The RNA Binding Domain of Jamestown Canyon Virus S Segment RNAs

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ABSTRACT:

Jamestown Canyon virus (JCV) is a member of the Bunyaviridae family, Orthobunyavirus genus, and California serogroup. Replication, and ultimately assembly and packaging, rely on the process of encapsidation. Therefore, the ability of viral RNAs (genomic and antigenomic) to interact with the nucleocapsid protein (N) and the location of this binding domain on the RNAs are of interest. The questions to be addressed are where is the binding domain located on both the viral and complementary RNA 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 (v) and complementary (c) S segment RNA, as well as 3′ deletion mutants of both viral and complementary RNA, nonviral RNA, and hybrid viral/nonviral RNA were analyzed for their ability to interact with bacterially expressed JCV nucleocapsid protein. RNA:nucleocapsid interactions were examined by UV crosslinking, filter binding assays, and 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 viral RNAs.

INTRODUCTION:

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,
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 $G_N$ and $G_C$, which are cleaved post-translationally, a nonstructural M protein, nucleocapsid, and the nonstructural S protein(14).

Like other negative-strand RNA viruses, replication of JCV takes place in ribonucleoprotein (RNP) complexes composed of the nucleocapsid protein, viral RNA (either genomic or antigenomic sense), and the RDRP. These RNP complexes have been observed to have a circular appearance (2, 21-23, 26, 27) due to the fact that 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 due to the fact that 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 twelve 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 stated a binding preference for double-stranded RNA but with
no real specificity (9). The next set of results with the Hantavirus genus suggests that while no
specific sequence was identified, full-length viral RNA was identified as a preferable target over
RNA containing only internal RNA sequences (29). Further analysis was completed which
indicated that nucleotides 1-39 were all that were necessary for RNA binding to occur (30).
However, more recent data suggest that the panhandle conformation resulting from the
basepairing 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, 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 viral RNA. This region is thought to
contain two stemloop structures which may be mediating the specificity of viral binding (20).
There is an obvious need for specific individual viral studies due to the dissimilarities found
throughout this viral family.

The study reported here examines both the viral and complementary RNA of Jamestown
Canyon virus 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 complementary RNA is also important for binding. The
3′ end of the genome, both viral and complementary, is not involved in RNA binding.
Transplanting 5′ end viral sequences into a nonviral background restores binding of that RNA to
nearly wild-type levels.

MATERIALS AND METHODS:
**Jamestown Canyon Virus N Protein Purification:** The S segment open reading frame that encodes the JCV N protein was cloned by RT-PCR with 5’ and 3’ primers engineered with *NcoI* and *XhoI* (restriction sites underlined). The 5’ forward primer was 5’- 
TTGCCATGGGGATTTGGTTT and the 3’ reverse primer is 5’-
TTTCTCGAGTGGAACCTTTAC. The amplified fragment was then cloned into the Qiagen pQE-TriSystem vector using the *NcoI* 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 8 histidines. This cloning created a fusion protein with the predicted mass of 27 kDa. Protein expression was induced by generating a small overnight culture (100 ml), transferring it to a 1 L culture, and allowing it to grow at 29°C until the OD$_{600}$ reached 0.8. The nucleocapsid protein was purified under native conditions using a Ni-NTA column chromatography (Qiagen). The lysis, wash, and elution solutions each contained 0.5% NP-40, 10% glycerol and the protein was eluted from the column with 400 mM imidazole (Sigma). Column fractions were analyzed by sodium dodecylsulfate-polyacrylimide gel electrophoresis (SDS-PAGE) and Western blot analysis. Fractions containing purified JCV N 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 Assay Reagent Kit (Pierce). Protein was stored at -80°C in small aliquots.

**Preparation of RNA Substrates:** The entire S segment of JCV N, both viral RNA (vRNA) and complementary RNA (cRNA), was amplified by RT-PCR with 5’ and 3’ primers
engineered with flanking $AatII$ and T7 promoter sequence and $BspEI$ restriction sites. The $JCVv5'$ forward primer is 5'-

$$CCGACGTCTAAATACGACTCATAAAGTAGTGCTCCACTGAA$$ and the $JCVv3'$ reverse primer is 5'-GCTCCGGATCTAGAAGTAGTGTAATCCACTTGAA. The $JCVc5'$ forward primer is 5'-ATGACGTCTAAATACGACTCATAAAGTAGTGTAATCCACTTG and the $JCVc3'$ reverse primer is 5'-CCTCCGGATCTAGAAGTAGTGCTCCACTG. The amplified segment was cloned into the $AatII$ and $BspEI$ sites (underlined above) within the pBR322 vector (New England Biolabs) (NEB). 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 $BglII$, $EcoRI$, $EcoNI$, $BglI$, $HpaII$, $EcoRV$, $AvalI$, $PsI$, $Scal$, and $BclI$. One nonviral RNA was generated from pGem7 zf(+) (Promega) linearized with $DraI$. In order to generate a second nonviral transcript closer in length to the Jamestown Canyon virus S segment, 991 nucleotides, the pGem7zf(+) vector was cut with the restriction enzyme $AlwNI$. After digestion with $AlwNI$, which produces a 3' overhang not compatible with transcription reactions, the vector was again digested with Mung Bean Nuclease (NEB) which removed the 3' overhang and created a blunt end. This allowed for a 912 nucleotide transcript to be produced. Using the Promega T7 transcription kit, [$\alpha^{32}P]$UTP radiolabeled transcripts were generated. Following DNA removal by digestion with RQ1 DNase (Promega), the transcripts were purified over BioRad spin columns to remove unincorporated nucleotides. RNA was examined on 0.25x TBE 1% agarose gels to demonstrate
that full-length or specifically truncated transcripts were produced. Purified RNA was stored at -80°C.

**Filter Binding Assay:** Filter binding reactions were carried out in a volume of 20 µl. RNA transcripts were heated to 100°C for 10 minutes 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 nM [$\alpha^{32}$P]UTP-labeled RNA along with 20 mM KCl, 20 mM NaCl, 10 mM MgCl$_2$, 50 ng of heparin sulfate, and 111.6 µM JCV N protein. The reactions were incubated at room temperature for 20 minutes and then were slot-blotted (BioRad, Hercules, CA) onto a prewetted nitrocellulose filter, washed 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.

**UV Crosslinking Assay:** Briefly, RNase-free components were combined on ice in a final volume of 20 µl: 10 mM Tris-HCl (pH 7.4), 1 U RNasin, 111.6 µM JCV N full-length protein, and 23 nM of [$\alpha$-$^{32}$P]UTP-labeled RNA along with 20 mM KCl, 20 mM NaCl, and 10 mM MgCl$_2$. In standard reactions JCV N protein was added to the reaction buffer last and incubated for 30 minutes at 25°C. Following incubation at 25°C, the reactions were placed on ice and crosslinked at 254 nm (UV Stratalinker) at a distance of 10.5 cm for 30 minutes. After crosslinking was completed, 5 µl RNase Cocktail (Ambion) was added to each reaction and incubated at 37°C for 15 minutes. Samples were heated to 95°C before being resolved on a 10-20% SDS-PAGE gel. Gels were either transferred to nitrocellulose membranes and Western blotted or were dried down and the results visualized by exposing the autoradiographic film.

**Competition Assay:** Using the filter binding assay, competition experiments were performed.
A constant amount of Jamestown Canyon virus nucleocapsid protein, 115 µM, was incubated with 23 nM of labeled RNA probe for 10 minutes at 25°C. Various concentrations (0-92 nM) of unlabeled RNA, which included vRNA, cRNA, and nonviral RNA, were added to the binding reaction and incubated for an additional 10 minutes. Reactions were then slot-blotted onto a nitrocellulose membrane, washed, and air-dried as described above. Signals were quantitated by a Molecular Dynamics PhosphorImager and analyzed by ImageQuaNT software.

Generation of 5' and 3' Halves of Either JCV V or C RNA in a Nonviral Backbone:

Generation of pGem7zf(+) clones containing either 5' or 3' halves of the viral/complementary (v/c) RNA began with the digestion of pGem7zf(+) for the 5' halves with the restriction enzymes AatII and EcoRI. The full-length v/c sequences in pBR322 were digested with AatII and EcoRI. The gel purified fragment containing either the 5' half of v or c was then ligated into the already digested pGem7zf(+) vector. The 3' halves of either full-length v or c in pBR322 were digested with EcoRI and BspEI restriction enzymes. The pGem7zf(+) vector was digested with EcoRI and XmaI, which produce compatible ends to 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 v or c 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.

RESULTS

Expression, Purification, and UV Crosslinking of Recombinant JCV Nucleocapsid

Jamestown Canyon virus nucleocapsid protein was generated with a C terminal 8 histidine tagged protein in E. coli and purified under native conditions using nickel affinity
chromatography. The nucleocapsid protein was eluted with 400 mM imidazole. Eluted fractions were dialyzed against a buffer containing 40 mM Hepes pH7.4, 300 mM NaCl, and 10% glycerol. Protein aliquots were stored at -80°C, but when thawed were able to retain activity and stability at 4°C for 4 weeks. Coomassie staining showed only a single band at the expected 27kDa (data not shown). The protein preparation underwent UV crosslinking to ensure the absence of any other RNA binding proteins. The nucleocapsid protein was incubated with in vitro transcribed full-length genomic sense RNA, full-length antigenomic sense RNA, or nonviral RNA. Both full-length genomic and antigenomic transcripts were engineered so that they contained authentic 5' ends and a 3' end that contained only one additional nucleotide making it possible for a panhandle conformation to occur. Nonviral RNA was 1256 nt in length, 256 nucleotides longer than either genomic or antigenomic RNA. Another nonviral RNA probe was generated which was 912 nucleotides in length. Although it was only 76 nucleotides shorter than full-length viral genomes, this probe had nearly identical binding 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 crosslinking (Figure 1) showed the presence of monomeric, dimeric, and tetrameric forms of RNA:nucleocapsid interactions for all three forms of RNA. In the absence of nucleocapsid protein, RNase digestion completely removed any nonviral RNA (Figure 1, Panel A, lane 5). Genomic RNA was not completely digested away (Figure 1, Panel A, 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 crosslinking data did confirm the absence of any additional RNA binding proteins in the nucleocapsid protein preparation, suggesting that all the interaction could be attributed to JCV nucleocapsid. The Western blot (Figure 1, Panel B) confirmed that the protein that was interacting with the RNA in the UV crosslinking was indeed Jamestown Canyon nucleocapsid 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 0-50 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. Ethylenediaminetetraacetic acid (EDTA) was then assessed; the addition of any EDTA dramatically decreased the binding. The next condition to be analyzed was that of salts, both sodium chloride (NaCl) and potassium chloride (KCl). 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 due to the fact that both are present in a natural infection. The range tested was 0-500 mM of each salt separately. While increasing mM 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 50-100 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 in other members of
the Bunyaviridae family (18, 20, 29, 30). Heparin sulfate and t-RNA, which are both
noncompetitive inhibitors, were tested from 0-1 µg. The t-RNA 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
concentration of protein was performed. The binding profile showed that binding reached its
maximum level when there was 5-6000 times more protein present than that of the RNA.

The Specificity of RNA:Nucleocapsid Interaction

To determine if the interaction between the various forms of RNA and the nucleocapsid
protein was specific or based solely upon electrostatic interaction, a competition assay (Figure
2) was performed. 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 µM of nucleocapsid and
increasing amounts (from 2.97–92 nM) of unlabeled competitor RNA. Nonviral RNA was not
an effective competitor of either genomic or antigenomic RNA (Figure 2 closed symbols). It
decreased binding at most 8%, but at higher concentrations acted like a nonspecific inhibitor
actually increasing the binding of both genomic and antigenomic RNA. This supports the
concept that nonviral RNA is capable of binding to nucleocapsid because when there is no other
RNA present which is specific for the nucleocapsid protein target, the nucleocapsid protein
would prefer to bind nonspecific RNA rather than no RNA at all. Genomic RNA and
antigenomic RNA were both specific competitors of binding, shown by decreased binding, thus
indicating the specificity that viral and complementary RNA have for the nucleocapsid protein (Figure 2 open symbols).

**Identification of Regions Responsible for RNA:Nucleocapsid Binding**

Since the competition assay confirmed that the interaction between viral and complementary RNA was preferred versus the interaction between nonviral RNA and nucleocapsid protein, the next question 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 (Figure 3, Panel A). Also included was a mutant missing the last 52 nucleotides of the 3′ end to test the necessity of a panhandle conformation versus a single-stranded RNA template for binding.

The *EcoNI* mutant on the viral RNA 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 could suggest that the RNA binding domain is located between nucleotides 126 and 520 of the viral RNA. 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 could be
explained if some of these transcripts (mutants AvaII, Scal, BclI, and PsiI) developing higher order structures that either form double-stranded molecules which are not able to bind the nucleocapsid protein or form structures which mask the binding region due to secondary/tertiary structures.

Doing similar experiments (Figure 3, Panel B) with the antigenomic (c) RNA 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 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 where binding returned to within 10% of wild-type levels. This suggests that secondary/tertiary structure may be masking the binding domain in the intermediate constructs. The return to nearly wild-type binding levels could also be due to the fact that these mutants contain nearly but not 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 is also possible that there is an even smaller region less than 52 nucleotides closer to the already reported 32-39 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 viral/complementary RNA contains the RNA binding domain.

**Ensure 3′ End of Viral and Complementary RNA 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 (Figure 4) was performed using labeled v/cRNA
with unlabeled v/c 3’ ends. As expected, neither v nor c 3’ halves were able to compete off full-length viral or complementary RNA supporting the idea that the 3’ halves are not involved in RNA binding.

**Rescue of Nonviral RNA by Replacement of Viral/Complementary Sequences**

To fully establish that the 5’ end of either the viral or complementary RNA contained the RNA binding domain, new nonviral transcripts were generated which contained either the 5’ half or the 3’ half of both viral and complementary RNA in the nonviral background (Figure 5, Panel A). After the new mutants were created, transcripts were generated and a filter binding assay was performed. 23 nM of each transcript was incubated with increasing amounts of nucleocapsid protein (92 to 184 µM) and then slot-blotted onto a nitrocellulose membrane and analyzed by phosphoimaging. The results coincide with what has been shown previously (Figure 5, Panel B).

The nonviral backbone containing the 5’ half of the complementary RNA was able to restore binding to complementary wild-type levels. The mutant containing the 5’ half of the viral RNA was able to restore binding to within 10% of wild-type viral levels. The mutants containing the 3’ halves of either viral or complementary RNA were unable to rescue binding and bound at wild-type nonviral levels. The ability to restore binding of nonviral RNA to that of wild-type viral and complementary levels with the 5’ ends of either viral or complementary sequences supports the idea that the 5’ half contains the RNA binding domain as well as the idea that the RNA is in a single-stranded conformation rather than double-stranded configuration. It also helps to address the idea that nonviral binding is a matter of being present rather than the premise that binding is simply proportional to the number of bases in the substrate.
DISCUSSION

The ability of the viral nucleocapsid 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 viral RNA:nucleocapsid interaction has been studied in other members of the *Bunyaviridae* family (9, 15, 17, 18, 20, 29, 30).

Results concerning what region is responsible for RNA interacting with nucleocapsid 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 within at least members of the *Orthobunyavirus* genus, identification of the RNA binding domain on both the viral and complementary strands of the S segment using UV crosslinking, filter binding, competition assays, and generation of rescue mutations was performed.

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

Initial competition assays show that viral and complementary RNA interaction with the nucleocapsid is preferred. Each transcript was able to be competed off when using unlabeled identical transcript but not when using unlabeled nonviral transcript. Labeled nonviral transcript
was easily removed by the presence of either viral 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 not only the ability to specifically bind viral RNA but
also to bind RNA nonspecifically (12, 13, 31, 33), suggesting that this is a normal part of
RNA:nucleocapsid protein interaction and that JCV nucleocapsid protein acts in a manner
similar to that of other viruses.

The 3′ deletion viral mutant data corroborate results obtained during the Bunyamwera
study, which suggest that RNA:nucleocapsid protein interaction is based upon single-stranded
RNA (20) rather than double-stranded RNA such as the panhandle formed by complementary
ends. This finding is also consistent with data from Hantaan virus which found that the first 39
nucleotides alone were sufficient for encapsidation (30). These results contrast significantly
with studies using Sin Nombre virus which indicated that the panhandle region, 32 nucleotides
from both the 5′ and 3′ ends of the genome, was sufficient to bind trimeric N (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 but not in any other members of the Bunyaviridae (20, 24, 30). The
crystallized nucleocapsid of another negative-sense RNA virus, influenza, 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 that importance of stemloops for binding (1, 3-5) but not the importance of double-stranded RNA. 

Other than with the hantavirus nucleocapsid in a trimeric conformation (17), this is the first time that the complementary RNA’s binding region has been examined. RNA binding between c and v is consistent in the concept that it is driven by single-stranded RNA rather than double-stranded RNA and that the region thought to retain the binding domain is in an identical region rather than in identical nucleotides. Since the nucleotides in both v and c regions would be complementary rather than identical but would be able to produce regions with matching structures, this suggests that the region is more structure than sequence driven.

One unique aspect when you compare complementary and viral binding is the difference in binding activity between the two strands. Viral RNA binds 1.5 times more efficiently than does complementary RNA. One possible explanation for this difference could be that the sequence contained within the viral RNA is slightly more recognizable to the nucleoprotein than the sequence in the complementary RNA. Viral RNA is the sense that is not only the precursor template for transcription and translation and replication but is also the form which is incorporated into the virion and would logically have a higher affinity for the nucleoprotein complex.

An explanation for the regions containing either 45 nucleotides on the v strand or 52 nucleotides on the c strand not acting in a manner similar to that of other viruses listed in the literature (20, 30) could have two possibilities. While these mutants encompass only noncoding region nucleotides, that region may be either too large or too small, not containing the entire region, thus resulting in instability of any secondary/tertiary structures present. This
region is most likely important since the ability to bind RNA increases to within 20% of wild-type levels. Further studies to increase the mutant to contain the entire noncoding region and then decrease it by 2-3 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 nucleocapsid protein at levels comparable to full-length v and c RNA by the addition of viral sequences is a novel finding among the Bunyaviridae. The ability to rescue binding is actually sequence specific since the 3′ ends of both the v and c strands did not alter binding at all. This phenomenon suggests that binding is preferential, not just a matter of adding additional 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, solidifies the concept of preferential binding. What remains consistent is the idea that the 5′ end of both viral and complementary strands of the S segment contains the RNA binding domain. This continues to be accurate across 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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**FIGURE LEGENDS**

**Figure 1. UV Crosslinking/Western Blotting of Purified JCV N to RNA.** Panel A shows P-labeled RNA probes SvRNA (lanes 1+4), ScRNA (lane 2), and nonviral RNA (lanes 3+5) that were incubated with (lanes 1-3) or without (lanes 4-5) purified, bacterially expressed, His-tagged JCV N protein, followed by UV crosslinking, RNase treatment, and SDS-PAGE. Panel
B illustrates His-tagged JCV N protein that was incubated in the absence (lanes 5-7) or in the presence of $^{32}$P-labeled RNA probes SvRNA (lanes 2+5), ScRNA (lanes 3+6), and nonviral RNA (lanes 4+7), as described for filter binding reactions. Following SDS-PAGE, the gel was blotted onto nitrocellulose and the membrane was analyzed directly by Western blotting using His-specific antibodies.

**Figure 2. RNA Competition Assay.** Each labeled RNA probe, 23 nM, v (circles), c (triangles), or nonviral (squares) RNA was incubated with 115 µM of purified JCV N for 10 minutes at 25°C. Increasing amounts of unlabeled RNA (0-92 nM), which included vRNA, cRNA (open symbols), and nonviral (closed symbols) RNA were added to the reaction and incubated for an additional 10 minutes before being slot-blotted onto a nitrocellulose membrane and retained labeled RNA was determined by phosphoimaging and analysis by ImageQuaNT. % RNA bound indicates the N protein bound by riboprobe; a decrease in percentage indicates the ability of competitor to replace labeled RNA. The data points represent the mean of three experiments and the error bars depict the standard deviation.

**Figure 3. Viral and Complementary 3′ Deletion Mutants.** Panel A shows 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. % RNA bound is (the amount of radiolabeled probe retained by the filter)/(the total amount of radiolabeled probe added to each reaction) x 100. Panel B shows the identical information about the complementary (white bars) S segment of the 3′ deletion mutations.
Figure 4. Competition Assay Using Unlabeled 3′ End of Viral and Complementary Segments. Each labeled RNA probe, 23 nM, v (circles) or c (triangles) RNA was incubated with 115 µM of purified JCV N protein for 10 minutes at 25°C. Increasing amounts of unlabeled RNA (0-92 nM), which included 3′vRNA and 3′cRNA were added to the reaction and incubated for an additional 10 minutes before being slot-blotted onto a nitrocellulose membrane and retained labeled RNA was determined by phosphoimaging and analysis by ImageQuaNT. % RNA bound indicates the N protein bound by riboprobe; a decrease in percentage indicates the ability of competitor to replace labeled RNA. The data points represent the mean of three experiments and the error bars depict the standard deviation.

Figure 5. Graphic Representation of Rescue Mutants and Filter Binding Results of Generated Rescue Mutants. Panel A: An illustration of rescue mutations generated using pGem (nonviral) backbone. Each picture depicts the placement of the viral or complementary sequences within the nonviral backbone. Panel B: The filter binding results for full-length (dashed line) viral RNA (circle), full-length complementary RNA (triangle), and nonviral RNA (square) compared to rescue mutations (solid lines) generated containing either the 5′ (closed symbols) or 3′ (open symbols) half of complementary or viral RNA. A constant concentration of RNA probe (23 nM) was incubated with increasing amounts of JCV nucleocapsid (92-184 µM). % RNA bound = (the amount of radiolabeled probe retained by the filter)/(the total amount of radiolabeled probe added to each reaction) x 100. The data points represent the mean of three experiments and the error bars depict the standard deviation.
Figure 2
# Optimization of Filter Binding

<table>
<thead>
<tr>
<th>Condition</th>
<th>Range</th>
<th>Optimum</th>
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<tr>
<td>MgCl$_2$</td>
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<td>5 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>0-10 mM</td>
<td>0 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>0-500 mM</td>
<td>20/20</td>
</tr>
<tr>
<td>KCl</td>
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<td></td>
</tr>
<tr>
<td>Heparin sulfate</td>
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<td>50 ng</td>
</tr>
<tr>
<td>Protein Concentration</td>
<td>1-15,000 nM</td>
<td>5-6000 nM</td>
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</tbody>
</table>

Table 1
Figure 3 A+B
Figure 5A

Panel A

- 5' vRNA 5' to EcoRI 520nt
- 5' vRNA EcoRI to 3' end 471nt
- 5' cRNA 5' end to EcoRI 467nt
- 5' cRNA EcoRI to 3' end 524nt
Figure 5 B