examined by autoradiography. Protein washes once with ice-cold buffer (40 mM HEPES [pH 7.4] and 100 mM NaCl), and air dried. Signals were quantitated by a Molecular Dynamics PhosphorImager and analyzed by ImageQuaNT software.

Generation of 5' and 3' halves of either JCV vRNA or cRNA in a nonviral backbone. Generation of pGem7zf(+) clones containing either 5' or 3' halves of the vRNA/cRNA began with the digestion of pGem7zf(+) for the 5' halves with the restriction enzymes AatII and EcoRI. The full-length vRNA/cRNA sequence in pBR322 were digested with AatII and EcoRI. The gel-purified fragment containing the 5' half of vRNA or cRNA then was ligated into the already-digested pGem7zf(+) vector. The 3' half of either full-length vRNA or cRNA in pBR322 was digested with EcoRI and BspEI restriction enzymes. The pGem7zf(+) vector was digested with EcoRI and XmaI, which produce ends compatible with BspEI so that ligation between fragments could occur. The 3'-half fragments were gel purified and ligated into the pGem7zf(+) vector. The resulting plasmids containing vRNA or cRNA 5' and 3' halves were sequenced to ensure that the correct viral sequences had been placed into the pGem7zf(+) vector without mutation. The hybrid transcripts were generated by linearizing the mutant DNA with DraI.
protein, RNase digestion completely removed any nonviral RNA (Fig. 1A, lane 5). Genomic RNA was not completely digested away (Fig. 1A, lane 4), but this could be due to the panhandle structure formed by the complementary ends of the genome and the fact that the RNases added were not capable of digesting double-stranded RNA. The UV cross-linking data did confirm the absence of any additional RNA binding proteins in the N protein preparation, suggesting that all of the interaction could be attributed to JCV nucleocapsid. The Western blot analysis (Fig. 1B) confirmed that the protein that was interacting with the RNA in the UV cross-linking was indeed JCV N protein. It also verified the presence of the monomers, dimers, and tetramers.

**Optimization of filter binding conditions.** In order to determine which binding conditions would create the most specific binding profile, a number of conditions were optimized (Table 1). These conditions also enabled the deduction of what types of RNA-nucleocapsid interaction were taking place. Magnesium chloride (MgCl₂) was tested in a range from 0 to 50 mM, with the optimum concentration being 5 mM. Increasing MgCl₂ beyond 5 mM did not have a huge effect on binding, suggesting that electrostatic interaction with the phosphodiester backbone does not contribute largely in the RNA-nucleocapsid interaction. EDTA then was assessed; the addition of any EDTA dramatically decreased the level of binding. The next condition to be analyzed was that of salts, both sodium chloride and potassium chloride. The protein preparation already adds at least 60 mM NaCl to the reaction mixture, which comes as a carryover from the dialysis conditions. Both NaCl and KCl were looked at because both are present in a natural infection. The range tested was 0 to 500 mM of each salt separately. While increasing millimolar amounts of both salts past 100 mM did decrease binding to some degree, it did not abrogate binding, thus implying that the ionic strength of the interaction is not based upon electrostatic interaction. Since 50 to 100 mM of either NaCl or KCl seemed to create the best profile, different concentrations of both salts together were analyzed. The addition of 20 mM of both NaCl and KCl together plus the additional 60 mM from the dialysis yielded the best binding profile. These results are consistent with what has been observed for other members of the *Bunyaviridae* family (18, 20, 29, 30). Heparin sulfate and tRNA, which are both noncompetitive inhibitors, were tested from 0 to 12 μg. The tRNA did not increase the specificity of the interaction, but 50 ng of heparin sulfate increased the binding potential of viral genomes and diminished nonviral interactions. Using 1 nM RNA, a binding profile with increasing concentrations of protein was performed. The binding profile showed that binding reached its maximum level when there was 5 to 6,000 times more protein present than RNA.

**Specificity of the RNA-nucleocapsid interaction.** To determine if the interaction between the various forms of RNA and the N protein was specific or based solely upon electrostatic interaction, a competition assay was performed (Fig. 2). Using labeled vRNA, cRNA, and nonviral RNA, each RNA was competed off with either unlabeled RNA of the same species or unlabeled nonviral RNA. A constant concentration of labeled RNA (23 nM) was incubated with 115 μg of nucleocapsid and increasing amounts (from 2.97 to 92 nM) of unlabeled competitor RNA. Nonviral RNA was not an effective competitor of either genomic or antigenomic RNA (Fig. 2). It decreased binding by at most 8%, but at higher concentrations it acted like a nonspecific inhibitor, actually increasing the level of binding of both genomic and antigenomic RNA. This supports the concept that nonviral RNA is capable of binding to RNA.
the nucleocapsid, because when there is no other RNA present that is specific for the N protein target, the N protein would prefer to bind nonspecific RNA rather than no RNA at all. Genomic RNA and antigenomic RNA both were specific competitors of binding, as shown by decreased binding, thus indicating the specificity that vRNA and cRNA have for the N protein (Fig. 2).

**Identification of regions responsible for RNA-nucleocapsid binding.** Since the competition assay confirmed that the interaction between vRNA and cRNA was preferred over the interaction between nonviral RNA and N protein, the next issue to address was where on the RNA the binding domain was located. Based upon the literature and preliminary experiments (data not shown), 3’ deletion mutants starting from the EcoRI restriction site (thus missing the entire 3’ half of the RNA molecule) were generated (Fig. 3A). Also included was a mutant missing the last 52 nucleotides of the 3’ end to test the necessity of a panhandle conformation versus that of a single-stranded RNA template for binding.

The EcoNI mutant on the vRNA strand, missing the 3’ 52 nucleotides, showed binding identical to that of wild-type viral RNA. This result indicates that the panhandle configuration is not necessary to facilitate binding and that single-stranded RNA may be the preferred template. The deletion of the 3’ half of the viral genome does not reduce binding at all, as evidenced by the EcoRI mutant, which bound at wild-type levels. This result further promotes the idea that single-stranded RNA is the state necessary for binding to occur. As further deletions are made toward the 5’ end, a decrease in binding occurs until the mutant (BglII) is reached, with only the last 45 nucleotides remaining at the 5’ end of the viral genome. These results suggest that the RNA binding domain is located between nucleotides 126 and 520 of the vRNA. The return of binding to within 20% of wild-type levels that occurs when only the 5’ 45 nucleotides of the genome are present is confounding, but it could be explained if some of these tran-

![RNA competition assay](https://jvi.asm.org/)

**FIG. 2.** RNA competition assay. Each labeled RNA probe (23 nM each) was incubated with 115 μM of purified JCV N protein for 10 min at 25°C. Increasing amounts of unlabeled RNA (0 to 92 nM), which included vRNA, cRNA, and nonviral RNA, were added to the reaction and were incubated for an additional 10 min before being slot blotted onto a nitrocellulose membrane, and the retained and labeled RNA was determined by phosphorimaging and analyzed by ImageQuaNT. % RNA bound indicates the percentage of N protein bound by the riboprobe; a decrease in percentage indicates the ability of a competitor to replace labeled RNA. The data points represent the means from three experiments, and the error bars depict the standard deviations.

![Viral and complementary S segment deletion mutants](https://jvi.asm.org/)

**FIG. 3.** Viral and complementary 3’ deletion mutants. (A) A graphic representation of the viral S segment 3’ deletion mutations (black bars) generated and examined for their ability to bind JCV N protein. Mutations are named after the restriction enzyme that was used to generate the 3’ end. The number of nucleotides in each mutant represents the length of each mutant. The percentage of RNA bound was determined with the following formula: 

\[
\% \text{RNA Bound} = \left(\frac{\text{amount of radiolabeled probe retained by the filter}}{\text{total amount of radiolabeled probe added to each reaction}}\right) \times 100
\]

(B) The representation is identical to that of panel A, except that the subject is complementary (white bars) S segment of the 3’ deletion mutations. nt, nucleotide.
scripts (mutants AvaII, Scal, BelII, and PsiI) develop higher-order structures that either form double-stranded molecules that are not able to bind the N protein or form structures that mask the binding region due to secondary/tertiary structures.

Similar experiments (Fig. 3B) with the antigenomic cRNA strand yielded similar results. Removal of the last 49 3'-terminal nucleotides did not decrease binding, supporting the hypothesis that single-stranded RNA is the preferred template. Removal of 476 nucleotides, as done with the cAvaII mutant, did not decrease RNA binding from wild-type levels. Once again, there was a decrease in binding as the mutations proceeded toward the 5' end until reaching the last 52 nucleotides, at which point binding returned to within 10% of wild-type levels. This suggests that a secondary/tertiary structure may be masking the binding domain in the intermediate constructs. The return to nearly wild-type binding levels also could be due to the fact that these mutants contain nearly all of the noncoding region, which extends to nucleotide 70, so the entire noncoding region may be necessary for fully functional binding to take place. It also is possible that there is an even smaller region less than 52 nucleotides closer to the already reported 32 to 39 nucleotides, which is all that is necessary for binding to occur, and that this region is free from secondary/tertiary structures masking the actual binding site. The one consistent fact is that the 5' half of the vRNA/cRNA contains the RNA binding domain.

The 3' end of vRNA and cRNA is not necessary for RNA binding. In order to support the conclusion that the 3' end of the RNA molecule is not necessary for RNA binding to occur, a competition assay (Fig. 4) was performed using labeled vRNA/cRNA with unlabeled vRNA/cRNA 3' ends. As expected, neither vRNA nor cRNA 3' halves were able to compete off full-length vRNA or cRNA, supporting the idea that the 3' halves are not involved in RNA binding.

Rescue of nonviral RNA by replacement of vRNA/cRNA sequences. To fully establish that the 5' end of either the vRNA or cRNA contained the RNA binding domain, new nonviral transcripts were generated that contained either the vRNA or cRNA contained the RNA binding domain, new nonviral RNA sequences. To fully establish that the 5' end of either the vRNA or cRNA contained the RNA binding domain, new nonviral transcripts were generated that contained either the 5' half or the 3' half of both vRNA and cRNA in the nonviral background (Fig. 5A). After the new mutants were created, transcripts were generated and a filter binding assay was performed. Each transcript (23 nM) was incubated with increasing amounts of N protein (92 to 184 μM) and then slot blotted onto a nitrocellulose membrane and analyzed by phosphorimaging and analysis by ImageQuaNT. % RNA bound indicates the percentage of N protein bound by the riboprobe; a decrease in the percentage indicates the ability of a competitor to replace labeled RNA. The data points represent the means from three experiments, and the error bars depict the standard deviations.

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DISCUSSION

The ability of the viral N protein to interact with both genomic and antigenomic RNA plays a vital role in viral replication. The idea that there may be a selective mechanism for vRNA-nucleocapsid interaction has been studied with other members of the Bunyaviridae family (9, 15, 17, 18, 20, 29, 30).

Results concerning what region is responsible for RNA interacting with N protein among the members of the Bunyaviridae are just as varied as the viruses themselves, even leading to disagreements when using the same viruses. In order to seek some clarity, at least for the members of the Orthobunyavirus genus, the identification of the RNA binding domain on both the viral and complementary strands of the S segment using UV cross-linking, filter binding, competition assays, and generation of rescue mutations was performed.

Binding conditions suggest that binding between vRNAs and the N protein are specific interactions. The UV cross-linking showed that there was an absence of contaminating RNA binding proteins and also that the RNA was capable of interacting with different oligomerization states of the N protein, such as dimers and trimers. This phenomenon of dimers and trimers also was seen when the Bunyamwera virus nucleocapsid gene was bacterially expressed (20). Recent studies using Sin Nombre virus N protein were done with a purified trimer (18), suggesting that various oligomerization states of the N protein are capable of binding vRNAs.

Initial competition assays show that vRNA and cRNA interaction with the nucleocapsid is preferred. Each transcript was able to be competed off with unlabeled identical transcript but not with unlabeled nonviral transcript. Labeled nonviral transcript was easily removed by the presence of either vRNA or nonviral RNA. Like Bunyamwera nucleocapsid, there is still
the ability to bind nonviral RNA in a nonspecific manner (20). A number of viral core/coat proteins have the ability not only to bind vRNA specifically but also to bind RNA nonspecifically (12, 13, 31, 33), suggesting that this is a normal part of RNA-N protein interaction and that JCV N protein acts in a manner similar to that of N proteins of other viruses.

The 3' deletion viral mutant data corroborate results obtained during the Bunyamwera study, which suggest that RNA-N protein interaction is based upon single-stranded RNA (20) rather than double-stranded RNA, such as the panhandle formed by complementary ends. This finding also is consistent with data from Hantaan virus studies, which found that the first 39 nucleotides alone were sufficient for encapsidation (30). These results contrast significantly with studies using Sin Nombre virus that indicated that the panhandle region, 32 nucleotides from both the 5' and 3' ends of the genome, was sufficient to bind trimeric N protein (18). The concept of single-stranded RNA as the binding template is justified by the fact that removing as few as 45 or even 54 nucleotides maintained binding. Even when the ability to form the panhandle is removed, binding is not immediately abrogated, thus demonstrating that panhandle RNA is not the template for encapsidation. While the idea that double-stranded RNA is important for encapsidation is present in the literature (9, 18), it has been shown only with members of the Hantavirus genus, not for any members of the Bunyaviridae (20, 24, 30). The crystallized nucleocapsid of another negative-sense RNA virus, influenza virus, which also has the hallmark ability to form a panhandle structure from complementary ends, illustrates that binding with its RNA occurs in a single-stranded manner rather than being double stranded (32). Other encapsidation signals from a variety of viruses have shown the importance of stem-loops for binding (1, 3–5) but not the importance of double-stranded RNA.

Other than that of the hantavirus nucleocapsid in a trimeric conformation (17), this is the first time that the cRNA's binding region has been examined. RNA binding between complementary and viral regions is consistent in that it is driven by single-stranded RNA rather than double-stranded RNA and that the regions thought to retain the binding domains are in

FIG. 5. Graphic representation of rescue mutants and filter binding results of generated rescue mutants. (A) An illustration of rescue mutations generated using a pGem (nonviral) backbone. Each bar depicts the placement of the viral or complementary sequences within the nonviral backbone. (B) The filter binding results for full-length vRNA (v), full-length cRNA (c), and nonviral RNA compared to those for rescue mutations containing either the 5' or 3' half of cRNA or vRNA, c5' and v5', 5' half of cRNA and vRNA, respectively. A constant concentration of RNA probe (23 nM) was incubated with increasing amounts of JCV nucleocapsid (92 to 184 μM). The percentage of RNA bound was determined with the following formula: [(amount of radiolabeled probe retained by the filter)/(total amount of radiolabeled probe added to each reaction)] × 100. The data points represent the means from three experiments, and the error bars depict the standard deviations. nt, nucleotides.
identical regions rather than in identical nucleotides. The idea that nucleotides in both viral and complementary regions would be complementary rather than identical but would be able to produce regions with matching structures suggests that the region is more structure than sequence driven.

One unique aspect of complementary and viral binding comparisons is the difference in binding activity between the two strands. vRNA binds 1.5 times more efficiently than does cRNA. One possible explanation for this difference is that the sequence contained within the vRNA is slightly more recognizable to the nucleoprotein than the sequence in the cRNA. vRNA is the sense strand that is not only the precursor template for transcription and translation and replication but is also the form that is incorporated into the virion, and it logically would have a higher affinity for the nucleoprotein complex.

There are two possible explanations for the regions containing either 45 nucleotides on the viral strand or 52 nucleotides on the complementary strand that do not act in a manner similar to that of other viruses listed in the literature (20, 30). While these mutants encompass only noncoding region nucleotides, that region may be too large or too small, not containing the entire region, thus resulting in the instability of any secondary/tertiary structures present. This region most likely is important, since the ability to bind RNA increases to within 20% of wild-type levels. Further studies to increase the size of mutant to contain the entire noncoding region and then decrease it by two to three nucleotides at a time could be done to determine if a finite region at the 5' end of the genome is responsible for RNA binding.

The ability of nonviral RNA to bind N protein at levels comparable to those of full-length vRNA and cRNA by the addition of viral sequences is a novel finding for the Bunyaviridae. The ability to rescue binding activity is sequence specific, since the 3' ends of both the viral and complementary strands did not alter binding at all. This phenomenon suggests that binding is preferential, not just a matter of adding nucleotides. The fact that the 5' ends, which added nearly the same amount of additional binding sites as the 3' ends, were able to increase binding while the 3' ends were unable to solidify the concept of preferential binding. What remains consistent is the idea that the 5' ends of both viral and complementary strands of the S segment contain the RNA binding domain. This continues to be accurate for most members of the Bunyaviridae studied thus far (20, 24, 30). The absolute discrete region.structure has yet to be identified and should be further characterized.

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