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The Conserved N-Terminal Region of Sendai Virus Nucleocapsid Protein NP Is Required for Nucleocapsid Assembly

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Sendai virus nucleocapsid protein NP synthesized in the absence of other viral components assembled into nucleocapsidlike particles. They were identical in density and morphology to authentic nucleocapsids but were smaller in size. The reduction in size was probably due to the fact that they contained RNA only 0.5 to 2 kb in length. Nucleocapsid assembly requires NP-NP and NP-RNA interactions. To identify domains on NP protein involved in nucleocapsid formation, 29 NP protein mutants were tested for the ability to assemble. Any deletion between amino acid residues 1 and 399 abolished formation of nucleocapsidlike particles, but mutants within this region exhibited two different phenotypes. Deletions between positions 83 and 384 completely abolished all interactions. Deletions between residues 1 and 82 and between residues 385 and 399, at the N- and C-terminal ends of the region from 1 to 399, resulted in unstructured aggregates of NP protein, indicating only a partial loss of function. Deletions within the C-terminal 124 amino acids were the only ones that did not affect assembly. The results suggest that NP protein can be divided into at least two separate domains which function independently of each other. Domain I (residues 1 to 399) seems to contain all of the structural information necessary for assembly, while domain II (residues 400 to 524) is not involved in nucleocapsid formation.

Nucleocapsids of Sendai virus, a model paramyxovirus, are large, helical, highly flexible ribonucleoprotein particles, approximately 15 nm in diameter and 1 μm in length. They contain the 15,384-nucleotide-long negative-stranded RNA genome and three different viral proteins. The major structural component of the nucleocapsid is the nucleocapsid protein NP (58 kDa), of which about 2,600 molecules tightly encapsidate the RNA. Encapsidation renders the RNA inaccessible to RNases and is required for it to serve as a template for the viral RNA-dependent RNA polymerase complex. The two proteins necessary for polymerase functions are the large protein L (251 kDa), which is considered the core of the polymerase complex, and the phosphoprotein P (65 kDa). Both of these proteins are present in only minor amounts on nucleocapsids (4). NP also binds M protein (7). Binding of M protein to nucleocapsids seems to modify polymerase activities (12, 13) and may also be required to mediate interactions between nucleocapsids and the viral envelope proteins during virus budding.

The paramyxovirus RNA polymerase is able to work in two different modes, and NP protein may be regarded as a regulatory factor that switches the polymerase from the transcriptive to the replicative mode. During transcription, intergenic start/stop signals on the viral genome are recognized, and thus a 54-nucleotide-long leader RNA and six capped, polyadenylated mRNAs are generated. Replication, as opposed to transcription, results in full-size antigenomic RNA, encapsidated by NP protein. Replication, but not transcription, requires ongoing synthesis of NP protein and concurrent encapsidation of the nascent RNA. Therefore, replication seems to depend mainly on the presence or the intracellular concentration of NP protein. In a recently developed in vitro replication system, the formation of NP-P and P-L complexes was found to be essential for replication (8). As yet, no formal proof exists that NP-P complex formation is also required in vivo, but at least in vitro an NP-P complex rather than NP alone seems to serve as a substrate for specific encapsidation of nascent viral genomic RNA.

NP protein thus plays a central role in paramyxovirus replication. Localization of domains responsible for NP-NP and NP-RNA (encapsidation) and NP/M (budding) interactions as well as NP/P and NP/polymerase (replication) interactions is critical for an understanding of regulatory events in the life cycle of paramyxoviruses. The size of NP protein predicted from the sequences initially published was 518 amino acids (16, 21). Resequencing of the strain Z NP gene (15) and peptide sequencing of NP proteins from Fushimi strains (18) indicated that NP protein contains 524 rather than 518 amino acids, with a C terminus even more acidic than predicted earlier. It is this sequence that all of our data refer to. We developed assay systems to study the functions of NP protein. Recently, we showed that NP protein binds P protein with high affinity in vitro, and we mapped domains involved in binding (7). Here we report an analysis of domains of NP protein participating in assembly of nucleocapsids. We show by electron microscopy and by biochemical methods that NP protein synthesized in mammalian cells assembles into RNA-containing nucleocapsidlike particles in the absence of other viral proteins. This phenomenon allowed us to map regions in NP which are involved in

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nucleocapsid assembly. On the basis of these data, functions of domains in NP protein are discussed.

MATERIALS AND METHODS

Cells and viruses. Sendai virus (strains Fushimi and D52) was propagated in embryonated chicken eggs and purified as described previously (7). Recombinant vaccinia virus containing the bacteriophage T7 RNA polymerase gene (vTF7-3) was kindly provided by B. Moss and grown in CV1 cells.

Transfection. Subconfluent CV1 cells in 60-mm-diameter culture dishes were infected with vTF7-3 at a multiplicity of infection of 5 in 1 ml of minimal essential medium. After 1 h at 37°C, 2 ml of Opti-MEM medium (GIBCO-BRL) containing 3 μl of plasmid DNA and 12 μl of Transfectace (GIBCO-BRL) was added. Cell extracts were prepared 24 h after transfection.

Construction of clones. For vaccinia virus T7 expression, the Sendai virus NP gene from pBS-NPrev (7, 18) was subcloned into pGem4 (Promega) (NP-Gem) and pTF7-5 (NP-TF), kindly provided by B. Moss. Each plasmid contains NP gene sequences from genome positions 54 to 1728. NP-pGem4 was used as the wild-type (wt) NP reference in all experiments.

Constructs presented in Fig. 5A were generated by different methods. Most deletions were introduced by overlap extension polymerase chain reaction (PCR) (6) and subsequent cloning of the mutated PCR fragments into NP-Gem (B, C, D, E, F, G, N, R, S, T, and U) or NP-TF (I, K, L, M, O, P, Q, and R). Mutagenesis by overlap extension involved the generation of DNA fragments that, by virtue of having incorporated complementary oligonucleotide primers in independent PCRs, could be fused anywhere along the gene sequence by combining them in a second primer extension reaction. Thus, for the generation of each mutation, two PCRs were performed, using two standard primers (M13 Universal and M13 Reverse) and two mutagenic primers. First, each mutagenic primer was used in combination with a standard primer, yielding two different PCR fragments. Then the two PCR fragments were gel purified, fused, and amplified in a second PCR using the two standard primers. The final PCR fragment was gel purified, cut with appropriate restriction enzymes, and cloned into NP-Gem or NP-TF. All mutants were sequenced. To minimize undesired nucleotide changes during PCR, high concentrations of template DNA (1 ng/μl) were used, and no more than 15 cycles of denaturation (1 min, 94°C), annealing (1 min, 45°C), and extension (1 min, 72°C) were performed. Reaction buffer contained 50 mM KCl, 10 mM Tris hydrochloride (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM deoxynucleoside triphosphate, 1 μM primers, and 2.5 U of *Taq* polymerase (Promega) in a final volume of 100 μl.

For construction of mutant A, at first a new *Bam*HI site upstream of the original AUG codon was generated at genome position 214 by site-directed mutagenesis with oligonucleotide NP-Bam (GCCAGAGGATCCCAGTCTCAG). *Sau*I sites present in the polylinker and at genome position 980 were used to subclone the fragment containing the newly created *Bam*HI site into the *Sma*I site of pBluescript SKII (Stratagene). Then two complementary synthetic oligonucleotides, carrying a 5′ end compatible with the *Not*I site and a 3′ end compatible with the *Bam*HI site, and a new AUG codon corresponding to genome position 219 (GGCCGC CCGGGAAAGTTCACGATGGCGCTA) were hybridized with each other and ligated into the *Not*I site and the *Bam*HI site. Finally, the subfragment was reintroduced as an *Acc*I-*Sau*I fragment into the *Sma*I-*Sau*I sites of NP-Gem.

Generation of mutations *Eco*RV-*Nco*I and *Bgl*II-*Bgl*II has been described previously (7). For generation of the Stop mutation, a stop linker was inserted into the single *Bal*I site.

Point mutations were introduced by site-directed mutagenesis of single-stranded phagemid DNA obtained from pBS-NPrev, using phosphorothioate-modified DNA (23). The mutagenic oligonucleotides used were 229/230-AI (TCA GCAAAGCGCTATATCTCTCTCATG), 229/230-RE (CAG CAAAGCCGTGAATCTCTCATG), 237/238-VL (GGTTGA GACCGTTCTGACTATGAA), 237/238-RE (GTTGAGAC CCGTGAGACTATGAAT), 489-G (AGACTCGCAGGAA GACGGCAAG), and 489-P (GAGACTCGCACCGAGACG GCAA).

Cell extracts, antibodies, and immunoprecipitation. To monitor protein expression in transfected cells, rabbit antiserum raised against detergent-disrupted Sendai virus or a monoclonal anti-NP antibody was used. Cell extracts were prepared by lysis in 50 mM Tris hydrochloride (pH 8.0)–150 mM NaCl–1.5 mM $MgCl_2$–1% Nonidet P-40–0.1% sodium dodecyl sulfate (SDS) (BDH) containing aprotinin (1 μg/ml) and phenylmethylsulfonyl fluoride (200 μg/ml) (7). Immunostaining was performed with alkaline phosphatase (Promega) or the enhanced chemiluminescence system (Amersham) according to the manufacturer's protocol. For electron microscopy and CsCl gradient analysis, cleared cytoplasmic extracts were prepared by hypotonic lysis in 10 mM Tris hydrochloride (pH 8.0)–0.1 M EDTA and subsequent centrifugation at approximately 10,000 × *g* for 10 min (22). Immunocoprecipitation was performed essentially as described by Pattnaik and Wertz (19). After transfection, cells were incubated in Dulbecco modified Eagle medium containing 50 μCi of [^{3}H]uridine per ml or in methionine-free Dulbecco modified Eagle medium containing 100 μCi of [^{35}S]methionine per ml for 16 h. Cell extracts were prepared in 10 mM Tris hydrochloride (pH 7.4)–10 mM EDTA–1% Nonidet P-40–0.4% sodium deoxycholate. Nuclei were removed, and supernatants were adjusted to 0.1% SDS. Extracts were incubated overnight with 60 μl of rabbit anti-NP antiserum at 4°C. Then 150 μl of 50% protein A–Sepharose in lysis buffer containing 0.1% SDS was added, and incubation was continued for 1 h at 4°C. Immune complexes were washed four times with lysis buffer containing 150 mM NaCl and 0.1% SDS, and NP protein was finally eluted in 200 μl of 10 mM Tris hydrochloride (pH 7.4)–100 mM NaCl–1 mM EDTA–0.5% SDS by boiling for 2 min. ^{35}S-labeled proteins were analyzed on SDS–10% polyacrylamide gels. ^{3}H-labeled RNA was purified by phenol-chloroform extraction, resolved in 1.2% formaldehyde agarose gels, and detected by fluorography.

CsCl gradients. All solutions for CsCl gradients were prepared in TNE (25 mM Tris hydrochloride, [pH 7.4], 50 mM NaCl, 2 mM EDTA). Cleared cytoplasmic extracts (0.4 ml) were layered onto 12-ml 20 to 40% CsCl gradients and centrifuged in a Beckmann SW41 rotor for 16 h at 30,000 rpm. To determine the relative amounts of unassembled versus assembled NP protein, separation in 4-ml step gradients (0.4 ml of 30% glycerol, 1.2 ml of 20% CsCl, 1.2 ml of 30% CsCl, and 1.2 ml of 40% CsCl) was performed in a Beckman SW60 rotor for 16 h at 36,000 rpm and 16°C. Subsequently, gradients were divided into seven fractions each containing 0.6 ml.

Electron microscopy. Electron microscopy was carried out as previously reported (22). Briefly, cytoplasmic fractions were spotted onto carbon-coated, glow-discharged grids and

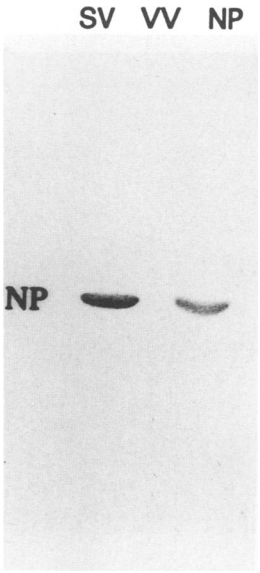


FIG. 1. Synthesis of NP protein in transfected cells. Extracts from Sendai virus-infected cells (20 μg; lane SV), from vTF7-3-infected cells (5 μg; lane VV), and from vTF7-3-infected cells transfected with NP-Gem (5 μg; lane NP) were separated by SDS-PAGE (10% gel). NP protein was stained on protein blots by using a monoclonal anti-NP antibody.

negatively stained with 2% uranyl acetate in water. Preparations were observed with a Philips EM 410 microscope at 580 kV or a Philips CM-12 microscope at 100 kV. For in situ immunolabeling, cells were embedded in Lowicryl K4M, sectioned, deposited on grids, and incubated with monoclonal anti-NP antibody followed by goat anti-mouse immunoglobulin G coupled to 5-nm colloidal gold particles (BioCell, Cardiff, United Kingdom).

RESULTS

NP protein assembles into nucleocapsidlike particles. Sendai virus NP protein was synthesized in mammalian cells by using the vaccinia virus T7 expression system (3). CV1 cells were infected with recombinant vTF7-3, providing bacteriophage T7 RNA polymerase, and subsequently transfected with plasmid NP-Gem, which contains the NP gene downstream of a T7 RNA polymerase promoter. The identity of NP protein was determined by immunostaining of protein blots (Fig. 1).

Immunofluorescence revealed that NP protein accumulated exclusively in the cytoplasm of transfected cells (data not shown); in contrast, measles NP protein was detected in the nucleus and in the cytoplasm of transfected cells (10). Electron microscopy after immunogold labeling of NP protein showed that gold particles accumulated on tubular structures in the cytoplasm of transfected cells (Fig. 2A) but not in vTF7-3-infected, untransfected cells (Fig. 2B). These tubular structures were further characterized by electron microscopic analysis of cleared cytoplasmic fractions from transfected cells and Sendai virus-infected cells. Extracts from transfected cells contained numerous structures with an overall morphology similar to that of Sendai virus nucleocapsids (Fig. 3A and B). These nucleocapsidlike particles also appeared as flexible rods, with the same diameter as authentic nucleocapsids. The only difference was that they were consistently much smaller than Sendai virus nucleocapsids. Quantification of this property was not deemed necessary, as the results were consistent with similar studies of measles virus nucleocapsids (22).

To determine the density of nucleocapsidlike particles, cytoplasmic fractions from transfected or Sendai virus-infected cells were separated on continuous 20 to 40% CsCl gradients. In these 12-ml gradients, NP protein from cells transfected with the NP gene banded at exactly the same density (1.31 g/ml) as did Sendai virus nucleocapsids (data not shown). To determine the relative amounts of assembled versus unassembled NP protein, extracts from transfected or Sendai virus-infected cells were separated on 4-ml CsCl step gradients. Gradients were subsequently divided into seven fractions, which were analyzed for the distribution of cellular proteins by SDS-polyacrylamide gel electrophoresis (PAGE) and silver staining and for NP protein by Western blotting (immunoblotting). In these step gradients, 98% of the proteins from uninfected control cell extracts were recovered in fractions 1 to 4. Virtually no cellular proteins sedimented into fraction 6 (data not shown). In this fraction, which represents the 30 to 40% CsCl interphase, approximately 70% of the NP protein from Sendai virus-infected cells was recovered, corresponding to the characteristic density of nucleocapsids (9). The residual 30% of NP protein found in the top fractions of the gradient probably represented unassembled monomeric NP protein. With extracts from transfected cells, on the other hand, NP protein accumulated almost exclusively in fraction 6; less than 5% of NP protein was detected unassembled in the top fractions (Fig. 3C). NP protein thus was proven to assemble into structures with high density. Their density on CsCl gradients was identical to that of authentic nucleocapsids.

Nucleocapsidlike particles are associated with RNA. To test whether nucleocapsidlike particles also contained RNA, we performed immunocoprecipitations by using a protocol developed by Pattnaik and Wertz to detect encapsidated replication products of vesicular stomatitis virus (VSV) (19). The immunoprecipitation was highly specific (Fig. 4A), and [^{3}H]uridine-labeled RNA was indeed precipitated by anti-NP antiserum only in NP protein-expressing cells, not in vTF7-3-infected cells (Fig. 4B). The RNA was heterogeneous in size and ranged from 0.5 to 2 kb. Radiolabeled RNA also cosedimented with NP protein in CsCl gradients. To determine the ratio of RNA versus protein, we quantified the relative amounts of [^{3}H]uridine and [^{35}S]methionine in double-labeled, CsCl gradient-purified nucleocapsids and nucleocapsidlike particles. Before and after micrococcal nuclease treatment (10 μg/ml, 30 min, 37°C [8]), aliquots were trichloroacetic acid precipitated, and the radioactivity was determined by liquid scintillation counting. The values for nucleocapsidlike particles and Sendai virus nucleocapsids were similar, varying in the range of 20% (data not shown). These results suggest that the relative RNA contents of Sendai virus nucleocapsids and nucleocapsidlike particles from NP-transfected cells are similar, but they do not completely exclude the possibility that the RNA can be encapsidated in a different way than viral genomic RNA even though nucleocapsidlike particles and Sendai virus nucleocapsids had the same density. However, the heterogeneity in size of nucleocapsidlike structures detected in the electron microscope corresponds well with the heterogeneity of the associated RNA, which also indicates that RNA is involved in the formation of nucleocapsidlike particles.

Analysis of NP gene deletion mutants for the ability to assemble. As nucleocapsidlike particles are similar to au-

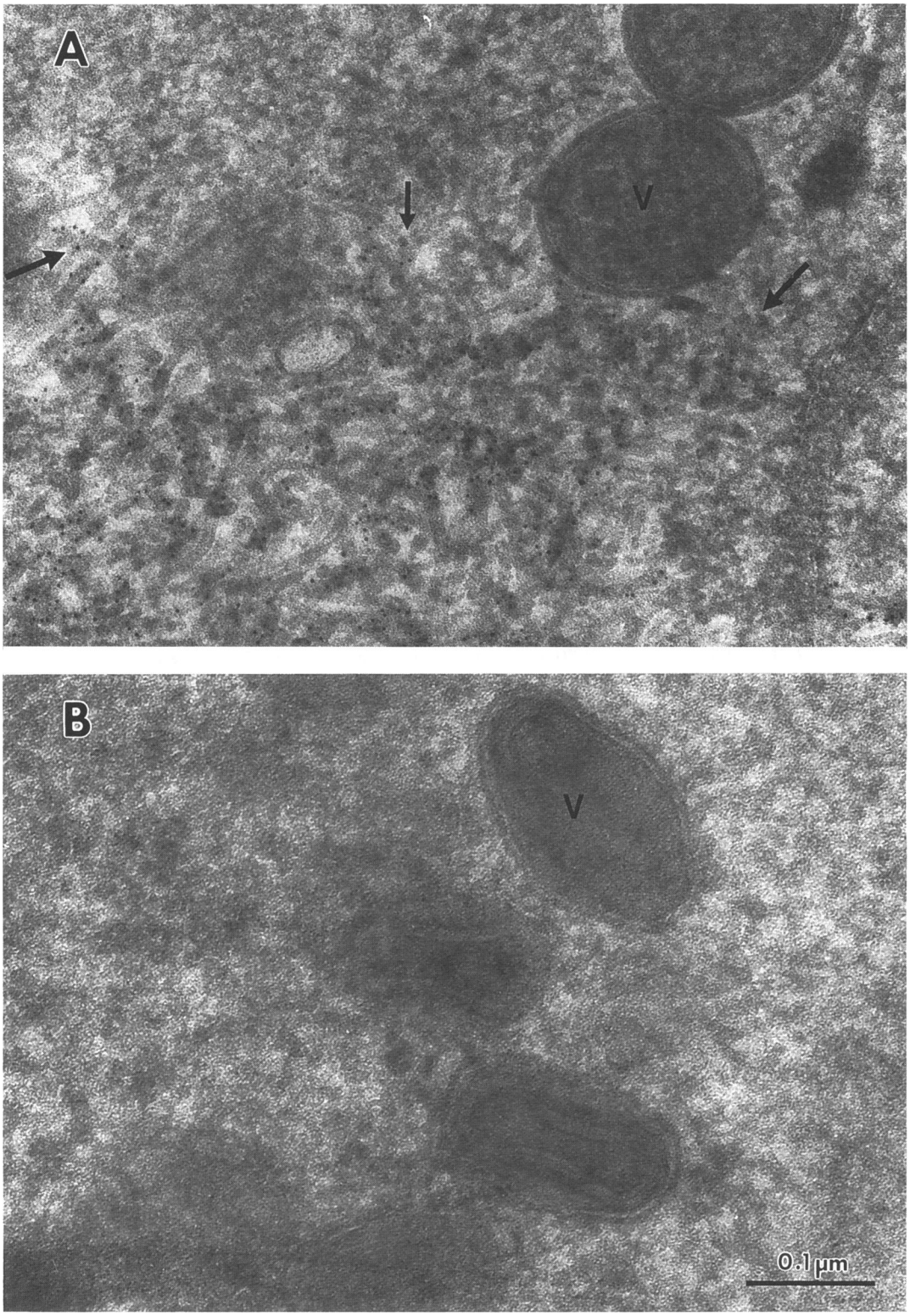


FIG. 2. Immunogold labeling. vTF7-3-infected cells transfected with NP-Gem (A) were fixed, embedded in epoxy resin, and cut into ultrathin sections. NP protein was detected by immunogold labeling using 5-nm goat anti-mouse gold beads after previous incubation with monoclonal anti-NP antibody. Arrows indicate nucleocapsidlike particles. Untransfected, vTF7-3-infected cells were used as the control (B).

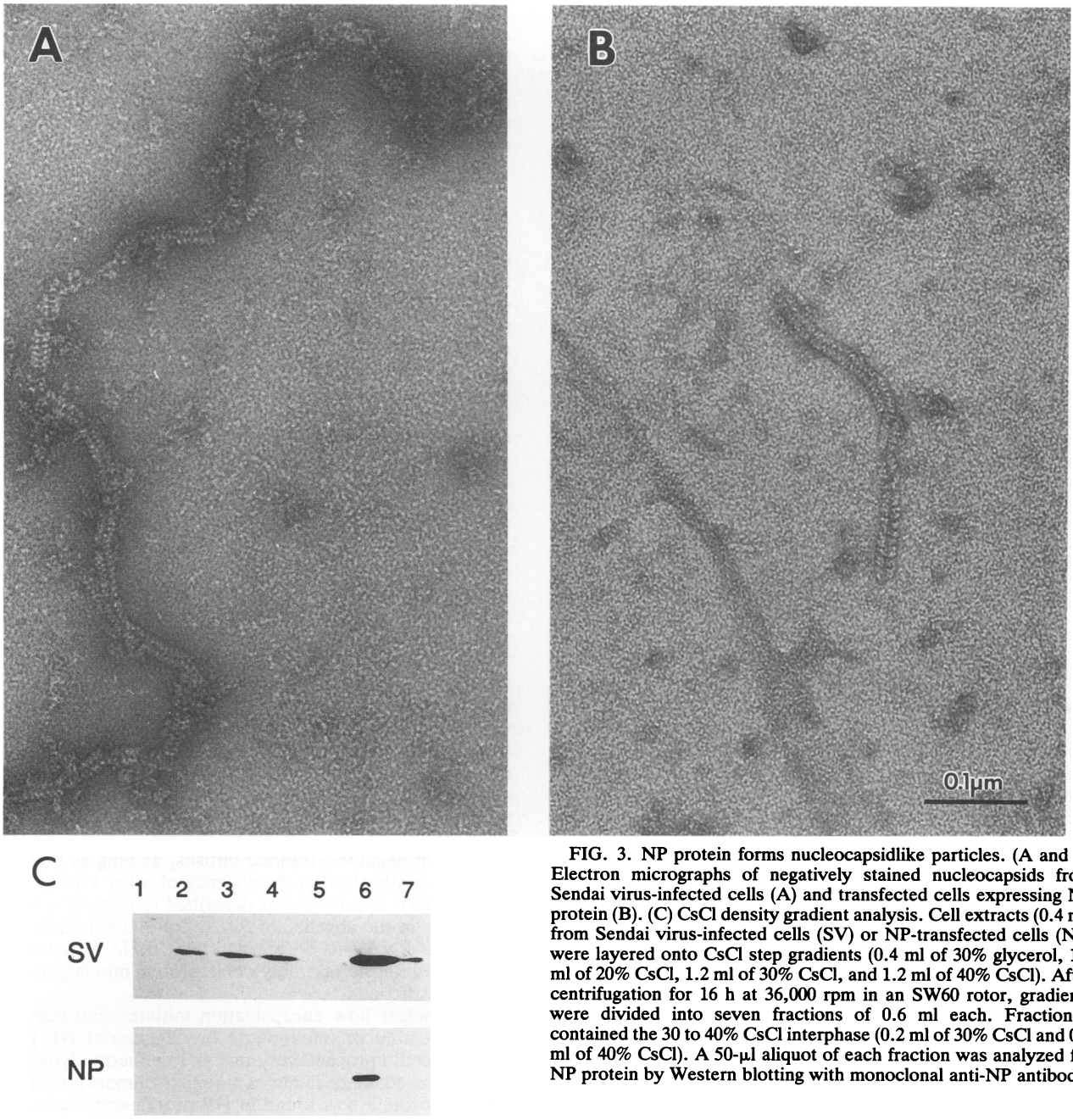


FIG. 3. NP protein forms nucleocapsidlike particles. (A and B) Electron micrographs of negatively stained nucleocapsids from Sendai virus-infected cells (A) and transfected cells expressing NP protein (B). (C) CsCl density gradient analysis. Cell extracts (0.4 ml) from Sendai virus-infected cells (SV) or NP-transfected cells (NP) were layered onto CsCl step gradients (0.4 ml of 30% glycerol, 1.2 ml of 20% CsCl, 1.2 ml of 30% CsCl, and 1.2 ml of 40% CsCl). After centrifugation for 16 h at 36,000 rpm in an SW60 rotor, gradients were divided into seven fractions of 0.6 ml each. Fraction 6 contained the 30 to 40% CsCl interphase (0.2 ml of 30% CsCl and 0.4 ml of 40% CsCl). A 50-μl aliquot of each fraction was analyzed for NP protein by Western blotting with monoclonal anti-NP antibody.

thentic nucleocapsids with regard to morphology, density, and association with RNA, it is reasonable to assume that domains involved in assembly of these particles also determine assembly of Sendai virus nucleocapsids. To localize these domains, we generated a set of 23 deletion mutants covering the whole NP gene (Fig. 5A). NP from deletion mutants was expressed in CV1 cells by using the vaccinia virus T7 system, and cytoplasmic cell extracts were analyzed by Western blotting with a Sendai virus antiserum. Most constructs produced high amounts of protein. Only mutants E and H gave barely detectable levels of NP protein (Fig. 5B). The same results were obtained when the mutants were expressed in other cell lines. Since, on the other hand, proteins from constructs E and H could be synthesized by in vitro translation (data not shown), low expression in vivo was probably due to their instability in mammalian cells. The migration of NP proteins from all the other mutants was according to their size except for mutant S, which induced the synthesis of a protein with an apparent molecular mass higher than that predicted from the sequence. Mutants Stop and U repeatedly produced lower amounts of protein than did wt NP. However, this did not impair subsequent experiments (see below).

All NP protein deletion mutants that allowed expression in transfected cells were then tested for the ability to assemble into nucleocapsidlike particles. On CsCl gradients, the mutants exhibited different phenotypes and were thus divided into three groups (Fig. 6A). NP protein from mutants A, B,

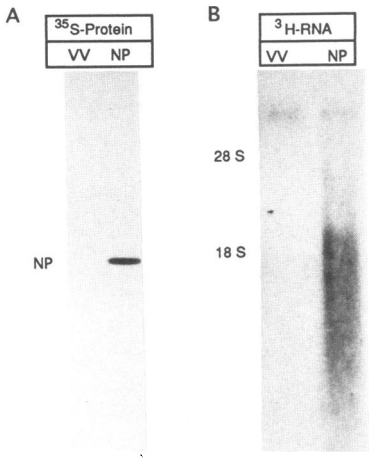


FIG. 4. Immunocoprecipitation of RNA associated with NP protein. vTF7-3-infected cells (lanes VV) and vTF7-3-infected, NP-Gem-transfected cells (lanes NP) were labeled with [^{35}S]methionine (A) or [^{3}H]uridine (B). NP protein was immunoprecipitated from hypotonic cell extracts by using an anti-NP serum as described in Materials and Methods. (A) Autoradiograph of [^{35}S]methionine-labeled proteins immunoprecipitated with monoclonal anti-NP antibody and separated on an SDS–10% polyacrylamide gel. (B) Autoradiograph of [^{3}H]uridine-labeled RNA immunocoprecipitated with NP protein and separated in a denaturing 1.2% formaldehyde agarose gel.

C, and S (group 1) in repeated experiments did not yield clearly resolvable peaks in the gradients but was distributed in equal amounts in the upper and lower parts of the gradients. NP protein from mutants D, F, G, I, K, *Bgl*II-*Bgl*II, L, M, O, P, Q, and R (group 2) was found exclusively in the top fractions of the gradients and did not sediment into 30% CsCl. NP protein from mutants T, U, *Eco*RV-*Nco*I, and Stop (group 3), on the other hand, was exclusively found in fraction 6 of the gradient and thus behaved like wt NP protein. In parallel experiments, all cell extracts were examined by electron microscopy. While no nucleocapsidlike particles were found in cell extracts containing NP protein from mutant groups 1 and 2, numerous nucleocapsidlike particles were detected with mutants belonging to group 3 (T, U, *Eco*RV-*Nco*I, and Stop) (Fig. 6B). Thus, NP from group 3 mutants, like wt NP protein, assembled into highly ordered regular and flexible structures similar to viral nucleocapsids. The behavior of group 2 mutants is probably due to an entire loss of the ability to assemble. To further examine the phenotype of group 1 mutants (A, B, C, and S), immunogold electron microscopy was performed on cytoplasmic cell extracts. With this technique, large aggregates were observed with group 1 mutants, but no regular, ordered structures were seen (data not shown). It is therefore improbable that these mutants form loose, unstable, or more fragile nucleocapsidlike particles that would disassemble under high-salt conditions while being separated in CsCl gradients. It is more likely that group 1 mutants form complex aggregates varying in density.

The data presented show that large parts of NP protein are involved in nucleocapsid assembly. Any deletion within the N-terminal 399 amino acids abolished formation of nucleocapsidlike particles. Even mutant N, lacking only seven amino acids in the N-terminal domain, was unable to assemble. Additionally, even changing the Leu-Val motifs at positions 229 to 230 and 237 to 238 to Arg-Glu completely abolished assembly, and only conservative replacements to Ala-Ile and Val-Leu, respectively, still allowed assembly (data not shown). In contrast, changing Glu at position 489 to Gly or Pro (data not shown), and deletions as large as 70 amino acids (*Eco*RV-*Nco*I) within the 124 carboxy-terminal amino acids, had no effect on the formation of these particles.

DISCUSSION

This report demonstrates that Sendai virus NP protein, synthesized in mammalian cells, assembles into nucleocapsidlike particles in the absence of viral genomic RNA and other viral proteins. Self-assembly of capsid proteins of different viruses is a common phenomenon. What discriminates negative-stranded viruses from other viruses is that in the former, the nucleocapsid is not only a structural component used to enwrap the genome but also a functional entity required for virus replication. We therefore analyzed nucleocapsid assembly in more detail.

The physical appearance of the nucleocapsidlike particles demonstrates that Sendai virus NP protein is the only viral component that determines the structure of nucleocapsids. We showed that nucleocapsidlike particles are associated with RNA 0.5 to 2 kb in length. Therefore, NP protein alone does not seem to determine the specificity of the process of encapsidation of RNA. The RNA associated with nucleocapsidlike particles probably derived from the pool of molecules present in the cell. This pool may include transcription products from the vaccinia virus genome and the transfected plasmid. Binding of nonspecific RNA to N proteins has been described for two other negative-stranded viruses, VSV and influenza virus (11, 14). In contrast to our experiments, these assays were performed in vitro. Also, for measles virus NP protein, the self-assembly into nucleocapsidlike particles was recently described (22). As these structures also contain RNA (21a), nonspecific encapsidation of RNA seems to be a common property of nucleocapsid proteins of negative-stranded viruses, as long as these proteins are synthesized in the absence of other viral proteins. Purified VSV N protein was described to form only disklike structures in the absence of RNA. Upon addition of leader RNA, this RNA was encapsidated (1). RNA may therefore be required for the assembly of NP protein into larger, highly ordered structures.

It is unclear how encapsidation initiates, but once initiated, assembly of (monomeric or oligomeric) NP protein subunits could proceed very fast and be thermodynamically highly favored, since in our experiments almost no unassembled NP protein was found in NP protein-expressing cells. Assembly may depend on the concentration of both NP protein and RNA. NP protein synthesized in low concentration in a reticulocyte lysate did not self-assemble (data not shown). On the other hand, in vaccinia virus-infected cells, nascent RNA not associated with RNA-binding proteins is present in high amounts and may be a driving force for formation of nucleocapsidlike particles.

Our results clearly show that NP protein alone is able to form nucleocapsidlike particles. However, at least in vitro, also P protein seems to be required for encapsidation of nascent genome RNA. Regarding the possible contradiction between these findings, it has to be considered that only the latter process is highly specific. In Sendai virus-infected cells, self-assembly of NP protein and nonspecific encapsidation of RNA must be inhibited. Inhibition may result from posttranslational modification of NP protein and/or from

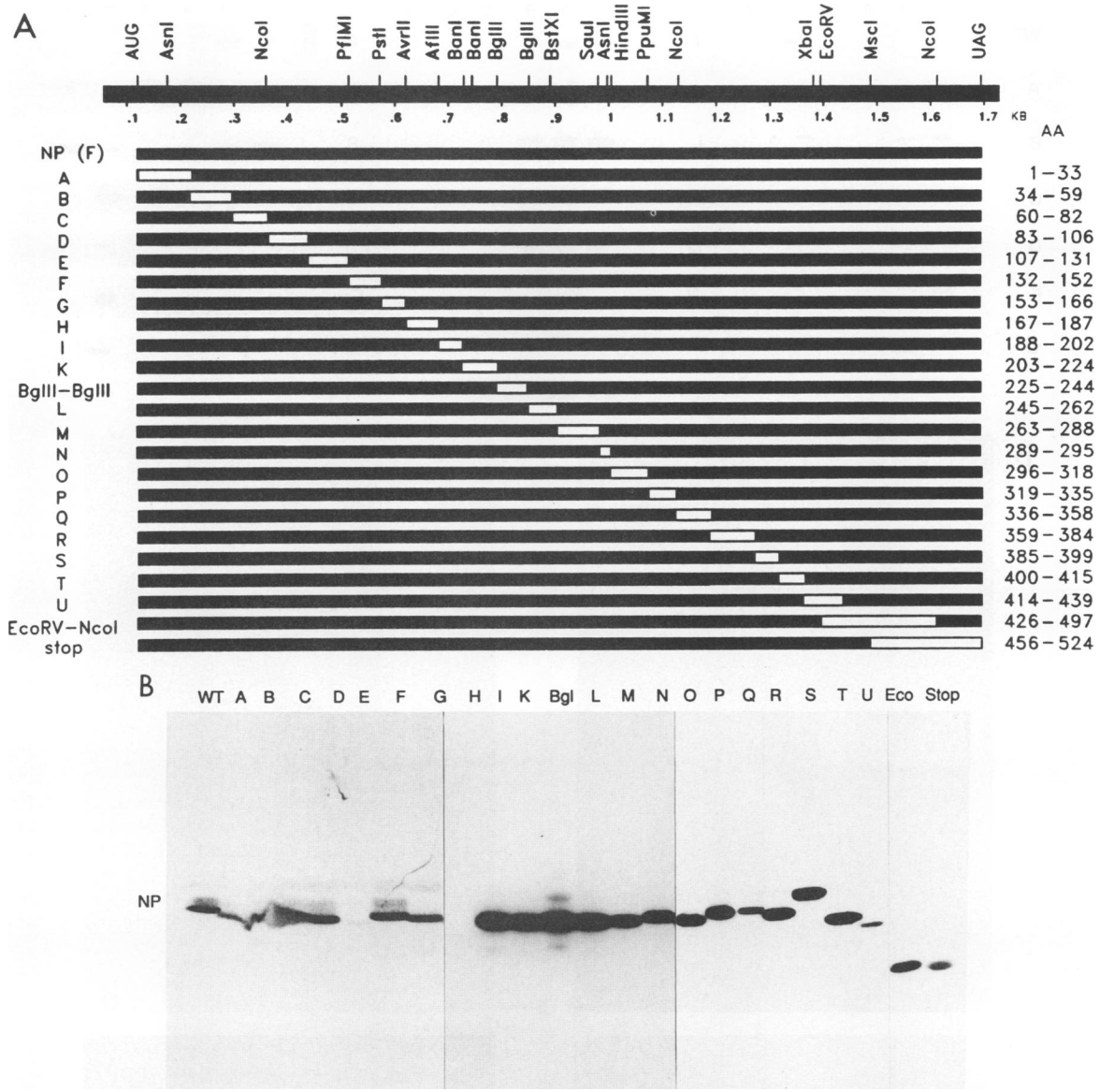
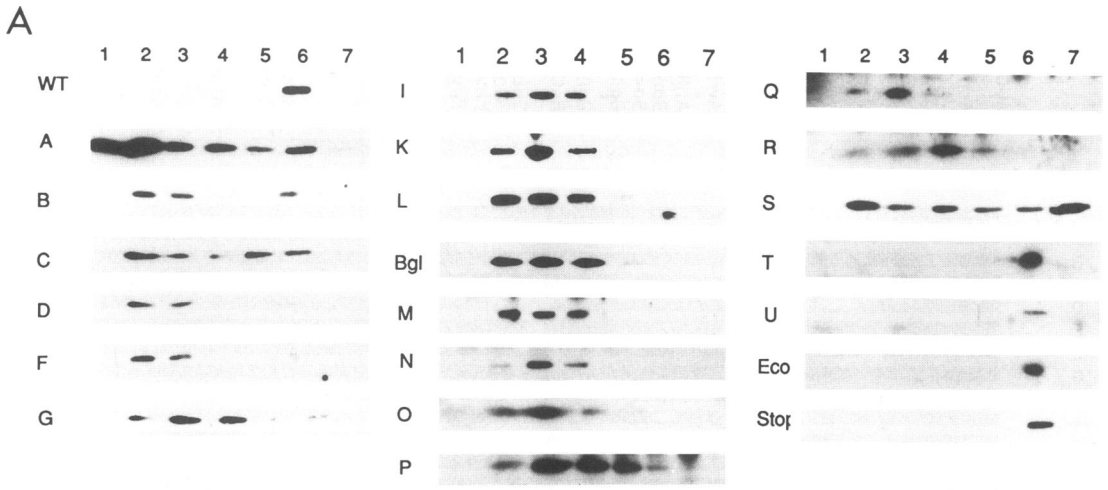


FIG. 5. Expression of NP gene deletion mutants. (A) Schematic representation of deletions in the NP gene. Positions of the first and last deleted amino acids (AA) are indicated. (B) Analysis of NP protein mutants. Cytoplasmic extracts from cells transfected with the mutated NP plasmids indicated at the top were analyzed by Western blotting with a Sendai virus antiserum. Lanes WT to G, H to N, and O to Stop represent individual blots exposed for slightly different times. Lane Eco is mutant *Eco*RV-*Nco*I; lane Bgl is mutant *Bgl*II-*Bgl*II.

complex formation with P protein. NP and P proteins bind to each other with high affinity (7, 8). We observed that coexpression of NP and P proteins largely increased the amount of unassembled NP protein to approximately 50% of the total amount of NP protein (1a). Therefore, P protein indeed inhibited self-assembly of NP protein similarly to the analogous VSV NS protein that prevented nonspecific encapsidation of RNA by complex formation with N protein (14). Specific encapsidation of viral genomic RNA may thus be due to the fact that the inhibitory function of P protein is released only under specific conditions. The NP-P complex, for example, may be resolved only when it contacts the nascent RNA attached to the polymerase complex.

We performed a deletion mutant analysis to identify functional domains on NP protein. Since no structural data were available, we covered the entire protein with deletions as small as possible and assayed for the assembly of nucleocapsidlike particles. Analysis of 21 deletion mutants and six mutants carrying point mutations indicated that the entire N-terminal region up to position 399 behaves largely like a single entity. Thus, NP protein can be divided into two domains, probably folding independently of each other and carrying out completely different functions, as summarized in Fig. 7. The border between the two domains maps between positions 385 and 399 (mutant S). Only domain I, the N-terminal region of NP protein, determines assembly of nucleocapsids. This part of NP protein is the one best conserved among paramyxoviruses and is largely hydrophobic, with a relatively high proportion of β-plated sheets predicted from the sequence. This entire region probably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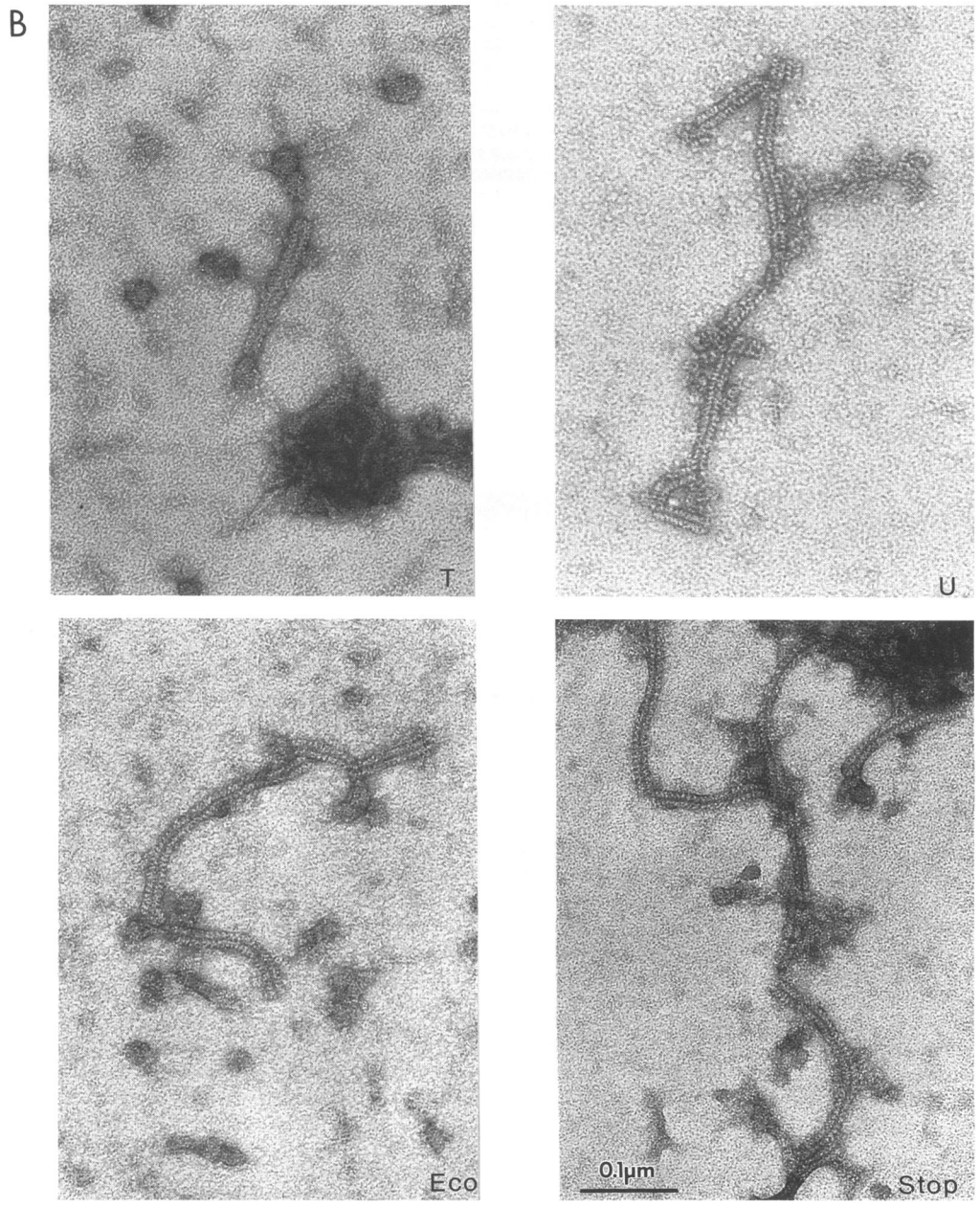
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0.1μm
Stop

FIG. 6. Mapping of domains on NP protein required for nucleocapsid assembly. (A) CsCl density gradient analysis of NP protein mutants. Cell extracts and gradients were prepared as described for Fig. 3C. Each mutant protein was analyzed in a separate gradient. Gradients were divided into seven fractions of 600 μl; 50 μl of each fraction was analyzed for NP protein by Western blotting with a Sendai virus antiserum and for detection with the enhanced chemiluminescence system. Exposure times differed for each Western blot, as they were adjusted to allow comparison of the relative amounts of NP protein in the different fractions of each gradient. Deletions are named as in Fig. 5B. (B) Electron micrographs of nucleocapsidlike particles formed by NP protein mutants T (T), U (U), *Eco*RV-*Nco*I (Eco), and Stop (Stop).

folds into a complex tertiary structure, which tolerates only few changes without losing its ability to assemble into nucleocapsids. Domain II, the C-terminal region, on the other hand, is not required for assembly (positions 399 to 524). It is poorly conserved, highly charged, probably much more flexible, and was earlier shown to reside on the outer surface of nucleocapsids. Tryptic digestion of nucleocapsids cleaves off a C-terminal 12-kDa polypeptide, the cleavage site probably being amino acid 410 (16, 17). The N-terminally truncated 48-kDa NP protein fragments still remain assembled as nucleocapsids, even after further cleavage into two polypeptides of 34 and 15 kDa (5). Deletions between positions 289 and 399, spanning the region where the respective additional cleavage sites reside, did not allow formation of nucleocapsidlike particles. Therefore, this region is essential for nucleocapsid assembly and only when assembled can be cleaved at sites presumably protruding from the surface of the tertiary structure without destabilizing nucleocapsids. From these data, it can also be inferred that the domains responsible for NP-NP and NP-RNA interactions, which are both required for nucleocapsid assembly, must reside in the N-terminal 399 amino acids.

NP from group 1 mutants A, B, C (positions 1 to 82), and S (positions 384 to 399) did not form any regular structures but accumulated in aggregates, some of which were dense enough to sediment into 30% CsCl. Therefore, it is possible that deletions in the C- and N-terminal parts of the region from 1 to 399 do not entirely disrupt the protein structure and the assembly process like mutations in the middle of this domain seem to do. Thus, either NP-NP or NP-RNA or both interactions would be only partially affected. However, it is striking that mutations A, B, C (positions 1 to 82), and S (positions 384 to 399) cover basic regions of NP protein, which are prime candidates for interactions with phosphates of the RNA. Basic amino acids at positions 60 to 72 and 107 to 115 have been previously proposed as RNA binding sites (16). An additional basic cluster exists at positions 391 to 397 (K-391ERLRHH-397). Thus, potential RNA binding sites are present on opposite ends of a presumably globular, highly structured region (positions 83 to 385). Nucleocapsid formation therefore possibly requires RNA binding by two separate regions of NP protein (i.e., 1 to 82 and 386 to 399) which both would have to cooperate in correct positioning of NP protein molecules for contiguous assembly. In a separate study, all of the NP protein deletion mutants described here have been analyzed for the ability to support replication of Sendai virus defective interfering virion genomes in vitro. Consistent with our results, all NP protein mutants that allowed formation of nucleocapsidlike particles allowed replication in vitro (2). Thus, domain I (positions 1 to 399) also contains all regions required for specific encapsidation of viral genomic RNA.

Domain II (amino acids 400 to 524) is not involved in assembly, so what are its functions? As it is exposed to the surface of nucleocapsids, it is suited for contacting the highly basic M protein, possibly via the acidic residues 418 to 440 (16), and it may be the region where the polymerase complex (P-L) binds. Recently, defective interfering virion rescue experiments were performed in which P and L genes as well as wt NP or the mutated NP gene were provided in *trans* by transfecting recombinant plasmids into defective interfering virion-infected cells. In these experiments, domain II of NP protein was essentially required for genome amplification, i.e., for the template function of nucleocapsids assembled from NP protein mutants (2). Nucleocapsids bind cell-free synthesized P protein in cosedimentation assays (20), and we showed that deleting the C-terminal 27 amino acids of NP protein decreases binding of P protein by 50% in protein overlay assays (7). This observation supports the idea that the C terminus of NP protein contains a binding site for P protein. However, we also reported that deletions N terminal of amino acid 426 completely abolished binding of P protein (7). Domain I may therefore be regarded as another binding site for P protein that overlaps with regions responsible for NP-NP and NP-RNA interactions. Thus, binding of P protein to domain I would present self-assembly of NP protein. On the other hand, after assembly of nucleocapsids, binding of P protein (or the P-L complex) to domain II, the carboxy terminus of NP protein, would allow access of the polymerase to the template RNA. This possibility is currently being analyzed in replication assays and cosedimentation assays of P protein and purified nucleocapsidlike particles.

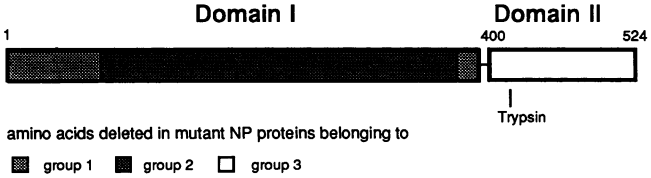

FIG. 7. Domains on NP protein and their possible functions. Domain I extends from amino acids 1 to 399 and is required for formation of nucleocapsidlike particles. Mutations in this domain produce two different phenotypes. Group 1 mutants (A, B, C, and S) still form aggregates but no regular structures. Group 2 mutants do not form any structures. Domain I overlaps with the region previously described as being essential for binding to P protein in vitro (7). Domain II extends from positions 400 to 524. It is not involved in nucleocapsid assembly. Further details are discussed in the text.

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