Characterization of the RNA Chaperone Activity of Hantavirus Nucleocapsid Protein

M. A. Mir and A. T. Panganiban*

Department of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, Albuquerque, New Mexico 87131

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Hantaviruses are tripartite negative-sense RNA viruses and members of the *Bunyaviridae* family. The nucleocapsid (N) protein, encoded by the smallest of the three genome segments (S), has nonspecific RNA chaperone activity. This activity results in transient dissociation of misfolded RNA structures, may be required for facilitating correct higher-order RNA structure, and may function in viral genome replication. We carried out a series of experiments to further characterize the ability of N to dissociate RNA duplexes. As might be expected, N dissociated RNA duplexes but not DNA duplexes or RNA-DNA heteroduplexes. The RNA-destabilizing activity of N is ATP independent, has a pH optimum of 7.5, and has an Mg^{2+} concentration optimum of 1 to 2 mM. N protein is unable to unwind the RNA duplexes that are completely double stranded. However, in the presence of an adjoining single-stranded region, helix unwinding takes place in the 3'-to-5' direction through an unknown mechanism. The N protein trimer specifically recognizes and unwinds the terminal panhandle structure in the viral RNA and remains associated with unwound 5' terminus. We suggest that hantaviral nucleocapsid protein has an active role in hantaviral replication by working cooperatively with viral RNA polymerase. After specific recognition of the panhandle structure by N protein, the unwound 5' terminus likely remains transiently bound to N protein, creating an opportunity for the viral polymerase to initiate transcription at the accessible 3' terminus.

Members of the *Hantavirus* genus of the family *Bunyaviridae* are spherical, enveloped viruses containing three negative-sense, single-stranded genomic RNA molecules (27). These three RNA segments, designated L, M, and S, encode a virion-associated RNA-dependent RNA polymerase (RdRp), the envelope glycoproteins (G1 and G2 or Gn and Gc), and nucleocapsid (N) protein, respectively. The sequences at the 5' and 3' termini of the hantaviral genome are complementary, and the termini are in "panhandle" conformation (Fig. 1). Hantavirus infection can cause two serious and often fatal human diseases, including hemorrhagic fever with renal syndrome and hantaviral pulmonary syndrome characterized by lung damage and cardiac dysfunction (26). Humans usually come into contact with hantavirus via rodent reservoirs through the inhalation of aerosolized excreta (28). More than 200,000 people in Europe and Asia are infected with hantaviruses annually, with a mortality rate of up to 10% for prototypical hantaviruses such as Hantaan virus.

The viral N protein plays a vital role in the hantaviral replication cycle. N is the most abundant viral component in both virions and infected cells and is the major antigen in early serological response in humans (12, 32). N has multiple functions in the hantaviral replication cycle. Although its three-dimensional structure has not been determined, functionally important regions of N have been identified in the N polypeptide. The RNA binding domain of N protein appears to be situated within a central conserved region from 175 to 217 amino acids (34). The C-terminal 141 amino acids are required

for the Golgi localization (23). Both N- and C-terminal regions have been implicated in homotypic N interaction, and putative coiled-coil motifs in the N-terminal region of N have been proposed to facilitate trimerization (1, 2, 13). N differentially interacts with minus-strand viral RNA (vRNA), plus-strand cRNA, and the mRNA. Cell culture-based experiments indicate that encapsidation requires full-length vRNA or cRNA molecules, since no mRNA molecules are observed in nucleocapsids (9). The termini of full-length vRNA, and likely the plus-strand cRNA, undergo substantial antiparallel base pairing, resulting in the formation of intramolecular "panhandle" structures. Unlike vRNA and cRNA, mRNA in the *Bunyaviridae* has a truncated 3' terminus. Since mRNAs lack these 3' terminal nucleotides, panhandle formation mediated by the terminal nucleotides would not occur in these molecules. Thus, the panhandle present in vRNA and cRNA but absent in mRNA may serve as a virus-specific encapsidation signal that allows selection of vRNA into nucleocapsids destined for virions. Several in vitro binding studies have been used to examine the specificity of interaction between N and RNA. These data indicate that N binds with specificity to a region near the 5' terminus (6, 10, 11, 19, 29, 30) but that the vRNA panhandle is the preferential substrate both necessary and sufficient for high-affinity binding by N (16, 17).

In addition to ostensibly playing a crucial role in specific recognition of vRNA during encapsidation, N probably also functions in correct viral genome replication. In infected cells, N colocalizes with L protein. Moreover, many in vitro and in vivo studies indicate that the nucleocapsid protein from diverse negative-sense RNA viruses has a role in viral RNA replication either by working in conjunction with the viral polymerase RdRp or by interacting with template RNA during replication (3, 4, 7, 14, 21).

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of New Mexico Health Sciences Center, Albuquerque, NM 87131. Phone: (505) 272-4214. Fax: (505) 272-9912. E-mail: apanganiban@salud.unm.edu.

FIG. 1. Tripartite hantaviral genome. The sequences of the 5' and 3' termini that form the general "panhandle" structure of the S, M, and L segments are shown.

N has an intrinsic RNA chaperone activity that may be important for the function of N in encapsidation and genome replication (15). RNA and protein chaperones facilitate the correct folding of RNA and protein substrates into correct functional higher-order structures. Examples of cellular RNA chaperones include hnRNP A1, cold shock protein CspA, host factor 1, protein Hfq, yeast LA protein, and the tumor suppressor protein p53 (8, 18, 20, 22). In addition to the hantaviruses (and presumably other members of the *Bunyaviridae* family), several other RNA viruses encode proteins with RNA chaperone activity. These include the nucleocapsid protein of human immunodeficiency virus type 1 (HIV-1), core protein of hepatitis C virus, and hepatitis delta antigen (5, 24, 33).

RNA chaperone activity involves the reiterative nonspecific dissociation of higher-order RNA structures to enable progressive refolding of RNA and generation of higher-order RNA structures with function. Thus, one activity indicative of the chaperone activity of N is the ability to dissociate duplex RNA (15). Here we characterize the RNA helix destabilization of Sin Nombre hantavirus (SNV) N protein. Specific recognition and subsequent unwinding of viral panhandle structure by SNV N protein suggest the role of this activity in viral replication.

MATERIALS AND METHODS

Oligonucleotides and enzymes. PCR primers were from Sigma Genosys. All restriction enzymes were from New England Biolabs. Hot mastertaq polymerase was from Eppendorf. DNase I and T7 transcription reagents were from Invitrogen. RNA purification and reverse transcription reagents were from QIAGEN. All other chemicals were obtained from Sigma.

Expression and purification of hantavirus N protein. *Sin Nombre* virus nucleocapsid protein was expressed as either a His₆-tagged or glutathione *S*-transferase (GST) fusion protein in *Escherichia coli*. Trimeric N protein was purified as described previously (17). HIV-1 Gag protein was also expressed as a His₆-tagged protein in *E. coli* and purified using Ni-nitrilotriacetic acid beads. The concentration of full-length N protein was estimated by N protein preparations on sodium dodecyl sulfate (SDS)-polyacrylamide gels and visual comparison with known amounts of a standard protein (bovine serum albumin [BSA]) following

Coomassie staining. The concentration of N was verified by using the Bradford protein assay.

Preparation of RNA substrates. The S segment gene containing intact, full-length SNV S segment genomic RNA was amplified by reverse transcription-PCR using appropriate 5' (5'-ATTGGTAATACGACTCACTATAGTAGTAGTATGCTCCTTGAA) and 3' (5'-TAGTAGTAGGCTCCTTGAG) primers. The 5' primer also contained a flanking T7 promoter. The amplified full-length S segment DNA was gel purified following amplification and used directly in T7 transcription reactions to produce the full-length S segment genomic RNA. [α -³²P]CTP-radiolabeled transcripts were produced from DNA templates by using a T7 transcription kit (MBI Fermentase). We used XmnI-digested pGEM-11Zf(-), which contained a T7 promoter, as a template for in vitro T7 transcription reactions to synthesize a nonviral RNA molecule of 1,980 nucleotides. Purification of RNA transcripts was performed using RNeasy and TRIzol reagents (QIAGEN). Purified RNA was stored at -20°C in 25- μ l aliquots for up to 2 weeks. RNA was quantified by determining the percent incorporation of [α -³²P]CTP into RNA using trichloroacetic acid precipitation. The total mass of RNA synthesized in T7 transcription reactions was calculated on the basis of the total number of moles of [α -³²P]CTP incorporated and average molecular weight of a nucleotide (320.5 g/mol). The specific activity of the RNA product was determined by trichloroacetic acid precipitation.

RNA dissociation assays. RNA molecules 40 to 60 nucleotides in length were synthesized by in vitro transcription with T7 polymerase in the presence of [α -³²P]CTP. These relatively short RNA molecules were either partially or completely complementary to either the SNV S segment or a nonviral RNA generated from pGEM-11Zf(-) (described above). Radiolabeled and unlabeled RNA molecules were mixed in a 1:10 molar ratio (labeled/unlabeled) in "binding" buffer (40 mM HEPES [pH 7.4], 80 mM NaCl, 20 mM KCl, 1 mM dithiothreitol [DTT]), heated at 95°C for 3 min, and annealed at room temperature (RT) for 3 h. Unhybridized radiolabeled RNA was removed from reaction mixtures using RNeasy. Heteroduplex RNA composed of labeled shorter RNA and unlabeled longer RNA was gel purified and used as a substrate in helix destabilization reactions. For typical reactions, such as those described in the legend to Fig. 2, the heteroduplexes contained a relatively long RNA (1,980 nucleotides in length) and a shorter radiolabeled RNA (60 nucleotides in length). The central 40 nucleotides of the shorter RNA were exactly complementary nucleotides 1440 to 1480 of the longer RNA, and 10 nucleotides at both the 5' and 3' ends of short, radiolabeled RNA were noncomplementary to the larger RNA. Standard RNA dissociation reactions (20 μ l) contained 75 nM N protein or HIV-1 Gag protein and 75 nM heteroduplex substrate RNA in binding buffer containing 1 mM Mg²⁺ unless otherwise indicated. Similarly, the molar ratio RNA/N was 1:1 unless otherwise noted. Reactions were incubated at 37°C and terminated by addition of 4 μ l of RNA sample buffer (100 mM Tris HCl, pH 7.4, 50 mM

FIG. 2. RNA helix dissociation activity of SNV N protein. (A) Purified SNV N protein expressed and purified from *E. coli* containing an N-terminal GST tag (lane 1) or a C-terminal His₆ tag (lane 2). Protein molecular mass markers are shown in lane 3. The theoretical molecular masses of GST-N protein and His₆-tagged N proteins are indicated, and the mobility of these N derivatives corresponds well to the size standards. (B) RNA helix dissociation assays. Heteroduplexes were formed as described in Materials and Methods. Lane 1 shows a gel-purified heteroduplex. Lane 2, an aliquot from a 20- μ l helix dissociation reaction containing a 1:1 RNA/protein ratio (75 nM heteroduplex RNA and 75 nM BSA); lane 3, 75 nM heteroduplex RNA and 75 nM human tissue factor pathway inhibitor-2 (TFPI-2); lane 4, 75 nM heteroduplex RNA and 75 nM biotin protein ligase; lane 5, heteroduplex heated at 95°C for 5 min before loading into gel; lane 6, 75 nM RNA heteroduplex and 75 nM GST-N; lane 7, 75 nM heteroduplex RNA and 75 nM His₆-N protein; and lane 8, 75 nM heteroduplex RNA and 75 nM HIV-1 His-Gag. RNAs were fractionated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). (C) Comparison of the helix dissociation assays of purified trimeric N and unfractionated N. Lane 1, a 12% SDS gel with purified heteroduplex; lane 2, heteroduplex preincubated with unfractionated N; lane 3, heteroduplex preincubated with purified trimeric N.

EDTA, 0.1% Triton X-100, 0.5% SDS, 50% glycerol, and 0.1% bromophenol blue). Samples were fractionated on 12% SDS gels. Gels were exposed to phosphorimager screens, and RNA dissociation was quantified by determining the relative amount of dissociated labeled RNA as well as labeled RNA that remained in heteroduplexes. Data points were fitted to a hyperbolic equation using the Origin 6 program (Microcal). Heteroduplex half-life ($t_{1/2}$) corresponds to the time point at which half of the duplex is unwound.

Preparation of RNA-DNA, DNA-RNA, and DNA-DNA templates. In some experiments, RNA-DNA heteroduplexes were used in helix dissociation assays in place of RNA-RNA heteroduplexes. For the synthesis of RNA-DNA heteroduplexes, unlabeled S segment or nonviral RNA was synthesized as described above and annealed with a 60-nucleotide single-stranded DNA molecule complementary in sequence to the RNA. These DNA molecules were end labeled with [γ -³²P]ATP using T4 kinase prior to heteroduplex formation. For the preparation of DNA-RNA heteroduplexes, a 40-nucleotide RNA molecule was annealed to a 2-kb DNA. A similar strategy was used for the synthesis of DNA-DNA heteroduplexes using a 40-nucleotide, end-labeled DNA annealed with a second DNA molecule 2 kb in length. All heteroduplexes were gel purified.

RNA filter binding assays. Thirty-two-nucleotide-long RNAs corresponding to either the 5' or 3' terminus of SNV S segment RNA were synthesized by *in vitro* T7 transcription and radiolabeled by incorporation of [α -³²P]CTP as described above. All protein-RNA binding reactions were carried out in binding buffer (40 mM HEPES [pH 7.4], 80 mM NaCl, 20 mM KCl, 1 mM DTT) at a constant concentration of RNA with an increasing concentration of N protein. Reaction mixtures were incubated at room temperature for 45 min and filtered through nitrocellulose filters under vacuum. The filters were washed with 10 ml of binding buffer, dried, and monitored by scintillation counting. Nonspecific retention of RNA was monitored by filtering the reaction mixtures that lacked protein. Dissociation constants were calculated by fitting the experimental data points into either hyperbolic or sigmoidal curves using the Origin 6 program (Microcal). The apparent dissociation constant (K_D) corresponds to the concentration of N protein required to obtain half saturation, assuming the complex formation obeys a simple bimolecular equilibrium. We assumed that plateau in the binding profile represents complete binding of RNA to allow the calculations at half saturation.

Reverse transcription reactions. Reverse transcription reactions using SNV S segment "minipanhandles" as templates used a primer complementary to the 3' end of the minipanhandle and were carried out by using reverse transcription reagents from Invitrogen following the manufacturer's protocol. Twenty-microliter reactions containing 10 nM SNV S minipanhandle, 500 nM RNA primer (UAGUAGUAGACUCCUUGAGAAGCU), and 0.5 mM deoxynucleoside triphosphates (dNTPs) with [α -³²P]dATP were heated at 65°C for 5 min and placed on ice to allow the annealing of the RNA primer to the 3' end of SNV minipanhandle. To determine the potential effect of N on primer annealing, increasing concentrations of N were added to reaction mixtures and incubated at room temperature for 20 min without heating and cooling steps. After incubation, 4 μ l of 5 $\times$ first-strand buffer, 2 μ l of 0.1 M DTT, and 1 μ l of RNase out were added to the reaction mixture, followed by incubation at 37°C for 2 min.

One microliter of Moloney murine leukemia virus reverse transcriptase was added and further incubated at 37°C for 50 min. Reactions were terminated by heating the samples at 70°C for 5 min, and samples were analyzed on polyacrylamide gels to characterize the products of reverse transcription.

RESULTS

General RNA helix dissociation activity of hantavirus N protein. Hantavirus N protein was expressed and purified as a fusion protein containing either an N-terminal GST tag or a C-terminal His₆ tag as described in Materials and Methods (Fig. 2A). We previously observed that N has the ability to nonspecifically dissociate RNA duplexes, which is indicative of its role as an RNA chaperone (15). We constructed an RNA heteroduplex composed of an RNA approximately 2,000 nucleotides in length and partially complementary 60-nucleotide-long labeled RNA. In this heteroduplex, the central 40 nucleotides of the 60-base RNA are complementary to a sequence near the middle of the longer RNA, and 10 flanking nucleotides at the 5' and 3' ends are not complementary. This heteroduplex was incubated with N protein at an RNA/N molar ratio of 1:1 in binding buffer containing 1 mM Mg²⁺ for 2 h and then analyzed by gel electrophoresis. As expected, we observed the dissociation of the two RNA strands, as evidenced by the liberation of the labeled RNA from its longer RNA (Fig. 2B). Moreover, N protein containing either the N-terminal GST tag or the C-terminal His₆ tag exhibited this activity. Similarly, HIV-1 Gag protein, another demonstrated RNA chaperone, had RNA helix dissociation activity, whereas negative controls, such as BSA, human tissue factor pathway inhibitor 2 (TFPI-2), and biotin protein ligase (BirA), did not (Fig. 2B). TFPI-2 and BirA were expressed in *E. coli* as His₆-tagged proteins and were purified the same way as His₆-tagged hantavirus N.

N protein forms stable trimers, and trimer formation is required for specific binding of N to the RNA panhandle that arises from base pairing of the terminal sequences (17). To compare purified trimeric N with unfractionated N, we purified trimeric N protein and used this purified trimer in the helix

FIG. 3. Effect of ATP, $MgCl_2$, and pH on the RNA helix dissociation activity of SNV N. (A) Effect of ATP on N-mediated helix dissociation. Lane 1 contains heteroduplex RNA incubated without added N protein, lane 2 contains the products of a reaction that contained RNA heteroduplex (as in Fig. 2) and N protein in the presence of 1 mM ATP, and lane 3 contains the products of a reaction without ATP. (B) Effect of $MgCl_2$ on helix dissociation. The graph shows the percent release of the radiolabeled shorter RNA from heteroduplex RNA by N at a range of $MgCl_2$ concentrations. (C) Effect of pH on the helix dissociation activity of N. The graph shows the percent release of the radiolabeled shorter RNA from the RNA heteroduplex as a function of pH.

dissociation assay. The results of this experiment also indicated that the trimer is able to dissociate the RNA heteroduplex (Fig. 2C). Thus, the trimer has the ability both to bind specifically with RNA containing the vRNA panhandle and to non-specifically dissociate duplex RNA.

Effect of NTPs, pH, and Mg^{2+} concentration on helix-destabilizing activity. In contrast to general RNA chaperones, RNA helicases typically require ATP as an energy source to mediate helix unwinding. Thus, we asked whether supplementing the helix-unwinding reaction with ATP would augment dissociation. We found that ATP did not measurably affect helix dissociation, indicating that helix destabilization by N protein is ATP independent (Fig. 3A). Similar results were obtained when helix destabilization was assayed in the presence and absence of the other three NTPs (data not shown).

RNA helicases also require divalent cations for their activity. To determine whether divalent cations are required for RNA helix dissociation and to determine the concentration and pH optima for RNA helix dissociation, we carried out a set of reactions in which the $MgCl_2$ concentration and pH were varied. These experiments indicated that $MgCl_2$ was required for efficient helix dissociation by N, with 1 or 2 mM $MgCl_2$ being optimal for activity. The pH optimum for the RNA dissociation activity was about pH 7.5 to 8.0 (Fig. 3B and C). Taken together, these data are consistent with the idea that the RNA helix destabilization activity of hantavirus N is an ATP-independent, Mg^{2+} -dependent reaction and indicate that N is an RNA chaperone rather than an RNA helicase.

The helix dissociation activity of N is RNA specific. Since hantaviruses are RNA viruses, it seemed likely that the helix dissociation activity of N is RNA specific. However, other known RNA chaperones from RNA viruses, such as retroviral nucleocapsid protein and even hepatitis C virus core protein, are able to facilitate the refolding of both RNA-RNA and RNA-DNA duplexes (5, 24). We synthesized a set of RNA-RNA, RNA-DNA, and DNA-DNA heteroduplexes (Fig. 4B) and used these as substrates for N in the helix dissociation assays. In the case of RNA-DNA duplexes, we used annealed

nucleic acids containing the 2-kb RNA and a short, labeled DNA as well as a 2-kb DNA annealed to a short, labeled RNA. The data from these experiments indicated that N protein is unable to facilitate the dissociation of duplexes that contain DNA in either strand (Fig. 4C). In contrast, we observed that HIV Gag facilitated the unwinding of the RNA-DNA duplex (data not shown). Thus, the helix-unwinding activity of hantavirus N protein is specific for duplex RNA structures.

Helix unwinding is not sequence specific and takes place in the 3'-to-5' direction. To further characterize the substrate requirements for dissociation, we synthesized different types of RNA heteroduplexes composed of a longer unlabeled RNA approximately 2 kb in length and a shorter radiolabeled cRNA 60 nucleotides in length (Fig. 5A). The central 40 bases of the shorter RNA were complementary to the 5' end of longer RNA, and 10 bases from both 5' and 3' ends were noncomplementary [Fig. 5A(a)]. We also created heteroduplexes in which the 10-base-long noncomplementary region of the shorter RNA from either the 5' end, the 3' end, or both ends was removed [Fig. 5A(b), (c), and (d)]. When these RNA duplexes were challenged with N protein, we observed the release of short RNA from each duplex, indicating that the presence or absence of single-stranded regions in the shorter RNA of the heteroduplex did not grossly affect helix unwinding. However, there was a slight increase in dissociation half-life for the heteroduplex lacking the 3' single-stranded region of the shorter RNA (Fig. 5B). It is also evident from these results that N does not require a single-stranded 5' terminus of long RNA for helix unwinding. We also examined the dissociation of similar heteroduplexes composed of two shorter RNA molecules having lengths of 60 nucleotides each in which the central 40 nucleotides were paired and 10 nucleotides at both termini were unpaired. These shorter heteroduplexes also gave similar results (data not shown).

We synthesized and examined the dissociation of four additional heteroduplexes [Fig. 5A(e), (f), (g), and (h)]. In the heteroduplex shown in Fig. 5A(e), the shorter RNA was complementary to the 3' end of the longer RNA molecule. This

FIG. 4. Helix dissociation activity of SNV N is specific for heteroduplexes composed solely of RNA. (A) Kinetics of N-mediated helix dissociation using a heteroduplex with both the longer and shorter nucleic acid composed of RNA [heteroduplex (a) of panel B]. The RNA heteroduplex was incubated with N protein for increasing lengths of time and fractionated on a 12% gel. $t = 0$, lane 1; $t = 10$ min, lane 2; $t = 20$ min, lane 3; $t = 40$ min, lane 4; $t = 80$ min, lane 5; $t = 160$ min, lane 6; and $t = 205$ min, lane 7. (B) Various RNA and DNA heteroduplexes used in helix dissociation assays. (a) Heteroduplex formed between a longer RNA molecule and a small, radiolabeled RNA 60 nucleotides long. (b) Heteroduplex containing the same "longer" RNA as in panel a but with a shorter labeled DNA. (c) Heteroduplex containing a longer DNA and the same shorter RNA as in panel a. (d) Heteroduplex composed solely of DNA. (C) N-mediated dissociation of the various heteroduplexes shown in panel B. Samples from helix dissociation reactions were fractionated on 12% SDS-PAGE and exposed to phosphorimager screen, as in panel A, and dissociation was quantified using phosphorimaging.

heteroduplex lacked an available single-stranded 3' end. Another contained a 3' single-stranded region 10 nucleotides in length [Fig. 5A(f)]. A third was composed of two short, exactly complementary RNA molecules 40 nucleotides in length [Fig. 5A(g)]. A fourth heteroduplex contained 10 centrally located single-stranded nucleotides flanked by double-stranded regions [Fig. 5A(h)]. All of these heteroduplexes were incubated with N protein, and results are presented in Fig. 5C. Both heteroduplexes that lacked a single-stranded 3' end [Fig. 5A(e) and (g)] were resistant to dissociation by N. All other heteroduplexes which contained either a single-stranded region 3' to a duplex or a single-stranded loop in the middle [Fig. 5A(f) and (h)] were dissociated by N. Thus, it appears that hantavirus N protein is a nonspecific RNA chaperone that requires a single-stranded 3' region adjacent to a base-paired region to initiate the helix-unwinding process.

N protein unwinds the panhandle structure of the hantaviral genome. The sequences at the 5' and 3' termini of the hantaviral genome are complementary and exist in the form of a panhandle structure (Fig. 1 and 6). Moreover, this predominantly double-stranded vRNA panhandle is the preferred RNA substrate for high-affinity *in vitro* binding by the N protein trimer (16, 17). This specific vRNA-N complex formation could serve as an initial structure for subsequent viral RNA encapsidation or initiation of genome replication. Initiation of genome replication would be expected to require at least tran-

sient panhandle dissociation. Thus, we asked whether the N protein trimer dissociates the panhandle after specifically binding to the panhandle. We synthesized a heteroduplex that resembles the viral minipanhandle composed of 32 nucleotides from both the 5' and 3' ends of SNV S segment RNA. Either the 5' sequence or the 3' sequence was radiolabeled prior to the formation of such heteroduplex "pseudopanhandles" (Fig. 6A). We observed that this heteroduplex was efficiently dissociated by N protein trimer (Fig. 6B). The half-life for the panhandle dissociation reaction was 12 min, whereas alternative nonviral templates were dissociated with an average half-life of 25 min. This somewhat more rapid dissociation of the panhandle relative to a nonviral heteroduplex may be due to the fact that the vRNA panhandle is bound with higher affinity than duplexes derived from other RNAs.

Since the N protein trimer binds with high affinity to the viral RNA panhandle and since trimeric N also dissociates the panhandle, it seemed likely that trimeric N would remain bound to either one terminus or both termini following dissociation. To examine the maintenance of interaction between trimeric N and viral RNA after dissociation of the pseudopanhandle, we carried out parallel reactions in which trimeric N was incubated for different time intervals using pseudopanhandles with radiolabeled 5' or 3' termini. Samples were passed through nitrocellulose filters, and radiolabeled RNA retained on the filter was quantified. In the absence of protein, RNA does not

FIG. 5. N-mediated helix dissociation requires a single-stranded region 3' to the duplex. (A) Several related RNA heteroduplexes were generated to characterize N-mediated helix dissociation. These include a molecule containing a shorter RNA complementary to the 5' terminus of a longer RNA with an additional 10 noncomplementary nucleotides at both the 5' and 3' ends (a). (b) A molecule similar to that in panel a but the 5' end of the shorter RNA is exactly complementary to the longer RNA. (c) The molecule is identical to that in panel a except that the 3' end of the shorter RNA is exactly complementary to the longer RNA. (d) A molecule similar to that in panel a except that both the 5' and 3' ends of the shorter RNA are exactly complementary to the longer RNA. As shown in the diagram, the shorter RNA of the molecules shown in panels a through d is complementary to the 5' end of the longer RNA. In contrast, in panels e and f, the shorter RNA is complementary to the region at or near the 3' end of the longer RNA. The heteroduplex in panel e is a 40-nucleotide radiolabeled short RNA complementary to the 3' end of the longer RNA, whereas that in panel f contains a smaller RNA that is complementary to the longer RNA beginning at a site 10 nucleotides from the 3' end of the longer RNA. (g) Heteroduplex composed of two relatively small RNAs (40 nucleotides in length) that are exactly complementary to each other. (h) Heteroduplex similar to that in panel g except that the central 10 bases of the heteroduplex are not complementary. (B) Kinetic dissociation profiles for heteroduplexes shown in panels a, b, c, and d of panel A. (C) Kinetic dissociation profiles for heteroduplexes shown in panels e, f, g, and h from panel A.

significantly bind to the filter but can be retained by virtue of stable interaction with trimeric N, which is efficiently retained on the filter. Retention of the labeled 5' or 3' terminus is shown in Fig. 6C. Since the pseudopanhandles are initially mostly double-stranded RNAs and are then dissociated by N, it might be expected that RNA could be retained on filters both in duplex form and following dissociation, provided that N remains stably bound with the RNA following dissociation. Over prolonged incubation times, there is marked preferential retention of the 5' terminus on the filter in comparison to that of the 3' end, indicative of stable protracted interaction be-

tween N trimers and the 5' terminal sequence (Fig. 6C). However, at a relatively early time point (10 min) following incubation with N protein trimer, both 5' and 3' ends are retained on the filters. These data are consistent with retention of both the 5' and 3' termini in the form of initial complex formation between N and the largely duplex panhandle. Then, as duplex dissociation ensues, the 3' end of the panhandle is not retained, as this end does not remain stably associated with N.

To further investigate the association of trimeric N with the termini, we carried out additional filter binding studies using the terminal 32 nucleotides from the individual 5' and 3' ends

FIG. 6. N-mediated dissociation of helices corresponding to the viral panhandle. (A) The heteroduplex formed by 32 nucleotides from 3' and 5' ends of the SNV S segment RNA. In panel i, the 3' nucleotides were radioactively labeled, whereas in panel ii the 5' nucleotides were labeled. Labeled nucleotides are indicated by bold lettering. (B) Kinetics for the dissociation of radiolabeled 3' and 5' nucleotides from the heteroduplexes shown in panels i and ii. (C) RNA filter binding data using the two heteroduplexes. Each heteroduplex was incubated with N protein trimer for the indicated time period, and protein-dependent retention of radiolabeled 3' nucleotides of the panhandle (shown by dark boxes) and 5' nucleotides of the panhandle (shown by open boxes) was measured. The molar ratio of RNA/N was 1:1. Panel D shows the results of an RNA filter binding assay with increasing concentrations of N protein trimer and either the 3'-terminal 32 nucleotides (open squares) or the 5'-terminal 32 nucleotides (filled squares). Measured dissociation constants for both RNAs are also shown. N protein was incubated with radiolabeled RNA for 45 min at room temperature before filtration through nitrocellulose filters.

of SNV S segment RNA. The corresponding binding profiles for trimeric N and these two RNAs are shown in Fig. 6D. The dissociation constants (K_D) for the 5' and 3' ends indicate that the 5' end of the viral RNA is recognized at higher affinity than is the 3' end. These data are consistent with previous observations (6, 19, 25, 29, 30) and the notion that high-affinity recognition of the panhandle is followed by panhandle dissociation and maintenance of N with the 5' end of the viral RNA. However, the binding affinity of trimeric N for intact panhandles (K_D of 25 to 30) (16) is superior to that observed for binding to either terminus alone.

Based on previous *in vitro* binding assays, it would be expected that nonviral RNA would not stably interact with trimeric N and be efficiently retained on nitrocellulose filters. Thus, the nonviral heteroduplex used in Fig. 4B(a) was similarly challenged with N protein, and the retention of dissociated short, radiolabeled RNA was monitored using a filter binding assay as described above. As expected, we observed that the retention of this short RNA was negligible (data not shown).

N can facilitate transcription initiation through the panhandle. Since dissociation of the panhandle would be expected to either precede or occur in concert with genome replication,

we postulated that N protein might unwind the panhandle and enable replication initiation at the accessible 3' terminus by the viral RNA-dependent RNA polymerase. To indirectly test this hypothesis, we asked whether N protein can facilitate primed transcription initiation by an alternative polymerase (reverse transcriptase) using the panhandle as a template. A minipanhandle RNA having 32 nucleotides from 5' and 3' ends of SNV S segment RNA separated by six uracil residues was used as a template for reverse transcriptase in the presence and absence of N (Fig. 7A). Typically, reverse transcription reactions involve thermal denaturation and annealing of RNA template and a primer. However, we incubated minipanhandle RNA at room temperature (without heating or cooling steps) with an RNA primer complementary to the 3' end of the panhandle in the presence or absence of trimeric N and reverse transcriptase. Under these conditions, N protein appeared to facilitate the annealing of the primer to the panhandle, resulting in the generation of a reverse transcription product (Fig. 7B, lane 2). In contrast, in the absence of trimeric N the thermodynamically stable panhandle structure was apparently not significantly dissociated and reverse transcription was significantly reduced (Fig. 7B, lane 1). However, reverse transcription was most robust following thermal denaturation to allow annealing

FIG. 7. Reverse transcription using SNV S minipanhandle as a template. Panel A depicts an SNV S segment minipanhandle containing 32 nucleotides from both the 3' and 5' end of S segment RNA separated by six uracil residues that comprise the loop sequence. An RNA primer complementary to the 3' end of this minipanhandle, which was used as a primer for reverse transcription, is also shown at the bottom of the minipanhandle. (B) Reverse transcription reactions using SNV minipanhandle and the RNA primer shown in panel A. SNV S minipanhandle template and RNA primer incubated at RT without thermal denaturation or addition of N protein are shown in lane 1. The SNV S minipanhandle template and RNA primer incubated at RT with N protein and without thermal denaturation are shown in lane 2. Reverse transcription products from a reaction with the SNV S minipanhandle template and RNA primer following thermal denaturation and primer annealing are shown in lane 3. (C) Reverse transcription products produced at different molar ratios of trimeric N and the SNV S minipanhandle in the absence of thermal denaturation. Samples were fractionated using 12% SDS-PAGE in panel B, whereas an 8% gel was used in panel C.

of the RNA primer to the minipanhandle (Fig. 7B, lane 3). To further examine the ability of trimeric N to potentially dissociate the panhandle and augment reverse transcription, we carried out reactions using a range of panhandle/N ratios. We observed that reverse transcription was most efficient at a panhandle/N ratio of about 1:10 to 1:15 (Fig. 7C). These data are consistent with the idea that N dissociates the panhandle to create an accessible 3' terminus that can be used for transcription initiation. Since N protein facilitated the activity of this heterologous polymerase, we suggest that N and hantavirus RNA polymerase may also work cooperatively in replicating the viral genome.

DISCUSSION

Hantavirus N protein has three interrelated activities of probable biological significance. First, the protein has the propensity to bind to viral RNA with specificity during the encapsidation process, which is mirrored *in vitro* by specific binding to the panhandle. Second, N can serve as an RNA chaperone to nonspecifically dissociate RNA duplexes, and this activity may help facilitate RNA panhandle formation *in vitro*. Finally, it appears that N is able to dissociate, at least transiently, the terminal viral panhandle. The data presented in this paper are directed principally at defining some of the parameters required for helix dissociation.

The termini in both minus- and plus-strand RNA molecules can undergo base pairing to form a panhandle structure. However, the hantaviral genome synthesized under in vitro conditions can misfold, and a significant proportion of such RNA molecules can potentially lack the characteristic panhandle structure at the terminus. Formation of the panhandle is likely

to be driven by both the viral RNA substrate itself and N. In particular, those nucleotides that comprise the panhandle have very few possible pairing partners (15). In vitro formation of the panhandle is substantially hindered by alternative, locally stable, intramolecular interactions (kinetic traps). N protein (an RNA chaperone) then facilitates escape of the RNA substrate from kinetic traps by repetitive nonspecific helix destabilization to enable the opportunity for the panhandle to form.

Under *in vitro* conditions, the panhandle structure formed in the negative-sense viral RNA molecule is specifically recognized by hantaviral N protein trimers, whereas panhandles formed by plus-strand viral RNA molecules are recognized at lower affinity (16). This specific interaction between the minus-strand panhandle and N protein might facilitate preferential encapsidation of the minus strand. Recognition of the minus- and plus-strand panhandle might also be involved in genome replication. Interestingly, we found that the RNA panhandle was more rapidly dissociated by N trimers than were other RNA duplexes examined in helix dissociation assays. This could be due to the fact that N binds with high affinity with the panhandle. In addition, following dissociation, N remains associated with the 5' terminus. Although purified trimeric N associates preferentially with the panhandle (17), N protein from related viruses (in the form of a mixed population of N with alternative subunit composition) binds preferentially with the 5' terminus of viral RNA (6, 19, 25, 29, 30). N probably has a role in the replication of the RNA genome to transiently dissociate the panhandle to render the 3' end of the genome accessible for replication initiation by the RdRp. During this process, N would remain in association with the 5' end, so that the accessible 3' end would be available for replication initiation.

Our results using purified trimeric N, which indicate that the protein dissociates the viral RNA panhandle and remains associated with the 5' end, are consistent with results we obtained previously using unfractionated N protein (15). This is significant from the standpoint that specific and high-affinity viral RNA panhandle binding is a characteristic of the N trimer, whereas unfractionated N does not discriminate between the panhandle and the single-stranded 5' end of the viral RNA (17). It is possible that only trimeric molecules of N protein are functional in a mixed population or that N protein undergoes trimerization while interacting with RNA. Regardless, it is apparent that high-affinity binding of trimeric N with the panhandle leads to both panhandle dissociation and retention of N with the 5' end of the panhandle.

The nonspecific RNA helix-unwinding (RNA chaperone) activity of N requires a single-stranded region situated 3' to the RNA duplex that will be dissociated by N. Thus, it appears that dissociation takes place in the 3'-to-5' direction and is attributable to either an enzymatic catalytic activity or displacement of the RNA duplex by cooperative single-stranded RNA binding that dissociates the duplex by invasion of the duplex. The latter process would be expected to require sufficiently large amounts of protein for concerted dissociation of duplex RNA. However, significant helix dissociation takes place at a 1:1 molar ratio of N and duplex RNA. These data are consistent with the idea that helix dissociation mediated by N works catalytically, in a way that does not require the cooperative binding of a large number of protein molecules. However, these data do not absolutely rule out the latter possibility. The exact mechanism for helix unwinding remains to be elucidated.

Helix unwinding does not require chemical energy. How is the helix-unwinding process driven? Hantavirus N protein contains a prototypical "disordered" domain that likely functions during RNA helix destabilization activity. Tompa and Csermely made the intriguing observation that the theoretical disorder among the known RNA chaperones far surpasses that of any other class of proteins (31). Disordered regions in polypeptides are unlikely to assume uniform configuration in a population of protein molecules. This disorder could serve two important functions. First, lack of consistent structure would enable chaperones to interact with a variety of substrate molecules at relatively low affinity, consistent with their ability to unfold many substrates. Second, binding of the disordered chaperone region to the misfolded RNA would result in transient ordering of the disordered region and concomitant unwinding of the duplex RNA in a process of "reciprocal entropy transfer." Thus, simultaneous ordering of the chaperone would provide the thermodynamic cost for unwinding the duplex RNA in the kinetically trapped misfolded RNA molecule.

For the negative-strand RNA viruses, including the *Bunyaviridae*, the paradigm is that intracellular viral RNA and cRNA are found in association with N protein. How might the RNA chaperone activity of hantavirus N function if the full-length viral RNAs exist as such nucleoprotein complexes in vivo? During relatively early antigenome and vRNA synthesis, the amount of intracellular N may not be sufficient to saturate the antigenome and vRNA, and the formation of functional higher-order structures, such as the panhandle, may require the chaperone activity of N. At later times during infection, rapid association of N with full-length RNA might enable N to dissociate local

kinetic traps prior to the formation of stable nucleoprotein complexes. Transient association with viral mRNA might also facilitate escape from kinetic traps and increase translation efficiency.

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