Mutations in the C-Terminal Loop of the Nucleocapsid Protein Affect Vesicular Stomatitis Virus RNA Replication and Transcription Differentially[∇]

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The 2.9-Å structure of the vesicular stomatitis virus nucleocapsid (N) protein bound to RNA shows the RNA to be tightly sequestered between the two lobes of the N protein. Domain movement of the lobes of the N protein has been postulated to facilitate polymerase access to the RNA template. We investigated the roles of individual amino acid residues in the C-terminal loop, involved in long-range interactions between N protein monomers, in forming functional ribonucleoprotein (RNP) templates. The effects of specific N protein mutations on its expression, interaction with the phosphoprotein, and formation of RNP templates that supported viral RNA replication and transcription were examined. Mutations introduced into the C-terminal loop, predicted to break contact with other residues in the loop, caused up to 10-fold increases in RNA replication without an equivalent stimulation of transcription. Mutation F348A, predicted to break contact between the C-terminal loop and the N-terminal arm, formed templates that supported wild-type levels of RNA replication but almost no transcription. These data show that mutations in the C-terminal loop of the N protein can disparately affect RNA replication and transcription, indicating that the N protein plays a role in modulating RNP template function beyond its structural role in RNA encapsidation.

Vesicular stomatitis virus (VSV) is the prototype member of the family *Rhabdoviridae* in the order *Mononegavirales* (47). The single-stranded, negative-sense RNA genome encodes five proteins: the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large polymerase protein (L) (43). The viral RNA genome is always found tightly associated with the nucleocapsid protein, both in virus particles and in the cytoplasm of infected cells (22, 45, 48). The N protein-encapsidated RNA genome, known as the ribonucleoprotein (RNP), is also associated with the viral P and L proteins (25, 44), which comprise the viral RNA-dependent RNA polymerase (RdRp) (36).

Only encapsidated genomes are recognized by the RdRp as the template for transcription of viral mRNAs and for genomic RNA replication (16, 17, 34). The RdRp enters the genome at or near the 3' end (14, 52) and transcribes five capped and polyadenylated mRNAs, one for each viral gene (15). Transcription is obligatorily sequential and polar, so that promoter-proximal genes are transcribed to higher levels than promoter-distal genes (1, 3, 15, 27). Once a critical concentration of viral N protein synthesis has been achieved, genomic RNA replication occurs (11, 40, 49). The genome is copied into a full-length, positive-sense antigenome that serves as the template to generate more negative-sense genomes (23). Both positive- and negative-sense replication products are encapsidated by the N protein and are thereby protected from digestion by RNases (45, 49); mRNAs are not similarly

encapsidated and remain nuclease sensitive (15). Beyond the requirement for N protein synthesis in genomic RNA replication, the precise factors that control polymerase participation in replication or transcription remain unclear. Two different polymerase complexes have been reported (42), and transcription and replication have been shown to initiate at distinct locations on the viral genome (52). Additionally, it was previously shown that the N protein mutant polR1 perturbed the ratio of products that initiated at the exact 3' end of the genome and at the N gene start (7), suggesting a possible role for the nucleocapsid protein in modulating transcription and replication.

Early attempts to study the function of the N protein by expressing it in bacterial cells in the absence of other viral proteins resulted in the formation of high-molecular-weight aggregates that failed to support viral RNA replication (8). The formation of N protein-encapsidated templates requires three factors: the N protein, the viral genomic RNA template, and the VSV P protein (10, 24, 29, 32, 33, 35, 41). The VSV P protein acts as a chaperone and maintains the N protein in a soluble and encapsidation-competent form (24, 41).

In later work, the N and P proteins were coexpressed in *Escherichia coli* as soluble proteins, and a high-molecular-weight complex that consisted of 10 molecules of N protein, 5 molecules of P protein, and 90 nucleotides (nt) of RNA was purified (20). The P protein was dissociated from the complex at low pH, leaving an encapsidated RNA that had a ring-like appearance by electron microscopy (6, 20). The purified N-RNA (RNP) complexes (20) were crystallized and used to solve the structure of the VSV nucleocapsid protein bound to RNA at 2.9-Å resolution (21). In each N protein monomer, the structure begins with a flexible N-terminal arm (residues 2 to 29) that descends into the N-terminal lobe and then ascends

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FIG. 1. Selecting target regions for mutagenesis in the VSV nucleocapsid protein. (A) Schematic of three monomers of the N protein viewed from outside the ring. The C-terminal loop and N-terminal arm participate in forming three long-range contacts: (i) the C-terminal loop of the left-hand monomer (green) contacts the adjacent C-terminal lobe of the central monomer (yellow), (ii) the N-terminal arm of the right-hand monomer (red) contacts the neighboring C-terminal lobe of the central monomer (yellow), and (iii) the C-terminal loop of the left-hand monomer (green) contacts the N-terminal arm of the right-hand monomer (red). (B) Illustration of the region where the C-terminal loop (green) and the N-terminal arm (red) converge on the underlying C-terminal lobe (yellow). Target residues within the C-terminal loop (green) are shown in a stick representation. (C) Residues selected as targets for mutagenesis in the C-terminal loop. The target residues in the C-terminal loop are shown in a stick representation, and alternating residues are colored red and blue. (D) Residues that contact the target amino acids. The left-hand figure depicts residue F348 and its "contact" amino acids in a stick representation. Residue F348 appears to contact residues V5 and R7 in the N-terminal arm (red), T246 and A255 in the C-terminal lobe (gold), and Q346 within the same C-terminal loop (green). The right-hand figure shows in silico modeling of residue 348 as alanine. This substitution is predicted to break all contacts with the N-terminal arm (red) and C-terminal lobe (gold). Panels B to D were generated in PyMOL (12).

into the C-terminal lobe (21). The C-terminal loop (residues 339 to 376) extends outward before turning back on itself toward the C-terminal lobe (21). The RNA chain is tightly sequestered in the cavity formed between the two lobes of the N protein; each N monomer binds 9 nt of RNA (21). Previous work demonstrated that some of the RNA bases could be chemically modified within the RNP, while the phosphate backbone was not modified (26, 28). These studies suggested that in the RNP complex the RNA backbone is protected but that some of the bases are accessible to solvent.

The N-RNA structure revealed an extensive network of interactions between N monomers on the RNA chain (21). The majority of the interactions occur at the hydrophobic side-to-side interface between neighboring monomers, primarily between contiguous C-terminal lobes (21). The N-terminal arm (N-arm) and C-terminal loop (C-loop) also participate in forming three long-range interactions that link N protein monomers along the RNA chain (21). The interactions are illustrated schematically in Fig. 1A and are as follows: (i) the N-terminal arm of the right-hand monomer extends to contact the neighboring C-terminal lobe of the central monomer; (ii) the C-terminal loop of the left-hand monomer extends to contact the neighboring C-terminal lobe of the central monomer; and (iii) the C-terminal loop of the left-hand monomer forms contacts with the N-terminal arm of the right-hand monomer, which is one position removed from it along the RNA chain (21). Zhang et al. showed by deletion of the N-terminal arm ($\Delta 1$ -22) or a portion of the C-terminal loop ($\Delta 347$ -352) that

these regions are important for N oligomerization and RNA encapsidation (53).

The 2.9-Å structure of the N protein in complex with RNA showed that the RNA is substantially buried between the lobes of the N protein (21). It is unclear how the viral polymerase gains access to the genomic RNA within the RNP complex to carry out transcription and replication. Recently, Green and Luo described the structure of the C-terminal domain of the P protein bound to the RNP and postulated that domain movement of the N protein might occur, in effect opening the cavity sequestering the RNA to allow the polymerase to gain access (19). We further hypothesized that the long-range interactions between the N-terminal arm, the C-terminal loop, and the C-terminal lobe regions of the adjacent nucleocapsid proteins (21, 53) might influence the postulated domain movement of the N protein to open the RNA binding cavity, which in turn would affect whether the RNA genome would become accessible to the viral polymerase.

Here, we have extended previous studies by investigating single-amino-acid mutations that were predicted by structure-guided analysis to disrupt interactions within the C-terminal loop, between the C-terminal loop and the N-terminal arm, or between the C-terminal loop and the C-terminal lobe. These predictions were based on an in silico analysis of the available structural data using the molecular graphics software PyMOL (12). In the present work, we describe the effects of specific amino acid substitutions in the nucleocapsid protein on its expression and ability to interact with the P protein and to

form functional RNP templates that support viral RNA synthesis. The results show that mutations in the C-terminal loop of the N protein produced templates with greatly varied abilities to carry out viral transcription and replication. Surprisingly, most of the N protein residues that were mutated in the C-terminal loop resulted in RNP templates that supported increased RNA replication without an equivalent increase in transcription, indicating that, in addition to its established role as a structural ribonucleocapsid protein that protects the viral genome (45, 49), the N protein plays a functional role in modulating the levels of RNA synthesis.

MATERIALS AND METHODS

Visualizing and selecting target residues for mutagenesis. The structure of the VSV N protein bound to RNA (21) was obtained from the Protein Data Bank (PDB identification number 2GIC) and visualized by using the molecular graphics software PyMOL (12). Six amino acids within the C-terminal loop of the N protein (A345, Q346, Q347, F348, C349, and T361) were selected for site-directed mutagenesis based on their proximity to the N-terminal arm of a neighboring N monomer.

Plasmid construction and mutagenesis. The plasmid encoding the VSV N protein under the control of a T7 promoter has been described previously (38, 39). Mutations were engineered into the N protein cDNA using the QuikChange (Stratagene) site-directed mutagenesis protocol. For each mutant, the entire N gene was sequenced to confirm that the correct mutations had been cloned.

Protein labeling, immunoprecipitation, and Western blotting. N protein expression levels were determined by transfecting plasmids encoding either the wild-type (WT) or mutant N proteins, in conjunction with a plasmid encoding P protein, into BHK-21 cells that were infected with the vaccinia virus T7 recombinant, vTF7-3 (18), as described previously (4, 51). At 14 to 16 h posttransfection, the transfected cells were treated with 10 μ g/ml of actinomycin D-mannitol (Sigma-Aldrich) for 30 min and then starved in medium lacking methionine for another 30 min before being exposed to 50 μ Ci/ml [35 S]methionine label (Perkin-Elmer) in medium lacking methionine for 30 min. Cytoplasmic extracts were prepared using lysis buffer (1% NP-40, 0.4% deoxycholate [DOC], 66 mM EDTA, 10 mM Tris-HCl, pH 7.4), and the proteins were resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels (77:1 acrylamide-bisacrylamide) (50). The gels were fixed and dried and then exposed to phosphor screens.

For immunoprecipitations, the lysates were treated with 2 μ l of a VSV monospecific polyclonal anti-P antibody or with 5 μ l of a monoclonal VSV anti-N antibody, 10G4 (a kind gift from D. Lyles, Wake Forest University) (5), overnight. The proteins were immunoprecipitated by the addition of 100 μ l of IgG-sorb (New England Enzyme Center) for 1 hour. The IgG-sorb-antibody complexes were washed two times with wash buffer (1% NP-40, 0.5% DOC, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4) and then resuspended in 2 $\times$ Laemmli sample buffer. The samples were resolved on 10% SDS-polyacrylamide gels (77:1 acrylamide-bisacrylamide) (50). The gels were dried down, and the proteins were visualized by phosphorimaging.

Alternatively, the immunoprecipitated protein samples described above were transferred to polyvinylidene difluoride membranes (Millipore) and probed with a rabbit polyclonal VSV anti-N (1:1,000) or anti-P (1:1,000) antibody. The proteins were detected using a goat anti-rabbit secondary antibody conjugated to Alexa Fluor 680 (1:5,000) (Invitrogen). The blots were scanned with the Odyssey scanner and software (Li-Cor Biosciences) according to the manufacturer's instructions.

Transfections and RNA labeling. To investigate the abilities of the mutant N proteins to support viral-RNA synthesis, BHK-21 cells were infected with a recombinant vaccinia virus (vTF7-3) that expresses T7 polymerase and then transfected with a plasmid expressing a subgenomic replicon of VSV, Δ Bgl22 (4 μ g) (51), as well as plasmids encoding the P (1.25 μ g) and L (1 μ g) proteins, and WT or mutant N proteins (4 μ g) (4, 51). At 14 to 16 h posttransfection, the cells were treated with 10 μ g/ml actinomycin D-mannitol (Sigma-Aldrich), and actinomycin D-resistant RNAs were labeled with 33 μ Ci/ml [3 H]uridine (Perkin-Elmer) for 5 h prior to harvest. Cytoplasmic RNAs were harvested in lysis buffer (1% NP-40, 0.4% DOC, 66 mM EDTA, 10 mM Tris-HCl, pH 7.4) and purified by phenol-chloroform extraction and ethanol precipitation. The 3 H-labeled RNAs were resolved on acid agarose-urea gels (pH 3.0) and detected by fluorography (38). Transfections were performed at 37°C or 31°C as stated.

Primer extension. For each transfection with WT or mutant N proteins, a fraction of the labeled RNA was retained for primer extension analysis. Antige-

TABLE 1. Target residues in the C-terminal loop, their predicted contact residues, locations of the contact residues within the N protein, and site-specific substitutions in the C-terminal loop

Target C loop	Location(s) of contact residue(s) ^a			Mutant	Phenotype ^b	
	N arm	C lobe	C loop		Replication	Transcription
A345		L250	Q347	A345R	WT	WT
Q346	I8	L250	F348 K354	Q346A	++++	—
Q347	I8	I249	A345	Q347A	+++	++
F348	V5 R7	T246 A255	Q346	F348A	WT	—
C349	K6		V350	C349A	++	++
T361				T361A	+	—

^a Contacts predicted to be lost upon mutagenesis are in boldface.

^b WT, WT levels; +, ++, +++, +++++, increased relative to WT; —, decreased relative to WT.

nomic replication was measured using a negative-sense oligonucleotide (5' GG ATCCTCATTTGCAGGAAGTTTGG 3') that anneals to positions 134 to 109 in the VSV genome and lies within the N gene. To measure genomic RNA replication levels, a positive-sense oligonucleotide (5' GGAATGATTGAATG GATCAATGG 3') was used. This primer anneals to positions 10964 to 10986 in the VSV genome and lies within the L gene. All of the primers (5 μ M) were end labeled using [γ - 32 P]ATP (0.5 μ Ci/ μ l; Perkin-Elmer) and T4 polynucleotide kinase (NEB) for 30 min at 37°C and then purified using the QIAquick nucleotide removal kit (Qiagen). Primers were annealed in excess to the labeled RNA and then extended with Moloney murine leukemia virus reverse transcriptase (Invitrogen) for 30 min at 42°C according to the manufacturer's instructions. The radiolabeled products were resolved on a denaturing 6% polyacrylamide gel containing 8 M urea and were visualized by autoradiography.

Nuclease sensitivity assay. Cytoplasmic extracts containing labeled viral RNAs were prepared as described above, using lysis buffer lacking EDTA (1% NP-40, 0.4% DOC, 10 mM Tris-HCl, pH 7.4). For each sample, one-fourth of the lysate was treated with micrococcal nuclease (NEB) or RQ1 DNase (Promega) according to the manufacturer's instructions. The RNAs were purified by phenol-chloroform extraction and ethanol precipitation, and the levels of genomic and antigenomic RNA were measured by primer extension as described above.

Quantitation. Genomic and antigenomic replication levels were quantified from PhosphorImager scans using ImageQuant software (Molecular Dynamics) and a model 860 Storm scanner. RNA synthesis levels were also determined by densitometric analysis of fluorographs using Quantity One software (Bio-Rad) and a PDI model 320i densitometer. In each case, the density in the control lane (lacking N protein) was subtracted as background. All of the experiments were repeated a minimum of three times.

RESULTS

Selection of residues for mutagenesis. The goal of this study was to investigate the roles of individual amino acids in the C-terminal loop of the N protein in RNA synthesis. We analyzed amino acids that had the potential to interact with other residues within the loop itself, with residues in the N-terminal arm, or with residues in the C-terminal lobe but that should not affect the majority of N protein interactions maintained by side-to-side contacts. The amino acids targeted for mutagenesis were A345, Q346, Q347, F348, C349, and T361, and they are illustrated in a stick representation in Fig. 1B (in relation to the N-terminal arm and the underlying C-terminal lobe) and Fig. 1C.

Structural analysis of the target residues. We determined the predicted interacting partners for each residue selected as a target for mutagenesis by analyzing the N protein structural data using the molecular graphics software PyMOL (12). Table 1 lists the putative contact residues for each of the target amino

acids, as well as their locations in the N protein. Each target residue in the C-terminal loop was individually replaced in silico with alanine, except A345, which was replaced with arginine. We predicted which amino acid contacts would be lost upon mutagenesis of the target residues to alanine (or arginine). In Fig. 1D, for example, residue F348 is predicted to contact residue Q346 within the same C loop. It also forms contacts with V5 and R7 in the N-terminal arm and with T246 and A255 in the C-terminal lobe. When we changed residue F348 to alanine in silico, if all the backbone contacts were to remain constant, F348A was predicted to lose contact with both the N-terminal arm and the C-terminal lobe (Fig. 1D). The contacts lost upon mutagenesis were predicted for each target residue (Table 1).

Generation and characterization of the mutant nucleocapsid proteins. Site-directed mutations were introduced into the N protein cDNA as described in Materials and Methods. To determine whether the mutant N proteins were expressed at levels comparable to those of the WT N protein, vTF7-3-infected BHK-21 cells were cotransfected with plasmids encoding mutant or WT N protein, along with a plasmid encoding the VSV P protein. Coexpression of the N and P proteins is required to prevent the N protein from forming large insoluble aggregates that do not support viral RNA replication (8, 9, 20, 24, 29, 33, 41, 46). The proteins were metabolically labeled with [³⁵S]methionine and then resolved by SDS-polyacrylamide gel electrophoresis (PAGE). All of the mutant N proteins were expressed at levels comparable to that of the WT (Fig. 2A), and the mutant protein F348A had altered mobility relative to WT N protein (Fig. 2A, B, and C).

We tested the abilities of the N protein mutants to interact with the VSV P protein by coexpressing WT P protein with WT or mutant N proteins in transfected cells. All of the mutant N proteins could interact with the VSV P protein, as confirmed by coimmunoprecipitation of both proteins with the anti-P antibody (Fig. 2B). Alternatively, the proteins were immunoprecipitated using a monoclonal antibody to N protein followed by Western blotting using antibodies to probe for the P or N protein (Fig. 2C, top and bottom, respectively). These experiments confirmed that the mutant N proteins retained the ability to interact with the P protein and were stable, as shown by Western blotting (Fig. 2C).

Effects of target N gene mutations on viral-RNA synthesis. To examine the effects of the N protein mutations on its ability to form RNP templates that supported transcription and genomic replication, viral RNA synthesis was reconstituted using the vaccinia virus T7-based subgenomic replicon system described previously (4, 51). A plasmid encoding the subgenomic replicon, ΔBgl22 (51), was used as the template for viral RNA synthesis. This replicon contains a single noncoding transcriptional unit that is flanked by the VSV leader and trailer regions at the 3' and 5' ends, respectively (Fig. 3A). The 3' leader region has complementarity with the 5' trailer region extended from the WT 8 bp to 22 bp (51). ΔBgl22 replicates genomic and antigenomic RNA and transcribes a single capped and polyadenylated mRNA (51) (Fig. 3A). This replicon was selected because it supports higher levels of RNA replication than replicons with WT termini and therefore allows detection of the replication products more readily (51). However, all of the results obtained with ΔBgl22 were con-

FIG. 2. Analysis of mutant N protein expression and N-P interactions. (A) BHK-21 cells were transfected with plasmids encoding the WT P protein and WT or mutant N protein. The proteins were labeled with [³⁵S]methionine 14 to 16 h posttransfection and then resolved on 10% SDS polyacrylamide gels as described in Materials and Methods. (B) The abilities of ³⁵S-labeled mutant N proteins to interact with WT P protein were assayed by immunoprecipitation (IP) using a monospecific polyclonal anti-P antibody (ab), followed by SDS-PAGE. (C) The abilities of N and P proteins to interact were also assayed by immunoprecipitation using a monoclonal anti-N antibody, followed by Western blot (WB) analysis to detect the N and P proteins. Mutant F348A exhibited a mobility shift relative to WT N, which is indicated by an asterisk in all three panels. -N and -P, no N and no P protein, respectively.

firmed using a replicon (p8) that had WT 3' and 5' termini (51) and by using a naturally occurring defective interfering particle as a template (data not shown).

The replicon system has an advantage over viral infections in that it allows independent evaluation of the effects of mutations in the N protein on transcription and replication. During VSV infections, viral replication is dependent on the transcription of viral mRNAs to produce the proteins required for replication (11, 40, 49); mutations that abrogate transcription cannot be recovered in virus. However, in the replicon system, the viral proteins required for replication are provided by cotransfection of plasmids expressing the N, P, and L proteins, which alleviates the requirement for de novo viral transcription prior to the onset of replication (4, 51).

To assess the effects of mutating residues in the nucleocap-

FIG. 3. Effects of target N gene mutations on viral RNA synthesis. (A) Schematic of the Δ Bgl22 subgenomic replicon used as the template for viral RNA synthesis. This replicon has the 3' and 5' signals required for transcription and replication and contains a single noncoding transcriptional unit (51). The subgenomic replicon is transcribed by T7 polymerase into a negative-sense RNA (-) that serves as the template for transcription of a single mRNA and for replication of a full-length antigenomic RNA (+) that serves as the template to generate more genomic RNA. (B) RNA synthesis was examined at 37°C in BHK-21 cells infected with the recombinant vaccinia virus vTF7-3 and transfected with plasmids encoding the WT P and L proteins, the Δ Bgl22 replicon, and WT or mutant N protein. The [3 H]uridine-labeled RNA products were resolved on acid agarose-urea gels (pH 3.0) and detected by fluorography (38). The replication products (Rep) and mRNA are indicated. Replication and transcription levels were determined by densitometric analysis of fluorographs using Quantity One software (Bio-Rad) and a PDI model 320i densitometer. The bar graphs show the means of three independent experiments plotted as percentages of the WT $\pm$ the standard error of the mean. (C) RNA synthesis at 31°C was examined as described for panel B. Transcription and replication levels were measured by densitometric analysis of fluorographs. The bar graphs show the transcription or replication levels expressed as percentages of the WT levels measured from a single experiment that was representative of three independent repeats. (D) Quantitation of genomic RNA replication supported by the target N gene mutations at 37°C. Genomic replication levels were measured by primer extension as described in Materials and Methods. The radiolabeled products were resolved on 6% SDS-polyacrylamide gels and detected on phosphorscreens using a model 860 Storm scanner. The primer extension product templated by the T7 primary transcript (T7) is separated from the genomic RNA primer extension product (G) by 2 nt. The product templated by the T7 transcript appears as a doublet because a proportion of the transcript was capped by the vaccinia virus capping enzyme. Quantities were measured using ImageQuant (Molecular Dynamics), and the density in the -N control lane was subtracted as background. Quantities are expressed as percentages of WT replication, and the values were normalized to the level of N mRNA T7 primary transcript from each N plasmid. The experiment was repeated three times, and the bar graph shows the means $\pm$ the standard errors of the mean. (E) Primer extension analysis of antigenomic RNA replication supported by the target N gene mutations at 37°C was done as described for panel D.

FIG. 4. Nuclease sensitivities of genomic and anti-genomic RNPs. BHK-21 cells were transfected with replicon Δ Bgl22 and plasmids expressing P, L, and the WT or mutant N proteins as described in the legend to Fig. 3. Cytoplasmic extracts of the transfected cells were prepared and treated with micrococcal nuclease (Dig) or left untreated (Un). Total RNA was purified by phenol-chloroform extraction. Genomic (A) and antigenomic (B) RNAs were detected using primer extension as described in the legend to Fig. 3 and Materials and Methods. In the top gel in panel A, the primer extension product templated by the T7 primary transcript (T7) is separated from the genomic-RNA primer extension product (G) by 2 nt. The product templated by the T7 transcript appears as a doublet because a proportion of the transcript was capped by the vaccinia virus capping enzyme. The majority of the T7 transcript was digested by micrococcal nuclease.

sid protein C-terminal loop on viral RNA synthesis, transfections were programmed with a plasmid encoding the sub-genomic replicon and plasmids encoding the WT L and P proteins and WT or mutant N proteins. Viral RNA synthesis was examined both by direct metabolic labeling and by strand-specific primer extension (Fig. 3B, C, D, and E). The major RNA-synthetic event was transcription; replication occurred at lower levels. The transcription product appears as a diffuse band due to unequal polyadenylation of the single mRNA (Fig. 3B and C) (4). The results presented in Fig. 3B show that N proteins with the mutations Q346A, Q347A, and C349A supported substantially higher levels of RNA replication at 37°C than the WT (600%, 300%, and 250%, respectively). For these residues, transcription was largely unaffected or was stimulated slightly (Q347A and C349A). In the case of Q346A, despite sixfold stimulation of replication and an increased number of genomic templates, transcription was decreased compared to the WT (Fig. 3B), indicating that mutation Q346A causes a large differential effect on replication and transcription. Interestingly, the N protein mutant F348A supported replication at levels similar to that of the WT, but transcription levels were only 5% of WT levels, again resulting in a differential effect on the two RNA synthetic processes. To assess whether these mutations might be temperature sensitive, we repeated the experiment at 31°C. We found that N protein mutants Q346A, Q347A, and C349A now stimulated RNA replication up to 10- to 12-fold without causing an equivalent increase in transcription (Fig. 3C), suggesting that these mutations are affected by temperature.

Since RNA replication was substantially stimulated by several of the C-terminal loop mutations, we investigated whether synthesis of the antigenomic (+) or genomic (−) replication products was preferentially affected. The viral antigenomic or genomic RNAs synthesized at 37°C were analyzed by strand-specific primer extension. The data in Fig. 3D and E show that the N protein mutants Q346A, Q347A, C349A, and T361A had increased the levels of both genomic and antigenomic RNA synthesis by two- to fourfold above the levels supported by WT N protein (Fig. 3D and E). These data indicate that mutations of the C-terminal loop of the N protein that disrupt intraloop contacts support higher levels of RNA replication than WT N protein.

Nuclease sensitivities of RNPs with mutant N proteins. A characteristic feature of VSV RNP templates is that the encapsidated RNA is protected from nuclease digestion (45, 49). The mutations we introduced into the N protein had the potential to affect certain long-range N-N interactions but were unlikely to affect the major side-to-side interactions. To determine whether the C-terminal loop mutations affected RNA encapsidation, we assessed the resistance of RNPs bearing the N mutations to digestion with micrococcal nuclease. Following digestion with nuclease, the RNAs were deproteinized and quantitated by primer extension analysis. As shown by the data in Fig. 4B, all of the viral antigenomic RNAs encapsidated by WT or mutant N proteins were completely protected from nuclease digestion (Fig. 4B) under conditions in which mRNAs were completely digested (data not shown). All of the genomic RNAs in WT or mutant N protein-containing RNPs were also protected from nuclease digestion, with the surprising exception of genomic RNPs encapsidated with the N protein mutant F348A, which were nuclease sensitive (Fig. 4A). This demonstration of a mutant protein that confers differential nuclease sensitivity in a strand-specific manner is unprecedented. This finding correlates with the RNA synthesis phenotype of F348A, in which genomic replication occurs, as the antigenomic template is stable (Fig. 4B); however, transcription and antigenomic replication are inhibited (Fig. 3B and E), which may be due to the genomic RNP template being nuclease sensitive and therefore defective.

Characterization of N proteins with mutations in residues that contact the target residues. We next investigated the effects of mutating residues predicted by our analysis to interact with, or contact, the target residues examined above in the C-terminal loop. The predicted contact residues located in the C-terminal loop, N-terminal arm, or C-terminal lobe (Table 1 and Fig. 5A) were examined for function by site-specific replacement by alanine, with the exception of A255, which was mutated to glycine.

We examined whether N proteins bearing mutations of the contact residues were expressed at levels comparable to that of WT N protein. The WT or mutant N proteins were coexpressed along with the VSV P protein in transfected cells (see Materials and Methods). The proteins were metabolically labeled with [35 S]methionine and resolved by SDS-PAGE (Fig.

FIG. 5. Analysis of N protein expression and N-P interactions of “contact” mutant proteins. (A) Schematic of the contact residues located in the C-terminal loop (green), N-terminal arm (red), and C-terminal lobe (gold) shown in a stick representation (generated in PyMOL [12]). (B) BHK-21 cells were transfected with plasmids expressing the WT P protein and WT or mutant N protein as described in the legend to Fig. 2A. The proteins were labeled with [35 S]methionine and resolved on 10% SDS-polyacrylamide gels. (C) The samples in panel B were analyzed for the abilities of the N and P proteins to interact by immunoprecipitation (IP) using a monospecific polyclonal anti-P antibody (ab). (D) The abilities of the N and P proteins to interact were also assayed by immunoprecipitation using a monoclonal anti-N antibody, followed by Western blot (WB) analysis to detect the N and P proteins. The mobility of the N protein mutant K354A was shifted slightly relative to the WT, as indicated by the asterisks in panels B, C, and D.

5B). All of the N proteins with mutations of the potential contact residues were expressed at levels comparable to that of the WT. N protein mutant K354A had altered mobility relative to the WT N protein (Fig. 5B, C, and D).

We also examined whether these mutant N proteins could interact with the P protein by immunoprecipitating the coex-

pressed labeled N and P proteins using a polyclonal, monospecific anti-P antibody (Fig. 5C) as described above. All of the mutant N proteins were able to interact with the P protein, since in each case the N protein was coimmunoprecipitated with the P protein by the anti-P antibody. Alternatively, we immunoprecipitated the coexpressed N and P proteins with a monoclonal antibody to the N protein and then assayed by Western blotting with antibodies specific to the P or N protein to determine whether the proteins were coimmunoprecipitated. As shown by the data in Fig. 5D, the P protein was coimmunoprecipitated along with the WT N protein, as well as all of the mutant N proteins, indicating that the mutant N proteins were able to interact with the P protein.

Effects of contact mutations on viral RNA synthesis. We tested whether the N proteins with mutations at the residues predicted to contact the target residues in the C-terminal loop could support viral replication and transcription using the subgenomic replicon system described above. In particular, we wanted to determine whether reciprocal mutation of the contact residues would recapitulate the phenotypes observed for the target mutants. Viral RNA synthesis was examined by metabolic labeling and by strand-specific primer extension (Fig. 6).

All the mutant N proteins supported RNA synthesis at 37°C, except L250A (Fig. 6A). Transcription levels supported by N proteins with mutations in the N-terminal arm (V5A and R7A) were reduced by 75 to 50% below the level of WT N protein. Replication levels at 37°C were low and were therefore measured by primer extension (Fig. 6C and D). Most of the contact mutants supported less genomic RNA replication than the WT at 37°C (Fig. 6C); antigenomic RNA replication levels were similar to that of the WT, except those of mutants R7A, L250A, and A255G, which were decreased (Fig. 6D).

The N protein with the C-terminal lobe mutation L250A was almost completely inactive at 37°C (Fig. 6A), but when RNA synthesis was examined at 31°C, this mutant supported greatly enhanced levels of RNA replication and transcription, indicating that the N protein L250A was temperature sensitive (Fig. 6B). N proteins with the mutations I8A, T246A, I249A, A255G, and K354A also supported higher levels of transcription than the WT at 31°C and slightly higher levels of RNA replication, which indicated they were also temperature sensitive (Fig. 6B). Taken together, these data, suggest that mutations that disrupt contact between the C-terminal loop and the N-terminal arm (V5A and R7A) result in templates that support decreased transcriptional activity.

DISCUSSION

For VSV and other negative-strand RNA viruses, the template for transcription and replication is an RNP complex consisting of the viral RNA genome bound by the N protein (16, 45). In this study, we examined the roles of individual amino acid residues located in the C-terminal loop of the N protein in forming RNP templates that support viral-RNA synthesis. Zhang et al. reported previously that the C-terminal loop and N-terminal arm of the N protein are required for RNA encapsidation (53). They showed that deletion of the N-terminal arm (Δ 1-22) or shortening of the C-terminal loop (Δ 347-352) resulted in N protein mutants that failed to oligo-

FIG. 6. Effects of "contact" N gene mutations on viral RNA synthesis. (A) Viral RNA synthesis supported by WT or mutant N proteins at 37°C. BHK-21 cells were infected with the recombinant vaccinia virus vTF7-3 and transfected with plasmids encoding the WT P and L proteins, the Δ Bgl22 replicon, and WT or mutant N protein. 3 H-uridine-labeled RNAs were resolved on acid agarose-urea gels (pH 3.0) and detected by fluorography (38). The replication products (Rep) and mRNA are indicated. Transcription levels were determined by densitometric analysis of fluorographs using Quantity One software (Bio-Rad) and a PDI model 320i densitometer. The bar graph quantitates transcription from each mutant transfection as a percentage of that of the WT and shows the means of three independent experiments $\pm$ the standard errors of the mean. (B) RNA synthesis at 31°C was examined as described for panel A. Transcription and replication levels were measured by densitometric analysis of fluorographs. The bar graphs show a representative experiment measuring transcription and replication levels as percentages of the WT for all of the target mutants at 31°C. The assays were repeated three times, and the bar graphs are from a single representative experiment. (C) Quantitation of genomic replication supported by the contact N gene mutations at 37°C. Genomic replication levels were determined by primer extension of the transfections described for panel A and as detailed in Materials and Methods. Quantities were measured using ImageQuant (Molecular Dynamics), and the density in the control (-N) lane was subtracted as background. Quantities are expressed as percentages of WT replication, and the values were normalized to the level of N mRNA T7 primary transcript from each N plasmid. The bar graph shows the means of three experiments $\pm$ the standard errors of the mean. (D) Quantitation of antigenomic replication supported by the contact N gene mutations at 37°C. Primer extension was performed as described in Materials and Methods. Antigenomic replication was expressed as a percentage of WT replication, and all values were normalized to the level of N mRNA T7 primary transcript from each N plasmid. The bar graph shows the means of three independent experiments $\pm$ the standard errors of the mean.

merize or encapsidate RNA but still bound the P protein (53). The current analysis of individual residues in the C-terminal loop extends these data by showing that mutations of single amino acids in the C-terminal loop do not primarily affect encapsidation but instead alter RNP template function.

The C-terminal loop residues we selected for study were chosen based on their location in the portion of the loop that most closely approaches the N-terminal arm of a neighboring N protein monomer (Fig. 1B). In addition, out of 37 amino acids in the C-terminal loop, only the selected target residues

FIG. 7. Contacts within the C-terminal loop shown by modeling WT contacts and mutants within the C-terminal loop in silico. (A) Cartoon of the C-terminal loop with the target residues illustrated in a stick representation. Alternating residues are colored green and blue. Residue Q346 contacts residues F348 and K354 on either side of the C-terminal loop. Residue Q347 also contacts A345, and C349 contacts the neighboring V350. (B) Cartoon of C-terminal loop residues where positions 346, 347, and 349 were replaced with alanines in silico. The loss of the glutamine side chain at position 346 breaks contact with residues F348 and K354 on either side of the C-terminal loop. Panels A and B were generated in PyMOL (12).

showed sequence conservation among rhabdoviruses (21). Many of these residues are also structurally conserved between VSV and rabies virus nucleocapsids (2, 21, 31).

Each of the target residues was individually replaced with alanine, except A345, which was replaced with arginine. For each target substitution, we predicted the WT contacts that would be lost upon removal of the side chain (Table 1). This analysis was based on the assumption that all of the backbone contacts in the nucleocapsid protein structure remained unchanged. Mutations of the N protein could affect viral RNA synthesis in two ways: (i) at the level of encapsidation of newly replicated genomic and antigenomic RNAs to form RNPs or (ii) at the level of RNP template function. Except for F348A, all of the mutations generated in the C-terminal loop resulted in functional RNP templates that were resistant to nuclease digestion, similar to WT RNPs (Fig. 4). These data indicate that mutagenesis of individual residues in the C-loop of the N protein results in mutant proteins that can still encapsidate nascent RNAs, unlike the deletion mutants reported previously (53). Thus, we conclude that the observed effects of the individual mutations are predominantly at the level of template function.

RNA replication is stimulated by mutations predicted to disrupt intramolecular contacts within the C-terminal loop. Since mutations generally disrupt function, it was surprising to find that three of the N protein mutations within the C-terminal loop (Q346A, Q347A, and C349A) supported substantially higher levels of RNA replication than WT N protein (Fig. 3). All three residues normally form contacts with other residues within the C-terminal loop (Fig. 7A), and the Q346A and Q347A mutations are predicted to break those intramolecular contacts (Fig. 7B and Table 1). Residue Q346 is positioned as an “anchor” residue in the loop by forming contacts with residues F348 and K354 within the same C-loop and stabilizes the turning back of the loop on itself (Fig. 7A). Replacing residue 346 with alanine was predicted to destabilize the C-loop by breaking the contacts between either side of the loop (Fig. 7B). Mutant Q346A templates supported the highest levels of genomic replication relative to WT N protein (Fig. 3).

Despite the substantial increases in RNA replication, mu-

nants Q346A, Q347A, and C349A did not support similar increases in viral transcription (Fig. 3B). Mutation Q346A, which resulted in the largest increase in replication levels and consequently the largest number of available genomic templates, had slightly reduced transcriptional activity relative to the WT (Fig. 3B). These data (summarized in Table 1) show that single-amino-acid changes in the C-terminal loop differentially affected the two RNA-synthetic processes of transcription and replication.

Mutagenesis of residues predicted to contact Q346 (F348A and K354A) resulted in RNP templates that supported replication levels similar to that of the WT N protein (Fig. 3 and 6). However, these reciprocal mutations were not predicted to break contacts within the C-terminal loop (Table 1). Consistent with this, the A345R substitution was not predicted to break intraloop contacts, and this mutation did not affect viral replication (Fig. 3). Mutation T361A had a minor effect on replication (Fig. 3). T361 was not predicted to contact any residues within the N protein. The structure of the C-terminal domain of the P protein in contact with RNP showed that residues located on the distal side of the C-loop are involved in binding the P protein (19). Residue T361 is the only target residue located on the distal side of the loop. All of the other target residues are located on the proximal side of the loop and are not positioned to affect P binding, and in fact, all of the mutant N proteins bound the P protein, as shown by the data in Fig. 2 and 5.

One of the most striking reciprocal mutations was the L250A substitution, which was predicted to disrupt the contact between the C-terminal lobe of the N protein and residue Q346 in the C-terminal loop. This mutant RNP template was nonfunctional at 37°C but supported a 10-fold stimulation of RNA replication and a 5-fold stimulation of transcription at 31°C (Fig. 6A), indicating that the effect of breaking contacts between the C-terminal lobe and the loop was temperature sensitive. The defect in transcription and replication at 37°C suggests that stabilizing the loop relative to the C-terminal lobe is important for viral-RNA synthesis. We speculate that this may affect N protein domain movement, which is postulated to allow polymerase access to the RNA within the RNP (19). At the lower temperature, there may be sufficient points of contact between the C-loop and the C-lobe for transcription and replication to be able to proceed, and greater flexibility of the RNP template may facilitate polymerase access and RNA replication.

It is interesting to speculate why mutations that result in increased levels of RNA replication are not found in nature. One possibility is that increased replication might result in decreased fidelity of the polymerase, thereby allowing the accumulation of mutations that may exceed the error threshold (30). Alternatively, abundant replication may lead to the formation of defective interfering particles, which could effectively terminate a natural infection (13).

Transcription is inhibited by mutations predicted to break contacts between the C-terminal loop and the N-terminal arm. Another C-terminal loop mutation that affected transcription and replication levels disparately was F348A. Mutant F348A templates supported RNA replication at levels similar to that of the WT N protein but were almost completely nonfunctional for transcription at 37°C (Fig. 3B). Mutation F348A was pre-

dicted to break contact with residues V5 and R7 in the N-terminal arm and residues T246 and A255 in the C-terminal lobe (Table 1 and Fig. 1D). Furthermore, mutations of residues in the N-terminal arm predicted to contact residue F348 (V5A and R7A) also exhibited greatly decreased transcriptional activity compared to the WT N protein (Fig. 6A). These results suggested that interactions between the C-terminal loop and the N-terminal arm (Fig. 1B and D) are important for supporting transcription from the RNP template. Disrupting the contacts between the C-terminal loop and the N-terminal arm may negatively impact the postulated domain movement of the N protein, rendering the RNP template less able to support viral-RNA synthesis.

We also observed that genomic RNPs consisting of mutant F348A were sensitive to nuclease digestion (Fig. 4A), suggesting that the genomic RNPs formed were unstable and might not provide functional templates for transcription. In contrast, the F348A antigenomic RNPs were nuclease resistant, like the WT RNPs (Fig. 4B), and this could account for the amplification of genomic RNA during replication. Recently, Nayak et al., identified several mutations within a conserved sequence located near the RNA binding cavity that resulted in RNP templates that supported genomic-RNA replication but were not competent to support transcription (37). They postulated that this was due to differential recognition of the mutant template by the viral "transcriptase" and "replicase," but the nuclease sensitivities of these mutant templates were not investigated.

In summary, analysis of the 2.9-Å structure of the nucleocapsid protein bound to RNA allowed the structure-guided mutagenesis of residues in the C-terminal loop predicted to form inter- and intramolecular contacts between N protein monomers. The data presented demonstrate that residues in the C-terminal loop can disparately affect the levels of viral transcription and RNA replication carried out by the RNP template. Several mutations within the loop, predicted to disrupt intramolecular contacts, directed increased levels of RNA replication, while transcription was inhibited or was unaffected. These results indicate that the N protein plays a functional role in RNA synthesis beyond its role in RNA encapsidation and protecting the viral genome from degradation. The residues identified here may be used to further probe the distinction between transcription and replication.

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