Importance of Hydrogen Bond Contacts between the N Protein and RNA Genome of Vesicular Stomatitis Virus in Encapsidation and RNA Synthesis[∇]

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Vesicular stomatitis virus (VSV) genomic RNA encapsidated by the nucleocapsid (N) protein is the template for transcription and replication by the viral polymerase. We analyzed the 2.9-Å structure of the VSV N protein bound to RNA (T. J. Green, X. Zhang, G. W. Wertz, and M. Luo, *Science* 313:357–360, 2006) and identified amino acid residues with the potential to interact with RNA via hydrogen bonds. The contributions of these interactions to N protein function were investigated by individually substituting the residues with alanine and assaying the effect of these mutations on N protein expression, on the ability of the N protein to interact with the phosphoprotein (P), and on its ability to encapsidate RNA and generate templates that can support transcription and RNA replication. These studies identified individual amino acids critical for N protein function. Nine nucleotides are associated with each N monomer and contorted into two quasi-helices within the N protein RNA binding cavity. We found that N protein residues that formed hydrogen bond contacts with the nucleotides in quasi-helix 2 were critical to the encapsidation of RNA and the production of templates that can support RNA synthesis. Individual hydrogen bond interactions between the N protein and the nucleotides of quasi-helix 1 were not essential for ribonucleoprotein (RNP) template function. Residue R143 forms a hydrogen bond with nucleotide 9, the nucleotide that extends between N monomers. R143A mutant N protein failed to encapsidate RNA and to support RNA synthesis and suppressed wild-type N protein function. These studies show a direct correlation between viral RNA synthesis and N protein residues structurally positioned to interact with RNA.

Vesicular stomatitis virus (VSV) is a negative-sense RNA virus and is the prototypic member of the *Rhabdoviridae* family (27, 39). The 11,161-bp genome is composed of five genes that encode the five structural proteins in the order nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, glycoprotein (G), and the large polymerase subunit (L) (1, 4, 36). These genes are flanked by 3' leader and 5' trailer regions, which are required for viral RNA synthesis (22, 25, 30, 37, 44). The RNA genome is always found associated with the nucleocapsid (N) protein, and this ribonucleoprotein (RNP) complex is the template for transcription and RNA replication (18, 38, 41). The RNA-dependent RNA polymerase (RdRp), which consists of the viral P and L proteins (28), can utilize only encapsidated genomes as a template; naked RNA is not recognized by the polymerase (11, 12). The N protein is maintained in a functional form by its association with the P protein and forms large insoluble aggregates when expressed in the absence of the P protein (8, 15, 19, 33, 34).

The RdRp directs the following two types of RNA synthetic reactions from the encapsidated genomic template: transcription and replication. Transcription is obligatorily sequential, initiating at a 3' proximal entry site (10, 45), and does not require de novo protein synthesis (27). Attenuation at suc-

sive gene junctions produces a gradient of mRNA products, N mRNA being the most abundant and L mRNA being the least (1, 4, 21, 40). Replication of the genome requires de novo synthesis of the N protein (3, 32). During RNA replication, the RdRp ignores the gene junctions to generate the antigenome, a full-length complementary copy of the negative-sense genome. The antigenome is also encapsidated by the N protein and is used as a template to synthesize negative-sense genomic RNA (38), which can be packaged into newly formed virus particles (27). The transition between transcription and RNA replication and the factors involved in this process are poorly understood.

The structure of the VSV N protein complexed with RNA has been solved at 2.9-Å resolution (16). The structure solved was a decameric ring of N monomers associated with 90 nucleotides of RNA; nine nucleotides are associated with each monomer (16). Each N monomer is composed of two lobes, and the RNA is enclosed in a cavity formed between the two lobes in a jaw-like structure (16). The interior of the RNA binding cavity is hydrophobic mainly, with positively charged residues located on the solvent exposed side of the cavity (16).

Within the RNA binding cavity, the nine nucleotides are contorted into two quasi-helices (16). The first quasi-helix is composed of nucleotides 1 to 4, which stack on top of each other facing the solvent (Fig. 1A). Nucleotide 5 is positioned in the opposite direction, facing the hydrophobic interior of the RNA binding cavity, and nucleotide 6 is flipped again to face the solvent. Nucleotides 7 and 8 face the interior of the cavity,

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FIG. 1. N protein amino acids predicted to form hydrogen bonds with RNA. (A) Top-down view of the nine nucleotides in each N protein monomer; each nucleotide is colored differently. Quasi-helix 1 (nucleotides 1 to 4) and quasi-helix 2 (nucleotides 5, 7 and 8) are indicated. (B) Predicted hydrogen-bonding amino acids (R143, K154, K155, A226, K286, S291, and R408) of the VSV N protein viewed facing the RNA binding cavity. The amino acids predicted to form hydrogen bonds are shown in a stick representation in red, with the predicted hydrogen bonds shown as black dotted lines. The remaining amino acids of the N monomer are shown in green in a ribbon form. The nine nucleotides associated with each individual N monomer are alternately shaded light and dark gray, with the backbone phosphates shown in orange. (C) Rotations (90°) of the image in panel B, showing side views of the amino acids predicted to form hydrogen bonds within the RNA binding cavity.

with their bases stacked under that of nucleotide 5. Nucleotides 5, 7, and 8 form quasi-helix 2, while nucleotide 9 bridges between neighboring N monomers (Fig. 1A) (16).

The nine nucleotides are tightly associated with each N monomer and are resistant to digestion by nuclease and to alkaline hydrolysis (27, 38). Chemical probing of nucleocapsids from infected cells and purified virions showed that the nitrogenous bases of the encapsidated nucleotides were accessible to chemical modification; however, the phosphate backbone was protected (20, 23). The N protein-RNA structure shows the RNA is tightly sequestered between the two lobes of the N protein, and several of the nucleotide bases face the interior of the RNA binding cavity (16). It is likely that at least some of the bases are inaccessible to the RdRp within the RNP structure. In order for the RdRp to gain access to the RNA template during transcription and RNA replication, it has been proposed that domain movement of the N protein occurs (14, 16, 46).

The N protein has been shown to affect the processes of transcription and RNA replication in addition to its role as a structural protein. A single amino acid mutation in the N protein, termed PolR, led to a substantial increase in the amount of read-through at the leader-N gene junction (7, 35). Investigation of a highly conserved region of the N protein (residues 282 to 291), located between the N- and C-terminal lobes near the RNA binding cavity, found that in spite of their sequence conservation, substitution of all but one of these residues with alanine produced mutants that supported RNA synthesis at close to wild-type levels (29). Additionally, recent studies have shown that mutations in the C-terminal loop of the N protein result in templates that support increased levels of RNA replication (17). Together, these data indicate that the N protein could be involved in modulating RNA synthesis.

Numerous positively charged amino acids with the potential to form hydrogen bonds are located within the RNA binding cavity, and several of these residues are highly conserved both

in sequence and in structure among members of the *Rhabdoviridae* (16, 26). We hypothesized that hydrogen bond interactions between the N protein and RNA might not only stabilize the RNP structure but also play a critical role in the function of the RNP template, particularly during the domain movement of the N-terminal lobe that is postulated to occur to allow the viral polymerase access to the RNA during transcription and replication (14, 16, 46). In the work reported here, we carried out a bioinformatic analysis of the N protein-RNA structural data and identified the amino acids located in the RNA binding cavity involved in hydrogen bonded N protein-RNA interactions. Individual mutations were engineered to disrupt the formation of the predicted hydrogen bond contacts, and the effects of these changes on N protein function were evaluated. These mutations produced a wide range of phenotypes and identified key residues involved in RNA encapsidation and template function. The data demonstrate the importance of N protein-RNA interactions, particularly hydrogen bonds, in RNP template function and indicate a wider functional, as well as a structural, role for N protein during the VSV life cycle.

MATERIALS AND METHODS

Plasmid constructions. The plasmids containing the VSV N, P, and L genes under the control of the T7 promoter and the Δ Bgl-22 subgenomic replicon have been described previously (31, 43). The QuikChange mutagenesis protocol (Stratagene, La Jolla, CA) was used to introduce mutations into the N gene to generate plasmids containing codon changes that resulted in single amino acid substitutions. Overlapping oligonucleotides (Sigma-Aldrich, St. Louis, MO) containing a GCA codon were used to introduce mutations to alanine into specific sites within the N plasmid. Alanine 226 was mutated to glycine using overlapping oligonucleotides containing a GGU codon. Sequence analysis (SeqWright, Houston, TX) of the entire N gene was done to confirm all mutations.

Transfections and metabolic labeling of RNA. BHK-21 cells were first infected with the vTF7-3 recombinant vaccinia virus (13) and then transfected with plasmids that encode a VSV subgenomic replicon, Δ Bgl-22 (4 μ g), and the VSV N (4.5- μ g), P (1.5- μ g), and L (1- μ g) proteins (43). The N plasmids containing single amino acid mutations to alanine were substituted for the wild-type N plasmid as indicated in the text. Transfections were performed at 31°C or 37°C and incubated for 16 h. Viral RNA synthesis was assayed by direct metabolic labeling or primer extension.

VSV RNA products were recovered from transfected cells as described previously (31, 43). Briefly, at 16 h posttransfection, cells were treated with 10 μ g/ml of actinomycin D-mannitol (Sigma-Aldrich, St. Louis, MO) and subsequently labeled for 5 h in media containing 33 μ Ci/ml of 3 H-uridine (PerkinElmer, Waltham, MA) at 31°C or 37°C. Cells were harvested in lysis buffer (1% NP-40, 0.4% desoxycholate, 66 mM EDTA, and 10 mM Tris-HCl at pH 7.4), and the nuclei were removed by centrifugation. RNA was purified from cytoplasmic extracts by phenol-chloroform extraction and ethanol precipitation. The extracted RNA was electrophoresed through a 1.75% agarose-urea gel (pH 3) and visualized by fluorography (31).

To analyze the effect of N protein mutations on RNA synthesis from an authentic defective interfering particle template (DI-T), BHK-21 cells were coinfecting with vTF7-3 recombinant vaccinia virus and VSV defective interfering (DI) particles. Following an hour-long adsorption period, the cells were transfected with plasmids encoding wild-type or mutant N protein and wild-type P and L proteins. Cells were incubated at 37°C and, at 5 h posttransfection, were treated with actinomycin D-mannitol (10 μ g/ml) and subsequently labeled with 3 H-uridine (33 μ Ci/ml) for 4 h at 37°C. Cytoplasmic extracts were harvested, and RNA was resolved by 1.75% agarose-urea gel (pH 3) electrophoresis and detected by fluorography (31).

Primer extension analysis. RNAs recovered from transfected cells as described above were also analyzed by primer extension. Polyacrylamide gel electrophoresis (PAGE)-purified oligonucleotides (Operon, Valencia, CA) were end labeled in the presence of [γ - 32 P]ATP (50 μ Ci/ml) using T4 polynucleotide kinase (Invitrogen, Carlsbad, CA) and purified from unincorporated label using a QIAquick nucleotide removal column (Qiagen, Valencia, CA). A negative-

sense oligonucleotide (5'-GGATCCTCATTTCAGGAAGTTTTGG-3') that anneals to positions 134 to 109 within the VSV N gene was used to detect transcription and antigenomic replication products from the Δ Bgl-22 subgenomic replicon. A positive-sense oligonucleotide (5'-GGAATGATTGAATGATCAATGG-3') that anneals to positions 10964 to 10986 within the VSV L gene was used to detect genomic products from the Δ Bgl-22 subgenomic replicon. The radiolabeled oligonucleotides were added in excess to RNA samples, and primer extension reactions were carried out for 30 min at 42°C using Superscript II reverse transcriptase, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The radiolabeled products of these reactions were electrophoresed on a 6% acrylamide sequencing gel containing 8 M urea and visualized by autoradiography (31).

Dominant negative assay. Equivalent amounts of the wild-type N plasmid and the plasmid encoding the R143A, R408A, or R143K mutants were cotransfected with the Δ Bgl-22 subgenomic replicon and the plasmids encoding the P and L proteins and incubated at 31°C overnight. To maintain the optimal N/P ratio within the transfected cells, both wild-type and mutant N plasmids were transfected at various concentrations, such that the combined N protein expression from both plasmids would maintain the optimal N/P ratios.

Nuclease sensitivity of encapsidated RNA. Cell lysates from transfections programmed with wild-type or mutant N protein and the P and L proteins were harvested in lysis buffer lacking EDTA (1% NP-40, 0.4% desoxycholate, and 10 mM Tris-HCl at pH 7.4). Then, 20 μ l of cell lysate was mock treated, or treated with micrococcal nuclease (NEB, Ipswich, MA), as per the manufacturer's specifications. RNA was purified by phenol-chloroform extraction and assayed by primer extension as described above.

Assay for the formation of N-P protein complexes. Cultures of BHK-21 cells were infected with vTF7-3 recombinant vaccinia virus (13) for 1 h. Infected cells were then transfected with plasmids encoding N protein (4.5 μ g) and P protein (1.5 μ g). N protein expression plasmids containing single amino acid mutations to alanine were substituted for the wild-type N plasmid, where indicated. Transfections were allowed to progress for 16 h at 37°C, at which time the media was replaced with 1 ml of methionine-free media for 30 min at 37°C. 35 S-labeled methionine was added at a final concentration of 50 μ Ci/ml (PerkinElmer, Waltham, MA) to the cells and incubated at 37°C for an additional 30 min. Cells were harvested in lysis buffer (1% NP-40, 0.4% desoxycholate, 66 mM EDTA, and 10 mM Tris-HCl at pH 7.4), and the nuclei were removed by centrifugation. The cytoplasmic extracts were separated into two fractions and assayed for total N protein or for the formation of N-P complexes by immunoprecipitation using 2 μ l of a monospecific, polyclonal antisera to the P protein. Protein-antibody complexes were precipitated with protein A-Sepharose (Sigma-Aldrich, St. Louis, MO). The immunoprecipitated N-P proteins or total cytoplasmic extracts were analyzed by electrophoresis on 10% polyacrylamide gels (77:1, acrylamide-bisacrylamide) (8).

Detection of N protein-RNA interactions by immunoprecipitation and Western blotting. BHK-21 cells were infected with vTF7-3 recombinant vaccinia virus and transfected with plasmids encoding the subgenomic replicon Δ Bgl-22 (4 μ g), the N protein (4.5 μ g), and the P protein (1.5 μ g) but not the L protein. Transfections were allowed to proceed for 16 h at 37°C. Cells were harvested in 400 μ l lysis buffer (1% NP-40, 0.4% desoxycholate, 66 mM EDTA, and 10 mM Tris-HCl at pH 7.4), and the nuclei were removed by centrifugation. To examine the relative N protein levels before immunoprecipitation, 4 μ l of lysate was retained for Western blot analysis. To determine the N protein levels after immunoprecipitation, another 4 μ l of lysate was treated with 8 μ l of a monoclonal antibody, 10G4 (a kind gift from Doug Lyles, Wake Forest University) (6), directed against the N protein. The N-antibody complexes were precipitated using protein A-Sepharose (Sigma-Aldrich, St. Louis, MO) as described above. The Sepharose A or lysates were resuspended in 2 $\times$ Laemmli sample buffer, and the proteins were resolved by electrophoresis on 10% polyacrylamide gels (77:1, acrylamide-bisacrylamide) (8). The proteins were transferred to polyvinylidene difluoride membranes (Millipore), and the membrane was probed using a rabbit polyclonal VSV anti-N antibody (1:1,000) and a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 680 (1:5,000) (Invitrogen, Carlsbad, CA). The blots were scanned with an Odyssey scanner (Li-Cor Biosciences), according to the manufacturer's instructions.

To measure RNA binding by the wild-type or mutant N protein, the remainder of the lysate was immunoprecipitated using 8 μ l of the 10G4 antibody (6), and the N-RNA-antibody complexes were precipitated using protein A-Sepharose (Sigma-Aldrich, St. Louis, MO), as previously described. RNA was isolated from precipitated N-RNA complexes by extraction with phenol-chloroform and ethanol precipitation. RNA was detected by primer extension, using a plus-sense primer (5'-GGAATGATTGAATGGATCAATGG-3'), which anneals to posi-

TABLE 1. N protein residues in the RNA binding cavity predicted by ENTANGLE to contact RNA via hydrogen bonds^a

Residue	Amino acid position	Hydrogen bond distance (Å)	Nucleotide
Arg	143	1.9	9
Arg	143	1.8	9
Lys	154	2.1	8
Lys	155	1.8	8
Ala	226	2.3	4
Lys	286	1.3	2
Ser	291	2.4	4
Ser	291	2.1	4
Ser	291	1.6	4
Arg	408	2.2	7

^a The nucleotide predicted to participate in the interaction and the hydrogen bond distances are given.

tions 10964 to 10986 in the VSV L gene to detect any encapsidated negative-sense T7 transcript.

Quantitation. A Storm 860 scanner and the ImageQuant program (Molecular Dynamics) were used to detect and quantify ³⁵S-labeled protein or ³³P-labeled primer extension products. ³H-labeled RNA was detected by fluorography and quantified using a PDI 320i densitometer and Quantity One (Bio-Rad) software. The density in the control lacking N protein was subtracted as background. Quantitations were based on the average values from a minimum of three separate experiments.

Structural analysis. The ENTANGLE program (2) (<http://www.bioc.rice.edu/~shamoo/project/entangle/install.htm>) was used to predict hydrogen bond protein-RNA interactions from the available N protein-RNA structure (16), which was obtained from the Protein Data Bank (PDB) website (PDB accession no. 2GIC). A hydrogen bond donor-to-acceptor cutoff distance of 3.9 Å and a hydrogen-to-acceptor cutoff of 2.5 Å were used. The PyMOL molecular graphics analysis program (DeLano Scientific LLC) (www.pymol.org) was used to visualize amino acid substitutions within the N protein-RNA structure (9). Figures in this work showing protein structure were generated using the PyMOL software.

RESULTS

Identification of potential RNA binding amino acids within the N protein. An in depth analysis of the VSV N protein-RNA structural data (PDB accession no. 2GIC) was performed using the program ENTANGLE (2), which predicts protein-RNA interactions. ENTANGLE identified seven amino acids (R143, K154, K155, A226, K286, S291, and R408) located within the RNA binding cavity that were predicted to form hydrogen-bonded interactions with the phosphate backbone of the RNA (Fig. 1B and C and Table 1), and their predicted interactions are described as follows: residues A226, S291, and K286 were predicted to interact with the nucleotides of quasi-helix 1 (nucleotides 1, 2, 3, and 4) and are located on two regions that descend down between the two lobes (Fig. 1B and C). These regions have been postulated to act as hinges to allow N protein domain movement during RNA synthesis (16). Residue K286 was predicted to form a hydrogen bond with nucleotide 2, and residues A226 and S291 were predicted to form hydrogen bond interactions with nucleotide 4 (Fig. 1B and C and Table 1).

Residues R143, K154, K155, and R408 were predicted to interact with the nucleotides of quasi-helix 2 (nucleotides 5, 7, and 8) and nucleotide 9 (Fig. 1B and C and Table 1). The long positive side chain of R408 descends from the C-terminal lobe toward the RNA and is positioned at the tip of the solvent-exposed side of the RNA binding cavity (Fig. 1B and C).

Residues R143, K154, and K155 are part of two alpha-helices within the N-terminal lobe (Fig. 1B and C). Both neighboring lysine residues K154 and K155 were predicted to form hydrogen bonds with nucleotide 8 (Fig. 1B and C and Table 1). A single hydrogen bond was predicted for both residues, but each residue was predicted to interact with a different oxygen atom on the phosphate backbone. The R143 residue was predicted to form a hydrogen bond with nucleotide 9, the nucleotide that extends between N monomers (Fig. 1B and C).

Although the solved N-RNA structure was composed of 10 monomers (16), the ENTANGLE program uses a single monomer to make predictions about protein-RNA interactions. We used the molecular graphics software PyMOL (9) to further analyze the interactions predicted by ENTANGLE in 5 out of the 10 monomers and determined that some of the residues identified by ENTANGLE can form hydrogen bonds in some monomers but not others. However, the majority of the predicted contacts were uniform around the ring. The ENTANGLE analysis of the structure provided the rationale for selecting amino acids for mutagenic analysis that are likely to be involved in RNA binding, but it did not rank the interactions in order of importance.

We substituted alanine *in silico* for each of the six residues predicted to make side chain hydrogen bonds to RNA. Analysis using PyMOL indicated that these mutations would disrupt hydrogen bond formation between each amino acid and the RNA. In the case of A226 where the hydrogen bond is contributed from the protein backbone, we substituted glycine, which could be considered as a control for altering protein structure rather than a test for disrupting a hydrogen bond. Together, the ENTANGLE and PyMOL analyses directed us to select the seven residues for mutagenesis.

N protein expression and interaction with the P protein.

The seven amino acids in the N protein predicted to form hydrogen bonded interactions with RNA were individually substituted with alanine, with the exception of A226, which was mutated into glycine by site-directed mutagenesis of a plasmid encoding the N protein (see Materials and Methods). Mutant N proteins were assayed for protein expression in comparison to the wild-type N protein by direct metabolic labeling. Cells were cotransfected with plasmids encoding either wild-type or mutant N protein and the P protein. Expression of the P protein is required to prevent the N protein from forming insoluble aggregates, which are unable to support RNA synthesis (19). At 16 h posttransfection, cells were labeled with [³⁵S]methionine and then harvested, and proteins present in cytoplasmic extracts were analyzed by electrophoresis on reducing SDS-PAGE gels (see Materials and Methods). The data depicted in Fig. 2A show that N proteins containing single amino acid mutations were expressed at levels similar to that of wild-type N protein.

The ability of the mutant N proteins to interact with the P protein was assayed by testing whether the N protein could be coprecipitated with the P protein following incubation with a monospecific, polyclonal antibody to the P protein. All of the mutant N proteins were able to interact with the P protein, as shown in Fig. 2B, and were coprecipitated at levels similar to those of the wild-type N protein (Fig. 2B), which suggests that these mutations did not disrupt the ability of the N protein to interact with the P protein.

FIG. 2. Expression and N-P interactions of N proteins containing single amino acid substitutions. (A) BHK-21 cells infected with vTF7-3 recombinant vaccinia virus were transfected with plasmids encoding the P protein and either wild-type or mutant N protein. Transfections were incubated at 37°C and, at 16 h posttransfection, labeled with [³⁵S]methionine. Cells were harvested, and proteins were resolved by SDS-PAGE, as described in Materials and Methods. (B) Interaction of the N proteins with P protein was measured by coimmunoprecipitation of N and P protein complexes using an anti-P antibody. [³⁵S]methionine-labeled cell lysates were generated as described in the legend to panel A and precipitated with Sepharose A beads. Coimmunoprecipitated N and P proteins were resolved by SDS-PAGE, as described in Materials and Methods. WT, wild-type.

Effects of mutations in the N protein on RNP template function. To test if the mutant N proteins could form functional RNP templates that support transcription and RNA replication, VSV RNA synthesis was reconstituted using the T7 transfection system as reported previously (31, 43). The vTF7-3 recombinant vaccinia virus-infected BHK cells were transfected with a subgenomic replicon (Δ Bgl-22) and plasmids encoding the VSV P, L, and wild-type or mutant N proteins. The Δ Bgl-22 subgenomic replicon contains the 3' leader and 5' trailer regions required for VSV RNA synthesis and produces a 368-nucleotide (nt) capped and polyadenylated mRNA and 478-nt genomic and antigenomic replication products (previously described in reference 43) (diagrammed in Fig. 3A). RNA synthesis was analyzed by direct metabolic labeling with ³H-uridine in the presence of actinomycin D, and RNAs were resolved by electrophoresis (see Materials and Methods).

The wild-type N protein and five of the seven mutant N proteins supported VSV RNA synthesis, albeit at different levels (Fig. 3B). The major RNA synthetic event supported by wild-type N protein was transcription of the single mRNA expressed from the subgenomic replicon (Fig. 3A), which appears as a diffuse band due to differential polyadenylation (Fig. 3B) (5). Genomic and antigenomic RNA replication occurred at lower levels, as shown by the data depicted in Fig. 3B. Analyses were carried out at 31°C, which optimizes replication levels. The R143A and R408A mutant N proteins were unable to support either transcription or RNA replication, as no actinomycin D-resistant RNA products were detected for these N protein mutants (Fig. 3B). In contrast, N proteins with mutations K154A, A226G, and S291A supported RNA replication and transcription at the wild-type levels or greater (Fig. 3B). N proteins with mutations K155A and K286A supported RNA

synthesis at 25 to 40% of the wild-type levels (Fig. 3B). RNA synthesis was also examined at 37°C (data not shown), and the patterns of synthesis supported by the mutant N proteins relative to wild-type N were found to be similar at 37°C compared to those at 31°C, although the overall levels of RNA replication were lower.

To test the effect of N mutations on the ability of the N protein to encapsidate nascent RNA and to examine the possibility that the mutant N proteins might differentially encapsidate the nascent T7 transcript generated during recovery of the template RNA from cDNA, we also examined RNA synthesis supported by the wild-type or mutant N protein using authentic defective interfering particles as templates. As shown by the data depicted in Fig. 3C, all of the mutant N proteins except R143A and R408A mutant N proteins supported replication of the defective interfering (DI) genomic and antigenomic RNAs, thereby confirming the data shown in Fig. 3B. These data also show that even with a natural template, the R143A and R408A mutant N proteins were unable to support RNA replication. Since these experiments were carried out using a natural, functional DI template encapsidated with wild-type N protein, they indicate that the defect in R143A and R408A mutant N proteins lies at the level of encapsidation of the newly synthesized RNA.

Nuclease protection of encapsidated RNAs. The nucleocapsid protein protects the encapsidated genomic and antigenomic RNAs from nuclease digestion (38, 42). To determine whether the N protein mutations affected nuclease protection, BHK cells were infected with vTF7-3 and subsequently transfected with a subgenomic replicon (Δ Bgl-22) and plasmids encoding the P, L, and wild-type or mutant N proteins. Cell lysates were harvested at 16 h posttransfection, and cytoplasmic extracts were divided in half; one half of the sample was treated with micrococcal nuclease, and the other half was left untreated. The RNA was then isolated by phenol-chloroform extraction, and the genomic and antigenomic RNAs were detected by primer extension. The data shown in Fig. 4A and B (top panels) show genomic and antigenomic RNA replication levels supported by wild-type N protein and each mutant N protein. These data confirm that all of the mutant N proteins, except R143A and R408A mutant N proteins, supported RNA replication. In addition, the data show that the effects of the mutations on genomic and antigenomic RNA synthesis were similar, with K155A and K286A mutant N proteins supporting reduced levels of RNA replication compared to those of wild-type N protein, while the remainder replicated at or near wild-type levels (Fig. 4A and B).

Digestion of the RNA replication products with micrococcal nuclease showed that all of the encapsidated replication products synthesized in the presence of wild-type or mutant N protein that supported replication were protected against nuclease digestion (Fig. 4A and B, bottom panels), whereas the majority of the initial T7 transcript (Fig. 4A) and mRNA (data not shown) were digested. These data show that the mutant N proteins that supported RNA synthesis encapsidated both genomic and antigenomic RNAs and that encapsidation conferred protection from nuclease digestion like the wild-type N protein.

Analysis of the ability of R143A and R408A mutant N proteins to bind RNA. The R143A and R408A mutant N proteins

FIG. 3. RNA synthesis supported by N proteins containing single amino acid mutations. (A) Schematic representation of the Δ Bgl-22 subgenomic replicon, showing the 3' leader (Le) and 5' trailer (Tr) regions required for transcription and replication and the single transcriptional unit positioned between these regions. The subgenomic replicon is initially transcribed by the T7 polymerase and serves as a template for subsequent viral replication of both antigenomic (AG) and genomic (Gen) RNA as well as for transcription of mRNA from the single gene. (B) BHK-21 cells infected with vTF7-3 recombinant vaccinia virus were transfected with plasmids encoding the Δ Bgl-22 subgenomic replicon, wild-type or mutant N protein, and wild-type P and L proteins. Transfections were incubated at 31°C and, at 16 h posttransfection, exposed to 3 H-uridine. Cells were harvested, and the RNA was extracted and analyzed by acid agarose-urea gel electrophoresis, as described in Materials and Methods. Transcription (mRNA) and replication (Rep) products are indicated (left). The gel shown is representative of three separate repeats, and the results are quantitated in the bottom panel, as described in Materials and Methods. Transcription and replication levels are expressed as percentages of the wild type, and values are the average results from three separate experiments. Error bars represent standard deviations. (C) RNA synthesis templated by DI particles. BHK-21 cells were infected with vTF7-3 recombinant vaccinia virus and wild-type VSV defective interfering particles (DI-T). After an hour-long adsorption period at 37°C, the inoculum was removed, and the cells were transfected with plas-

FIG. 4. Nuclease protection of encapsidated RNAs. BHK-21 cells infected with vTF7-3 recombinant vaccinia virus were transfected with the Δ Bgl-22 subgenomic replicon and plasmids encoding the wild-type P and L proteins and wild-type or mutant N protein. Transfections were incubated at 31°C and harvested at 16 h posttransfection. Cytoplasmic extracts were digested with micrococcal nuclease or left untreated. RNAs present in the digested or undigested extracts were measured by primer extension. (A, B) Genomic (Gen) (A) and antigenomic (B) products from the Δ Bgl-22 subgenomic replicon. RNAs were quantified as described in Materials and Methods, and the level of RNA in the digested samples was expressed as a percentage of the undigested RNA. The primer extension product templated by the T7 primary transcript (designated T7) is separated from the genomic RNA (Gen) primer extension product by 2 nt (31). This product appears as a doublet because a portion of this transcript is capped by the vaccinia virus-capping enzyme. Since the majority of the initial T7 transcript remains unencapsidated, it is digested by nuclease. Nd., none detected.

failed to form templates competent for RNA synthesis, and experiments using functional DI templates indicated that their defect was at the level of encapsidation of nascent RNA. Arginine 143 is positioned to form a hydrogen bond with the phosphate backbone of nucleotide 9 (Fig. 5A, upper left panel), which is the nucleotide that extends between the N

mids encoding wild-type or mutant N protein and wild-type P and L proteins. At 5 h posttransfection, cells were treated with actinomycin D and exposed to 3 H-uridine for 4 h at 37°C. Cytoplasmic extracts were harvested, and the RNA was extracted and analyzed by acid agarose-urea gel electrophoresis, as described in Materials and Methods. Genomic (Gen) and antigenomic (AG) replication products supported by mutant N proteins from the authentic DI template were detected by fluorography and quantified, as described in Materials and Methods, and expressed as percentages of the wild type (31).

FIG. 5. Effect of R143A and R408A mutations on N protein-RNA interactions. (A) Modeling of residues R143 and R408 of the VSV N protein and the R143A and R408A mutations, showing the loss of the predicted hydrogen bonds after mutation to alanine. The R143 and R408 residues and the R143A and R408A mutations are shown in stick form (red), with the predicted hydrogen bonds shown as black dotted lines. The remaining amino acids of the N monomer are shown in ribbon form (green). The nucleotides are alternately shaded light and dark gray, with the backbone phosphates shown in orange. (B) RNA bound by wild-type (WT) or mutant N protein. BHK-21 cells infected with vTF7-3 recombinant vaccinia virus were transfected with the Δ Bgl-22 subgenomic replicon and plasmids encoding the P protein, wild-type N protein, or R143A or R408A mutant N protein but not the L protein. Transfections were incubated at 37°C, and cytoplasmic extracts were harvested at 16 h posttransfection. RNA bound by wild-type or mutant N protein was assayed by immunoprecipitation using anti-N antisera. After immunoprecipitation, phenol-chloroform extractions were performed, and immunoprecipitated T7 transcript (T7) RNA from the subgenomic replicon was detected by primer extension, as described in Materials and Methods. Quantitation of the results with the standard errors of the means from three representative experiments is shown. The level of signal in the no N control lane was subtracted as background. (C) Western blot analysis of the total N protein levels in transfected cells at the time of harvest and after immunoprecipitation (IP). Western blotting was used to determine the levels of the N protein present in the cells transfected with wild-type or mutant N protein at the time of harvest and after immunoprecipitation.

monomers. This residue could be crucial to maintaining the structure of the RNA at the end of quasi-helix 2 (Fig. 1 and 5A). Arginine 408 is located in the center of the RNA binding cavity on the lip of the C-terminal lobe of the protein, reaching down into the RNA cavity to interact with the phosphate backbone of nucleotide 7 (Fig. 5A, lower left panel). The nitrogenous base of nucleotide 7 faces the interior of the RNA binding cavity, but the phosphate backbone of nucleotide 7 is located parallel to that of nucleotide 6 at the solvent-exposed side of the RNA binding cavity. This suggests that residue R408 is involved in stabilizing this region of the RNA chain as it turns from the solvent-exposed conformation of nucleotide 6, the “bulge” nucleotide, to that of nucleotide 7, which is in the opposite orientation, facing toward the interior of the protein (Fig. 1 and 5A). Both R143 and R408 are positioned to be key in stabilizing RNA in the cavity.

To test whether these mutations affected the ability of the N protein to interact with RNA, transfections were programmed with the subgenomic replicon (Δ Bgl-22) and plasmids encoding the P protein and wild-type or mutant N protein but not the polymerase L protein. The T7 transcript of the subgenomic

replicon will be expressed, and a portion will be encapsidated, but in the absence of the L protein, it will not be replicated. The formation of these N-RNA complexes was probed by asking whether the initial T7 transcript could be immunoprecipitated with anti-N protein antisera. The RNA present in immunoprecipitated complexes was extracted with phenol and assayed by primer extension (Fig. 5B). The T7 transcript synthesized in the presence of wild-type N protein was precipitated by the anti-N antisera, indicating that it interacted with RNA (Fig. 5B). The R143A and R408A mutant N proteins coprecipitated a quarter of the wild-type RNA levels, indicating that their ability to interact with RNA was compromised (Fig. 5B). A low level of T7 RNA was detected in the control lane lacking N protein, and for the quantitation, this signal was subtracted as background (Fig. 5B). The relative levels of N protein expressed by the wild-type or mutant N protein in the transfections before immunoprecipitation and the levels of N protein immunoprecipitated were examined by Western blotting. Figure 5C shows that R143A and R408A mutant N proteins were expressed in similar amounts and immunoprecipitated in equivalent amounts to those of wild-type N protein,

which suggests that the decreased levels of T7 transcript detected by primer extension (Fig. 5B) are due to decreased RNA binding and are not due to differences in the level of mutant N protein expressed or the ability of the mutants to be immunoprecipitated.

Dominant negative effect of R143A and R408A mutant N proteins. To further characterize the R143A and R408A mutant N proteins, we investigated whether these mutant proteins interfered with the ability of the wild-type N protein to function by cotransfecting wild-type and mutant N plasmids. As previously stated, the P protein maintains N in an encapsidation-competent form, and the N/P ratio is critical to support optimal viral RNA synthesis (19). The optimal N/P ratio for the subgenomic replicon system was achieved by transfecting the correct amount of each plasmid (4.5 μ g N plasmid to 1.5 μ g P plasmid). To determine the effect of coexpressing the R143A and R408A N proteins with wild-type N protein while maintaining the optimal N/P ratio, we coexpressed equal amounts of wild-type N plasmid (2.25 μ g) and (R143A or R408A) N protein plasmid (2.25 μ g) with plasmid encoding the P protein (1.5 μ g) and the L protein (1.0 μ g). The wild-type N protein supported both genomic and antigenomic RNA replication and transcription (Fig. 6A, lane 4), as measured by strand-specific primer extension, whereas the R143A and R408A mutant N proteins did not support viral RNA synthesis (Fig. 6A, lanes 5 and 7). When equal amounts of the wild-type N and R143A plasmids were cotransfected, no RNA synthesis was observed (Fig. 6A, lane 6). In contrast, coexpression of equal amounts of wild-type and R408A N proteins resulted in RNA synthesis (Fig. 6A, lane 8). To control for the effect of possible alterations to the N/P ratios from having half of the normal level of functional N protein, we examined the levels of RNA synthesis supported by transfecting half of the level of wild-type N plasmid (2.25 μ g) with half as much P plasmid (0.75 μ g) or transfecting half as much N plasmid (2.25 μ g) with the normal level of P plasmid (1.5 μ g) (Fig. 6A, lanes 2 and 3, respectively). As shown by the data depicted in Fig. 6A, these alterations resulted in minor decreases in the levels of RNA replication and transcription but did not abrogate RNA synthesis, indicating that the reduction in RNA synthesis caused by cotransfecting equal amounts of wild-type and R143A N proteins was not due to altered N/P ratios. Since cotransfection of equal amounts of wild-type and R143A N proteins supported no RNA synthesis (Fig. 6A, lane 6), these data suggest that the R143A N protein exerted a dominant negative suppression of wild-type N protein function.

To explore the extent of the R143A inhibition, we examined the minimum amount of R143A N protein required to inhibit wild-type N protein function. The R143A plasmid was transfected in increasing amounts relative to wild-type N plasmid, always keeping the total level of N plasmid constant. The data depicted in Fig. 6B show that RNA synthesis supported by wild-type N protein was inhibited as the amount of R143A transfected was increased to 25% or more of the total N protein.

Conservative substitutions for N protein residues R143 and R408. To assess the specificity for arginine residues at positions 143 and 408 in the N protein, we also conservatively substituted these residues with lysine. Unlike alanine, both lysine and arginine possess a long, positively charged side chain. The

FIG. 6. Dominant negative effect of N protein mutations. (A) RNA synthesis supported by wild-type N protein alone or in the presence of R143A and R408A mutant N proteins. BHK-21 cells infected with vTF7-3 recombinant vaccinia virus were transfected with the Δ Bgl-22 subgenomic replicon and plasmids encoding wild-type P and L proteins and wild-type or mutant N protein. Lane 1, no N plasmid control; lane 2, 2.25 μ g WT N plasmid plus 0.75 μ g P plasmid; lane 3, 2.25 μ g WT N plasmid plus 1.5 μ g P plasmid; lane 4, 4.5 μ g WT N plasmid plus 1.5 μ g P plasmid; lane 5, 4.5 μ g R143A N plasmid plus 1.5 μ g P plasmid; lane 6, cotransfection of equal amounts of both wild-type N plasmid (2.25 μ g) and R143A N plasmid (2.25 μ g) plus 1.5 μ g P plasmid; lane 7, 4.5 μ g R408A N plasmid plus 1.5 μ g P plasmid; lane 8, cotransfection of both 2.25 μ g wild-type N plasmid and 2.25 μ g R408A N plasmid plus 1.5 μ g P plasmid. Primer extension was used to examine the expression of encapsidated genomic, antigenomic, and mRNA products from the Δ Bgl-22 subgenomic replicon, as detailed in Materials and Methods. The primer extension product templated by the T7 primary transcript (designated T7) from the subgenomic replicon is separated from the genomic RNA primer extension product by 2 nt (31). The T7 product appears as a doublet because a portion of this transcript is capped by the vaccinia virus-capping enzyme. (B) To examine the extent of the R143A dominant negative effect on wild-type N protein, increasing amounts of R143A plasmid were cotransfected with the wild-type N plasmid while maintaining the optimal N/P ratio. Primer extension was used to detect the expression of encapsidated antigenomic and mRNA products from the Δ Bgl-22 subgenomic replicon, as detailed in Materials and Methods.

R143K and R408K mutant plasmids were generated by site-directed mutagenesis of the wild-type N expression plasmid (see Materials and Methods). Direct metabolic labeling showed that the N proteins carrying either the R143K or

FIG. 7. Conservative substitution of arginine with lysine at positions 143 and 408. (A) Expression of R143K and R408K mutants. BHK-21 cells infected with vTF7-3 recombinant vaccinia virus were transfected with plasmids encoding P protein and either wild-type N protein or mutant N protein (R143K or R408K). Cells were incubated at 37°C and, at 16 h posttransfection, labeled with [³⁵S]methionine. Cells were harvested, and labeled proteins were resolved by SDS-PAGE as described in Materials and Methods. (B) Interaction of the N protein with the P protein was measured by coimmunoprecipitation of the N and P protein complexes using an anti-P antibody. [³⁵S]methionine-labeled cell lysates, transfected as described above, were incubated with anti-P antibody and precipitated with Sepharose A beads. Coimmunoprecipitated N and P proteins were resolved by SDS-PAGE as described in Materials and Methods. (C) RNA synthesis supported by wild-type or R143K or R408K mutant N protein. BHK-21 cells infected with vTF7-3 recombinant vaccinia virus were transfected with the ΔBgl-22 subgenomic replicon and plasmids encoding the P protein, the L protein, and wild-type or mutant N protein. Primer extension was used to detect the expression of genomic (Gen), antigenomic (AG), and mRNA products and the initial T7 transcript (T7) from the ΔBgl-22 subgenomic replicon, as detailed in Materials and Methods. (D) RNA synthesis was examined in the presence of wild-type N protein and R143K mutant N protein. Half of the optimal levels of wild-type N plasmid (2.25 μg) and mutant R143K N plasmid (2.25 μg) plus P (1.5 μg) were cotransfected into BHK-21 cells to maintain the required N/P ratio, and primer extension was used to detect the expression of encapsidated genomic (Gen), antigenomic (AG), and mRNA products and the initial T7 transcript (T7) from the ΔBgl-22 cDNA, as detailed in Materials and Methods. The primer extension product templated by the T7 primary transcript (designated T7) is separated from the genomic RNA (Gen) primer extension product by 2 nt (31). The T7 product appears as a doublet because a portion of this transcript is capped by the vaccinia virus-capping enzyme.

R408K mutation were expressed at wild-type levels (Fig. 7A), and immunoprecipitation with anti-P antibody showed that both mutants interacted with P protein (Fig. 7B). When assayed for the ability to support RNA synthesis in the subgenomic replicon system, the N protein carrying the R143K mutation was unable to support viral transcription or RNA replication (Fig. 7C). However, the N protein carrying the R408K mutation supported transcription and RNA replication approximately at wild-type levels (Fig. 7C). These data suggest that the presence of an arginine residue at position 143 is critical for N protein function, but substitution of arginine 408

with lysine is tolerated. The ability of the R143K mutation to exert a dominant negative effect in the presence of the wild-type N protein was also examined. In contrast to coexpression of the wild-type N protein with the R143A N protein, coexpression of the wild-type N protein with the R143K mutant N protein resulted in only a limited reduction in the levels of transcription and RNA replication of the wild-type N protein (Fig. 7D).

DISCUSSION

The structure of the VSV nucleocapsid protein bound to RNA, solved to 2.9-Å resolution (16), was used in conjunction with the software ENTANGLE (2) to identify N protein residues predicted to contact RNA. Using this structure-based analysis, mutations were designed to specifically disrupt predicted hydrogen bonds between the N protein and the RNA. The identified residues were distributed throughout the RNA binding cavity and interacted with both of the quasi-helices formed by the nine nucleotides associated with each N monomer (Fig. 1B and C). The data showed that hydrogen bond interactions with quasi-helix 2 were critical to N protein function, whereas the hydrogen bond contacts with the nucleotides of quasi-helix 1 were not essential for N protein function and RNA synthesis.

Residues A226, K286, and S291 were predicted to interact with quasi-helix 1 and are located on two regions positioned between the N and C-terminal lobes that are postulated to be “hinge” regions (16). Many of the residues within these two regions are conserved among the rhabdoviruses, particularly the residues that are part of the GLSSKSPYSS sequence (residues 282 to 291), which was previously proposed to be an RNA binding domain in the rabies nucleocapsid protein (24). Although mutations A226G and S291A do not represent major amino acid changes, the localization of these residues to a highly conserved region of the N protein suggested they might play a functional role in RNA encapsidation. When these mutant N proteins were tested for the ability to form functional templates, only the K286A mutant N protein supported lower levels of RNA synthesis than the wild-type N protein (Fig. 3B). Substitution of these three residues did not abrogate N function, despite their sequence conservation among rhabdoviruses. This “hinge” region of the N protein is hydrophobic, and it is possible that maintaining the hydrophobic pocket, rather than individual hydrogen bonds, is of greater importance to N protein function. Nayak et al., investigated residues in the GLSSKSPYSS sequence and reported that K286A mutant N protein was able to support RNA replication but, using an indirect reporter gene assay, not transcription at 37°C, although low levels of transcription were detected at 33°C (29). We found that the K286A mutant supported RNA replication and transcription at both 31°C and 37°C at approximately 40% of the wild-type levels and that none of the mutations reported here affected transcription and replication differentially.

In contrast to the residues contacting quasi-helix 1, the residues contacting the nucleotides in quasi-helix 2 were critical for maintaining RNP template function. Residues R143, K155, and R408 are predicted to form hydrogen bond contacts with nucleotides 5, 7, and 8 of quasi-helix 2 and nucleotide 9. These nucleotides are positioned to face the interior of the RNA

binding cavity, and mutation of R143, K155, and R408 to alanine produced N proteins unable to or greatly reduced in their ability to support RNA synthesis (Fig. 3). The failure of these N protein mutants to support RNA synthesis suggests a requirement for hydrogen bonds to maintain these nucleotides in the correct conformation. Without these hydrogen bond interactions, the nucleotides may be unable to adopt the required conformation to achieve the two quasi-helical structures within the RNA binding cavity.

Two of the mutants, R143A and R408A mutant N proteins, were reduced in their ability to bind RNA (Fig. 5B) and were unable to support encapsidation or RNA synthesis (Fig. 3B and C). These residues are also highly conserved among the rhabdoviruses, and both residues were structurally conserved in the rabies virus N protein (26). R143 is predicted to stabilize the conformation of nucleotide 9, which is located at the junction between N monomers. Neither mutation to alanine nor a conservative mutation to lysine was tolerated at this position, suggesting a strict requirement for arginine at this position. Disruption of the hydrogen bond contact from R143 may negatively affect the ability of N protein to anchor the RNA chain in this region. We speculate that the dominant negative effect of the R143A mutation may be due to the formation of multimers with wild-type N protein that cannot elongate the RNP. The R143A N protein may be unable to stabilize the RNA chain, as it transitions between N monomers at nucleotide 9. The reduced dominant negative suppression of wild-type N protein by R143K compared to that by R143A may suggest that the R143K mutant N protein interacts less readily with the wild-type N protein or that the wild-type N protein induces correct protein folding.

The hydrogen bond between R408 and the phosphate backbone of nucleotide 7 is positioned to stabilize the turn from nucleotide 6, which faces the solvent-exposed side of the RNA binding cavity, and nucleotide 7, which is oriented in the opposite direction toward the hydrophobic interior of the protein (Fig. 1 and 5A). The hydrogen bond formed by R408 may also be required to maintain nucleotide 6 in its "bulge" conformation. The reason for the conformation of nucleotide 6 is unknown, but it is uniquely positioned at the tip of the solvent side of the RNA binding cavity. Substitution of the arginine at position 408 for lysine resulted in a functional N protein mutant, suggesting that the hydrogen bond formed by lysine was able to stabilize the RNA chain around nucleotides 6 and 7, allowing for the formation of the two quasi-helical RNA structures (Fig. 7C). The R143A and R408A mutant N proteins were both compromised in their ability to interact with RNA compared to that of wild-type N protein (Fig. 5B), and the use of authentic DI particles as templates indicated that mutation of residues R143A and R408A rendered the N protein unable to encapsidate nascent RNA (Fig. 3C).

The K154 and K155 residues were both predicted to form hydrogen bond interactions with nucleotide 8 in quasi-helix 2 (Fig. 1B and C). The K154A N protein replicated to near wild-type levels and was transcribed slightly better than the wild type (Fig. 3B). When we examined the individual N monomers within the decameric ring of the solved N-RNA structure, the K154 residue was found to exist in several altered conformations, some of which were positioned at a distance unable to support hydrogen bond formation. The *in silico* analysis and

functional data both suggest that the K154 residue is flexible and does not contribute significantly to RNA binding. In contrast, the K155A mutant N protein was severely debilitated in its ability to support RNA synthesis (Fig. 3B). Structural comparison between VSV and rabies N protein showed conservative homology at position 155, with an arginine residue present in the rabies N protein; however, no amino acid able to form a hydrogen bonding interaction was located at a position equivalent to K154 in the rabies N protein structure (26). This suggests the importance of stabilizing the RNA chain in the second quasi-helix by the hydrogen bonding contact formed by K155 but not by the interaction predicted for K154.

In summary, the work reported here demonstrates the importance of hydrogen bond contacts between the N protein and the RNA genome in RNP template function. Mutation of residues that form hydrogen bond contacts with the phosphate backbone of the nucleotides 5, 7, and 8 in quasi-helix 2 and nucleotide 9 were shown to be critical in the interaction of the N protein with RNA to permit encapsidation and the formation of functional RNP templates. These residues may form key contacts that allow for the correct folding of the quasi-helix 2 nucleotides to face the interior of the RNA binding cavity and stabilize the position of nucleotide 9 as it connects the RNA chain between adjacent N monomers. Conversely, maintaining the individual hydrogen bond interactions between the N protein and the nucleotides of quasi-helix 1 (nucleotides 1 to 4) was not critical to RNP template formation and function. These data indicate that the hydrophobic nature of the interior of the RNA binding cavity that surrounds quasi-helix 1 may be more important for stabilization of quasi-helix 1 than individual hydrogen bonds. The requirement for the nine nucleotides to be associated with each N monomer in a quasi-helical structure remains unknown. However, this work provides evidence that key hydrogen bond contacts with the phosphate backbone of the RNA are required to maintain the functional RNP complex and indicates their importance in maintaining the structure of the RNA within the RNP template.

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