Characterization of the Hantaan Nucleocapsid Protein-Ribonucleic Acid Interaction*

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William Severson‡, Laurel Partin§, Connie S. Schmaljohn¶, and Colleen B. Jonsson‡‡

From the ‡Graduate Program in Molecular Biology, §Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, New Mexico 88003 and the ¶Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland 21702

The nucleocapsid (N) protein functions in hantavirus replication through its interactions with the viral genomic and antigeneic RNAs. To address the biological functions of the N protein, it was critical to first define this binding interaction. The dissociation constant, Kd, for the interaction of the Hantaan virus (HTNV) N protein and its genomic S segment (vRNA) was measured under several solution conditions. Overall, increasing the NaCl and Mg2+ in these binding reactions had little impact on the Kd. However, the HTNV N protein showed an enhanced specificity for HTNV vRNA as compared with the S segment open reading frame RNA or a nonviral RNA with increasing ionic strength and the presence of Mg2+. In contrast, the assembly of Sin Nombre virus N protein-HTNV vRNA complexes was inhibited by the presence of Mg2+ or an increase in the ionic strength. The Kd values for HTNV and Sin Nombre virus N proteins were nearly identical for the S segment open reading frame RNA, showing weak affinity over several binding reaction conditions. Our data suggest a model in which specific recognition of the HTNV vRNA by the HTNV N protein resides in the noncoding regions of the HTNV vRNA.

Hantaviruses are tripartite negative-sense RNA viruses in the Bunyaviridae family (1). The viral RNA segments, L, M, and S, encode an RNA dependent RNA polymerase, two envelope glycoproteins, and nucleocapsid (N)1 protein, respectively. In mature virions, multiple copies of the N protein coat each of the genomic segments (vRNAs) to form three distinct ribonucleoprotein complexes, which may also include the viral polymerase. The N protein has been suggested to play a functional role in replication and/or transcription, perhaps by providing a role in replication and/or transcription, perhaps by providing a mechanism for controlling the amount or rate of macromolecular synthesis. Precedent exists for such activities with analogous proteins of other negative-strand RNA viruses such as those in the Rhabdoviridae, Paramyxoviridae, and Orthomyxoviridae families. The N protein of vesicular stomatitis virus (VSV) modulates the transition from transcription to replication (2), while the influenza nucleoprotein is required for RNA synthesis (3–5), and antitermination during viral mRNA synthesis (6). In contrast to influenza nucleoprotein (7, 8), selective encapsidation of viral genomic or leader RNAs has been reported for the N proteins of VSV (9, 10) and rabies virus (11). For hantaviruses, however, little is known regarding the mechanism for encapsidating viral RNA or the role that N protein plays in regulating viral transcription and replication.

The proposed activities of the hantavirus N protein in the viral life cycle rely on differential interactions with the three types of viral RNAs, the vRNA, the virus complementary (cRNA) and the messenger RNA (mRNA). The mechanism for selective encapsidation of the vRNA and cRNA by the N protein remains a central question in the hantavirus replication cycle as well as those of other members of the family Bunyaviridae. Cell culture-based experiments with Bunyamwera virus suggested that encapsidation requires full-length vRNA or cRNA because mRNA was not found in nucleocapsids (12). The mRNA is quite distinct in structure and sequence from the vRNA; with truncated 3′ termini and extended 5′ termini observed on all members of the Bunyaviridae. The 3′ truncation prevents base pairing of the complementary sequences at the 3′ and 5′ termini of the vRNA and cRNA and results in the inability of the mRNA to form panhandle structures. Thus, while vRNA and cRNA can form noncovalently, closed circular structures, mRNA cannot (13). This observation led to the suggestion that this gene region may play a role in assembly of the nucleocapsids, however, no sequences or secondary structures in any of the viral RNAs have been defined (14). Further, no readily identifiable RNA binding motifs have been located in N proteins, although deletion mapping of Hantaan (HTNV) and Puumala virus (PUUV) N proteins identified a nonspecific RNA binding domain in the carboxyl-terminal 93 amino acids (15). This region showed no apparent specificity for viral RNA. The N protein clearly functions in the viral replication cycle through its interactions with the viral nucleic acids and with other structural proteins. The biochemical and molecular determinants that promote interactions between the N protein and the viral RNAs have not been defined. To begin to address the biochemical basis of N protein and viral RNA interactions, we investigated the first event in the assembly process, the binding step. We report procedures for obtaining highly purified hantavirus N protein and for measuring its interactions with RNA. Using these methods, we defined the dissociation constant for HTNV N protein-RNA interactions under several solution conditions. In addition, we explored the ability of a heterologous N protein from Sin Nombre virus (SNV) to interact with HTNV-derived RNAs and a nonviral RNA.

EXPERIMENTAL PROCEDURES

Hantavirus N Protein Purification—The S segment open reading frame (S ORF) representing the HTNV N protein was cloned by poly-

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1 The abbreviations used are: N, nucleocapsid; SNV, Sin Nombre virus; HTNV, Hantaan virus; PUUV, Puumala virus; DTT, dithiothreitol; VSV, vesicular stomatitis virus; PAGE, polyacrylamide gel electrophoresis; S ORF, S segment open reading frame; GEMSA, gel electrophoretic mobility shift assay.

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erase chain reaction amplification with 5' and 3' primers engineered with flanking Ndel and XhoI sites. The amplified HTNV S ORF was cloned into the EcoRI site of pFLAG to create a N-terminal fusion of the epitope. Enterokinase can cleave the FLAG peptide from the HTNV N protein and will leave an amino-terminal Leu, (predicted mass 48.2 kDa). The plasmid expression vector, pSNV-N PET-1, (gift of Brian Hjelle, University of New Mexico) contains the entire ORF representing the SNV N protein (isolate 3H226). The ORF is cloned into the HindIII and XhoI sites of the pET23b expression vector, with an N-terminal fusion of the T7 leader and a C-terminal fusion of a hexahistidine tag; thus, the SNV fusion protein had a predicted size of 51.4 kDa.

The expression, purification and enterokinase cleavage of soluble HTNV N-FLAG were performed as described previously (16). The enterokinase-cleaved HTNV N protein fractions were stored at −80 °C in 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.1 mM glycine. Prior to analysis had a predicted size of 51.4 kDa.

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The expression, purification and enterokinase cleavage of soluble HTNV N-FLAG were performed as described previously (16). The enterokinase-cleaved HTNV N protein fractions were stored at −80 °C in 20 mM Tris, pH 8.0, 150 mM NaCl, and 0.1 mM glycine. Prior to analysis had a predicted size of 51.4 kDa.
say). After removing unbound RNA by RNase digestion, the reaction was examined by SDS-PAGE. The optimal conditions for forming complexes were determined earlier by extensive titrations of ionic strength, metal, and reducing agents in these UV cross-linking assays with the N protein (21). Titrations experiments revealed an increase in the concentration of ribonucleoprotein complexes with increasing amounts of HTNV N protein and a constant level of HTNV vRNA (Fig. 2A). Complexes were not detectable at 0.73 ng/μl (Fig. 2A, lane 2); however, efficient cross-linking was observed at 7.3 ng/μl (Fig. 2A, lane 3) and 14.5 ng/μl (Fig. 2A, lane 4). At 29 ng/μl, the reaction became saturated, as determined by quantitation from the PhosphorImager analysis (Fig. 2A, lane 5). We estimate that the concentration of the N protein required to saturate the reaction is greater than a 4000-fold excess of what would be necessary to encapsidate the vRNA with 1 protein per 10 nucleotides. These results are similar to capseidation experiments previously published for VSV nucleocapsid interactions (10).

Using the same approach and binding conditions, a titration of purified SNV N protein to a constant amount of HTNV vRNA was examined (Fig. 2B). The highest level of signal was observed when the concentration of SNV N was 58 ng/μl (Fig. 2B, lane 5). In addition, efficient cross-linking was observed at the concentrations of 7.3 ng/μl (Fig. 2B, lane 2), 14.5 ng/μl (Fig. 2B, lane 3), and 29 ng/μl (Fig. 2B, lane 4). The protein concentrations that provided saturation levels for HTNV and SNV N proteins were used in all subsequent UV cross-linking experiments. Finally, the UV cross-linking studies revealed that the structure of the ribonucleoprotein complexes promoted by HTNV and SNV N proteins were clearly distinct on the HTNV vRNA template. HTNV complexes were sensitive to digestion with the double-stranded nuclease, RNase V1, but not with the single-stranded nuclease RNase T1. In contrast, SNV complexes were only sensitive to RNase T1 digestion.

**Effect of Ionic Strength on the Dissociation Constant of HTNV Ribonucleoprotein Complexes**—The UV cross-linking experiments confirmed the absence of other RNA-binding proteins in N protein preparations, and defined the basic conditions suitable for further studies of N protein-vRNA interactions. However, the UV cross-linking assay is limited in that the accessibility of the nucleocapsid to RNase digestion as well as the activity of the RNase may vary under different ionic strength conditions. As exemplified by the nucleocapsid proteins of Sendai virus and VSV, increases in the ionic strength of the microenvironment may increase the tightness of nucleocapsid coiling, which can increase the resistance of the nucleocapsid to digestion with RNase (22). Therefore, using the reaction conditions defined in the UV cross-linking experiments, we developed and used a filter binding assay to explore the dissociation constant (Kd) for formation of N protein-RNA complexes under several ionic strength conditions (Fig. 3A). For comparison, we examined the effect of ionic strength on nucleocapsid formation using the UV cross-linking assay (Fig. 3B).

Filter binding experiments were performed with increasing

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2 W. Severson and C. B. Jonsson, unpublished results.
RNA Binding Activity of the HTNV N Protein

The effect of increasing ionic strength on the RNA binding activity of HTNV N protein was measured using filter binding (A) and UV cross-linking assays (B). In panel A, binding reactions were assembled in 5 mM MgCl2 in addition to standard reaction components as described under “Experimental Procedures.” 32P-Labeled RNA was incubated with the indicated molar concentrations of N protein (x axis). The amount of radioactively labeled N protein retained on the filter was calculated relative to the maximum radioactivity retained in each experiment (y axis). In panel B, binding reactions were assembled in 5 mM MgCl2, subjected to UV cross-linking, separated by 12% SDS-PAGE, and subjected to autoradiography and PhosphorImager analysis as described under “Experimental Procedures.”

Effect of Magnesium on the Formation of HTNV N Protein-vRNA Complexes—In addition to monovalent ions, divalent ions such as Mg2+ can compete with a RNA-binding protein for electrostatic-based interactions along the phosphodiester backbone of an RNA molecule. The ability of the metal ion to compete with the protein essentially reflects nonspecific protein-RNA interaction, as recently demonstrated in the interactions of the HIV-1 nucleocapsid and the tRNA3Lys (25). To explore the requirement for a metal ion in the in vitro binding reaction of the HTNV N-protein and vRNA, we examined the effects of MgCl2 on the RNA binding activity of the HTNV N proteins (Fig. 4, A and B).

In experiments in which binding reactions contained a constant level of HTNV vRNA and a constant amount of HTNV N protein (Fig. 3A), NaCl concentrations were titrated from 0.05 to 1.0 M. From the resulting Klotz plot, a \( K_d \) of 25 nM was calculated for the 50 mM NaCl binding isotherm. At 100 mM NaCl, the smallest dissociation constant, 14 nM, was observed. The \( K_d \) values observed at 0.25, 0.50, and 1.0 mM NaCl were 32, 42, and 47 nM, respectively (Table I). These experiments were repeated with a 3-fold higher concentration of vRNA (the upper limit allowed in our filter binding assay) and one-half the amount of N protein (Table I). The results were similar to those obtained with the standard assay conditions. In both experiments, the \( K_d \) for the HTNV N protein-vRNA interactions did not vary substantially as the ionic strength was increased in the binding reaction buffer. This suggests nonelectrostatic contributions to formation and/or stability of the ribonucleoprotein complex.

Using conditions identical to those in the filter binding assays, NaCl concentrations were titrated and evaluated for their effect on complex formation of the HTNV N protein and HTNV vRNA by UV cross-linking. PhosphorImager analysis of the resulting N protein-RNA complex revealed the highest level of RNA binding at 100 mM NaCl (Fig. 3B, lane 3). The level of binding decreased minimally, 1.2-fold, as the ionic strength was decreased to 50 mM. As the NaCl concentration was increased from 0.25 to 0.50 M NaCl the level of protein-RNA complex fell 1.1-fold (Fig. 3B, lane 4) and 1.5-fold (Fig. 3B, lane 5), respectively. RNase V1 displays optimal activity in reactions containing up to 0.5 M NaCl; therefore, as expected, no complex was observed at the 1.0 M NaCl concentrations (Fig. 3B, lane 6). In summary, both UV and filter binding assays showed a similar level of binding of the vRNA by the N protein over a wide range of ionic strength, 50–500 mM. While the UV cross-linking assay was limited to these lower NaCl concentrations, the filter binding assay clearly revealed a similar stability of the N protein-RNA complex at 100 mM and 1 M NaCl concentrations. Thus, the results suggest that electrostatic interactions are not a predominant factor in complex formation. In conjunction with the differential susceptibility of the HTNV N-vRNA complex to RNase V1 and RNase T1, these results suggest the major contributions to complex stability may be through short range van der Waals or dielectric interactions between the N protein and the phosphodiester backbone or nucleobases within single-stranded regions of the vRNA.

Effect of NaCl concentration of HTNV N protein-vRNA complex formation

| Reaction condition | (vRNA) substrate \( K_d \) |
|-------------------|------------------|
| 1 ng              | 34 ± 8           |
| 3 ng              | 12 ± 0           |

Table I

Table I summarizes the results of \( K_d \) values determined at concentrations of MgCl2 ranging from 1 to 8 mM. As the MgCl2 concentration was increased, we noted a small shift in the binding isotherms toward an increase in \( K_d \) (Fig. 4A). The \( K_d \) for each HTNV N protein-RNA complex was calculated from the isotherms presented in Fig. 4A, and plotted against the MgCl2 concentration at which it was determined (Fig. 4B). The \( K_d \) values determined at concentrations of MgCl2 ranging from 1 to 8 mM were similar. The \( K_d \) observed in the absence of MgCl2, 53 nM, was only slightly smaller. The highest \( K_d \) was observed when 10 mM MgCl2 was included in the reactions, and represented a 2.4-fold increase in the \( K_d \) as compared with the \( K_d \) at 8 mM MgCl2. Overall, these studies do
not support a major role for MgCl$_2$ in promoting the interaction of the HTNV N protein and HTNV vRNA.

Specificity of the N Protein for Viral and Nonviral RNAs—A central question in the hantavirus replication cycle is how the N protein selectively encapsidates vRNA and cRNA, but not mRNA. To address this question, we explored the $K_d$ for HTNV and SNV N proteins complexed with two different viral RNAs, the full-length HTNV vRNA and an RNA representing the open reading frame of the HTNV S segment (S ORF RNA). The S ORF RNA is derived from the cRNA and does not contain the terminal complementary regions, which are expected to form the panhandle structure characteristic of vRNA and cRNA. The $K_d$ for the formation of a complex between the HTNV N protein and a nonspecific 67-nucleotide RNA (control RNA) was also examined. As described above, the addition of 1–8 mM MgCl$_2$ in the binding reaction did not reduce the affinity of the HTNV N protein for HTNV vRNA. However, in the following, we decided to examine the binding of the three RNAs in the presence and absence of MgCl$_2$ to explore its effect on alternative RNA substrates.

Binding isotherms for the HTNV N protein and the three RNA substrates were defined for four different reaction conditions: 1) 40 mM NaCl (Fig. 5A), 2) 40 mM NaCl and 1 mM MgCl$_2$ (Fig. 5B), 3) 80 mM NaCl and 20 mM KCl (Fig. 5C), and 4) 80 mM NaCl, 20 mM KCl, and 1 mM MgCl$_2$ (Fig. 5D). The dissociation constants for each binding isotherm are summarized in Table II. At low ionic strength, we observed little difference in the $K_d$ for HTNV N protein and the three RNA substrates, although the $K_d$ for the control RNA substrate was 3-fold higher (Fig. 5A, Table II). The addition of 1 mM MgCl$_2$ to the binding reaction with 40 mM NaCl enhanced the affinity of the HTNV N protein for the vRNA as compared with the S ORF and control RNAs (Fig. 5B, Table II). The greatest difference in binding of the templates by the HTNV N protein was observed in experiments that increased the ionic strength of the binding reaction to 100 mM (4:1 NaCl:KCl). In these assays, we noted an approximate 5-fold increase in the preference of the HTNV N protein for its vRNA as compared with the S ORF and control RNAs (Fig. 5D, Table II).

We used the same approach to examine the effects of ionic strength on the stability of the heterologous SNV N protein and HTNV RNAs. In contrast to the HTNV N protein-vRNA interactions, increasing the ionic strength of the binding buffer increased the dissociation of the SNV N protein-vRNA interaction approximately 3-fold (Table III). A comparison of the $K_d$ for binding reactions containing 80:20 mM NaCl/KCl with no MgCl$_2$ and those with 1 mM MgCl$_2$ revealed an approximate 10-fold increase in the $K_d$ in the presence of the metal. The interaction of both the HTNV and SNV N proteins with the S ORF reflected a similar dissociation constant (compare Tables II and III).

Finally, we wanted to determine whether the histidine tag affected binding to RNA and the observed dissociation constants. The HTNV N protein was expressed in Escherichia coli, and purified from the soluble fraction as a FLAG fusion protein (16). After purification, the FLAG peptide was removed by enterokinase cleavage, and binding isotherms for the three RNA substrates were determined. Filter binding assays were performed with the reaction conditions noted as optimal for binding in Table II (80 mM NaCl, 20 mM KCl). The $K_d$ values, calculated from the resulting binding isotherms, were 32 ± 14 for the vRNA, 107 ± 26 for the ORF RNA, and 250 ± 38 for the control RNA. These results were similar to those defined for the HTNV N protein with the hexahistidine tag (Table II). However, the $K_d$ obtained for the control RNA with the enterokinase-cleaved HTNV N protein was approximately 2-fold lower than the $K_d$ obtained for the hexahistidine tag HTNV N protein. This suggests that the hexahistidine tag may provide a slight amount of stability toward the binding of nonspecific nonviral RNA.

DISCUSSION

The N proteins of various RNA viruses have distinct biological roles in the viral replication cycle such as encapsidation, interactions with membrane proteins for virus assembly, as well as regulation of viral RNA synthesis. In addition to encapsidation of the viral RNAs, the hantavirus N protein may also

![Diagram](Image)
play an important role in the regulation of replication and transcription (1). Little is known, however, regarding the structure of the RNA binding domain and its intrinsic biochemical properties and requirements for RNA interaction. To initiate detailed biochemical studies of this protein and its interactions, we developed methods to purify the hantavirus N protein. Our purification scheme resulted in greater than 95% homogeneity of the full-length HTNV and SNV N proteins. The protocols developed to purify the N protein are substantially different from those reported in an earlier brief report (15). A major

TABLE II

Dissociation constants for HTNV N protein with various RNAs

| Reaction condition | Substrate | vRNA | s ORF | Control |
|--------------------|-----------|------|-------|---------|
| 0 mM MgCl₂, 40 mM NaCl | nM       | 140 ± 7 | 140 ± 11 | 430 ± 21 |
| 1 mM MgCl₂, 40 mM NaCl | nM       | 70 ± 4  | 120 ± 3  | 310 ± 27 |
| 0 mM MgCl₂, 80:20 mM NaCl/KCl | nM | 35 ± 1  | 110 ± 12 | 110 ± 3  |
| 1 mM MgCl₂, 80:20 mM NaCl/KCl | nM | 53 ± 8  | 270 ± 32 | 260 ± 15 |

TABLE III

Dissociation constants for SNV N protein with various RNAs

| Reaction condition | Substrate | vRNA | s ORF |
|--------------------|-----------|------|-------|
| 1 mM MgCl₂, 40 mM NaCl | nM       | 34 ± 6 | 110 ± 22 |
| 0 mM MgCl₂, 80:20 mM NaCl/KCl | nM | 11 ± 1  | 200 ± 28 |
| 1 mM MgCl₂, 80:20 mM NaCl/KCl | nM | 110 ± 9 | 170 ± 9  |

Fig. 5. Saturation binding curves of various RNA substrates and the HTNV N protein under different solution conditions. Binding reactions were assembled in 40 mM NaCl (A), 1 mM MgCl₂ and 40 mM NaCl (B), 0 mM MgCl₂, 80 mM NaCl and KCl (C), and 1 mM MgCl₂, 80 mM NaCl and KCl (D) in addition to standard reaction components as described under "Experimental Procedures." 32P-Labeled RNA (●, vRNA; ●, s ORF RNA; *, control RNA) was incubated with the indicated molar concentrations of HTNV N protein (x axis). The amount of radioactively labeled N protein retained on the filter was calculated relative to the maximum radioactivity retained in each experiment (y axis).
difference in the two protocols is the use of NaCl throughout our purification scheme. During development of the optimal strategy for purification of HTNV and SNV N proteins, we found that addition of NaCl to the extraction buffer greatly increased the solubility of the N protein (21). In addition, our protocol allows for the complete refolding of the N by eliminating urea with extensive dialysis, and the inclusion of EDTA and the reducing agent, DTT, in the refolding of the protein. Thus, our strategy should result in a protein with a more native conformation than was reported earlier. In support of this, the same strategy has been used with great success to obtain highly active retrovirus enzymes such as the HIV-1 RNase H (26), as well as the murine leukemia virus (27) and human T-cell leukemia virus (17) integrases.

No comprehensive studies have addressed N protein binding in the family Bunyaviridae. Gel electrophoretic mobility shift assays (GEMSA) were used to characterize the RNA binding activity of N proteins of the Tospovirus tomato spotted wilt virus, and the hantavirus PUUV N proteins (15, 28). The RNA binding activity of the HTNV N protein was also examined following refolding of the protein on nitrocellulose filters (15, 28). The effect of ionic strength, metal ion, and redox conditions on the binding of RNA has not been previously reported for any hantavirus N protein. Therefore, in the development of these assays, we were interested in obtaining quantitative tools to measure conditions affecting RNA binding and to compare the binding of various RNAs under a variety of solution conditions. Herein, we have presented two assays, UV cross-linking and filter binding, with which to study hantavirus N protein-RNA interactions. A wide range of protein concentrations were noted to be effective for RNA binding in both assay systems and were found to be similar to the concentrations used in PUUV N protein GEMSA (15). The effect of metal ions on the interaction of the HTNV N protein and its vRNA was not notable until large concentrations of Mg$^{2+}$ were added to the binding buffer. However, when we compared the binding affinity of the HTNV N protein with alternative substrates, a clear preference was observed for the vRNA in the presence of the metal ion. Metal ions can play a role in RNA-protein interactions by enabling specific RNA folding and stability (24, 29). We suggest that addition of Mg$^{2+}$ may have increased secondary structures in the vRNA template and thereby reduced the nonspecific interactions based on structural affinities. Alternatively, Mg$^{2+}$ may have competed with the protein for nonspecific van der Waals interactions with the phosphodiester backbone (25). The metal ion may act to mimic the presence of a protein factor (e.g., hantavirus L protein), and thereby enhance the binding specificity of the N protein with vRNA and cRNA as opposed to mRNA. Precedent for this type of mechanism has been shown with the VSV and rabies virus P proteins, which increase the specificity of N protein encapsidation for their genomic or leader viral RNAs (11, 30). Examination of the effect of ionic strength on the binding reactions by filter binding and UV cross-linking assays showed that HTNV ribonucleoprotein complexes were stable to a wide range of ionic strength. This suggests that close range nonelectrostatic forces such as van der Waals and not electrostatic interactions play a predominant role in N protein-vRNA interactions. The lack of salt dependence in complex formation has been reported for other RNA-binding proteins such as the R17 coat protein (31), and the S15 (32) and L11 ribosomal proteins (33). Clarification of the mechanisms underlying these finding awaits a more thorough kinetic and thermodynamic analysis of the N protein and the vRNA.

In addition to defining the parameters for encapsidation of the HTNV vRNA by HTNV N protein, we were interested in determining whether the HTNV vRNA could be encapsidated by a heterologous N protein from a distant member of the Hantavirus genus, SNV. HTNV and SNV N proteins share 63% identity, and represent two distinct phylogenetic classes of hantaviruses (34). The N complexes formed by HTNV and SNV were distinct as revealed by their differential susceptibility to RNase V1 and RNase T1. The comparison of the binding isotherms for the HTNV or SNV N proteins and three RNA substrates revealed a preference for the HTNV N protein for its vRNA as compared with the other RNA substrates tested. Our results suggest that specific interactions, albeit with moderate affinity, exist between the HTNV N protein and the terminal regions of the vRNA and cRNA. These results are similar to those reported for the rabies virus N protein (11), in which a 3-fold greater binding was shown for the viral leader RNA than the N mRNA. In their analysis, Yang et al. also examined the binding of the vRNA to a nonviral control RNA. As observed for the hantavirus N protein, the vRNA had a greater affinity for a viral than a nonviral RNA. The studies reported herein are also in agreement with previous observations with La Crosse virus in which the signal for encapsidation was suggested to be in the terminal complementary nucleotides (13) and in a separate study, which showed the mRNA is not a preferred substrate for encapsidation (14). However, further analysis is required to define where within the untranslated region the specificity occurs. Previously, it was reported that the binding of the PUUV N protein to a vRNA was nonspecific as noted in a tRNA competition assay, and when compared with binding to a PUUV RNA, which contained only the PUUV ORF (15). We offer a different interpretation of the PUUV N protein GEMSA results. First, the authors observed an increase in the retardation of genomic S-RNA with the addition of increasing amounts of protein. We interpret this finding to suggest the presence of multiple binding sites and cooperativity (35). In addition, in the previous study, the authors indicated that GEMSA showed reduced binding of PUUV N protein and vRNA in the presence of tRNA. However, without quantitation, we do not find this interpretation compelling. Further, the presence of the tRNA appeared to have little impact on the formation of the higher ordered complexes as the concentration of N protein was increased. The second line of reasoning for their conclusions for an overall lack of specificity of the N protein for its vRNA template came from comparing its binding with the PUUV ORF RNA, for which they reported little change in the GEMSA pattern when compared with the vRNA or tRNA competition. If one examines the retardation of all the complexes, however, one can clearly observe a reduction in the mobility of the PUUV N protein-PUUV S ORF RNA complexes as compared with the others. Overall, we suggest that the conditions for vRNA specificity by the PUUV N protein were impossible to accurately ascertain with the GEMSA analysis performed. Finally, in the earlier study, the authors seemed to discount the importance of the GEMSA using a construct representing the flanking regions of the PUUV ORF and the PUUV N protein to show substrate preference. Interestingly, at all concentrations of PUUV N protein examined, a large aggregate is observed (well shift) in the case of the PS2-PS2+ RNA, partially double-stranded RNA. We offer that these experiments suggest a region of the vRNA with a higher affinity for the N protein. Further, we suggest that these interpretations taken in conjunction with our data support a model in which binding of the N protein to its vRNA proceeds through cooperative interactions among the N proteins that initiate in the terminal regions of the RNA, possibly at the 5′ end.

These studies suggest the HTNV N protein relies primarily on nonelectrostatic forces to recognize structural and/or se-
sequence determinants in its vRNA and cRNA. Further, the reduced affinity of the SNV N protein for the HTNV vRNA suggests unique structures and/or sequences in the RNA and/or protein have evolved for these two viruses. Future work will address the site of encapsidation of the vRNA and the thermodynamics and kinetics underlying the interaction between the N protein and viral RNAs. The solution conditions we have defined herein will also be useful for mapping the RNA binding domain that renders specificity to the vRNA.

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