An Amino-Terminal Domain of the Sendai Virus Nucleocapsid Protein Is Required for Template Function in Viral RNA Synthesis

TINA M. MYERS† AND SUE A. MOYER*

Department of Molecular Genetics and Microbiology and Department of Pediatrics, University of Florida, College of Medicine, Gainesville, Florida 32610

Received 13 June 1996/Accepted 15 October 1996

The nucleocapsid protein (NP) of paramyxoviruses play important and unique roles in viral genome RNA replication. The viruses contain nonsegmented, single-stranded negative-sense RNA genomes that are tightly associated with the nucleocapsid protein, forming an RNase-resistant helical nucleocapsid (RNA-NP), which is the template for RNA synthesis (16, 18; for a review see reference 24). The Sendai virus nucleocapsid is approximately 15 nm by 1 μm, with 2,564 molecules of the NP protein (524 amino acids [aa]) associated with the genomic RNA in a ratio of 1 polypeptide per 6 nucleotides (6). The NP protein must interact with itself, an NP-NP interaction, and with the viral RNA (RNA-NP) within the nucleocapsid. Biochemical evidence for NP-NP binding was shown by the self-assembly of the NP protein into nucleocapsidlike particles which apparently contained nonspecific cellular RNA (4). Previously, a soluble NP0-P complex was identified as the substrate for encapsidation of nascent genome RNA during replication (21). The polymerase complex, consisting of the phosphoprotein (P) and the large (L) proteins, is associated with the nucleocapsid template via the P moiety and catalyzes both transcription and replication (3, 22, 27, 30, 31).

Deletion analysis of the Sendai virus NP protein has shown that the domains required for the NP protein to function in RNA replication in vitro and to self-assemble into nucleocapsidlike particles which must reside within the N-terminal 400 aa of NP. This region is largely hydrophobic, with a few clusters of charged residues (aa 60 to 71, 107 to 129, and 391 to 397) (4, 12, 28). Two of the charged clusters have been postulated to be RNA-binding domains (aa 60 to 72 and 107 to 115), since the basic nature of the majority of these amino acids could lead to an interaction with the phosphate backbone of the RNA (4, 28).

Although there are no direct data, some of these residues are conserved among the parainfluenza virus NP proteins but not generally among other members of the paramyxovirus family (26).

To identify residues of the NP protein required for these various interactions, we selected clustered charge-to-alanine mutagenesis because of the likelihood of targeting surface residues, which are potential sites for protein-protein or protein-RNA interactions (1, 2, 10, 35). Previously charge-to-alanine mutagenesis has been used to identify residues on the surface of proteins important for protein-protein interactions (1, 2, 34), for catalytic activities (14), and to create mutants having temperature-sensitive (ts) phenotypes (14, 17, 35). Based on this approach, we present evidence that charged residues in the
N terminus of the Sendai virus NP protein are required for the nucleocapsid to function as a template in viral RNA synthesis.

MATERIALS AND METHODS

Cells and viruses. Wild type Sendai virus and the Sendai virus defective interfering (DI) particle, DI-H (Harris strain), were propagated in the allantoic fluid of 9-day-old embryonated chicken eggs as described previously (21). Recombinant vaccinia virus containing the gene for phage T7 RNA polymerase (VVT7) (15) was grown in Vero cells. Protein and RNA synthesis were performed in human A549 cells (American Type Culture collection).

Plasmids and antibodies. Plasmids pGEM-NP, pGEM-P/C, and pGEM-L were described previously (11). Plasmids pBS-NP and pTM1-GST-P, containing the glutathione S-transferase (GST) gene fused in frame to the Sendai virus P gene (GST-P), were described before (references 9, and 8, respectively). All of the viral genes were cloned downstream of the phase T7 promoter. A DNA clone of DI-H was obtained by reverse transcription-PCR of nucleocapsid RNA isolated from A549 cells coinfected with Sendai virus and DI-H as described previously (5). The hepatitis delta virus ribozyme and T7 terminator, generously provided by L. A. Ball (University of Alabama) (29), were subcloned 3’ to the DI-H sequence to create pSPDH-H. The sequence of the DI-H clone was confirmed by restriction nucleonuclease mapping and sequencing. Transcription of the DI-H clone by T7 RNA polymerase generates a full-length plus-sense DI-H RNA with the correct termini. Immunoprecipitation and immunoblotting used the following antibodies: a rabbit anti-Sendai virus (o-SV) antibody (7) and a rabbit anti-L (α-L) antibody specific for the Sendai virus L protein (21).

Construction of NP mutants. Charge-to-alanine mutagenesis targeted aa 107 to 129 of NP, generating six mutants containing the alanine substitutions shown in Fig. 1 and 2. PCR was used to create the mutants NP108, NP111, NP114, NP121, and NP126, using two overlapping complementary mutagenic oligodeoxy- nucleotide primers and two standard outside oligodeoxynucleotide primers (Table 1) with pGEM-NP as the template by the method of Higuchi et al. (13). The PCR products were digested with EcoRI and AflII and subcloned into those sites in pGEM-NP. For screening of mutant clones, each mutagenic primer introduced a new silent restriction site as shown in Table 1. The mutant NP146-6, containing a single charge-to-alanine substitution at aa 107 of NP, was created by using pBS-NP and an oligonucleotide-directed in vitro Mutagenesis System Version 2.1 kit (Amersham) according to the manufacturer’s protocol. A KpnI- AflII DNA fragment from pNP146-6 was subsequently subcloned into those sites in pGEM-NP, creating pNP107. The mutations in each clone were confirmed by double-stranded dideoxy sequencing of the entire subcloned fragment.

In vitro RNA synthesis and encapsidation. Subconfluent A549 cells in 60-mm diameter dishes (approximately 4.8 × 10⁶ cells) were infected with VVT7 at a multiplicity of infection of 2.5 PFU/cell and transfected with one or more plasmids at 37°C. Unless indicated otherwise, the amounts of plasmid transfected per dish were as follows: wt or mutant pGEM-NP, 2 μg; pGEM-P/C, 5 μg; and pGEM-L, 0.5 μg. Cytoplasmic cell extracts were prepared at 18 h post transfection (p.t.) by homogenization in 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol, 10 units/ml RNase inhibitor (Promega), and the RNA was assayed as indicated.

FIG. 1. Pulse-chase analysis and in vitro replication with the mutant NP107. (A) Amino acid sequences (aa 107 to 130) of wt NP and mutant NP107 proteins with the underlined letter changed to Ala. (B) VVT7-infected A549 cells were transfected in duplicate with no plasmids (−) or the wt (WT) or mutant NP (107) plasmid, as indicated at the top. The cells were pulse-labeled (P), and extracts were prepared immediately or following a chase (C) as described in Materials and Methods. Immunoprecipitation with α-SV antibody, and analyzed by SDS-PAGE. The position of the NP protein is indicated. (C) VVT7-infected cells were transfected with no plasmids (−) or the P and L plasmids together with the indicated wt or mutant NP plasmid. Cytoplasmic cell extracts were prepared and incubated with D and E (D) and subcloned into the presence of T7 promoter. The nucleocapsid proteins were purified, and RNA was extracted and analyzed by gel electrophoresis as described in Materials and Methods. The position of the DI-H RNA is indicated, and the amount of product DI-H RNA was quantitated on a PhosphorImager and calculated relative to the value for wt NP as 100% (A).

FIG. 2. In vitro DI-H RNA synthesis with the charge-to-alanine mutant NP proteins. (A) Amino acid sequence (aa 107 to 130) of wt and mutant NP proteins with the boldface underlined letters changed to Ala and the percent replication relative to that of wt NP as 100%. (B) wt or mutant NP plasmids were transfected as indicated in the figure legends, samples of per 30 min in methionine- and cysteine-free medium, and cell extracts were prepared immediately (pulse) or following an overnight chase with medium containing 10-fold excess methionine and cysteine. For overnight labeling, the cells were labeled in medium containing 0.1× unlabeled methionine and cysteine. Nonlabeled P-40 cell extracts were prepared, and samples were immunoprecipitated with the appropriate antibody (1 μg) as indicated in the figure legends and collected with inactivated Staphylococcus aureus Cowan strain as described previously (7). For analysis of NP-p-P complex formation, the wt or mutant NP and pTM1-GST-P (1 μg) plasmids were transfected as indicated in the figure legends, samples of extracts (75 μl) were either immunoprecipitated or incubated with glutathione-Sepharose 4B beads (15 μl per reaction; Pharmacia Biotech) for 15 min at 4°C and washed according to the manufacturer’s protocol, and the immunoprecipitated and bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9% gel).

% REP LICITION

### MATERIALS AND METHODS

#### Cells and viruses

Wild type Sendai virus and the Sendai virus defective interfering (DI) particle, DI-H (Harris strain), were propagated in the allantoic fluid of 9-day-old embryonated chicken eggs as described previously (21). Recombinant vaccinia virus containing the gene for phage T7 RNA polymerase (VVT7) (15) was grown in Vero cells. Protein and RNA synthesis were performed in human A549 cells (American Type Culture collection).

#### Plasmids and antibodies

Plasmids pGEM-NP, pGEM-P/C, and pGEM-L were described previously (11). Plasmids pBS-NP and pTM1-GST-P, containing the glutathione S-transferase (GST) gene fused in frame to the Sendai virus P gene (GST-P), were described before (references 9, and 8, respectively). All of the viral genes were cloned downstream of the phase T7 promoter. A DNA clone of DI-H was obtained by reverse transcription-PCR of nucleocapsid RNA isolated from A549 cells coinfected with Sendai virus and DI-H as described previously (5). The hepatitis delta virus ribozyme and T7 terminator, generously provided by L. A. Ball (University of Alabama) (29), were subcloned 3’ to the DI-H sequence to create pSPDH-H. The sequence of the DI-H clone was confirmed by restriction nucleonuclease mapping and sequencing. Transcription of the DI-H clone by T7 RNA polymerase generates a full-length plus-sense DI-H RNA with the correct termini. Immunoprecipitation and immunoblotting used the following antibodies: a rabbit anti-Sendai virus (o-SV) antibody (7) and a rabbit anti-L (α-L) antibody specific for the Sendai virus L protein (21).

#### Construction of NP mutants

Charge-to-alanine mutagenesis targeted aa 107 to 129 of NP, generating six mutants containing the alanine substitutions shown in Fig. 1 and 2. PCR was used to create the mutants NP108, NP111, NP114, NP121, and NP126, using two overlapping complementary mutagenic oligodeoxy- nucleotide primers and two standard outside oligodeoxynucleotide primers (Table 1) with pGEM-NP as the template by the method of Higuchi et al. (13). The PCR products were digested with EcoRI and AflII and subcloned into those sites in pGEM-NP. For screening of mutant clones, each mutagenic primer introduced a new silent restriction site as shown in Table 1. The mutant NP146-6, containing a single charge-to-alanine substitution at aa 107 of NP, was created by using pBS-NP and an oligonucleotide-directed in vitro Mutagenesis System Version 2.1 kit (Amersham) according to the manufacturer’s protocol. A KpnI- AflII DNA fragment from pNP146-6 was subsequently subcloned into those sites in pGEM-NP, creating pNP107. The mutations in each clone were confirmed by double-stranded dideoxy sequencing of the entire subcloned fragment.

#### In vitro RNA synthesis and encapsidation

Subconfluent A549 cells in 60-mm diameter dishes (approximately 4.8 × 10⁶ cells) were infected with VVT7 at a multiplicity of infection of 2.5 PFU/cell and transfected with one or more plasmids at 37°C. Unless indicated otherwise, the amounts of plasmid transfected per
TABLE 1. Oligodeoxynucleotide primers

<table>
<thead>
<tr>
<th>Mutant(s)</th>
<th>Mutagenic primer</th>
<th>Standard outside</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP108</td>
<td>SM212(+)CATAGAGGCCGCGGCTAAGAGGACG</td>
<td>SM030(+)TAATACGACTCCTATAG</td>
<td>NarI</td>
</tr>
<tr>
<td>NP111</td>
<td>SM213(+)CTCCCTGAGCCGCGCTATATGTGG</td>
<td>SM123(+)GGAAGCTTGGGGCC</td>
<td>BosFl</td>
</tr>
<tr>
<td>NP121</td>
<td>SM216(+)GGAGCCGGACCAGCGGTTCATTAGTGAAGACG</td>
<td>SM217(+)AAATGAAGGCGGCTATGGCCTCCATCTTAGG</td>
<td>NaeI</td>
</tr>
<tr>
<td>NP126</td>
<td>SM219(+)TTCCATAGGCCGGTCCACATATCGATGACG</td>
<td>SM220(+)GTATGGAACATATGCGGACCCACAGAATGG</td>
<td>SalI</td>
</tr>
<tr>
<td>NP146-6</td>
<td>SM146(+)CTACACATAGCGGAAAGACCC</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

* The plus or minus sign refers to the messenger sense or genomic sense, respectively, of the oligonucleotide, and the sequences are written 5′→3′. The restriction sites used for cloning and/or screening are underlined.

Self-assembly of NP proteins and polymerase binding. To measure self-assembly of the wt and mutant NP proteins, cells were infected and transfected as described above with the wt and mutant NP plasmids, and Nonidet P-40 cytoplasmic cell extracts were prepared between 18 and 20 h.p.t. The cell extracts were fractionated on separate CCl step gradients as described previously (4) in an SS55 rotor at 36,000 rpm for 16 h at 4°C. Fractions (0.714 ml) were collected from the top, and the pellet was resuspended in the last fraction. A portion of each fraction (25 µl) was analyzed by immunoblotting with an α-SV primary antibody and an alkaline phosphatase-conjugated secondary antibody. The density of each fraction was determined with a refractometer.

To determine polymerase binding to nucleocapsids, VVT7-infected A549 cells were transfected with no plasmids (mock) or the wt or mutant NP plasmid (2 µg) (self-assembled nucleocapsid), and multiple dishes were cotransfected with the P and L plasmids (5 µg of each) (polymerase). At 5.5 h.p.t., the cells were incubated for 3 h with Tran35S-label (66 µCi/ml) in methionine- and cysteine-free medium, and cytoplasmic extracts were prepared (22). The mock and the self-assembled wt or mutant nucleocapsids were purified by pelleting through 30% (vol/vol) glycerol (5 ml) in an SS55 rotor at 50,000 rpm for 90 min at 4°C, and the pellets were resuspended. Equal samples (100 µl) of the polymerase extract were incubated for 1 h at 30°C either with the purified mock or assembled nucleocapsid samples or with polymerase-free, purified DI-H RNA-NP. The samples were sedimented through glycerol (22), the pellets immunoprecipitated with α-SV and α-L antibodies, and the proteins were separated by SDS-PAGE (7.5% gel).

RESULTS

Effect of charge-to-alanine mutagenesis of the NP protein on Sendai virus RNA replication in vitro. We constructed charge-to-alanine mutations in the region from aa 107 to 129 (Fig. 1 and 2) as described in Materials and Methods to test for possible functions of this site in viral RNA replication. Initially, a single mutation (Glu to Ala, E107A) at aa 107 (NP107 [Fig. 1A]) was constructed. VVT7-infected cells were transfected in duplicate with the plasmids for wt NP or NP107, pulsed with Tran35S-label, and chased as described in Materials and Methods. Immunoprecipitation of the proteins showed that wt NP and NP107 were similarly expressed in the pulse (Fig. 1B, lanes 3 and 5) and both were relatively stable to an overnight chase (lanes 4 and 6). The other bands are vaccinia virus proteins that were nonspecifically immunoprecipitated with the antibody as seen in the VVT7-infected but not transfected cell extracts (Fig. 1B, lanes 1 and 2). To test the biological activity of the mutant, extracts of cells expressing wt NP or NP107 together with the P and L polymerase proteins were incubated with dd DI-H and assayed for genome RNA synthesis and encapsidation in vitro as described in Materials and Methods. Analysis of the nucleocapsid RNA products showed that replication with the NP107 mutant was actually somewhat better (141%) than that with wt NP (Fig. 1C, lanes 3 and 2, respectively). As expected, activity was dependent on the expression of the viral proteins, since no product was observed in an extract of VVT7-infected but not transfected cells (Fig. 1C, lane 1). Amino acid 107 is, therefore, not essential for the function of the protein in this assay.

Based on these results, we decided to change clustered groups of charged residues to Ala in the remaining five mutants (Fig. 2A) in an attempt to produce stable proteins with mutant phenotypes. The NP108, NP111, NP114, NP121, and NP126 proteins were all relatively stable, as shown by pulse-chase (data not shown) and immunoblot (Fig. 2D and E) analyses. These mutants were then tested for activity in DI-H RNA replication in vitro. Compared to replication of the DI-H template with wt NP protein (100% [Fig. 2B, lane 2]), mutants NP108, NP114, and NP121 gave nearly equal or equivalent activity (Fig. 2B, lanes 3, 5, and 6). RNA synthesis and encapsidation with NP111 and NP126 were somewhat more reduced but still significant, at 50% of levels for wt NP (Fig. 2B, lane 4, and Fig. 2C, lane 2, respectively). The proteins were all expressed in these extracts, as shown by immunoblot analysis (Fig. 2D, lanes 2 to 6; Fig. 2E, lanes 1 and 2). These data show that the charged residues encompassing aa 107 to 129 in the NP protein can be changed in clusters to Ala without drastically affecting the ability of the proteins to encapsidate nascent RNA in vitro.

NP0-P complex formation is conserved in the mutant NP proteins. One of the requirements for Sendai virus RNA replication is the formation of the encapsidation substrate, the NP0-P complex (13, 21). Since all of the mutants were active in vitro to some degree, we expected that they were forming NP0-P complexes, but this needed to be confirmed based on previous results with the measles virus nucleocapsid (MV N) protein (8). In this case, we showed that the MV N protein supported Sendai virus encapsidation in vitro, yet this activity did not depend on complex formation between the MV N and Sendai virus P proteins. To assay the NP0-P complex, we used a fusion protein (GST-P) containing the GST protein linked to the N terminus of P and measured the co-binding of NP with GST-P to glutathione-Sepharose beads.

The wt and mutant NP plasmids were transfected individually and together with the GST-P plasmid into VVT7-infected cells and incubated with Tran35S-label. Immunoprecipitation of cell extracts showed that significant levels of wt NP and
GST-P were expressed (Fig. 3A, lanes 2 to 4). As a positive control, GST-P expressed alone bound to the beads (Fig 3B, lane 4), while proteins from VVT7-infected but not transfected cells did not (Fig. 3B, lane 1). The faster-migrating bands (Fig. 3B, lane 4) are apparently proteolysis products containing GST. Wild-type NP protein formed a complex with GST-P, since NP cobound to the beads when coexpressed with GST-P (Fig. 3B, lane 3) but not when NP was synthesized alone (lane 2). The NP protein does not form a complex with GST alone (data not shown); therefore, the cobinding of NP with GST-P is specific for the P moiety of the fusion protein. Thus, this assay also measures NP-P-P complex formation which had previously been demonstrated by blotting (20), coimmunoprecipitation (21), and cosedimentation (13, 21). Similarly, each of the mutant proteins was synthesized (data not shown) and formed complexes with the P protein to the same extent as wt NP, as evidenced by their cobinding to beads in the presence, but not the absence, of GST-P (Fig. 3C).

The mutant NP proteins can self-assemble into nucleocapsid-like particles. In addition to the NP-P complex, NP interacts with itself and self-assembles into nucleocapsid-like particles (4). We wanted to test if the differences in the encapsidation ability of the mutants correlate with their degree of self-assembly. The individual wt or mutant NP proteins were expressed in cells, and extracts were banded on CsCl step gradients. Fractions were collected, and samples were analyzed by immunoblotting as described in Materials and Methods. Consistent with previous data (4), the majority of wt NP self-assembled into nucleocapsid-like particles that banded in fraction 5 at the 30 to 40% interface (Fig. 4B) like that of authentic nucleocapsids (4, 25). Unassembled NP protein was previously shown to sediment in fractions 1 to 4 under these conditions (4). VVT7-infected, mock-transfected cells showed no viral protein (Fig. 4A), as expected. Each of the mutants banded similarly to wt NP protein (Fig. 4C to G), suggesting that each self-assembled into nucleocapsid-like particles. For NP126 (Fig. 4G), the densities of fractions 4 and 5 in this particular gradient were close, and so some protein was found in fraction 4 as well. These data show that the substitution of Ala for the charged residues between aa 107 and 129 did not seem to disrupt the NP-NP interaction, and so the 50% decrease seen in replication in vitro for two of the mutants is apparently not due to this function of the protein.

Charge-to-alanine mutagenesis of the NP protein disrupts replication in vivo. One limitation of the in vitro replication-encapsidation assay is that it does not measure if the genome RNA encapsidated by the mutant protein can subsequently be used as a template for further rounds of replication. To measure this template function, we used a DI-H clone (pSPDI-H) (see Materials and Methods) as described previously (12). Following cotransfection of the DI-H plasmid with the NP, P, and L plasmids into VVT7-infected cells, plus-strand DI-H RNA is transcribed by T7 RNA polymerase and encapsidated by the expressed NP protein. The plus-strand DI-H RNA-NP is then replicated by the viral proteins to amplify the template. Replication of the endogenous template with the wt proteins was detected following preparation of cell extracts by labeling in vitro with \([\alpha-^32P]CTP\) (Fig. 5B, lane 2). The DI-H clone is not replicated in the absence of the Sendai virus proteins (Fig. 5B, lane 1), as expected. Compared to replication with wt NP as 100%, the RNAs encapsidated by the mutants NP107, NP108, and NP111 showed somewhat reduced (71, 68, and 69%, respectively [Fig. 5B, lanes 3 to 5]), but still significant levels of
replication of the amplified endogenous templates. DI RNAs encapsidated by mutants NP114 and NP126, however, were significantly impaired in template function (28% and 18%, respectively [Fig. 5B, lanes 6 and 8]), while RNA encapsidated by NP121 was completely inactive (Fig. 5B, lane 7). Immunoblot analysis on samples of the cell extracts demonstrated that the wt and mutant NP proteins were similarly expressed (Fig. 5C, lanes 2 to 8). Although the NP114, NP121, and NP126 proteins were capable of encapsidating the nascent RNA during the first round of replication using DI-H encapsidated with wt NP as a template (Fig. 2), the product RNAs encapsidated by these mutants were defective to various degrees as templates for further rounds of replication.

Clustered charge-to-alanine mutations in a variety of proteins have been shown to yield a proportion of wt mutants (14, 17, 35). We tested if the NP mutants defective in vivo, NP114, NP121, and NP126, have a temperature conditional phenotype by incubating the infected and transfected cells at 37 or 32°C. Nucleocapsid product RNAs were detected by Northern analysis with a DI probe as described in Materials and Methods. This method of analysis does not require the temperature shift between expression in cells (37°C) and the assay (30°C) that was necessitated by the in vitro experiments (Fig. 5). An unlabeled in vitro replication product from dd DI-H provides a marker (Fig. 6B, lane 10). There was no DI RNA in cells transfected only with the DI-H plasmid, showing that genome amplification was dependent on expression of the viral proteins (Fig. 6B, lane 1). The nucleocapsid RNA product produced with wt NP was reproducibly about threefold greater at 32°C than at 37°C (Fig. 6B, lanes 2 and 3). Replication with NP114, however, was about threefold greater at 37°C than at 32°C (Fig. 6B, lanes 5 and 4, respectively), suggesting that this mutant has a cold sensitive (cs) phenotype. In vivo replication with NP126 was significantly inhibited at both temperatures (5 and 20% [Fig. 6B, lanes 8 and 9]). Although these data are also suggestive of a cs phenotype for NP126, at these low levels of replication, this remains speculative. The mutant NP121 was completely inactive in the replication of genomic-length RNA at both temperatures (Fig. 6B, lanes 6 and 7). There were, however, some smaller RNA products produced with NP121 that may represent incomplete replication products which were not detected in the analysis in vitro. Immunoblot analysis showed that the wt and mutant NP proteins and P protein were all expressed (Fig. 6C). The amount of NP121 in this experiment was somewhat reduced, but this would not account for the lack of activity of this protein in vivo. The other bands are vaccinia virus proteins staining nonspecifically in the ECL assay.

Binding of the RNA polymerase to the wt and mutant self-assembled nucleocapsidlike particles. One requirement for viral RNA replication is the recognition of and binding of the viral polymerase complex through the P moiety to the template (3, 22, 27, 30, 31). To determine if the template defects were due to the inability of the polymerase to bind to the mutant nucleocapsids, proteins representing each defective phenotype seen in replication in vivo, significant (NP111), reduced (NP126), and no (NP121) activity, were self-assembled by the expression in VVT7-infected and transfected cells in the presence of Tran35S-label. The radiolabeled nucleocapsidlike particles were purified by sedimentation and incubated with extracts expressing the radiolabeled P-L complex and, as a positive control, with purified polymerase-free DI-H (RNA-NP template). The samples were then sedimented through glycerol, and the pellets were collected and immunoprecipitated to monitor the proteins associated with the nucleocapsids.

The P and L proteins copelleted and, therefore, bound in the presence of both the viral RNA-NP template and the wt nucleocapsidlike particles (Fig. 7, lanes 6 and 1, respectively), with only a small amount of the polymerase proteins pelleting
encapsidate in vitro (Fig. 2 to 4).

than at 32°C with the wild-type NP protein. The results indicate that the NP protein has identified a region from aa 114 to 129 that was required for the nucleocapsid to function as a template in RNA replication (Fig. 5 and 6) but was not required for the initial round of RNA encapsidation (Fig. 2). It was somewhat surprising that changing a total of 13 charged aa to alanine in these mutant proteins had little effect on in vitro encapsidation (Fig. 1 and 2), since deletion analysis of the NP protein by Curran et al. (12) had shown that the entire region from aa 1 to 400 was required. In fact, a deletion of residues encompassing this region (aa 107 to 131) gave an unstable protein. These conflicting results are likely due to the more drastic effect that deletions could have on protein structure as opposed to the Ala substitutions. The mutant proteins encompassing aa 107 to 129 appear overall to be correctly folded, since each formed both NP-P and NP-NP complexes and could support RNA encapsidation in vitro (Fig. 2 to 4).

When RNA encapsidation and amplification were assayed entirely at 37°C (Northern assay) as compared to encapsidation and amplification at 37°C followed by an in vitro assay at 30°C (in vitro labeling), some template function was restored to the mutant nucleocapsid containing NP114 (Fig. 5 and 6). In addition, NP114 replication at 37°C was about threefold better than at 32°C, suggesting that this mutant has a cs phenotype. In fact at 37°C, NP114 is twofold more active than wt NP. It appears that the conformation of NP114 at 30°C inhibits its ability to function in the template, but an active structure is maintained at 37°C. Both ts and cs mutants have been described previously in charge-to-alanine-scanning mutants (14, 17, 35).

One possibility for the observation that the nucleocapsids formed with the mutants NP114, NP121, and NP126 were defective as templates for replication (Fig. 5 and 6) could be that the RNA polymerase could not bind. Binding of the P-L complex to the template is mediated through the P protein (22, 27, 30, 31). We showed that the viral polymerase (P-L complex) did bind to NP121 and NP126 self-assembled nucleocapsids at wt levels (Fig. 7), suggesting that this is not the template defect. Interestingly, it was shown previously that four deletions within the C-terminal 124 aa of NP had defective template functions similar to those exhibited by NP114, NP121, and NP126, and they also formed NP-NP complexes and encapsidated RNA in vitro (12). A P binding site has been mapped within the C terminus of the self-assembled NP protein between aa 440 and 524 (3, 32), and so the mutants containing deletions of aa 426 to 497 or 456 to 524 could not replicate in vivo because the polymerase could not bind. However, the mutants with deletions of aa 400 to 415 or 414 to 439, like NP114, NP121, and NP126, could not replicate in vivo either yet bound the P protein.

In combination, these data suggest that at least two sites are required for template function not related to polymerase binding. One is located in the C-terminal 400 to 439 aa, and the other is from aa 114 to 129. The similar phenotypes of mutants in both regions suggest that they may form a single domain in the mature protein. While aa 114 to 129 are quite conserved in the paramyxoviruses, this is not the case for the other paramyxoviruses. Only aa 121 has a conserved basic charge in 13 different NP proteins (26). These data suggest that a basic amino acid is the crucial one in mutant NP121, which had altered two additional charged but nonconserved residues. In other NP proteins, different regions may be involved in the template function.

Several models can be envisioned for the role of these residues in template function, depending on whether these sites form one domain or two and whether the bases are exposed or protected in the nucleocapsid. If the bases are covered by the NP protein and aa 114 to 129 and 400 to 439 form a single domain in the NP protein, perhaps this domain is required for the transient displacement of NP to allow the viral polymerase access to the template during RNA synthesis (12). RNA synthesis does not appear to change the nuclease-resistant character of the template; therefore, the displacement of NP must be minimal and/or something else may take its place. Perhaps the NP protein is transiently removed by its binding to the P subunit of the RNA polymerase, via aa 114 to 129 and 400 to 439, with the L subunit filling the gap. Following synthesis, the displaced NP protein would be returned to the template. It had indeed also been proposed that in vesicular stomatitis virus, the NS (P protein) protein may mimic the template RNA and act as a transient binding site for the N protein (23).

Alternatively, the mutations in the template function domain may have resulted in an increased affinity of NP for either the RNA (NP-RNA interaction) or the adjacent NP proteins (NP-NP interaction) which might prevent the temporary displacement of the mutant NP protein from the RNA or from adjacent NP proteins. This increased affinity would not affect in vitro replication utilizing a viral RNA template that is encapsidated by wt NP protein. If the bases are accessible on the surface of the nucleocapsid, where the NP protein does not

![FIG. 7. The viral polymerase binds to wt and mutant self-assembled nucleocapsidlike particles. VVT7-infected cells were transfected with no plasmids (—) or the wt or the mutant NP plasmid (nucleocapsidlike particles), or separately transfected with the P and L plasmids (polymerase), and incubated with Tran^35S-labeled and cell extracts were prepared. The mock extract and the nucleocapsidlike particles were purified by pelleting through glycerol. Equal aliquots of the polymerase extract were incubated with mock (lane 5), each of the purified nucleocapsidlike particles (NUL; lanes 1 to 4), and purified polymerase-free DI-H (lane 6). The samples were pelleted through glycerol, and the bound proteins were analyzed by SDS-PAGE. The positions of the NP, P, and L proteins are indicated.](http://jvi.asm.org/)
need to be displaced, alternative, as yet undefined interactions which these NP mutations have disrupted must be occurring. One possibility is that the mutated residues are involved in the intra- rather than intermolecular interactions of NP and are required for NP assembly in nucleocapsids to undergo conformational changes during viral RNA synthesis.

In summary, by charge-to-alanine mutagenesis, we have constructed six stable mutant NP proteins which have identified a second region (aa 114 to 129) of the NP protein, in addition to the previously identified C-terminal 400 to 439 aa, which is required for template function of the nucleocapsid. Further studies will be necessary to determine the nature of the defect and if these two sites form one domain or fulfill separate functions.

ACKNOWLEDGMENTS

We thank Sherin Smallwood and Cheryl Zack for technical assistance and Sandra Horikami for helpful discussions and advice. This work was supported by grant AI 14594 from the National Institutes of Health.

REFERENCES