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The RNA binding and RNA chaperone activity of Sin Nombre virus nucleocapsid

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**THE RNA BINDING AND RNA CHAPERONE ACTIVITY OF
SIN NOMBRE VIRUS NUCLEOCAPSID**

BY

BRADLEY A. BROWN

B.S., Biochemistry, Texas Tech University, 2002

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biomedical Sciences**

The University of New Mexico
Albuquerque, New Mexico

August 2008

DEDICATION

For Whitney and my Family

ACKNOWLEDGEMENT

I would like to thank Nito for giving me the opportunity to work in his lab for the past five years. His guidance and patience these years has been the cornerstone of my education. Nito's knowledge and dedication to science has provided an excellent environment for learning and developing as a scientist. I will be forever in his debt for his advice, time and most of all friendship.

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The RNA Binding and RNA Chaperone Activity of Sin Nombre Virus

Nucleocapsid

by

Bradley A. Brown

B.S., Biochemistry

Doctor of Philosophy in Biomedical Sciences

ABSTRACT

Sin Nombre Virus (SNV) is a member of the hantavirus genus in the

Bunyaviridae family. Hantaviruses contain a tripartite negative sense RNA

genome with individual segments that form panhandle structures through

intramolecular interaction between the 5' and 3' termini. This panhandle

structure is recognized at high affinity by trimeric viral nucleocapsid protein (N),

and this interaction is likely to be important for specific encapsidation of viral RNA

(vRNA) into nucleoprotein complexes in cells, for incorporation of vRNA into

assembling virus particles, and for initiation of genomic vRNA replication. N is

also capable of unwinding RNA/RNA duplexes as an RNA chaperone. This

function is critical for rescuing kinetically trapped misfolded RNAs. We examined

the regions of N required for high affinity binding to the viral RNA panhandle and

isolate the domain responsible for RNA helix unwinding using a set of mutations

in the SNV *n gene*. These data indicate that components of both the N- and C-

termini of the N are necessary and sufficient for vRNA panhandle recognition. It

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is possible to functionally replace the C-terminal region with a foreign trimerization domain, the T4 phage fibritin domain, and maintain high affinity binding. Thus, N trimerization, mediated by the C-terminus, in conjunction with a putative RNA binding site in the N-terminus, appears to be required for panhandle recognition. In contrast, the RNA chaperone function is not dependent on trimer formation. The domain responsible for RNA helix destabilization resides within the putative disordered region of the N-terminal 100 amino acids of N. Surprisingly, rescue of trimer formation with the addition of the T4 fibritin domain abolishes RNA chaperone activity. We demonstrate here that the N-terminus of SNV N facilitates both high affinity vRNA binding and RNA chaperone activity.

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Chapter 1: Introduction

Sin Nombre Virus

Sin Nombre Virus (SNV) is a member of the hantavirus genus in the family *Bunyaviridae*. Viruses in *Bunyaviridae* are classified into Hantavirus, Nairovirus, Orthobunyavirus, Phlebovirus, and Tospovirus genera. They are enveloped viruses approximately 80-100 nM in diameter and have a tripartite negative sense RNA genome (Fig. 1).

The 5' and 3' ends of the three genomes segments contain triplet repeats. These triplet repeats, along with adjacent bases, facilitate the folding of the vRNA and aid in the creation of panhandles. The panhandles are formed when the 5' and 3' ends of the molecule fold back and interact with each other through imperfect hydrogen bonding (Fig. 1). These panhandles function in replication and presumably packaging of the ribonucleoprotiens (RNPs) into the virion. The three genome segments referred to as S, M and L, each encode one structural polypeptide. The S-segment encodes the viral nucleocapsid (N) whose main function is binding of genomic RNA. Recently, an approximately 14 KDa protein coded for in the +1 reading frame from the S-segment has been reported for both Tula virus (TULV) and Puumala virus (PUUV) (45). This protein, designated non-structural S (NSs) affects the activity NF-κB and interferon promoters in Cos-7 cells (45). No homologous protein has been reported for SNV.

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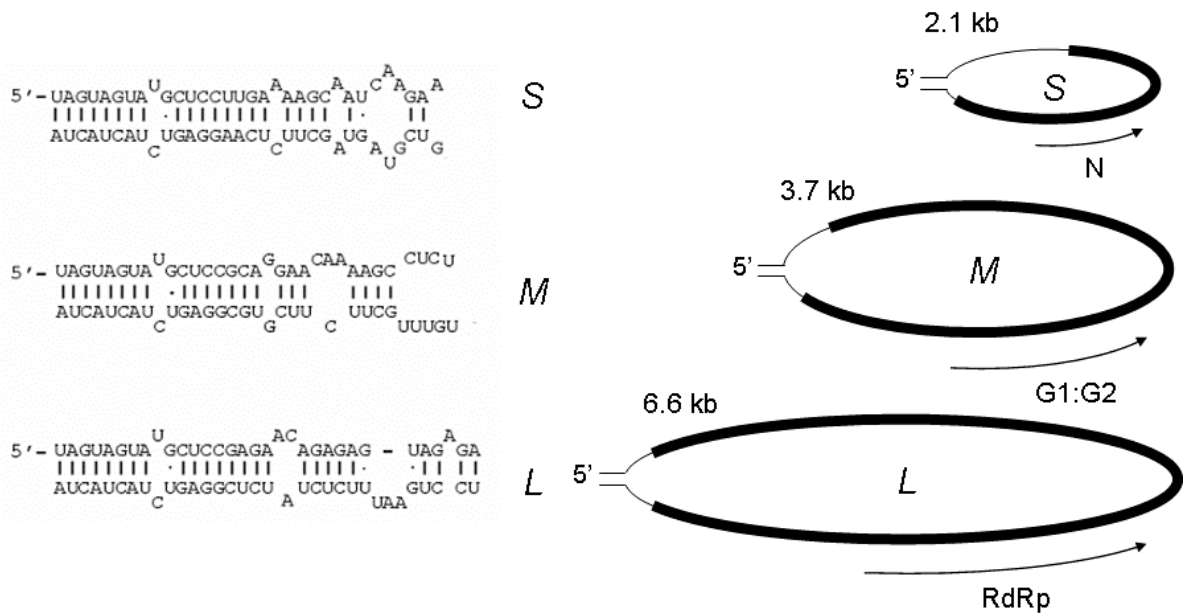
FIG. 1. SNV genome displaying panhandle nucleotide pairing. The SNV genome is composed of three segments named S, M and L with each segment encoding one polypeptide. Each segment forms a panhandle when the 5' and 3' ends of the segment fold back and interact with each other. Each panhandle is unique in secondary structure but is specifically recognized by SNV N.

The M segment encodes the glycoprotein precursor (GPC). The GPC is translated as one continuous peptide and then co-translationaly cleaved into G1 and G2. The cleavage site for Hantaan virus (HTV) GPC has been mapped to a conserved WASSA amino acid sequence in the middle of the peptide (60). After cleavage G1 and G2 interact to form a heterodimer, required for co-localization to the golgi (103). For *Bunyaviridae*, budding takes place at the golgi, with the exception of SNV which buds from the plasma membrane (36).

The L segment encodes the viral RNA dependent RNA polymerase (RdRp) responsible for cap snatching, transcription and replication of the genome (32). The RdRp, a 250 kDa protein, is presumed to be membrane associated and localizes to the perinuclear region in TUUV infected cells (52). During replication the RdRp or associated components initiates the change between transcribing mRNA and replicating vRNA from cRNA.

Medical Importance of Sin Nombre Virus

SNV is an important human pathogen that was first recognized during an outbreak in the southwestern United States in 1993 (79). Hantaviruses cause two forms of human disease. HTV, PUUV and other "old world" viruses are the causative agents for hemorrhagic fever with renal syndrome (HFRS). HFRS causes vascular leakage primarily affecting the kidneys and potentially causing acute renal failure. SNV and Andes viruses (AV) are the prototypical "new world" hantaviruses that cause hantavirus cardiopulmonary syndrome (HCPS) which

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carries a mortality rate of approximately 35%. The symptoms of HCPS involve vascular leakage primarily in the lungs and heart. Hantaviruses are unique in the *Bunyaviridae* family because they are transmitted to humans from rodents, which are the main reservoir in nature. Other members of *Bunyaviridae* are associated with arthropods as their reservoir in nature. Each species of hantavirus is closely associated with a particular species of rodent, which shows no apparent signs of hantavirus-induced disease. SNV is carried by *Peromyscus maniculatus*, and is transmitted to humans through inhalation of aerosolized excreta. There have been reports of person-to-person transmission of AV but no such transmission has been reported for SNV (66, 85). In addition, SNV has been listed as a possible bioterrorism agent increasing the need for further understand the molecular virology of the virus.

Hantavirus Entry

The receptor for pathogenic hantaviruses, that cause HFRS and HCPS is $\alpha_v\beta_3$ integrin (Fig. 2) (46). Attachment occurs through an Arginine-Glycine-Aspartate (RGD) independent mechanism (33, 35). Non-pathogenic hantavirus such as Prospect Hill virus (PHV) enter using a different set of receptors. The use of a different entry receptor for non-pathogenic hantaviruses has been postulated to be the reasons for a lack pathogenesis exhibited by PHV (Fig. 2) (35).

Internalization of the virus is mediated through a clathrin dependent mechanism. Entry appears to proceed through the endosomal and lysosomal pathways and requires low pH for productive infection. Treatment of cells with

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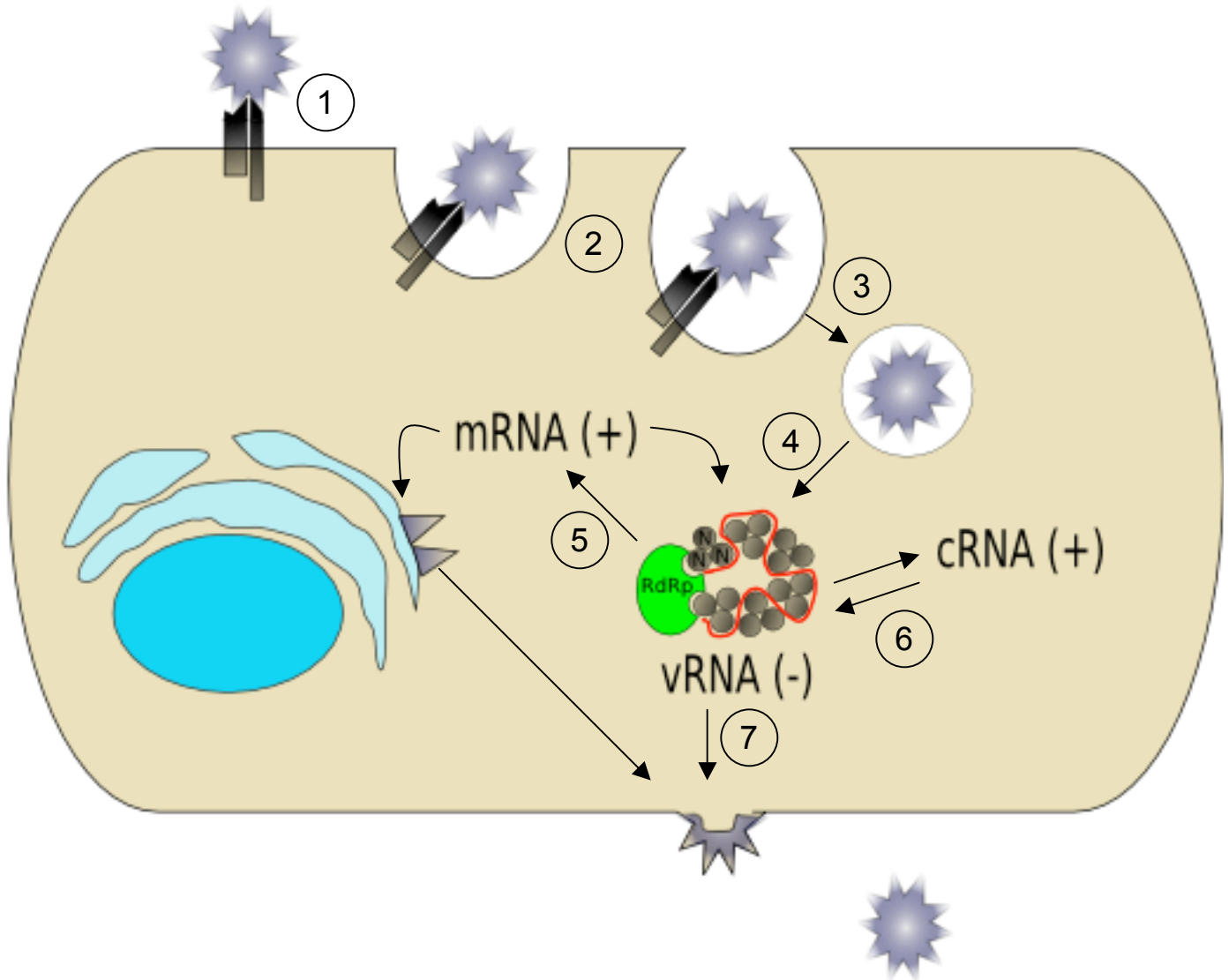
FIG. 2. Schematic representation of replication of SNV. 1. SNV binds its receptor $α_v\beta_3$. 2. The virus is internalized using a clathrin dependent mechanism. 3. The virus particle is shuttled into the endosomal and lysosomal pathway. 4. The low pH of the endosome uncoats and releases the RNP into the cytosol. 5. mRNA is synthesized in newly infected cells reaching detectable levels within 2

hours. 6. Six to eight hours post infection negative sense vRNA is synthesized. 7. For SNV the G1 and G2 initiate budding from the plasma membrane.

agents that increase the pH of endosomal and lysosomal compartments show a drastic reduction in number of cells infected (46). Small decreases in pH cause conformational changes in the surface glycoproteins for Uukuniemi Virus (UUKV) (82). The decrease in pH in endosomes is believed to release the viral RNP complex into the cytoplasm, through membrane fusion between the virion and vesicle.

Hantavirus Replication

Bunyaviruses replicate exclusively in the cytoplasm of infected cells, unlike Influenza which replicates in the nucleus (106). For SNV, positive sense mRNA is synthesized first by the viral ribonucleoprotein (RNP) in newly infected cells (25, 47). The mRNA is made using a "cap snatching" mechanism to prime the synthesis of its own RNA similar in mechanism to influenza. In the prime and realign mechanism a capped 5' 7-me-GTP mRNA from the host interacts with the 3' end of the vRNA. During this interaction the polymerase cleaves the host mRNA (between 2-17 nucleotides) and uses the small capped RNA as a primer to begin transcribing viral mRNA. After transcribing 3 to 5 bases of new mRNA the polymerase slips back and realigns the newly synthesized bases with the vRNA template, then continues elongating its mRNA transcript (32). This realignment is made possible by the triplet repeats at the ends of each of the template vRNA segments (32). Transcription continues through each of the open reading frame and then is terminated before the end of the segment. The viral mRNA contains no poly A tail unlike influenza and rhabdoviruses (32).

Later in replication the RNP switches from transcription of mRNAs to cRNA synthesis. Unlike mRNA, cRNA is not capped and is full length. This cRNA is then used by the RNP to make vRNA that is suitable for packaging into virions. For influenza, the switch between mRNA and cRNA synthesis is mediated through a difference in tertiary structure between cRNA and vRNA panhandles (86). The PB1 peptide, from the heterotrimeric influenza RdRp, is responsible for the recognition of cRNA and vRNA so it is assumed that the RdRp from SNV is also responsible for the recognition of the promoter in the vRNA panhandle (110). The La Cross virus (LCV), another member of the *Bunyaviridae* family, regulates its replication by encapsidation of not only the vRNA but also the cRNA and mRNA (39). This effectively limits the translation and transcription of these encapsidated segments leading to lower levels of virus protein in the cell. A similar phenomenon has not been demonstrated for SNV. However, SNV N does show an affinity for cRNA so it is possible that SNV uses a similar mechanism to control its RNA synthesis (74).

Bunyaviridae RNA synthesis requires active translation to be carried out in the host cell. Both puromycin and other translation inhibitors are known to inhibit active transcription *in vitro* and in infected cells. This observation has led to a model where active translation of the segments being transcribed is required to remove secondary structure that forms in the newly transcribed mRNA. The model for this resembles prokaryotic translation, where ribosomes are required to be actively translocating along the mRNA to remove secondary structure (39).

When ribosomes are not translating mRNA, secondary structures arise in the RNA. These secondary structures inhibit RdRp activity resulting in premature termination of mRNA transcripts (4).

Virus Budding

The two glycoproteins G1 and G2 are the primary candidates for initiating virion formation. The glycoproteins from UUK virus are both necessary and sufficient for forming virus like particles (84). This result is in disagreement with previous results for HTV, which requires N along with G1 and G2 for formation of virus like particles (VLP) (13). The ability for G1 and G2 alone to form virus like particles is different from other negative strand viruses such as semliki forest virus, which uses its nucleocapsid to initiate budding and influenza which requires a minimum of four proteins for virus like particle formation (53). For UUK virus the virion itself is given shape (T≈12) by the glycoproteins (82).

Bunyaviruses lack a matrix protein so presumably there is a direct interaction between the RNP and the G1-G2 heterodimer. For UUKV this interaction has been demonstrated between G1 and N (83).

Nucleocapsid: N-N Interaction

SNV N is a homotrimeric 48 kDa protein with an overall neutral charge (pI=6.8). The primary pool of N in virions and infected cells are assembled as trimers (1, 48). N proteins from the different hantaviruses are highly conserved (Fig. 3). PUUV and TULV N are primarily trimeric *in vivo* similar to SNV (48). The

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interaction between N monomers appears to be electrostatic by its susceptibility to high ionic strength. In addition N-N interaction is enhanced by the addition of divalent cations (48). This interaction for TULV N is also mediated by multiple contacts along N. N-N Interaction takes place across five major regions along the length of N. The most important region for trimer formation appears to be near the C-terminus from amino acids 392 to 396 (50). This region is also predicted to form a coiled-coil structure from amino acids 367-388 that facilitates multimerization. The tryptophan at position 388 plays a critical role in the stabilization of N-N interaction as mutation of tryptophan at 388 to alanine completely disrupts N-N interaction. This residue is conserved among many hantaviruses (49).

N-N interaction is also facilitated through interactions in the N-terminus. Predicted coiled-coils in the first 75 amino acids are present in many of the different hantaviruses and are thought to play a role in self association (1). These first 75 amino acids when expressed separately form higher order oligomeric structures *in vitro* (2). The N-terminal 73 residues have been crystallized with the help of the T4 phage fibritin domain and form a α-helical coiled-coil structure. The crystal structure showed no evidence for intermolecular association between the N-terminal 73 residues of N (16). The predicted coiled coil structures in the N-terminus of TULV are nearly identical to that of SNV. Removal of the first 78 residues from the N-terminus of TULV N causes reduced association between N molecules. In addition two residues from TULV N are important for maintenance
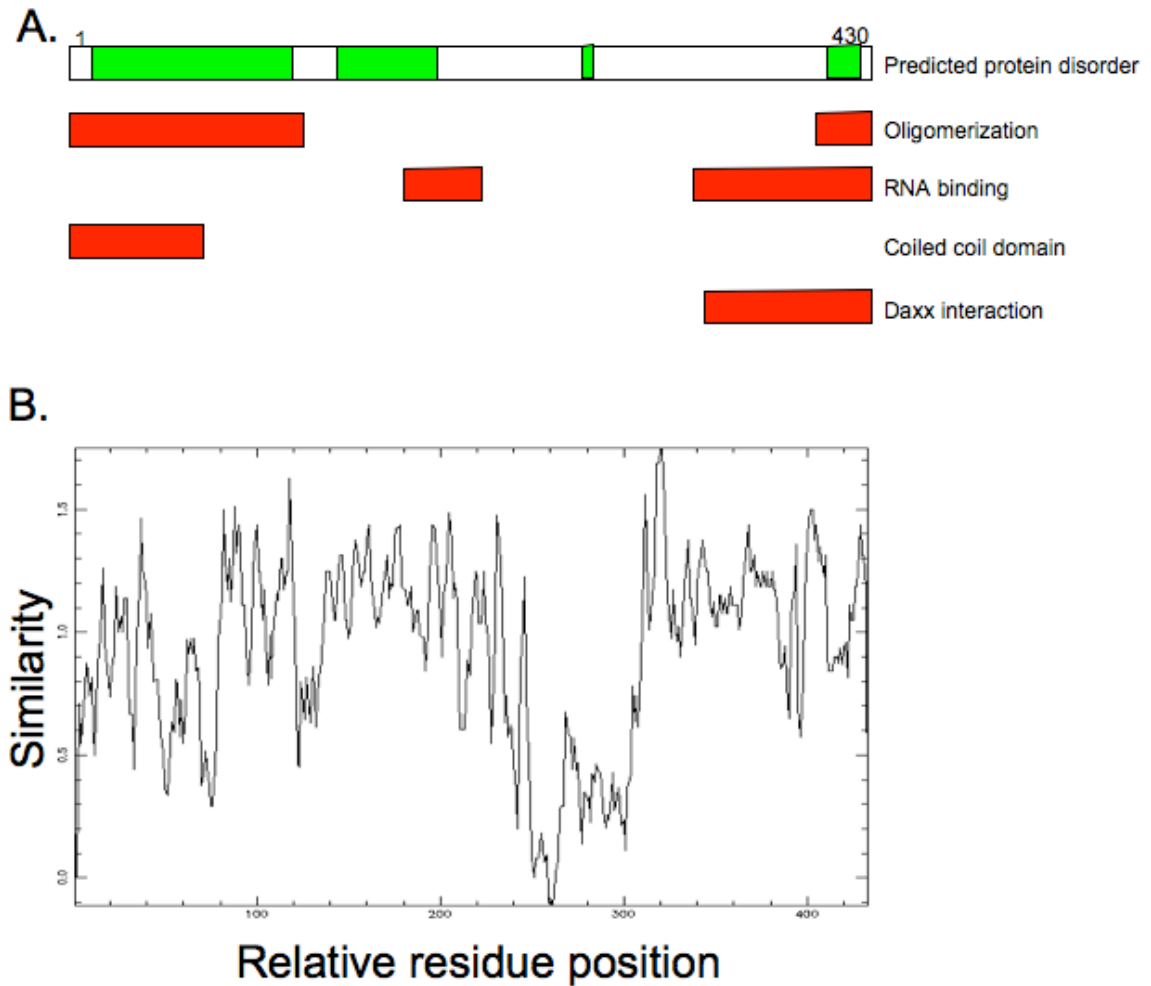
FIG. 3. Schematic representation of Hantavirus N. A. Regions of known interaction for hantavirus N. Oligomerization domain compiled for data from TULV, PUUV, SNV and HTV ((1-3, 48-50, 115). The RNA binding domain was mapped for PUUV and HTV (37, 101, 113). The crystal structure for coiled coil domain demonstrates intramolecular bonds (16) while the region for Daxx interaction is described in (58). B. Represent graphical representation of similarity

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in amino acid sequence between different Hantavirus nucleocapsids, represented

in the analysis PHV, TULV, SNV, PUUV, by Plotcon (EMBOSS).

of the interaction appear to be valine at position 51 and leucine at position 58, that are both conserved between SNV and TULV (3, 115). These N-terminal interactions do not appear to be as critical as the C-terminal interactions for individual association. Even with removal of the first 78 amino acids N-N interaction is still detectable, whereas deletion of the coiled coils at the C-terminus abolishes all detectable N-N interaction (3, 49). Intramolecular N-N interaction is required for proper internal localization of N in cells (3).

A role for both the N and C termini in multimerization of N proteins is also a demonstrated for other members of *Bunyaviridae*. Tomato Spot Wilt Virus (TSWV), genus tospovirus, contains both N-terminal and C-terminal domains capable of N-N interaction. Deletion of the N-terminal 39 amino acids of TSWV N abolishes interaction with itself but is still capable of interaction with wild type N supporting the head to tail model for arrangement of these molecules (109). This same N and C terminal requirement is also demonstrated for bunyamwera virus (BUUNV) (genus orthobunyavirus). For BUUNV, N-N interaction is facilitated by the N-terminal 10 amino acids and C-terminal 17 (55). Hantaviruses N has a different stoichiometry of multimerization compared with N from other bunyaviruses. For BUUNV and Rift Valley Fever Virus (RVFV), N the basic subunit composition appears to be dimeric while hantaviruses N is trimeric (54, 55).

N from hantaviruses binds to cellular proteins that could effect the host's response to infection. One of these cellular proteins that N interacts with is the

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apoptosis enhancer DAXX (58). DAXX has multiple roles inside the cell but primarily functions as transcription repressor (58). The interaction of N with DAXX could facilitate an anti-apoptotic response in cells.

Hantavirus N also interacts with both small ubiquitin-like modifier (SUMO) and ubiquitin conjugating enzyme-9 (Ubc9), which attaches SUMO to proteins enzymatically. The interaction of N with Ubc9 and SUMO has been mapped to amino acids 188-191 for HTV (62). The role of N-Ubc9-Sumo-1 interaction in viral replication remains unexplained. N interaction with Ubc9 does show some correlation with the subcellular localization of N and possibly a N-Ubc9-Sumo-1 complex interacts with DAXX affecting the cellular response to infection (62).

Nucleocapsid RNA Binding

After an RNA virus invades a cell it must of package its own genome into virions and exclude other RNAs present in the cell. RNA viruses also must distinguish between their genome and anti-genome which both contain similar higher order terminal RNA structures. Negative sense RNA viruses often recognize their vRNA by interaction with the 5' and 3' ends alone or in panhandle configuration. For SNV this job is presumably handled by N, which is capable of discriminating between not only its vRNA and cellular RNA but also the cRNA produced by the virus (74). A similar interaction mediated by HIV NC recognizes encapsidation signals in its vRNA (67-69). This interaction allows for efficient packaging of the viral genome into new particles. Mutations in HIV psi encapsidation region lead to decreased packaging efficiency (67). These RNA

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protein interactions are critical to the replication and packaging of RNA viruses. Typically the higher affinity of a protein for its substrate RNA is correlated to higher specificity. If it were not for specific association between hantavirus N and vRNA the vRNA would be subject to degradation, inefficient replication/transcription and ineffective packaging.

A primary role of SNV N is to recognize and bind to genomic vRNA. N recognizes its own vRNA by interaction with the panhandle formed when the 5' and 3' ends of individual segments interact. This presumably creates a very specific 3-dimensional structure that is recognized by the N that is virus specific (Fig. 4) (75). The RNA binding of SNV N to its vRNA causes a conformational change in the protein that is detectable through changes in the tryptophan fluorescence of N (74). This specific interaction has been mapped to a central region from 175-217 for HTV N (101, 113). However, these results are in contrast with earlier results that showed the C-terminus of HTV and PUUV N were responsible for RNA binding (37). For other members of *Bunyaviridae* multiple RNA binding regions have been mapped to the N and C-terminus of N (94).

Perhaps the most characterized protein-RNA interactions are those for the bacteriophage MS2, Qß and R17 (18-20, 95, 112). These coat proteins have disassociation equilibrium constants of ≈1 nM (MS2) and ≈3.6 nM (R17) for their specific operators (19, 112). The operators for these viruses form stem loop structures, which can be considered analogous to the panhandles formed by the hantavirus segments.

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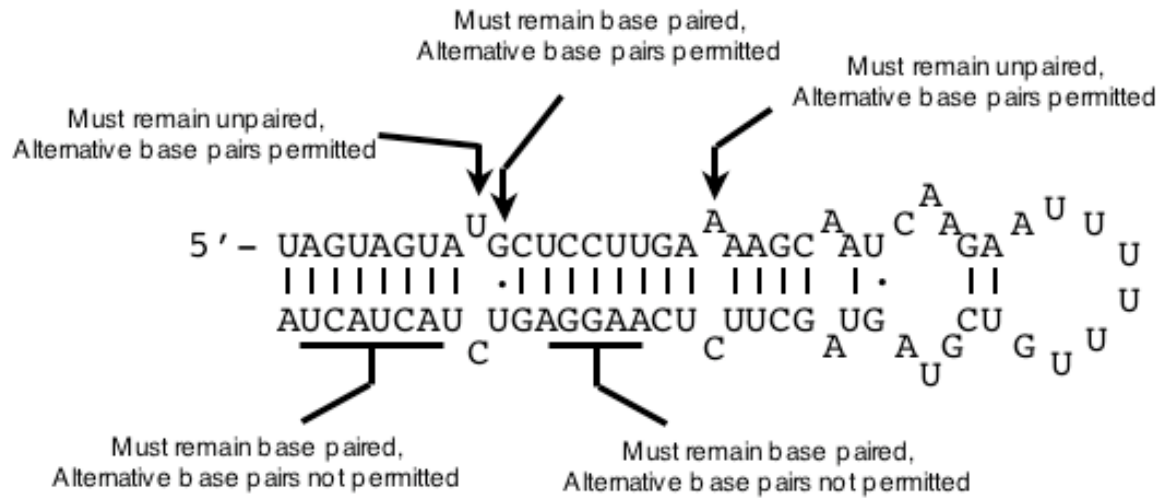
FIG. 4. Important bases in recognition of SNV panhandle by N. The base

pairing of the triplet repeats present on the 5' and 3' termini are required for

interaction with N. The U residue at 9 cannot be basepaired but alternative

bases are tolerable in binding to vRNA. Finally, the A at position 19 must all

remain unpaired however alternative base pairs are also permitted (74).

The nucleocapsid protein (Np), from negative sense RNA virus, Influenza is capable of binding vRNA with affinity of 20 nM (8). However, Influenza Np demonstrates little sequence preference since it also binds tRNA with similar affinity (8). The primary role of Np is protection of the vRNA. Each vRNA segment from influenza forms an RNP that consists of polymerase (PA, PB1, PB2), Np and vRNA. A similar structure is presumed for SNV RNPs. NS1 is a dimeric double stranded (dsRNA) binding protein from influenza which functions in attenuation of host response to viral infection, through interaction with host cellular proteins and binding of dsRNA. NS1 requires dimer formation for its interaction with RNA, which is facilitated by α-helices formed in the N-terminal 73 amino acids of the protein (70, 111). SNV N also forms α-helices in the N-terminal 75 amino acids of N (16) which may be involved in binding of vRNA.

The retroviral NCs, including that of HIV-1, are some of the most well-studied nucleocapsid peptides of RNA viruses. The crystal structure of HIV NC has been determined. NC interacts with 4 hairpins that are present on the psi encapsidation region on the genomic RNA (10, 68). This interaction is facilitated by two Zinc finger motifs on NC leading to a NC-vRNA binding affinity of Kd≈90 nM (40). The recognition of the psi encapsidation region by NC is required for proper encapsidation of the vRNA (11). The psi encapsidation signal is analogous to the panhandles formed by many negative sense RNA viruses, which are used for encapsidation.

The interaction between SNV N and its vRNA panhandle is presumed to be the critical in initiating the packaging step of the vRNA. It is this interaction that presumably selects the vRNA for encapsidation and discriminates against packaging non-viral RNAs. This mechanism is conserved for all members of genus hantavirus is likely expandable to the *Bunyaviridae* family, and perhaps found in other families of minus strand viruses.

RNA Chaperones

RNA chaperones are a broad class of molecules that facilitate the proper folding of RNAs (99). RNA is a highly flexible structure that is able to assume many different conformations that lead to a broad range of functions for the molecule. From a large pool of potential structures, RNA must assume a very limited number of 3-dimensional conformations that are active for biological function. Misfolded RNA generally is not as thermodynamically stable as the RNA in active conformation. However, the kinetics of unwinding misfolded RNA is relatively slow on a biological time scale. Because RNA in solution does not have sufficient energy to overcome these "kinetic traps" RNA chaperone proteins are needed to assist with the unfolding of misfolded RNA.

Having one of three activities *in vitro* identifies RNA chaperones. Classical chaperone activity is considered the ability to actively promote correct RNA/RNA interaction. For example, this activity is characterized by the catalysis of a dsRNA from two single stranded RNAs at a higher rate than the two complementary RNAs alone (90). Obviously If the two sequences share enough complementarity

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this reaction will occur in the absence of protein but it is accelerated with an RNA chaperone present (88). In the case of the annealing of + and – sense Tar hairpin sequence from HIV, the function of the chaperones is to not only to promote annealing but also remove any intramolecular folding of the RNAs so that they are capable of interaction with each other (44). The model for protein assisted RNA duplex formation requires either two RNA binding proteins capable of interaction to bring RNAs together, or a protein with two individual RNA binding sites. In the latter case, when RNA is bound by one site the second site must be available for binding. The binding to the second site increases the local concentration of RNA thereby facilitating duplex formation (89).

A second related activity of RNA chaperones can be to facilitate strand displacement (90, 99). The first step in this process is the unwinding of the initial RNA/RNA duplex. This function requires that the protein is capable of binding to and unwinding dsRNA into single stranded RNAs. The generation of a new more stable RNA/RNA duplex can be obtained in two ways. First, disassociation of the duplex simply allows the RNA to interact with different RNA molecules that is potentially a more stable pairing partner. This is the way proteins such as ribosomal protein S1 functions since these chaperones show no RNA annealing activity (9, 89). If the protein is capable of binding two RNAs and forming duplexes, it is likely that it uses this ability to facilitate the interaction between the two newly unwound RNAs.

To be classified as a functional RNA chaperone the protein does not need to contain both of the functions described previously but is required to have at least one these activities. RNA helix destabilization (RHD) activity is the primary activity measured when strand displacement is used to assay for chaperone activity. The ability of proteins to serve as RNA chaperones greatly increases pool of functional RNAs in a system.

A useful assay for RNA chaperone activity is monitor acceleration of ribozyme activity for both the R3 and HH16 ribozyme (90, 99). This activity can detect a combination of the two previously described functions for chaperone activity. An RNA chaperone can facilitate the proper folding of the ribozyme by destabilization of misfolded RNA to attain its active conformation. A chaperone can also increase the annealing step of the substrate to the ribozyme. HIV NC is capable of increasing annealing by 20 fold over a ribozyme in absence NC (42). RNA chaperones do not affect the catalytic step of cleavage of the substrate RNA into product but increases disassociation of cleaved product from the ribozyme allowing for increased turnover. HIV NC increases disassociation of products from the ribozyme by 20 to 30 fold over the ribozyme alone (42). This enhancement of ribozyme activity is a hallmark function of RNA chaperones because it measures both the annealing and disassociation RNA chaperone activities.

Because of the vast number of structures that RNA can form it is not surprising that a conserved consensus sequence common to RNA chaperones

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has not been reported. However, it is thought that regions of the proteins that are generally disordered (protein structure cannot be resolved) are important in chaperone function. These disordered domains are thought to function in two ways. The disordered regions can potentially bind a broad range of RNA molecules. This is an advantage when dealing with RNA since many semi-stable conformations can be formed. The disorder allows recognition of these varied RNA conformations, allowing one protein to potentially facilitate many different RNAs in folding.

Many protein and RNA chaperones contain a high level of disorder. The predictor of natural disorder (PONDR®) estimates a level of disorder along a linear polypeptide (97). The prediction for known RNA chaperone and protein chaperones show large regions of predicted disorder (107). Disordered regions of proteins, that are highly dynamic, have increased entropy. Since many RNA chaperones generally function using an ATP independent mechanism they must unwind the RNA duplex without using chemical energy. When a disordered protein binds RNA, becoming more ordered, the decrease in entropy is transferred to the RNA , increasing the entropy of the RNA which is predicted to facilitate unwinding of the duplexed RNA (107). This entropy transfer model proposes to link protein disorder to RNA chaperone activity.

SNV N is an RNA chaperone that is capable of resolving misfolded structures in RNA by unwinding RNA/RNA duplexes (73). HIV NC represents one of the most well characterized RNA chaperones (42, 108). HIV NC is a

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disordered protein (47% disorder) that is capable of accelerating ribozyme activity, promoting strand annealing and displacement (42). This activity can be mapped to disordered regions in the N and C-terminus of the protein as removal of both of these regions reduces or ablates its RNA chaperone activity (43). NC chaperone activity functions in packaging of the viral genome by facilitating the vRNA dimerization (30, 31).

Flaviviridae are a diverse family of positive sense RNA viruses that share similar genomic structure. The primary RNA binding protein of this family is the core protein. However, there is very little homology between core proteins in members of this family. However, they are similar in that they do share long regions of predicted disorder and are capable of binding RNA (44). These domains are sufficient for RNA chaperone activity since all tested core proteins exhibit chaperone function (44). The ability for the core proteins to survive extreme heat and still function support the hypothesis that chaperone activity is linked to protein disorder (44). HCV core will also facilitate DNA/DNA duplex formations in addition to RNA/RNA annealing activity. In contrast, SNV N is only functional on RNA/RNA duplexes that contain a unpaired 3' base (72). In addition the activity of N to unwind duplexes is pH dependent, requiring a pH between 6.5 and 8. SNV N is also capable of unwinding the panhandles formed by the genome segments. During the unwinding of the panhandles N stays preferentially bound to the 5' end of the disassociated panhandle. This activity implies that N may play a role in transcription of the SNV genome. In the model described by

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Mir and Panganiban (72) the association of N with the free 5' end would allow the 3' end to associate with the RdRp for transcription (Fig. 5). In addition, to this it is likely that chaperone activity plays a role in restructuring of the RNP in preparation for packaging into virions.

SNV N is a multifunction protein. Its recognition of RNA has been extensively studied for other members of the hantavirus genus (101, 113). Its role as an RNA chaperone has increased our understanding of the role N in replication. Despite these advances the role of trimer formation on RNA binding and chaperone activity has not been characterized. Examining the link between multimerization and RNA chaperone activity will expand our understanding of N-RNA interactions for not only SNV but also for the entire hantavirus genus and possibly has implications for all negative sense RNA viruses.

A — newly synthesized Linear vRNA substrate in nucleocapsid form

B — panhandle formation mediated by N and low *P-num* nucleotides

C — Specific, high-affinity interaction between N and the panhandle

D — N-mediated panhandle dissociation (N remains with 5' end)

E — replication initiation

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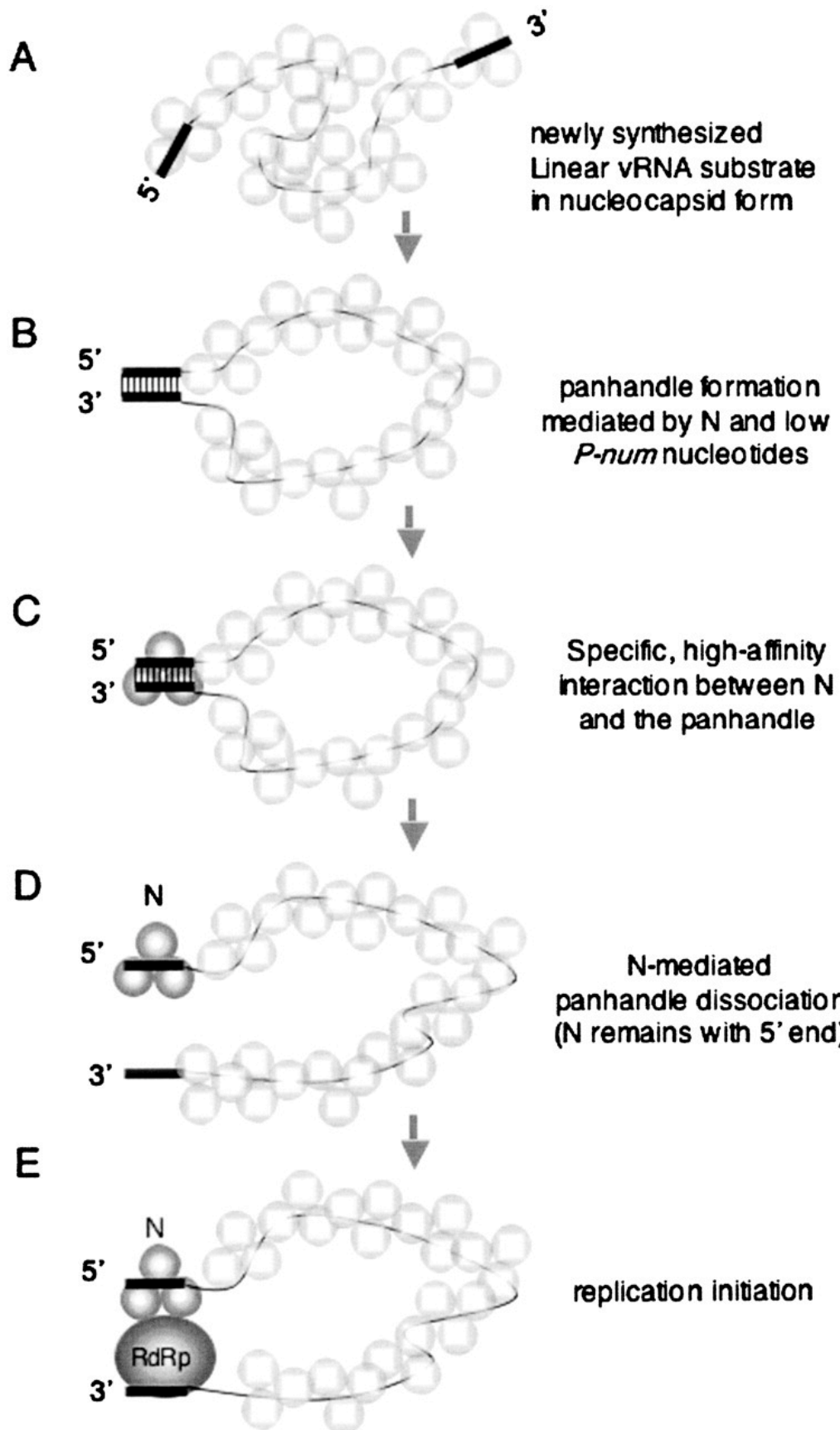
FIG. 5. Proposed mechanism for RNA chaperone activity of N during replication. Figure borrowed from (73). Newly synthesized RNA has a greater chance of being caught in a kinetic trap since the 5' and 3' ends of the RNA are first and last synthesized respectively. N coats the new RNA and removes secondary structure. After the proper formation of the panhandle a N trimer recognizes this region and unwinds the RNA. After unwinding N stays associated with the 5' end and the RdRp is free to associate with the now unwound 3' end to being transcription.

CHAPTER 2: Materials and Methods

Plasmids. All N peptides were cloned into the pTri-Ex 1.1 Vector from Novagen. The vector contains the T7 promoter for expression in the e.coli cell line Rossetta pLys I cell line. In addtion the pTri-Ex 1.1 plasmid contains a CMV enchanced ß-actin promoter for expression in vertebrate cells. The pADS plasmid containing the untagged SNV N contains and CMV enchancer and promoter for expression in vertebrate cells.

Cell Lines. All cell culture experiments were carried out in HeLa cells. The protein was expressed for purification from Rossetta pLac I cells from Novagen. The cells contain a plasmid expressing pLac I from a chloramphenicol resistant plasmid. The Rossetta cells also contain a T7 polymerase under the control of an IPTG inducible promoter.

N peptides. N-His Δ 403-430 were generated by digestion with N-His with HindIII and BamHI. The linearized plasmids were incubated with Klenow fragment (NEB). The DNA was ligated and transformed into *e.coli* strain DH5α. N-His Δ238-430 was generated by PCR amplification of the SNV N with BSQRD 2.1F and BSQRD 2.6R. The PCR product was digest with Nco I and Hind III and cloned into the same sites on pTri-Ex 1.1 (Novagen). N-His Δ1-108 was generated by digesting N-His with Nco I and Sap I followed by treatment with Klenow fragment (NEB) and T4 ligase (NEB). N-His Δ1-218 was generated by PCR amplification of the n gene with BSQRD 2.7F and BSQRDD 2.1R. The PCR product was cut with Nco I and HinDIII and ligated into pTri-Ex 1.1. N-His Δ175-

313 was created by PCR amplification of n gene using primers, Segment1F primer and Segment1R primer. This PCR product was cut with Nco I and EcoRI and cloned into pTri-Ex 1.1. The 3' end of the *n gene* was PCR amplified with primers 2.10F and Segment2R cut with EcoRI and Hind III and cloned into corresponding sites in N-HisΔ175-313. For N-His-175F the foldon was attached by amplification of the foldon domain from template T4Foldon-v2 with PrimerFoldonF and PrimerFoldonR. The PCR product was cut with EcoRI and HindIII, and cloned into the corresponding sites on SNV N Δ175-313.

N-His 1-100 and N-His 100-200 were generated by PCR amplification of the N gene from N-His. For N-His 1-100 was amplified using primers 2.13 R and 2.13F then digested with Nco I and Eco RI. N-His 100-200 was amplified with 2.14R and 2.14F after which it was digested with Nco I and Eco RI. The PCR products were then ligated into the pTri-ex 1.1 vector (Novagen). N-His Δ1-175 was amplified with 2.1F and 2.15R. The PCR fragment was digested with Nco I and Hind III and cloned into pTri-Ex 1.1.

Purification of N. All N mutants were purified by Ni affinity chromatography. The proteins were expressed in Rosetta pLys I cells (Novagen) with each peptide being optimized for expression following induction. Cells were lysed overnight in His Lysis Buffer (100mM NaH2PO4, 10 mM Tris•Cl, 8 M Urea, .5 M NaCl, 10mM ß-mercaptoethanol (BME) with 10mM Imidazole, at pH 8.0. Lysates were centrifuged at 10,000g for 30 minutes to remove insoluble material and filtered through a .2μM filter. The cleared and filtered lysate was then bound

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to 5 mL HisTrap HP column (Amersham Biosciences). The column was then washed with lysis buffer with increasing concentrations of Imidazole ranging from 50 to 500 mM Imidazole. Fractions were collected and run on a SDS gel to identify fractions containing N. The purified protein was then renatured by stepwise dialysis with buffers lacking urea and BME. The renatured protein was concentrated using Centriplus concentrators (Amicon) by spinning at 3,000g. The protein was quantitated by bradford assay and the proteins were stored at -80ºC in RNA binding buffer RNA binding buffer.

RNA. The large RNA used in the helix destabilization assay was transcribed from pT-GFP after being cut with Pvu II. The cut plasmid was purified by agarose gel electrophoresis and transcribed using the T7 express RNA transcription kit (Promega). The transcription reaction was then treated with DNase for 30 minutes at 37ºC and purified by Trizol reagent (Invitrogen).

The smaller "probe" RNA was transcribed from the PCR reaction using primers pT-GFP-F and pT-GFP-R. The RNA was transcribed directly from the PCR product by the T7 transcription (Fermentas). Then DNAse treated for 30 minutes at 37ºC. The RNA was purified by Trizol reagent (Invitrogen).

RNA for RNA binding was synthesized using a T7 RNA polymerase kit (Fermentas) with S-segment mini-panhandle template generated by PCR amplification as previously described in (5). RNA was purified on RNeasy columns (Qiagen) and eluted in 50 μl of RNAse free water. The RNA concentration was determined by TCA precipitation and was stored at -80 ºC.

RNA Duplex formation. RNA/RNA duplexes were generated by taking equal molar amounts of the large GFP RNA and 60 nt Probe and heating them together at 95º C for 5 minutes. The duplex was then incubated at 42ºC for 3 hours. Finally, the duplex was purified from free 60 nt probe using RNeasy columns (Qiagen). The duplex was then stored at -80 for up to 2 weeks.

RNA helix destabilization assay. Standard RHD assays were carried out in 20 μl reactions. RNA/RNA duplex were incubated with varying amounts of protein and 1x RNA chaperone buffer (40 mM HEPES at pH 7.4, 80 mM NaCl, 20 mM KCl,10 mM MgCl and 1 mM DTT). The reaction mixture was incubated at 37ºC for 30 minutes and 10μl of 2x Laemmli buffer was added to stop the reaction. The RNA was then loaded onto a 12% acrylamide gel and electrophoresed at 200 V for 2 hours. The gel was exposed to a phosphor storage screen to detect radioactivity and RNA unwinding was quantified by the appearance of dissociated probe at the bottom of the gel using Image Quant software (v.5.4).

Prediction of Protein Disorder. The SNV N protein disorder was calculated using the predictor for natural disorder (PONDR ®) using the VL-XT network. (57, 96, 97)

R3 Ribozyme and S14 substrate. The ribozyme R3, template was generated by PCR of HH template , PrimerF-HH-S14 and PrimerR-HH (12). The PCR product was directly transcribed by the T7 polymerase (Promega), DNAse treated and purified by nucleotide removal (Qiagen). The S14 ribozyme substrate

template was amplified by PCR of the S14 template with PrimerF-HH-S14 and Primer R-S14. The product was DNAse treated and purified by nucleotide removal (Qiagen).

Ribozyme enhancement assay. 10 nM of R3 in RNA chaperone buffer was added to 30 nM S14 substrate and varying N concentrations in 5μl. The reaction was incubated at 37ºC for 30 minutes and stopped by the addition of 5μl 25 mM EDTA and 5μl Laemmli buffer and incubated on ice until loading. The samples were electrophoresed in a 9% acrylamide, 7M urea ,TBE gel for 2 hours. The exposed to a storage phosphor screen overnight and quantitated by Image Quant software (v. 5.4).

Calculation of percent active molecules. A technique similar to that described by Carey et al. (18) was used for determination of the percent of active N molecules in preparations. Using S-segment mini-panhandle, which is bound at high affinity by wild type N, we performed RNA excess binding with the different mutants. Complexes of N and RNA were allowed to bind for 15 minutes at room temperature and then applied to a nitrocellulose filter under vacuum and the amount of radiolabeled RNA retained on the filter by virtue of binding to N was determined. For simplicity, we calculated the percent of active molecules for each protein preparation assuming a binding stoichiometry of 1:1, since some of the N mutations affected trimerization, although we previously observed wild type trimeric N binding with a stoichiometry of 1 trimer to 1 panhandle (75)

Filter Binding Assays. Filter binding was carried out as previously described (75). 10 pM radiolabeled RNA was added to each reaction with varying concentration of protein. RNA protein complexes were allowed to incubate at 25°C for 15 minutes and then applied to nitrocellulose filters that had been equilibrated in RNA binding buffer . The complexes were applied to the nitrocellulose filter and washed with 1.5 mL of RNA binding buffer. Retained RNA was measured using a scintillation counter.

Intermolecular N-N assays. The plasmid pADS, which expresses wild type untagged N, and plasmids expressing His tagged N were cotransfected into HeLa cells by effectene reagent (Qiagen) when the cells were 70% confluent. The cells were harvested 48 hours after transfection and lysed with mammalian lysis buffer (50 mM NH2PO4, 300 mM NaCl, .5% Tween 20, pH 8.0). The cell lysates were then freeze and thawed twice and centrifuged for 30 minutes at 16,000xg. The cleared lysate was applied to Ni:NTA spin columns (Qiagen). The N-His proteins were bound, washed and eluted in 200 μl then frozen. 50 μL of the eluate was then added to 25 μL 2X laemmli buffer and separated by SDS page. Proteins were detected by Western blot using anti-N antibody (1:3000) and developed by BCIP/NBT.

Gel Filtration Chromatography. Protein samples were run on an Econo-column (Bio-Rad) (3cm x 30cm) packed with Ultrogel AcA 34 resin (Sigma) with RNA binding buffer. Thirty-five fractions, 1 mL, were collected and analyzed by

western blot using anti N antibody (1:3000) to visualize the N containing fractions. Protein standards were detected by coomassie staining.

Determination of Binding Rates. 10 pM S-segment mini panhandle was added to 3 nM N-His (corrected for percent active). After the addition of RNA 90 μl aliquots were spotted on nitrocellulose filters at intervals listed in the individual figures. The CPM were plotted and the on rate was estimated.

The off rate was calculated by the addition of 10 pM S-segment mini-panhandle to 3 nM N-His. The reaction was allowed to reach equilibrium at which time 100 fold excess of unlabeled S-segment mini panhandle was added to the reaction. 90 μL aliquots were removed at the time intervals listed in figures and were plotted to determine the half-life of N-S-segment binding.

Table 1. Primers

Primer Name	Primer Sequence
2.13 R	GACGAATTCTTATTTCCATATCTCAATGATGAC
2.13F	
	AAGGAGATATCCCATGGGCACCCTCAAAGAAGTGCAA
2.14R	TTTGAATTCTTTGTAACGGAACCTTCCGGGA
2.14F	AAACCATGGAAGTCCTTGATGTAAATTCCA
2.1F	GATCCCCGGAATCCATGGGCACC
BSQRD 2.6R	GGGCACCGTGCAGCAAGCTTGTCATCAATC
2.7F	CAGTCCGCCATGGGTGTGAT
2.1R	GAACCTCCCGGGAGTAATCTCATC
Segment1F	GAGATATACCATGGTCACCCT
Segment1R primer	ACCGGAATTCGTCTTGGCTTACGTATTCCA
2.10F	ATTCTACCAGTGAATTCTAAGGGTAC
Segment2R	GCGGCCGCAAGCTTTAAAGT
PrimerFoldonF	AAACGAATTCTAGGTTATATTCCGCAAGCG
PrimerFoldonR	TTTAAGCTTCAGAAAGGTAGACAGCAGCAC
pT-GFP-F	ACTACTTAATACGACTCATATAGGG
pT-GFP-R	CCTTGGGGCGGACTGGGTGCTCTGGT
T4 Foldonv2	GGTTATATTCCGGAAGCGCCGAGAGATGGTCAGGCGTA TGTGAGAAAGATGGTGAATGGGTGCTGCTGTCTACCTT CTG
HH-Template	TAATACGACTCACTATAGGGCTCTGTCCGTTTGCTGGCA ACTGCAAGGGTCTAGACCCACACTCTCTGATGAGTCCG TGAGGACGAAACTACTTCCTGCA
PrimerF-HH-S14	ACACTAATACGACTCACTATAGGG
PrimerR-HH	TGCAGGAAGTAGTTTCGTCCT
S-14 Template	
	TAATACGACTCACTATAGGGCTCTGTCCGTTTGCTGGCA ACTGCAAGGGTGTAGTTAAAGTAGTAAGAGTGTCTGCA
Primer R-S14	TGCAGACACTCTTACTACTTTAA

Chapter 3: The Effects of Trimer Formation on RNA Binding

Introduction

Hantavirus cardiopulmonary syndrome (HCPS) and hemorrhagic fever with renal syndrome (HFRS) are caused by infection with hantaviruses. HCPS has a fatality rate of 35% and is caused by a spectrum of viruses closely related to the North American prototype hantavirus, Sin Nombre Virus (SNV) (21). Old world hantaviruses, such as Hantaan Virus (HTV) and Puumala Virus (PUUV) are associated with HFRS (21). Although other members of the *Bunyaviridae* family are arboviruses, hantaviruses are transmitted directly from a narrow range of rodent hosts. For SNV this host is the deer mouse, *peromyscus maniculatus* (21).

Hantaviruses are enveloped viruses containing three negative sense RNA genome segments designated S, M, and L. The L segment encodes the viral RNA dependent RNA polymerase (RdRp), which is responsible for the replication and transcription of the viral genome. The M segment encodes the Glycoprotein precursor, which is cotranslationally cleaved into the mature viral glycoproteins, G1 and a G2 (60). G1 and G2 act together to facilitate virus attachment to $a_V\beta_3$ integrin (35) with entry taking place via a clathrin-dependent pathway (33, 35). The S segment encodes the viral nucleocapsid protein (N). This is the most abundant protein in the virion and is found in association with the viral RNA (vRNA) intracellularly and in virus particles.

Multiple studies with diverse members of the *Bunyaviridae* family including members of the hantavirus (37) (100, 102), the bunyavirus (81), and tospovirus genera (94) indicate that N recognizes vRNA with specificity. N interacts with the terminal vRNA "panhandle" that arises through imperfect but substantial intramolecular hydrogen bonding of complementary nucleotides at the 5' and 3' vRNA termini of the S, M and L segments (74, 75). The interaction between vRNA and N is probably important in the replication and encapsidation of both the vRNA and the antigenomic copy RNA (cRNA), which also contains a terminal panhandle that is recognized by N (39, 74). N spontaneously forms ≈150 kDa trimers *in vivo* and *in vitro* (1, 75). Homotrimerization of N is likely to be necessary for specific binding to the vRNA panhandle of each genome segment. Specific binding to the panhandle causes a marked but uncharacterized conformational change in N (71, 75). N also functions as an RNA chaperone. The chaperone activity of N is ATP independent and unwinds RNA/RNA duplexes. RNA dissociation begins within 3' single stranded regions adjacent to duplexes (72, 73).

An RNA binding domain for HTV N has been mapped to a limited central region of N corresponding to amino acids 175-217 (113). In contrast, earlier work indicated that the C-terminus (amino acids 335-429) of HTV N is required for interaction with RNA (37). Regions required for trimer formation of N were initially mapped to areas in both the N- and C- termini of the protein (2). However, the first 75 amino acids of SNV N protein have been crystallized and 3-

dimensional imaging indicates that the N terminus primarily contains coiled coil structures that participate in intramolecular interactions and are probably not the primary domain responsible for trimer formation (16). We wanted to further test the hypothesis that trimer formation is required for panhandle recognition and characterize the requirements for correct recognition of vRNA by N. Based on examination of a panel of mutants in SNV N, we find that binding to the vRNA panhandle requires both the N- and C- termini of N. The principle role of the C-terminus appears to be in trimerization, which is necessary for panhandle recognition, as this region can be replaced by a heterologous trimerization domain. An N-terminal region of N can be conditionally designated as responsible for vRNA panhandle recognition of this region, in trimeric form, is necessary and sufficient for panhandle binding.

Results

Intermolecular N interaction. We created a set of mutants in the SNV *n gene* to more thoroughly test the hypothesis that trimerization is important for vRNA panhandle recognition by N, and to further characterize the facets of N required for correct vRNA binding (Fig. 6). The Sin Nombre Virus (SNV) *n gene* was subcloned and expressed such that the peptide contained a C-terminal 8x histidine tag. To determine the effect of these mutations on the ability of N to self-associate we first used a metal affinity chromatography assay. The mutant peptides containing C-terminal histidine tags were co-expressed in human cells along with wild type N lacking a histidine tag (Fig. 6). The lysates from transfected cells were applied to Ni:NTA columns to recover mutant peptides by virtue of their histidine tags. Retention of both the wild type and mutant peptides on Ni-NTA columns was then assessed by western analysis using polyclonal anti-N antibody. In this assay, wild type, untagged N would not be expected to be recovered from columns unless it is intermolecularly associated with the tagged N peptide.

As expected, wild type, untagged N was not retained on columns when expressed in the absence of N derivatives lacking a histidine tag, (Fig. 7A). Also as shown in Fig. 7, wild type N co-purified with some of the retained mutant proteins indicative of intermolecular interaction. In particular, it was possible to delete the C-terminal 27 amino acids of N-His (Δ403-430) without affecting interaction with wild type N (Fig. 7A). In contrast, ablation of the adjacent
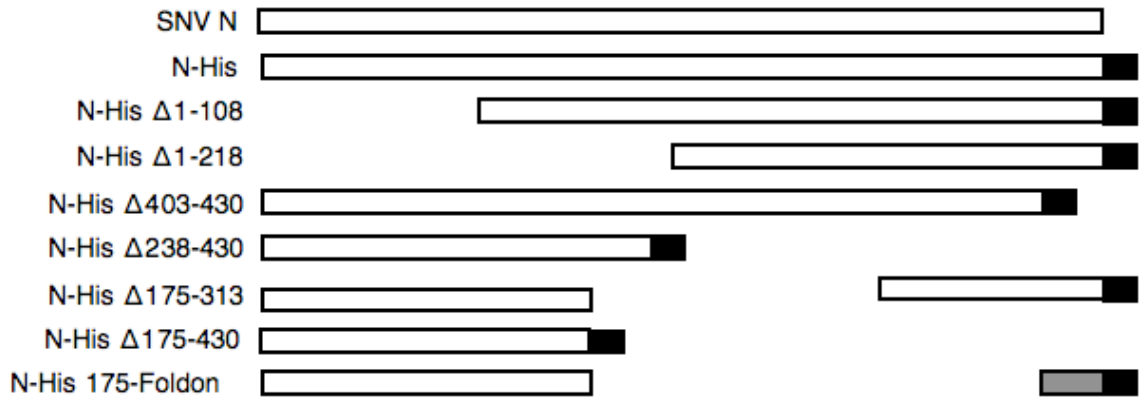
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FIG. 6. Schematic representation of the mutants generated for RNA binding. SNV N is the wild type protein expressed by pADS. N-His Proteins contain a C-terminal HSV and His tag represented by the region shaded black. The N-His mutants were subcloned and were capable of expression in *e.coli* and mammalian expression systems.

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region (Δ 347-430 and Δ 238-430) resulted in N derivatives that were unable to associate with wild type N in this assay (Fig. 7A). These data are consistent with published data indicating that a relatively short region from 347 to 403 of N is required for intermolecular N-N association and, likely, trimerization (104). Loss of approximately 100 amino acids from the N terminus of N, including the well characterized coiled-coil region (16), did not eliminate the ability of Full length N to interact with N-His Δ1-108 as evidenced by the retention of wild type N on Ni-NTA columns by N-His Δ1-108 (Fig. 2A). It should be noted that since the primary epitopes recognized by the anti-N antibody are clustered within the N-terminal region of N (114, 115), making it was difficult to directly detect mutants such as Δ 1-108. However, since retention of full-length wild type N is dependent on co-expression of N-derivatives containing a 8x histadine tag, the presence of wild type N is indicative of interaction with Δ 1-108. As expected the removal of the central domain, N-His Δ175-313, from N had no affect on the ability of these N molecules to interact with wild type N (Fig. 7A). This is consistent with published data for both SNV and other hantaviruses. Overall, our data corroborate complementary "two-hybrid" assays, which indicate that a region down stream of amino acid 108 but not requiring amino acids 175-313 are involved in trimerization of SNV N (104). This is not to say that trimerization occurs through binding to that region.

vRNA panhandle binding. We next characterized the different N peptides we had generated with regard to their ability to bind with vRNA.
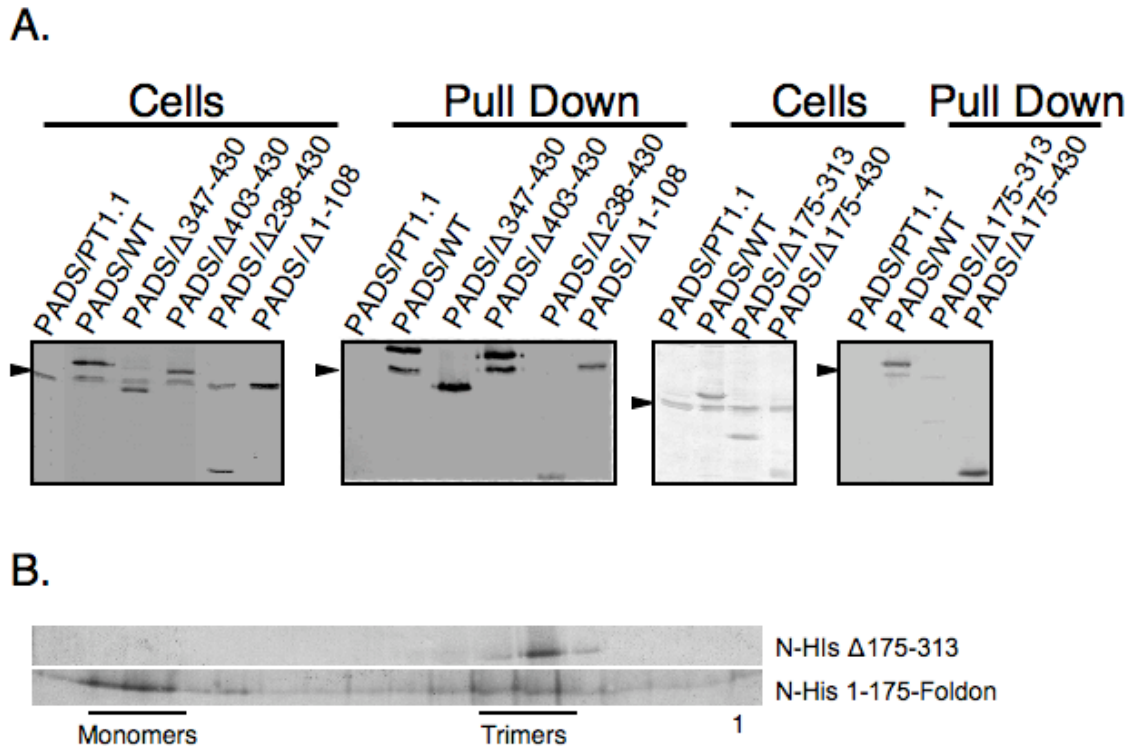
FIG. 7. Protein Protein interaction of full length N protein with N-His mutants. (A) Western blot of N-His pull down, pADS expresses SNV N without any modification. PT 1.1 is the parent vector of all the N-His mutants and expresses a small 8x histidine tagged polypeptide. The black arrow marks the size of wild type SNV N protein. The western blot from the cell lysate in HeLa cells showing the proteins that were loaded onto the column. N-His peptides that remained on the Ni:NTA column were detected by western blot. (B) Western blot of fractions from gel filtration chromatragraphy. 1 represents the first fraction to come off of the column.

Previous work indicates that a small RNA containing the terminal nucleotides of a vRNA segment in panhandle configuration ("mini-panhandle") is sufficient in cis for high affinity recognition by N. Thus, we used nitrocellulose filter binding assays to determine the Kd of the various N peptides with synthetic mini-panhandle S segment RNA. To obtain a more accurate Kd for the various N mutants we calculated the percent of active molecules for the various peptide preparations as described in Materials and Methods.

Wild type N interacted with mini-panhandle RNA at a Kd of 0.1 nM, which is lower then previous reports including those from our lab (71, 74, 100, 101, 113) (Fig. 8, Table 1). The higher affinity that we observe is almost certainly due to correction for the percentage of active molecules since determination without this correction yielded a Kd for N binding to the mini-panhandle similar to previous reports. We observed that mutations lacking the central region of SNV N (e.g. Δ175-313), exhibited vRNA binding similar to or better than that of wild type N (Table 1). In contrast, mutations in the C-terminal region that affect intermolecular N association also exhibited reduced RNA binding affinity; Δ238-430 was markedly reduced in RNA binding affinity whereas Δ403-430 did not (Table 1). In addition, deletion of the N-terminal 108 amino acids (Δ1-108) significantly decreased the binding affinity of N for the vRNA panhandle (Table 1), whereas loss of an additional 100 amino acids from the N terminus (Δ1-218) eliminated detectable binding. Taken together these data support the idea that amino acids
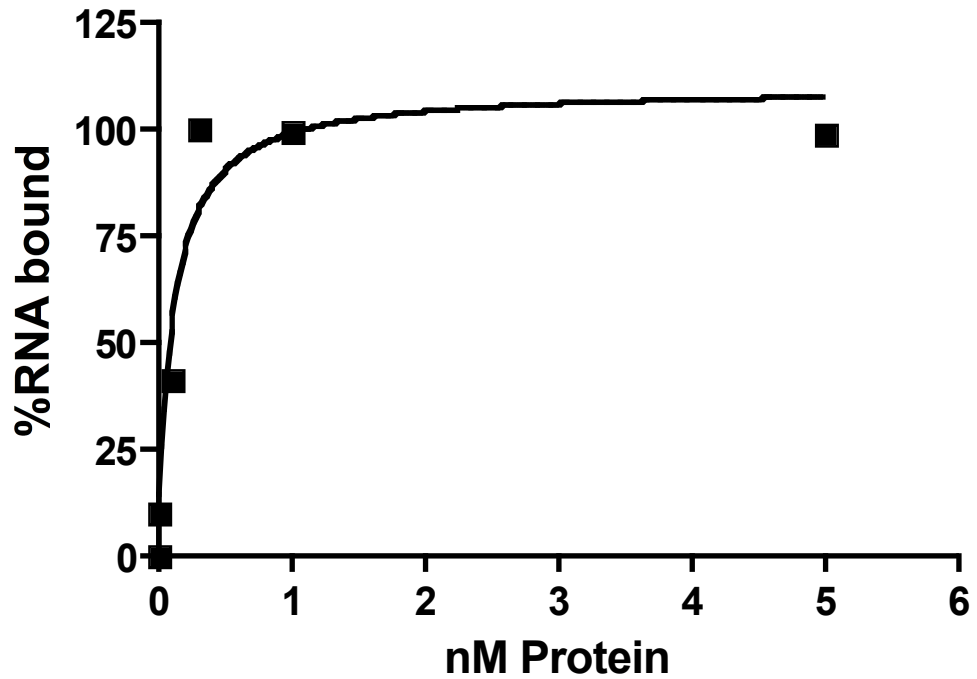
FIG. 8. Representative binding curve for N-His binding to S-segment mini-panhandle. 10 pM of panhandle was incubated with increasing amounts of N-His. The N-Mixture was incubated and applied to a nitrocellulose filter. The filter was then washed with RNA binding buffer and measured for CPM retained on the filter.

Table 2. K_d for different N-His proteins
measured by nitrocellulose filter binding
with correction for percent active molecules

N Protein	k_d (nM)
N-His	≤ 0.1
N-His Δ1-108	≤ 8.0
N-His Δ1-218	No Binding
N-His Δ403-430	≤ 0.2
N-His Δ238-430	≥ 20
N-His Δ175-313	≤ 0.1
N-His Δ175- 430	≥ 30

in the N terminus along with the C-terminus, which is required for intermolecular N association, are required for high affinity binding to the mini-panhandle.

Restoration of trimer formation restores high affinity RNA binding to N. Recognition of vRNA by N could possibly require the concerted activities of either one or more RNA binding domains as well as intermolecular interaction between N molecules. As discussed above, there was correlation between the ability of N to intermolecularly associate and to bind to vRNA with high affinity; Δ403-430 bound to S segment mini-panhandle RNA with high affinity whereas Δ175-430 and Δ238-430 interacted with vRNA at markedly lower affinity (Table 1). We attempted to more directly evaluate the role of N trimerization in vRNA binding by replacing the C-terminal region of N with a well characterized foreign trimerization peptide, and by assessing the ability of such a chimeric N peptide to bind to vRNA. The T4 phage fibritin trimerization domain mediates the trimerization of a T4 tail fiber protein (56), and has been used to direct peptide trimer formation when joined to alternative peptides (29, 104). This twenty seven amino acid long "foldon" domain was used to replace the intrinsic multimerization domain present near the C-terminus of wild type N. Mutant N-His 175F Foldon (1-175F) appends the foldon domain to the C-terminus of the N-terminal 175 amino acids of N. Thus, this chimera contains the apparent high affinity RNA binding region of N but lacks the C-terminal multimerization region. To determine whether the foldon domain mediated trimerization of the chimeric peptide, the protein expressed from N-His 175F was examined by gel filtration

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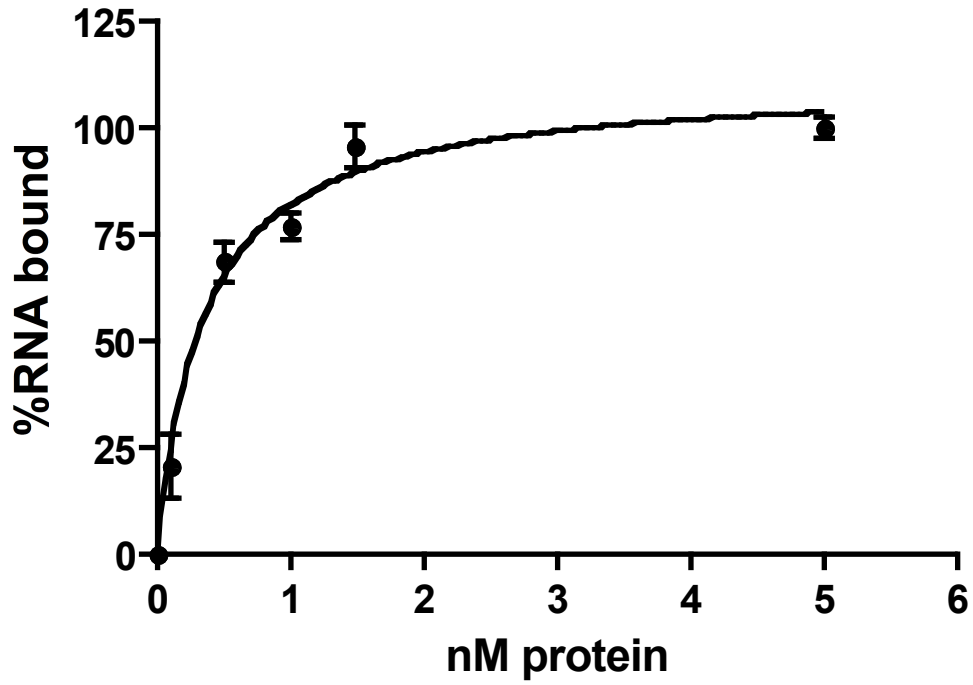**FIG. 9. Binding of N-His175F to S-segment mini panhandle.** 10 pM S-segment mini panhandle was incubated with increasing amounts of 175 F. The protein concentration was determined by calculation of active molecules in the protein preparation.

Table 3. K_d for different His-N proteins
measured by nitrocellulose filter binding

N Protein	k_d (nM)
N-His Δ175F	≤ 0.3
N-His Δ175- 430	≥ 30

chromatography (GFC). The foldon chimera was found in a trimeric form of approximately 70kDa while a population remained in monomeric form (Fig. 7B). As a control, the N mutant Δ175-313 was examined on the same column. The rate of elution of this mutant was indicative of trimer formation as expected. These data are consistent with the ability of the foldon domain to facilitate trimerization. Significantly, N-His 175F exhibited high affinity for the vRNA panhandle. In contrast, a control N derivative composed of only the first 175 amino acids of N-His Δ175-430 displayed significantly reduced affinity for the panhandle (Fig. 9, Table 2). These data indicate that trimerization, mediated by the foldon domain can enable high affinity binding to the panhandle. Further, these data indicate that a segment of N, near the N-terminus, is primarily responsible for panhandle recognition in trimeric configuration.

A chimeric N with the N-terminal 175 amino acids maintains specificity for vRNA. To further characterize the mutant N-His 175F we tested binding specificity for the vRNA panhandle. Using nitrocellulose filter binding, N-His and N-His 175F were pre-bound to radiolabeled vRNA, and subsequently increasing amounts of yeast RNA ($\approx$ 300 nucleotides) was added as a nonspecific competitor. 175F demonstrated specificity for vRNA panhandle in a manner indistinguishable from N-His (Fig. 10). The vRNA could be competed off by the addition of an excess of non-labeled S-segment mini panhandle vRNA (Data not shown). This provides further evidence that the region from 1-175 contains the necessary domain(s) for specific interaction with vRNA and that

maintenance of trimer formation is required for high affinity and specific vRNA binding.

Kinetics of vRNA binding by N-His proteins. N-His 175F restored high affinity RNA binding presumably through the interaction of three N-termini facilitated by trimer formation. To further examine this interaction we determined the association and dissociation rate for N-His, N-His Δ175-313, and N-His 175F (Fig. 11 and 12).

The first time point, 10 seconds, consistently showed more then 70% of the vRNA was already bound to protein (Fig. 11). Within the limits of detection the rate of binding for full length N-His is indistinguishable from that of N-His Δ175-313 and N-His 175F. The binding for all three peptides reached its maximum within 60 seconds. This binding was stable for up to 36 minutes at room temperature with no decrease in percent RNA bound (data not shown).

We examined the dissociation kinetics of the RNA by competition with unlabeled panhandle vRNA. N-His, N-His 175F, and N-His Δ175-313 showed similar disassociation rates with all mutants exhibited less than 10% RNA binding by 4 minutes (Fig. 12). This provides further evidence that the binding of 175F to the vRNA is comparable to the full length N.
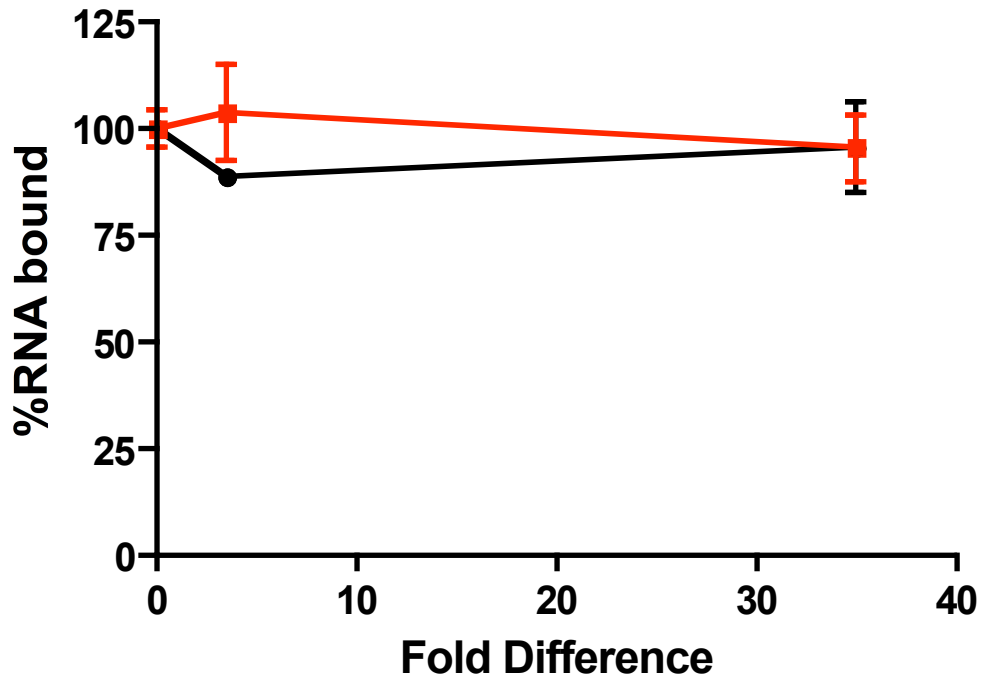
FIG. 10. Specificity of N-His 175F and N-His for vRNA. 10 pM vRNA was

incubated with 5 nM N-His 175 F ( ) or N-His (●) for 10 minutes at 25ºC. After

10 minutes increasing concentrations of non-viral RNA was added and incubated

for 10 minutes at 25ºC to compete with binding for the vRNA.

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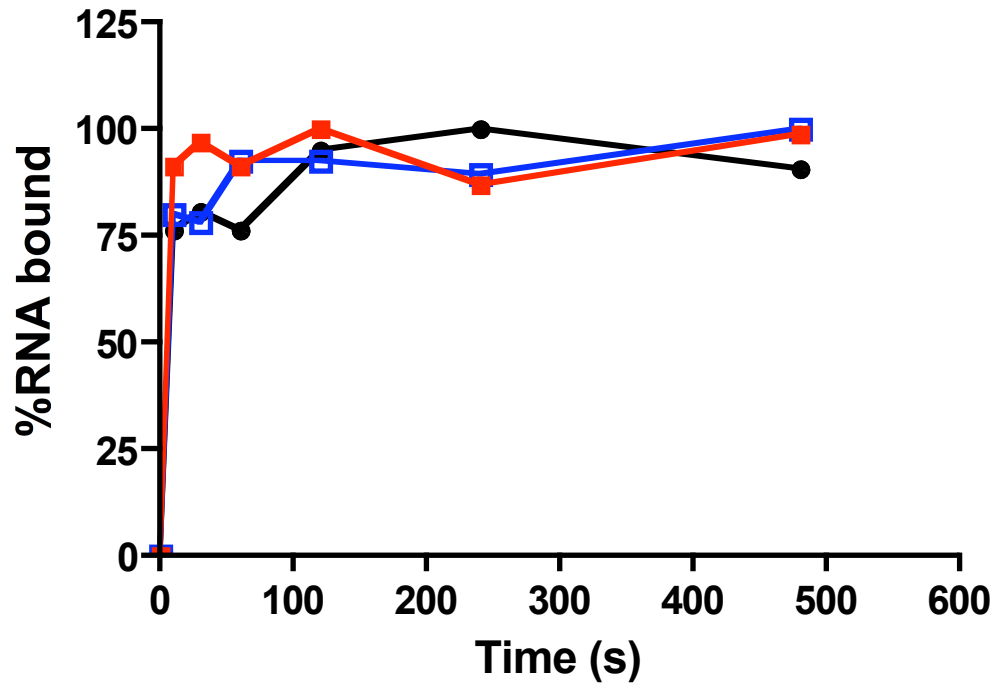
FIG. 11. Rate of association of S-segment mini-panhandle with N-His.

The kinetics of association was determined for N-His (●), N-His Δ175-430 (■)
and N-His 175F (□). 5nM protein was incubated with 10pM vRNA and removed at
the time points indicated. The removed vRNA was spotted on a nitrocellulose
filter and washed with 1.5 mL of RNA binding buffer and the amount of RNA that
remaining was determined.
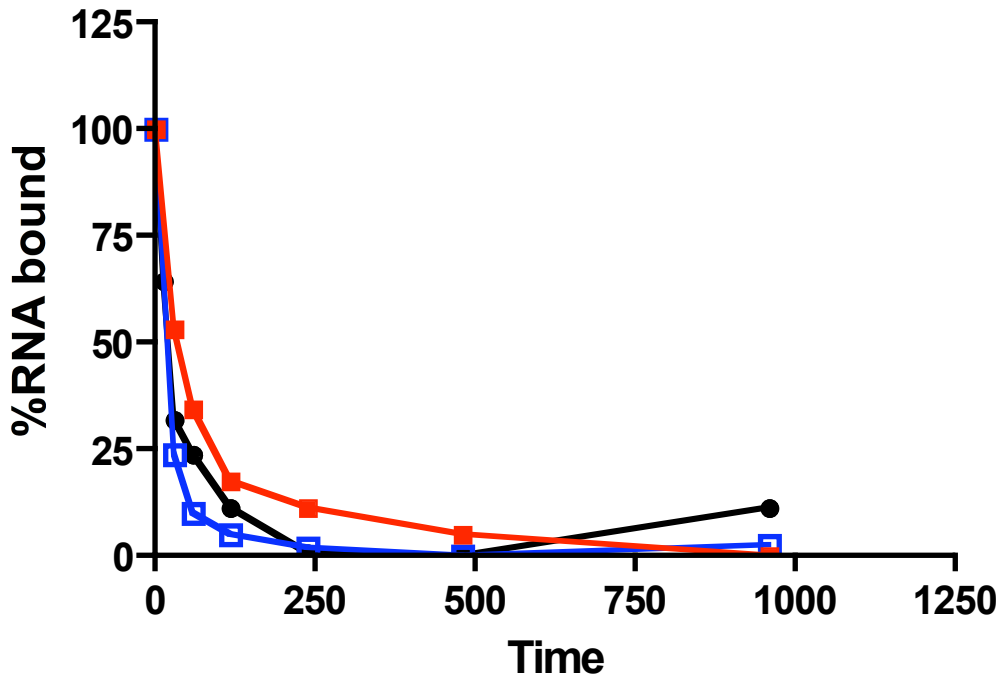
FIG. 12. The rate of disassociation of S-Segment mini-panhandle from N-His. The half life was determined for N-His (●), N-His Δ175-430 ( ) and N-His 175F ( ).10 pM radiolabled S-segment mini-panhandle were incubated with 5 nM active protein for five minutes at room temperature. At time 0 a 90 μL aliquot was removed and spotted on a nitrocellulose filter and washed with 1.5 mL of RNA binding buffer. 10 nM unlabeled S-segment mini-panhandle was added to the and 90 μl aliquots were taken at the time points given. They were spotted on nitrocellulose and washed with 1.5 mL of RNA binding buffer after which the CPM were determined.

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Discussion

Our data indicate that the N- and C-terminal regions of SNV N are required for vRNA panhandle recognition. The C-terminus ≈322 mediates trimerization of N while a region near the N-terminus can be provisionally considered to house an RNA binding site necessary for panhandle recognition. The C-terminal trimerization region of SNV is located upstream of amino acid 108 but is not contained withing the region from 175-313. This is consistent with previously reported data for both SNV and TUUV (1, 48, 50, 59). Moreover, our data are consistent with studies indicating that deletion of the putative coiled coil domains of this region eliminates intermolecular interaction of N (1, 48, 50, 59, 115). The N-terminus of N also contains coiled-coil domains (2). However, our data indicate that the region containing those domains (1-108) are dispensable for N-N interaction. These data are consistent with those obtained with TUUV, which indicated that deletion of the first 100 residues does not abrogate intermolecular N-N association in a two hybrid assay (59). Moreover, recent three-dimensional structure analysis indicates that the N-terminal coiled-coils are involved in intramolecular contacts meaning they are not involved in trimerization of SNV N(16).

The observation that of a short foreign trimerization peptide can functionally substitute for the C-terminal region of N without diminution of binding affinity for the panhandle indicates both that trimerization is required for vRNA panhandle recognition and that the C-terminus itself does not harbor an intrinsic

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high affinity RNA binding domain. This 27 amino acid foldon domain, which is sufficient for trimer formation (29, 38), has been used previously to drive trimerization of other proteins such as collagen (29) and the rabies virus glycoprotein ectodomain (104). In our experiments trimerization can be mediated by the foldon domain.. Trimerization was not as efficient as that exhibited by N harboring its native trimerization domain. Nonetheless, trimer formation was efficient enough to restore and facilitate high affinity binding to the vRNA panhandle. Studies with other RNA binding proteins, such as NS2 from influenza virus, have also demonstrated a need for intermolecular protein complex formation for RNA binding (111).

A region of N situated within the N-terminal 175 amino acids of N is also required for high affinity binding to the vRNA panhandle as evident by high affinity binding by Δ175-313 and Δ175F. Residues 1 to 175 are necessary but not sufficient for high affinity binding as this peptide must be in trimer conformation for proper function. Trimerization of N is likely necessary for generation of the proper quaternary structure for residues 1 to 175 allowing for high affinity binding to the vRNA panhandle.

The HTV RNA binding domain has been mapped to a small region spanning amino acids 175-217 (113). In contrast, prior work indicated that a region of N required RNA binding was located 327-433 (37). Surprisingly, we found that deletion of the central region in SNV N had no apparent effect on high affinity panhandle recognition. The conclusion that the central conserved domain

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is the primary region of N responsible for vRNA recognition was based on analysis of HTV rather than SNV. However, it seems unlikely that the RNA binding domain(s) of SNV and HTV N are substantially different from each other since the two proteins are so homologous. We do think that the published data indicate that this central region is able to bind to RNA. Severson et al. found that binding of full length N is specific for S segment RNA and that binding was of high affinity (Kd of approximately 15 - 50 nM) (100); this is an affinity similar to our measured binding affinity for trimeric N with the panhandle without the correction for percent of active molecules (74), (75). However, in their binding experiments with various deletion mutants, dissociation constants were not actually determined and binding was assessed by determining the amount of protein required for maximal binding. In some instances, binding required μM concentrations of N (113).

Similarly, RNA binding experiments with peptides corresponding to amino acids 175-217 are carried out at high (50 μM) peptide concentration (101). Thus, this region of N does bind RNA but at an affinity that may be four orders of magnitude less than that exhibited by full length N. This central region could potentially have an important function in one of the other two known activities of N, either in N-associated RNA chaperone activity (71) or N-mediated translation initiation (Mir and Panganiban, unpublished data), which both involve lower affinity binding of N to RNA. Our data is also consistent with data reported for tomato spotted wilt virus (TSWV) (94). For TSWV N, both the N and C-terminus

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are capable of binding RNA while the central region of TSWV N was not required for RNA interaction. Our results are similar with the earlier HTV work (37) and that conducted for TSWV, since the C-terminus plays an important role in RNA binding owing to its capacity to mediate trimerization (94).

There are multiple diverse functions associated with N. This includes its role in vRNA recognition during encapsidation, its structural role as the capsid protein in the virion, its ability to facilitate refolding of viral and nonviral RNA, a role in genome replication in conjunction with the viral polymerase, and its capacity to preferentially facilitate translation of viral mRNA. However, a common feature of each of biological functions of N is that specific or nonspecific RNA binding is central to N's activity. It would be economical for the virus to use a common RNA binding domain for several functions. On the other hand, it seems probable that specific interplay between several regions of N will be required in conjunction with one or more RNA binding domains to mediate these diverse functions.
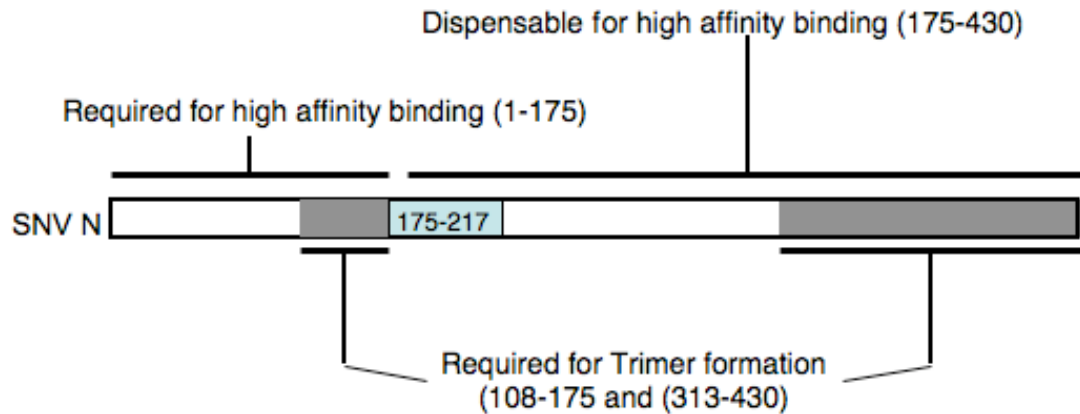
FIG. 13. A schematic diagram of regions involved SNV N RNA binding. The first 175 amino acids of the protein are required for RNA interaction. High affinity binding is achieved with the addition of the C-terminal trimerization domain from between 108 and 175 or 313 and 430. Replacing the region from 175 to 430 with the Foldon domain from T4 bacteriophage restored high affinity RNA binding. Region 175-430 is dispensable for RNA binding, assuming trimerization can be maintained. The Blue region from 175-217 was previously reported to be important in binding of the HTV N to S-segment vRNA.

Chapter 4: Determination of RNA chaperone domain for SNV N.

Introduction

Hantaviruses represent an important group of rodent-borne human pathogens. They are the causative agents of hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS). The primary cause of HCPS is Sin Nombre Virus (SNV) of the family *Bunyaviridae*, genus hantavirus. SNV is carried by *permiscus maniculatus* and is transmitted to humans through the inhalation of aerosolized excreta (15, 98, 114).

SNV is an enveloped virus with three negative sense RNA genome segments named L, M and S based on their relative size. The virus enters cells through $\alpha_V\beta_3$ integrin. The virus is uncoated and replication takes place in the cytoplasm of the cell (33-35). The L segment encodes the RNA dependent RNA polymerase (RdRp) (14). The M segment encodes the Glycoprotein Precursor (GPC) that is co-translationaly cleaved into G1 and G2 during replication. Both G1 and G2 are required for budding of the virus from the plasma membrane (60, 105).

The S segment encodes the viral nucleocapsid (N) protein and forms homotrimers *in vitro* and *in vivo* (1, 2, 16, 48, 50). N is a multifunctional RNA binding protein with a primary role in recognition and protection of the vRNA genome (71, 74, 75). N recognizes the RNA genome through interaction with the viral panhandle, which is created from non-continuous base paring between the

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5' and 3' end of individual segments (74, 75). In addition to the recognition of vRNA, N also functions as an RNA chaperone capable of helping RNA reach its active conformation (72, 73). Recent work indicates that N is also a translation initiation factor that can replace cap-binding complex (Mir and Panganiban in press)

RNA itself is a multifunctional molecule capable of enzymatic activity, storage of genetic of material, and even direct inhibition of proteins in the cell (93). However, RNA can function in these roles only in correct higher order conformations, which can be impeded by misfolding into energetically stable kinetic traps (41). RNA chaperones play a key role in allowing the proper folding of RNA so that it can reach its active conformation (99). RNA chaperones accomplish this by facilitating the interaction between different bases or by melting improperly formed base pairs (99). However, RNA chaperones constitute a diverse set of proteins and no consensus sequence has been linked to RNA chaperone function. The ability to interact with a large variety of RNA structures along with the ability to provide the energy to facilitate proper RNA folding, has led to a model that posits disordered regions of the protein are responsible for proper chaperone function (22, 107).

HIV NC and HCV core are two very potent RNA chaperones that facilitate the proper folding of RNA (12, 23, 44). These molecules contain predicted disordered domains that are responsible for their chaperone function. These disordered domains can facilitate the reformation and destabilization of base

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pairs in the absence of ATP (73). The entropy transfer model hypothesizes that disordered regions of protein with high entropy can transfer their entropy to the RNA molecule simultaneously disordering the substrate RNA and ordering the protein (107).This allows the RNA to escape kinetic traps and to refold into the proper conformation.

In this study we demonstrate that a putatively highly disordered region in the N terminus of SNV N mediates RNA chaperone activity. In contrast to the requirement for N trimerization in vRNA panhandle recognition, N-mediated RNA chaperone activity does not demand trimerization of N. We demonstrate that the predicted disordered region in the N-terminal 100 amino acids is both necessary and sufficient for RNA chaperone activity through destabilization of RNA/RNA duplexes.

Results

Putatively disordered domains and helix destabilization by N. SNV N is an RNA chaperone with RNA helix destabilization (RHD) protein activity. Previously we found that N facilitates RHD in a concentration-dependent manner (73). Using GFP RNA and a 60 nucleotide long complementary probe we measured the RHD activity of N unwinding on the RNA/RNA duplex (Fig. 14 A).

It has been postulated that disordered amino acid segments are required for the activities of RNA chaperones (107). The predictor for naturally disordered regions (PONDR®) predicts disorder along a linear polypeptide sequence (57, 97). SNV N contains a minimum of two major regions of putative high disorder (residues 18-94 and residues 144-190) both situated in the N terminus (Fig. 14 B). In addition, smaller pockets of potentially disordered amino acid sequence are predicted in the middle and C-terminus (276 to 287 and 407 to 424) of N. This prediction provided a starting point for examining regions of N required for SNV RHD activity.

RHD activity of N and C-terminal disordered regions. We first determined whether the N or C terminus of the protein functions independently in the RHD assay. N-His Δ238-430, which lacks the C-terminus of N, and N-His Δ1-217, which lacks the N terminus of N, have both been previously shown to exhibit diminished interaction with vRNA panhandle (Fig. 15) (17). N-His Δ238-430 SNV had near wild type levels of RHD activity (Fig. 16 A). In contrast, N-His Δ1-217 lacked RNA chaperone function. This result is generally consistent with the

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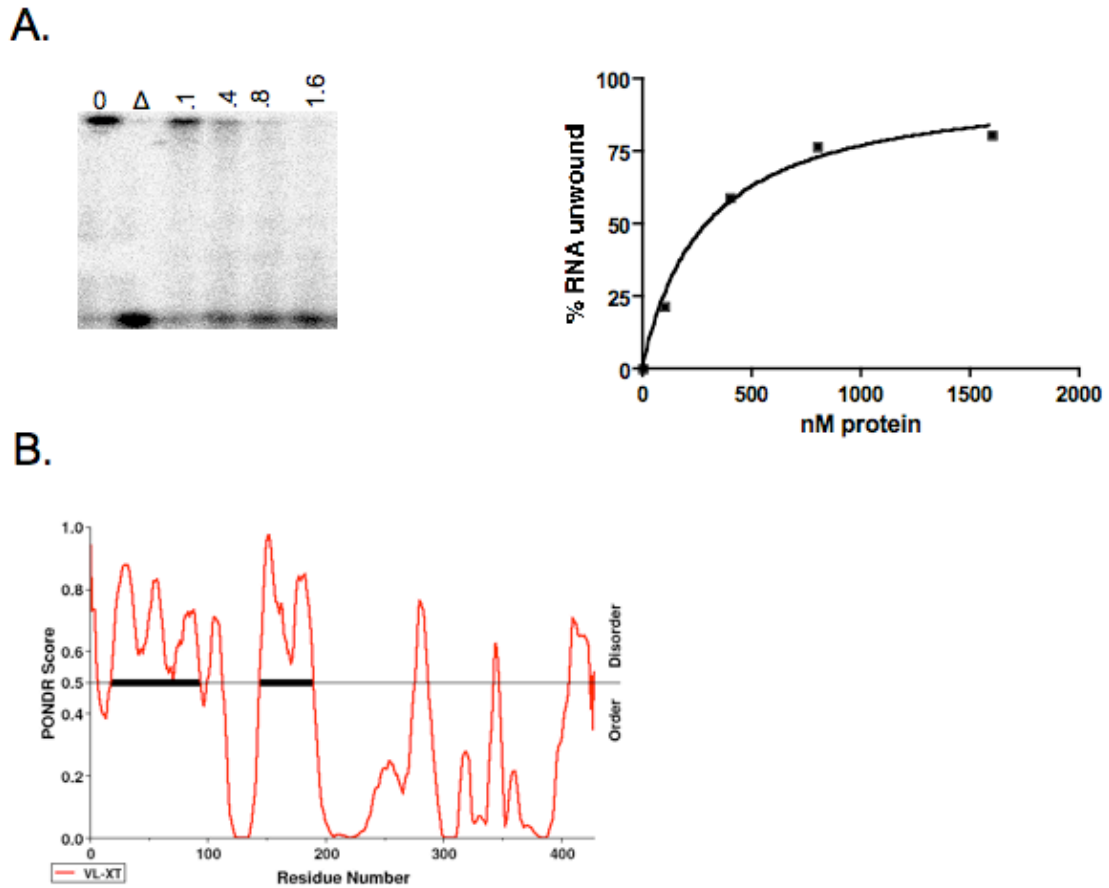
FIG. 14. SNV N predicted disorder and RHD activity. A. The RHD activity for

wild type SNV N. Increasing amounts of SNV N are added to ≈ 10 nM GFP

RNA/RNA duplex and incubated for 30 minutes at 37º C. The reaction was then

stopped and free probe was separated from duplexed probe by SDS-PAGE. The

gel was then quantitated and plotted for increase in probe RNA released from the

duplex. B. PONDR® prediction of disorder for SNV N using the VL-XT

parameters for prediction.

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Table 4: PONDR® VL-XT disorder prediction

Predicted Disorder Segment	Avg. Strength
18-94	0.6874
100-112	0.635
144-190	0.7466
276-287	0.5783
407-424	0.6436
Longest Disrodered Region	77
Overall percent disordered	42.06

FIG. 15. SNV N Mutants. SNV Mutants to map the domain required for chaperone activity. All the mutants contained a 8x C-terminal His tag to aid purification from *e.coli*. The green box represents regions predicted to be disordered by PONDR® score above .5.

idea that predicted peptide disorder is required for RNA chaperone function, since the most extensive region of disorder is predicted to be in the N-terminal half of N. This C-terminal region of N, while containing small regions of disorder, is apparently unnecessary for RHD activity.

Hantaan virus (HTV) contains an RNA binding domain for S-segment that has been mapped to amino acids 175 to 217 (113). This region also binds RNA at concentrations similar to those required for RNA chaperone activity (113). It seems plausible then that this region might function in RHD activity. N-His Δ175-430 contains the first 175 amino acids of the N-terminus and its complement, N-His Δ1-175, contains residues 175 to 430 including the region corresponding to HTV RNA binding domain (Fig. 15). N-His Δ1-175 was able to mediate significant RHD activity. However this interaction was less efficient than the activity of wild type RHD (Fig. 16 B). In contrast, N-His Δ1-175, demonstrated no detectable RHD activity (Fig. 16.B). Thus, the residues from 175-217 may enhance RHD activity since deletion of this region somewhat diminished helix unwinding.

In sum, the first 175 amino acids of N are sufficient for significant RHD activity, and the N-terminal 238 amino acids of N appear to be fully sufficient for RHD activity. Residues 1-175 contain the longest regions of predicted disorder (18-94 and 144 to 190), which are proposed to function in RNA chaperone activity. Thus, the results are consistent with a requirement for protein disorder for chaperone activity.
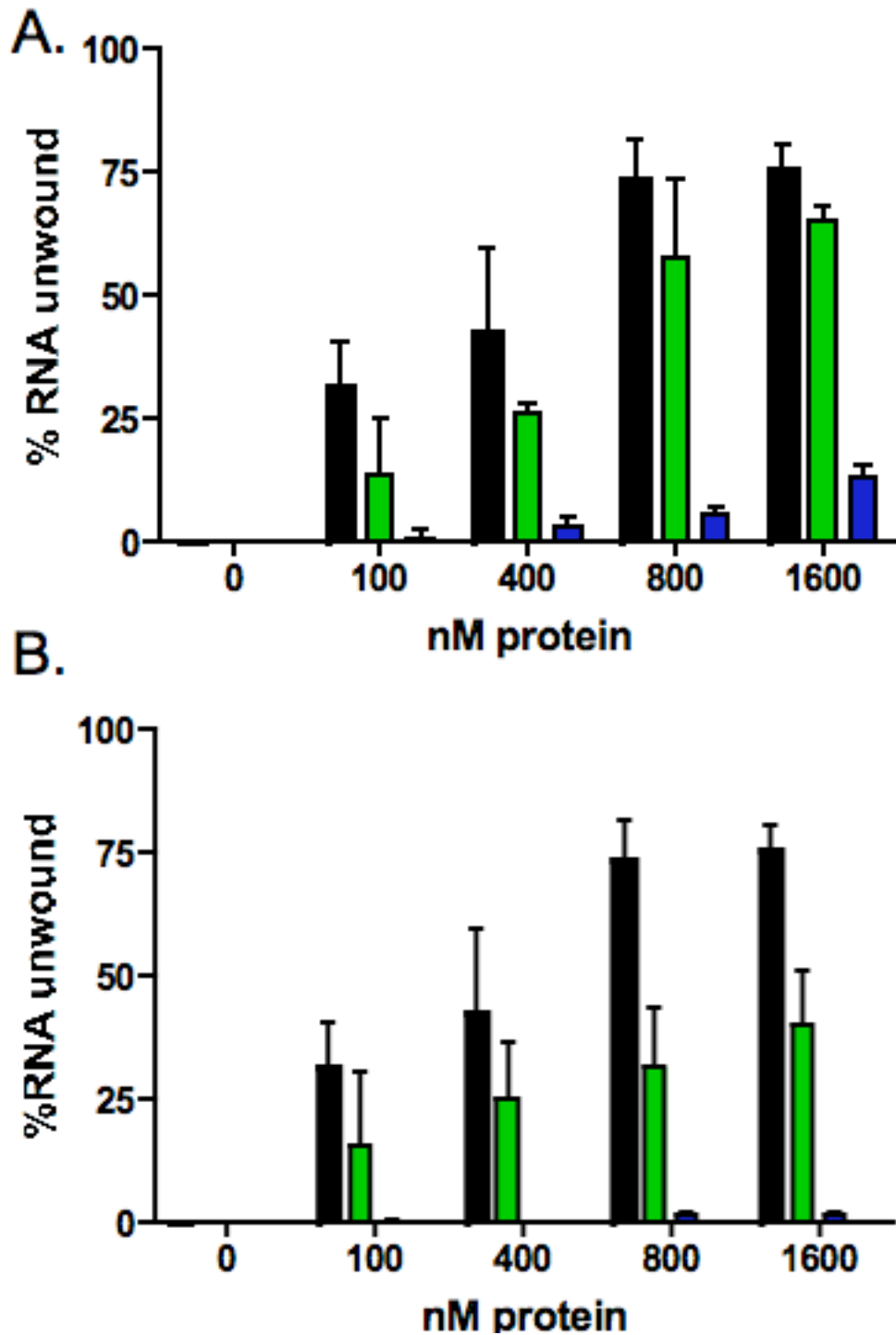
FIG. 16. Effects of N and C-terminal deletions on the RHD activity of SNV N.

A N-His (Black ▮), N-His Δ238-430 (Green ▮) and N-His Δ1-217 (Blue ▮) were

incubated with 10nM RNA duplex and assayed for RHD activity. B. Increasing

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amounts of His N (Black ▮), N-His Δ175-430 (Green ▮) and N-His Δ1-175 (Blue ▮)

were assayed were for RHD activity.

Amino acids 1-100 containing the predicted disordered domain (18-94) are responsible for RHD activity. Assignment of the RHD domain to the N terminal 175 amino acids raises the question of whether either of the two predicted disordered regions near the N-terminus, amino acids 18-94 or 144-175 or both are required for helix disassociation. Thus, we constructed two peptides containing either amino acids 1-100 (N-His 1-100) or amino acids 100-200 (N-His 100-200) that were assayed for their ability to disassociate an RNA/RNA duplex. We found that N-His 1-100 was functional in helix destabilization but was slightly less active than full length N (Fig 4). In contrast residues 100-200 of N displayed only limited activity in unwinding the RNA/RNA duplex. The first 100 amino acids, which contain the longest continuous region of predicted disorder, contains helix unwinding activity of SNV N (Fig 4).

The addition of the T4 fibritin trimerization domain inhibits the RHD activity of the first 175 amino acids. Previously we demonstrated that trimerization of N is required for high affinity recognition of the vRNA panhandle and that the first 175 amino acids of SNV N are sufficient for panhandle interaction in trimeric form in the absence of the native trimerization domain of N (17). In contrast RHD mediated by N appeared to occur efficiently without trimerization of N, since both N-His Δ238-430 and N-His Δ175 to 430 display RHD activity and are deficient in N-N interaction. Moreover, the foldon domain from the T4 bacteriophage fibritin protein was able to restore high affinity binding to the first 175 amino acids from SNV N (17). To see whether restoration of
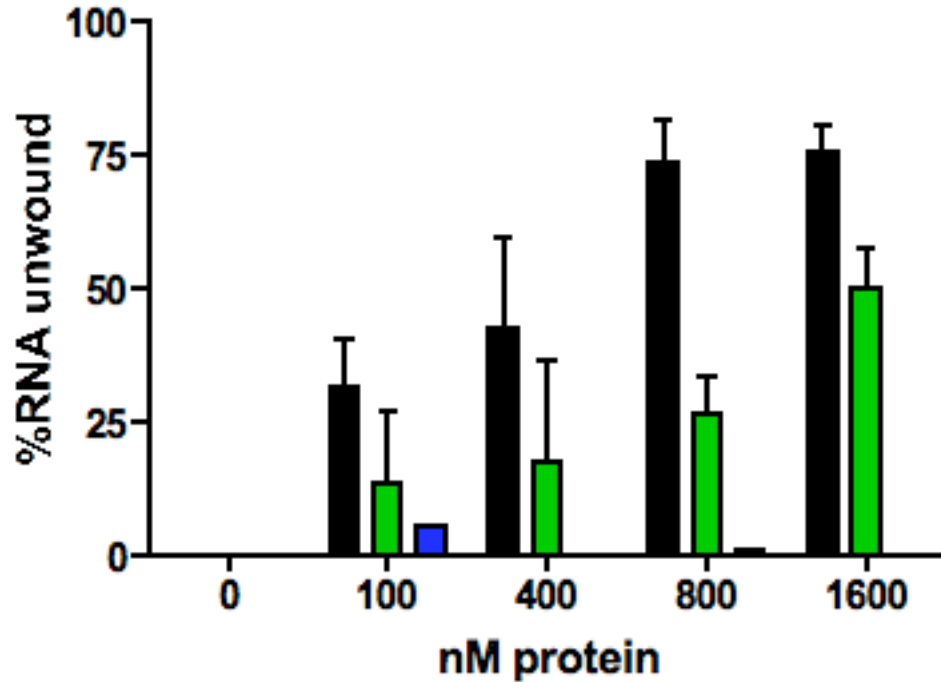
FIG. 17. Comparison of polypeptides containing N terminal disordered domains 18-94 and 144-190 RHD activity. N-His (Black ▮) compared with N-His 1-100 (Green ▮) and N-His 100-200 (Blue ▮) over increasing protein concentration after incubation at 37º C for 30 minutes.

trimerization for the first 175 amino acids affected RHD we examined N-His Δ175-313 and the N-His 175 Foldon (N-His 175F). N-His Δ175-313 contains the SNV trimerization domain, while N-His 175F contains the 27-residue foldon domain to facilitating trimer formation (Fig 2). Surprisingly, the addition of the foldon trimerization domain profoundly inhibited the ability of the first 175 amino acids to function in RHD (Fig 5). Whereas N-His Δ175-313 functioned in RHD, though less efficiently than full length N (Fig. 5). The decrease in activity cannot be accounted for by the loss in the RHD domain or in the loss in vRNA binding of 175F, since it contains the first 100 amino acids required for disassociation and binds vRNA at near wild type level. In conclusion the addition of the highly structured foldon domain from T4 bacteriophage abolishes RNA helix destabilization activity of the first 175 amino acids.

N peptides active in RHD activity also enhance ribozyme activity. It is possible that the RHD activity of N is its ability to coat RNA. A stable coating of the GFP, probe or both RNAs would result in RHD activity but would not allow the RNA to refold, a hallmark chaperone activity. Using a ribozyme enhancement assay to further examine if N-His, N-His 1-100 and N-His 175F would rule out the possibility of stable coating as a mechanism for RNA helix disassociation. RNA chaperones accelerate activity of ribozymes by increasing the rate at which substrate associate, and product disassociate from the ribozyme, they can increase ribozyme turnover allowing for enchantment of the ribozyme activity. N-His is able to enhance ribozyme activity by up to 40% over the ribozyme alone

(Fig 6 A and B). This enhancement is conferred by the N-terminal first 100 amino acids of N (Fig 6 C). Similar to the RHD activity amino acids 1-100 demonstrate diminished activity when compared with full length N. N-His 175F demonstrates a loss in RNA chaperone function similar to the results for RHD activity (Fig 19C). The enhancement of ribozyme activity supports the results from the RHD assay linking the putatively disordered N-terminal 100 amino acids to RNA chaperone function.

Temperature dependence of SNV N RHD. RNA helix destabilizing chaperones function by removing secondary structure in RNA that has become kinetically trapped. Lowering the temperature reduces the energy in the system available for helix unwinding. Incubation of reactions at 4º C for 30 minutes reduced the efficiency of RHD by N compared with the same activity at 37ºC (Fig. 20). This decrease in activity is not due to a loss in its ability to bind RNA at decreased temperatures (data not shown).

Processivity of SNV N chaperone activity. RNA chaperones are a broad class of molecules capable of facilitating rearrangement of different RNA structures into functional forms. DEAD/DEHD box helicases are ATP dependent enzymes that are capable unwinding RNA processivly from the 3' to 5' end (51, 76). Processivity has not been demonstrated for any independent RNA chaperones. Because N binds RNA but is inefficient at unwinding RNA at 4ºC, we tested whether SNV N is capable of processivity unwinding RNA/RNA duplexes.

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Incubation of N and RNA together for five minutes at 4ºC is sufficient for RNA binding but limits chaperone activity. Increasing the temperature to 37ºC restores RHD activity. By adding excess GFP RNA during this switch it is possible to determine whether N can unwind RNA duplexes to which it is pre-bound before moving on to another RNA. SNV N was unable to unwind a duplex after an increase in RNA concentration after initial binding. The inability to unwind the RNA duplex after pre-binding is consistent with the possibility that N is not processive when unwinding an RNA/RNA duplex.

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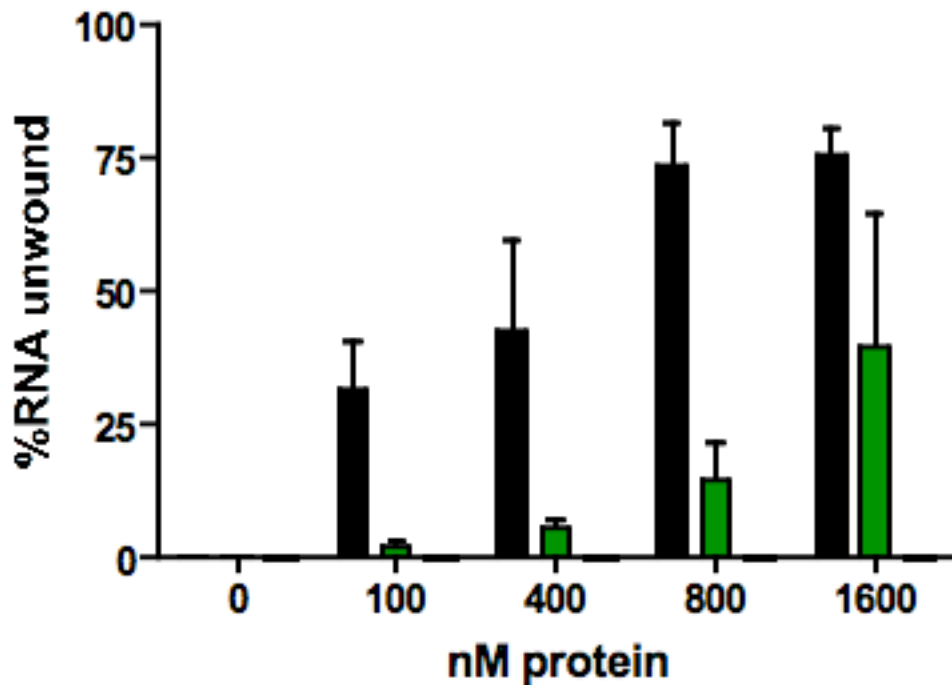
FIG. 18. The effect of trimerization on RHD activity. N-His (Black ▮) activity compared with the trimer forming N-His Δ175-313 (Green ▮) and N-His175F (Blue ▮). N-His 175F forms a trimer facilitated by the addition of the foldon fromT4 fibritin domain.
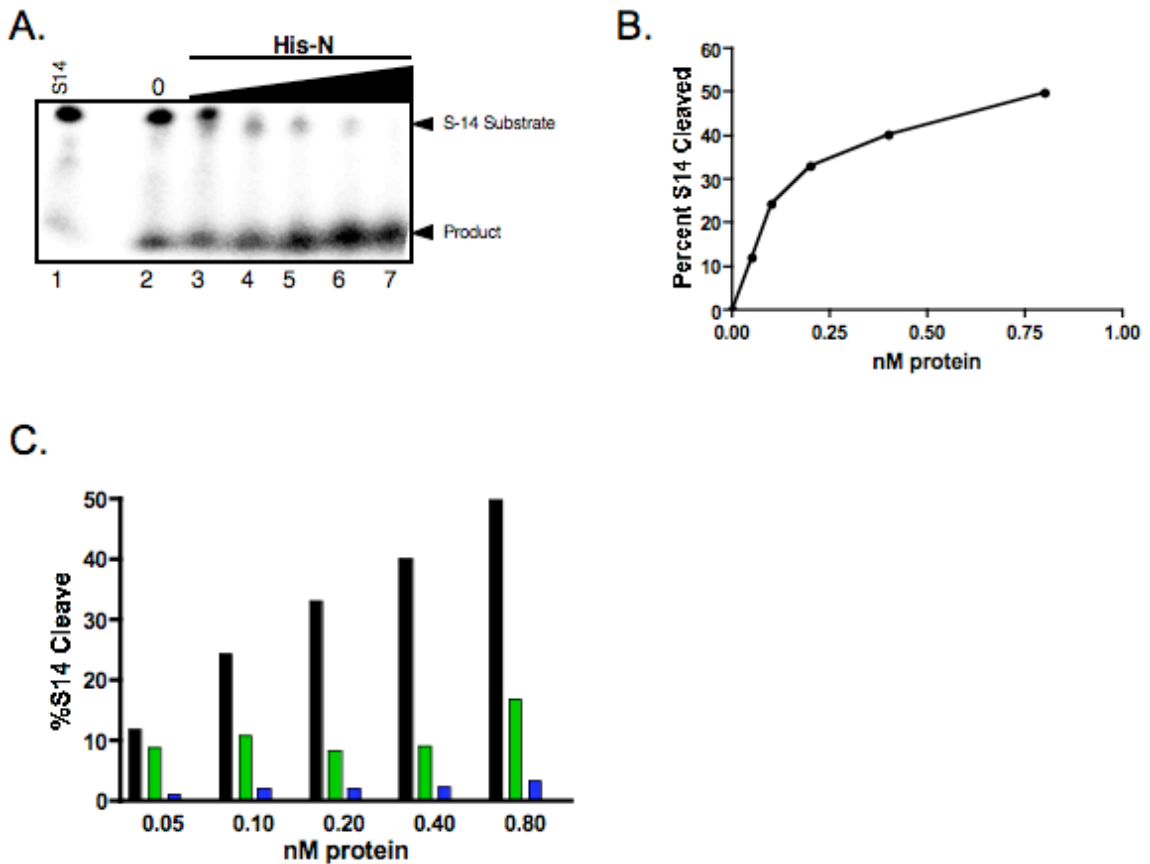
Figure 19. Ribozyme enhancement activity of SNV N. A representative gel examining N's ability to enhance ribozyme cleavage. 10 nM R3 ribozyme was incubated with 30 nM S14 substrate for 30 minutes at 37ºC (lane 2). Increasing concentrations of N (lane 3-7). Lane 1 is 30 nM S14 incubated with 0.8 nM N-His for 30 minutes at 37ºC in the absence of R3 ribozyme. B. The data from A quantified and the percent increase in S14 cleavage as a result of increasing N concentration was plotted. C. Ribozyme enhancement activity of N-His (Black), N-His 1-100 (Green) and N-His 175F (Blue) measured in the ribozyme enhancement assay. Concentrations of 0.5, 0.1, 0.2, 0.4, and 0.8 nM of the three

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N-peptides were incubated with R3 and S14. The increase in activity over R3 and

S14 without protein was measured.
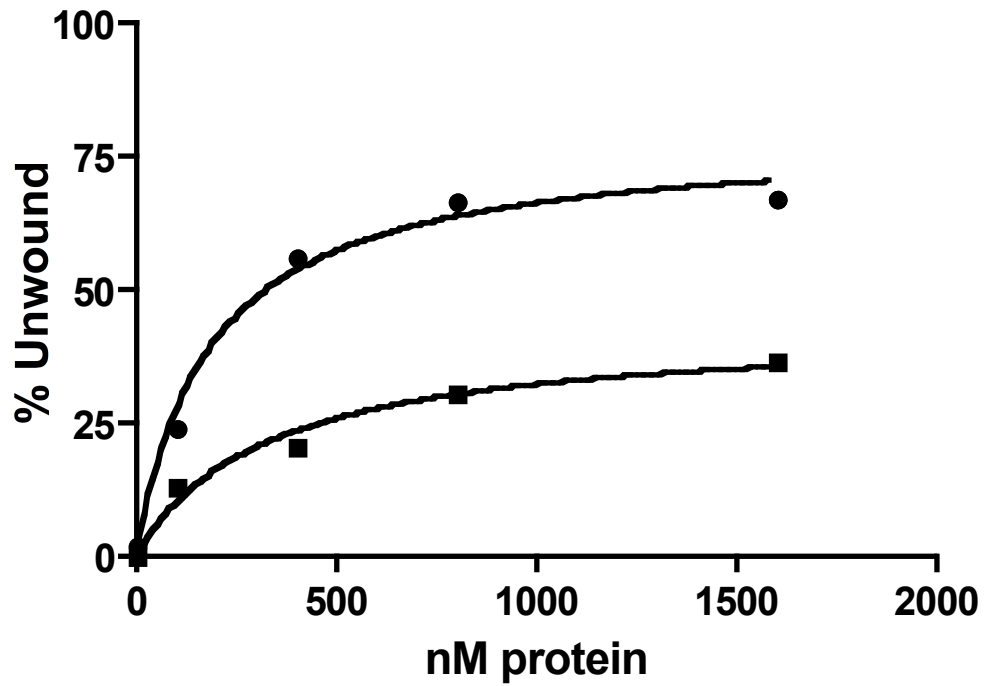
FIG. 20.Temperature dependence of SNV N RNA chaperone activity.

Increasing amounts of N-His were incubated with 10nM RNA/RNA duplex at

either 37ªC (●) or 4ºC (■). Reactions were stopped by the addition of 2x Lameli
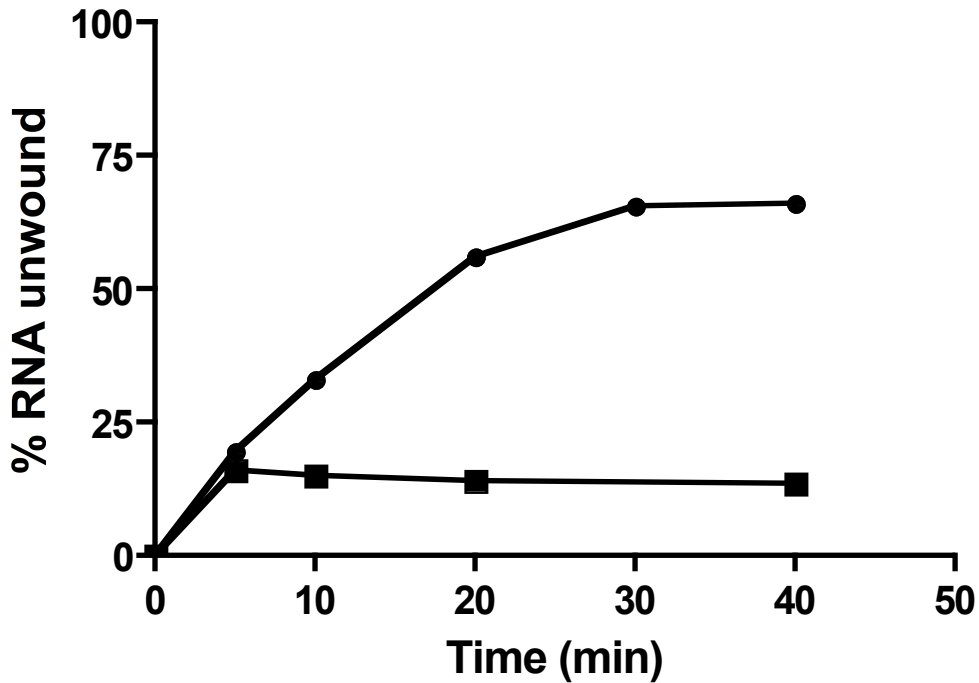
buffer.

FIG. 21. Processivity of SNV N RNA helix destabilizing activity. RNA was added to 800 nM N-His and incubated at 4°C for 5 minutes. After the initial incubation of five minutes RNA was added to one set (■) or RNA binding buffer was added (●). The two reactions were then moved to 37°C. At each time point 90 μl of sample was removed and the reaction was stopped by the addition of 2x lameli buffer and were promptly chilled on ice. Finally the samples were loaded on an SDS gel and assayed for helix destabilization activity.

Discussion

RNA chaperones play an important role in rescuing misfolded RNA from thermodynamically stable kinetic traps. The entropy transfer model proposed by Tompa and Csermely proposes that intrinsically disordered proteins are capable of unwinding stable RNA/RNA duplexes through entropy transfer (107). During entropy transfer disordered regions of the protein would transiently interact with misfolded, but ordered, RNA. After interaction the chaperone would decrease in entropy while concurrently increasing the entropy of the RNA/RNA duplex. This transfer of entropy enables unfolding of the RNA allowing the RNA to refold into its active secondary or tertiary structure. Previously, SNV N was demonstrated to work as an RNA chaperone by binding misfolded S-segment vRNA, unwinding the misfolded RNA an allowing the panhandles to form (73). Using an RHD assay and mutations in the *n gene* we have identified domains of SNV N responsible for the RNA chaperone activity. Full length N at a concentration of 400 nM is able to efficiently disassociate a majority of the RNA/RNA duplex (Protein: Nucleotide ratio of 1: .5). This protein to nucleotide ratio is similar to that reported for HCV core and WNV core for similar strand displacement activity (23, 43). SNV is predicted to be a largely disordered protein that is capable of unwinding RNA/RNA duplex. This is in keeping with the general model proposed by Tompa and Csermly.

PONDR® identified five domains of potentially significant disorder in SNV N (Table 1). Analysis for HCV core and HIV NC have predicted similar

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regions of disorder (24, 42, 61). The N-terminus from 1-238 contains the two longest predicted disordered regions and is sufficient for RNA chaperone activity (Fig 3 A). N-His Δ 238-430 is also incapable of forming multimers, evidence that unlike high affinity binding to vRNA, trimerization is not a requirement for RHD.

The ability to crystallize a protein requires regions of constant structure. Disordered domains cannot be crystallized because multiple conformations (disorder) are present in the protein population. Significantly, for SNV N the crystal structure has been determined for the first 73 amino acids (16), and this includes a very large portion of the putative N-terminal disordered domain from residues 18-94 (Fig 1 A). We think this apparent paradox can be explained by the fact that, to aid in the crystallization of SNV N the N-terminal 94 residues were attached to the T4 fibritin domain to aid in purification. The T4 fibritin domain also contains the foldon domain which facilitates trimer formation of heteralgous proteins (16).

Trimer formation is necessary for high affinity binding of N to its vRNA panhandle. However, trimer formation appears to be nonessential for N-mediated RHD activity. The first 175 amino acids lack the trimerization domain. Further, the addition of the T4 fibritin foldon domain restores trimer formation and high affinity RNA binding to the first 175 amino acids (38). In contrast, appending the foldon domain to the N-terminal 175 amino acids abolishes RHD activity. We think it likely that the lack of chaperone activity may be explained by the loss of disorder conferred by the adjacent highly ordered foldon domain. Perhaps, this is

manifested by limitation of the chaperone domain's ability to move freely in solution due to the presence of the foldon domain. Limited movement may lower the number of conformational states of the protein decreasing the entropy it can transfer to an RNA/RNA duplex. A second possibility is that the addition of the foldon domain directly induces order within the first 175 amino acids. This mechanism could be similar to HCV core, which is highly disorder but becomes more ordered with the addition of n-dodecyl ß-D-maltoside or interaction with lipid droplets (43). An interesting possibility is that formation of trimeric N creates a more ordered protein that is responsible for vRNA binding, and that the monomeric protein is more disordered than the trimer and functions as an RNA chaperone. Specific binding of the vRNA would seem to mandate that there are unique contacts between the vRNA and N. An alternative hypothesis is that it remains reasonable chaperone function is not dependent on a disordered region of the protein. The first 73 amino acids could be important for interaction. The charged residues on the N-terminal of N, in an ordered configuration, would mediate both specific panhandle binding as well as chaperone activity. However, such a model does not easily account for the strong negative effect of the foldon domain on chaperone activity and the strongly positive effect on panhandle binding. The increase in ribozyme activity provides evidence of a transient association between RNA and N (Fig 6). The transient interaction between N , S14 and R3 increases the ribozyme activity. N does not promote strand annealing (M. Mir personal communications) so ribozyme enhancement is a

result of strand displacement. Interestingly, the increase of ribozyme activity for N-His 1-100 is diminished compared to full length. This suggests another region of N is capable of enhancing RNA chaperone activity.

HTV N contains a S-segment RNA binding region from amino acids175-217, which has been shown to interact with RNA (113). However, this region is not sufficient for RHD as evident by the inability of N-His Δ1-175 to unwind RNA/RNA duplexes (Fig 3 B). While residues 175 to 213 lack RHD activity, they could play a role in enhancing chaperone activity mediated by the N-terminus of N. In particular, a mutant lacking this region was less active then full length N; mutant N-His Δ175-430 was able to significantly unwind an RNA/RNA duplex but was not as active as full length N.

It is reasonable to assume that all Hantavirus Ns contain a disordered region on their N-terminus. The PONDR® scores for HTV and PUUV, both old world hantavirus, demonstrate the same pattern of disorder in the N-terminus as SNV N (Fig 7). However, N-terminal disordered domains in N are not a characteristic of the other members of *Bunyaviride* such as bunyawera or tomato spot wilt virus (TSWV) (Fig 7 and Data not shown). It is possible that chaperone function is not found through the virus family. Hantavirus N is larger in size then its homologs in the other genera of *Bunyaviridae* (≈48 kDa and ≈25 kDa respectively). This predicted difference in N chaperone activity may explain why translational dependence mRNA transcription has not been described for any member of the hantavirus genus. Other members of *Bunyaviridae* require active

translation to disassociate inappropriate transcription termination signals that form in the nascent mRNA. In absence of translation premature termination of transcription occurs (4). It is enticing to envision that hantavirus N replaces the ribosome in this scenario removing secondary structure in newly transcribed mRNA.

A full understanding of the role of chaperone activity in hantavirus replication requires an understanding of mechanism. We have mapped the activity to the first 100 amino acids and to the first predicted disorder domain for SNV N. This activity could play an important function in genome replication, packaging and transcription of viral genomes, and requires further in vivo characterization to determine the scope of its biological function.

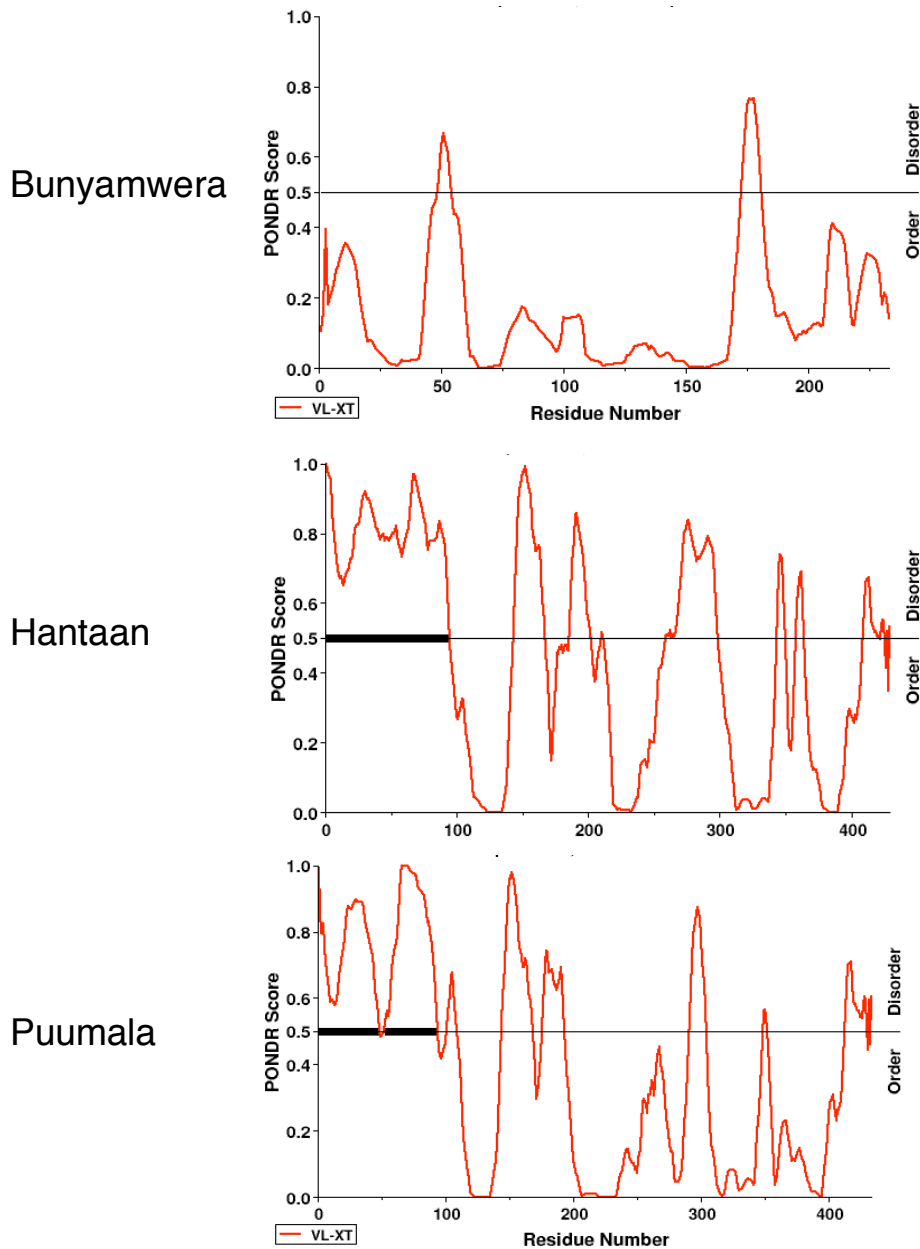Bunyamwera

Hantaan

Puumala

FIG. 22. PONDR® predictions for other members of the hantavirus genus and *Bunyaviridae*. VL-XT predictions for HTV (Old World) and PUUV (Old World) and bunyamwera (genus orthobunyavirus). All hantavirus N's examined contain a predicted region of disorder in the N-terminus.

Chapter 5: Future Directions

Expanded characterization of SNV N-RNA interaction. SNV N recognizes the panhandles present on vRNA. This recognition is dependent on trimerization of the N-terminal 175 amino acids. This observation provides the foundation for further examination of N-RNA interaction for hantaviruses. The segregation of the vRNA binding domain from the trimerization domain allows for examination of mutants that effect only RNA binding and not N:N interaction.

Influenza NS2 requires dimer formation for binding of dsRNA. This quaternary structure creates an RNA binding domain that consists of six α-helices. The use of α-helices to facilitate RNA binding is unusual since most RNA binding proteins interact with RNA through ß-sheets (78). The N-terminal 75 amino acids of SNV N form an α-helix despite the being predicted to be disordered by PONDR®(16). It is possible that hantavirus N uses α-helices for interaction with vRNA. Trimer formation brings the helices, from different N molecules, into close proximity increasing the concentration of charged amino acids available to bind the panhandle. Mutational analysis of the amino acids that form the helices could prove important in determining the domain used to recognize the vRNA. The results from such a study would probably be applicable to all members of the hantavirus genus.

The role of SNV N in encapsidation of vRNA during virion packaging. UUK virus requires only the expression of the GPC to form virus like particles. G1 interacts with N through its cytoplasmic tail (83). This interaction between N and

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G1 presumably targets the RNP for encapsidation. We have demonstrated that hantavirus N specifically recognizes panhandle structures *in vitro* (71). However, the role of N interaction with panhandles *in vivo* has not been characterized. N peptides, such as the ones used in previous studies, that have lost the ability to specifically bind vRNA but that maintain proper conformation allowing interaction with G1, G2 and RdRp could prove useful in dissecting the role of N in virion assembly. We have characterized the requirement for RNA binding and RNA chaperone activity of N. These findings could serve as tools in the examination of the role of N in the encapsidation of vRNA for all members of the hantavirus genus.

Two conflicting mechanisms are proposed for packaging of the viral segments for influenza. One asserts that the genome segments are packaged randomly (26). In this model the viral RNPs are packaged indiscriminately into the budding virion. This model is supported by the ability of the influenza virion to package nine genomes instead of the usual eight (26).

An alternative model of selective incorporation implies that encapsidation signals on each RNP are responsible for selective loading of the different segments into the virion. Electron microscopy has shown that for both the filamentous and spherical strains of influenza, eight RNPs are encapsidated in virions in a regular pattern (80). This model is supported by the apparent hierarchy of virion packaging, where efficient encapsidation of segments requires PB2 (77).

The encapsidation signals for influenza segments are located on both the 5' and 3' ends of individual segments, similar to the panhandles for SNV (63, 64, 77, 80). The Influenza RdRp is responsible for recognition of these ends for transcription, replication and presumably encapsidation since the influenza Np has little sequence specificity (27, 28, 65, 86, 87). The similarities in segment structure between influenza and SNV make it enticing to apply the influenza packaging model to SNV. Using N peptides characterized for RNA interaction in combination with mutations in the vRNA could help determine which regions of the RNP (N, RdRp, RNA) of SNV N that are capable of recognizing the panhandles formed by its genome and therefore likely responsible for recruiting the RNPs for incorporation into virions.

Interaction of N with RdRp. The panel of mutants characterized thus far for multimerization, RNA binding and RNA chaperone activity could prove useful in further characterizing the interactions between viral proteins and N. Influenza RdRp identifies the panhandles of each segment and distinguishes between the cRNA and vRNA conformation (86). It is currently unknown for the family *Bunyaviridae* whether the RdRp recognizes vRNA or whether this is the responsibility of N. The role of panhandles in replication has been examined for other members of *Bunyaviridae* (5-7) (84). These studies describe mutations of vRNA and observe a change at the transcriptional or translational level, but do not discriminate between the roles of N and RdRp. A conformational change in N trimers has been observed when N interacts with vRNA (74). It is possible that

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the RdRp has little recognition for the panhandle of vRNA and that changes in N upon binding of vRNA and cRNA facilitate the interchange from mRNA to vRNA transcription. Examining the interaction between RdRp, N and RNA could identify potential targets for inhibitors for viral transcription and replication.

Interaction of N with cellular proteins. Hantavirus N effects a myriad of cellular responses through the interaction with various cellular proteins. Interaction with apoptosis enhancer, DAXX, is mediated through the C-terminal one-third of N (58). N also interacts with actin promoting skeletal rearrangements (92). These interactions are different between the "new world" and "old world" subgroups of hantaviruses (91). This discrepancy between interactions could explain why new world and old world hantaviruses cause different forms of disease. A further examination of the domains involved in binding of actin is warranted. The mutants examined previously provide an excellent starting point for further characterization of this interaction.

N is a translation enhancer capable of increasing translation of capped mRNAs both *in vitro* and *in vivo* (Mir and Panganiban, submitted). N increases translation through interaction with the 40S ribosomal subunit, bypassing the need for cap binding complex eIF4F. It is possible that translational enhancement could also be assisted by the chaperone activity of N by maintaining a suitable mRNA template. The domains responsible for interaction of N with the 40 S ribosomal for interaction with capped mRNAs remains unidentifed. The current panel of N derivatives characterized for their RNA interactions could prove useful

in determining which domain(s) of N is responsible for 40S and cap binding. The novel interaction between N and the 40S subunit might be linked to viral pathogenesis and may be applicable to all members of the family *Bunyaviridae*.

The role of protein disorder in RNA chaperone activity. RNA chaperones are a diverse class of proteins that rearrange RNA structure and promote proper RNA folding. RNA chaperone activity is predicted to be associated with disordered domains. Use of disordered domains allows for recognition of multiple conformations of RNA. Disorder is also the cornerstone of the entropy transfer model. The entropy transfer model suggests that RNA chaperones unfold RNA by becoming more ordered and decreasing entropy, while simultaneously causing RNA to be come more disordered there by increasing entropy. (107). After unfolding the RNA is free to fold again to reach its active confirmation.

SNV N is an RNA chaperone that is capable of unwinding RNA and allowing it to refold into its proper confirmation (73). The N-terminal 100 amino acids are sufficient for RHD activity. This region is also predicted to be highly disordered, consistent with the current model for RNA chaperone function. However, the predicted region of disorder is in conflict with the current crystallographic data for SNV N (16). The predicted disorder and crystal structure regions overlap except for a small region from 74-96 that is disordered, characterized by a lack of a resolvable crystal structure (16).

The 24 amino acids from 74 to 96 are the most likely candidates for SNV N chaperone function. It is possible that this small region of SNV N is sufficient for RNA chaperone activity, However, this region has an overall negative charge and is unlikely to interact with RNA. It is likely that residues 74 to 96 function in conjunction with an RNA binding motif, which together comprise the RNA chaperone domain. This region of N is conserved among hantaviruses and presumably will possess similar functions. The disorder and location of these 22 amino acids of N suggests a role in RNA chaperone activity and warrants further examination.

The addition of the foldon domain restores high RNA binding to multimerization deficient mutants while inhibiting RNA chaperone function. This loss of RNA chaperone activity cannot be attributed to a loss of the RNA chaperone domain or RNA binding. It could prove fruitful to determine the reason that the foldon domain disrupts RNA chaperone activity while restoring high affinity binding. Inhibition of chaperone activity is possibly caused by steric hindrance of the N-terminus, which in the entropy transfer model requires dramatic rearrangement to become ordered. N-His Δ175-313 is a functional RNA chaperone. The length of the C-terminal trimerization domain potentially provides increased flexibility of the peptide. This same flexibility is not present when the smaller foldon domain is used to facilitate trimer formation of N. This lack in flexibility might be required to maintain entropy and allow it to be transferred to the RNA.

An alternate explanation for the loss in chaperone activity is that N itself has both a disordered and ordered state. In this model, protein function is dictated by which state the protein is currently occupying. In the ordered state, trimeric N is responsible for the recognition of SNV vRNA through interaction with panhandle. Monomeric N, in this model, would be more disordered since the molecule has not been restrained by trimer formation. The monomeric proteins maintain RNA binding but lack the high affinity for vRNA of the trimer. The monomeric population of N contains a high amount of disorder and is a powerful RNA chaperone. This chaperone activity is possibly used to maintain vRNA that is readable by the RdRp.

One possibility, which cannot be ruled out, is that RNA chaperone function is a result of multimerization along a single stranded template. SNV N is capable of unwinding RNA/RNA duplexes that have a free 3' end (72). This observation implies that SNV invades from single stranded regions of RNA to unwind a duplex. This characteristic of unwinding is similar to that observed in the DExH/D box Helicase NPH-II (51). Similar to NPH-II, N is unable to bind DNA and is also unable to disassociate both RNA/DNA and DNA/DNA duplexes. It is enticing to invoke a similar mechanism for N where N invades double stranded regions of RNA through a single stranded region similar to NPH-II. However, N is an ATP independent chaperone so it is assumed that it would invade the duplexed region by cooperatively binding and multimerizing from the 3' to the 5' end of the RNA.

Identification of the mechanism of unwinding is important to understanding how all ATP independent RNA chaperones function in their role of facilitating RHD.

The role of SNV N chaperone activity during RNA transcription and replication. Other members of the *Bunyaviridae* family require ongoing translation for mRNA synthesis to occur. This dependence on active translation is necessary to repress secondary structures that form in the newly synthesized mRNA causing premature termination of RdRp transcripts. The removal of secondary structure during mRNA synthesis is the responsibility of the translocating ribosome (4). This need for active translation is not affected by increasing the amounts of any bunyavirus proteins.

To date no report of a translation requirement for hantavirus transcription has been reported. It is possible that hantaviruses are able to overcome this need for active translation by an increase in the amount of N present in the system. The N of other *Bunyaviridae* members are not predicted to be disordered to the extent of hantavirus N. The hantavirus N could possibly melt secondary structures that cause premature termination of mRNA allowing for transcription of full length viral mRNA. Identifying whether hantavirus N is capable of preventing premature termination in either the BUUNV system or in cell culture for SNV would confer an applicable role to the RNA chaperone activity SNV N *in vivo.*

Finally, this work provides a foundation for a more exacting look at the interaction between N and vRNA. N is required for replication and is conserved among all hantaviruses. This makes N an attractive target for therapeutics which

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could aid in the treatment of various hantavirus related diseases. A further

understanding of N will provide the necessary foundations for possible treatments

of hantavirus related diseases.

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