Conformational Maturation of Measles Virus Nucleocapsid Protein

ADRIAN F. GOMBART, AKIKO HIRANO, AND TIMOTHY C. WONG*

Department of Microbiology, University of Washington School of Medicine, Seattle, Washington 98195

Received 23 February 1993/Accepted 5 April 1993

We have obtained a polyclonal antiserum, N-BE, against the denatured, amino-terminal half of the measles virus (MV) nucleocapsid (N) protein and a monoclonal antibody (MAb), N46, which recognizes a conformation-dependent epitope in the same region. Amino acid residues 23 to 239 were required and sufficient for the formation of the conformational epitope. Using these antibodies, we show that the N protein of MV is synthesized as a relatively unfolded protein which first appears in the free-protein pool. This nascent N protein undergoes a conformational change into a more folded mature form. This change does not require the participation of other viral proteins or genomic RNA. The mature N protein does not accumulate in the free-protein pool but is quickly and selectively incorporated into the viral nucleocapsids. The mature N protein is a target for interaction with the phosphoprotein (P protein) of MV. This interaction interferes with the recognition of the N protein by the N46 MAb. This suggests that the association with the P protein may mask the binding site for the N46 MAb or that it induces a conformational change in the N protein.

Measles virus (MV) belongs to the family *Paramyxoviridae*. Its nonsegmented, negative-sense RNA genome is encapsidated by the virus-encoded nucleocapsid (N) protein to form a helical ribonucleoprotein complex called a nucleocapsid (26). In paramyxoviruses as well as the prototypic negative-stranded RNA virus vesicular stomatitis virus (VSV), viral RNA synthesis requires the nucleocapsid as a template and both the phosphoprotein (P protein) and polymerase (L protein) as enzymatic components (3, 8, 12, 19, 22).

The dynamic interactions between the N protein, viral RNA, and other viral components involved in the formation of the MV nucleocapsids are not well understood. In MV-infected cells, only genomic- and subgenomic-length viral RNAs which possess a 56-nucleotide (nt) leader sequence are encapsidated by the N protein (6). This suggests a specific interaction between the N protein and leader-containing MV RNA. However, when the N protein was overexpressed from a vaccinia virus recombinant containing the N gene of MV, the N protein was found to form nucleocapsid-like structures (43). These structures may represent cellular RNA nonspecifically encapsidated by the N protein (43). This raises the possibility that other MV gene products influence the encapsidation specificity of the N protein. A clear knowledge of the properties of the N protein is necessary for understanding its function.

In this study, we used subcellular fractionation, expressible cloned genes, and antibodies to study posttranslational conformational changes in the N protein of MV and to study its interactions with the viral genomic RNA and other viral proteins.

MATERIALS AND METHODS

Cells and viruses. The Nagahata strain MV (45), used for all the infection experiments in this study, was propagated in African green monkey kidney CV-1 cells in minimal essential medium (MEM; GIBCO/BRL) containing 10% newborn calf serum. The recombinant vaccinia virus (VTF7-3) engineered

to express the T7 RNA polymerase (13) was obtained from American Type Culture Collection and propagated in human cervical carcinoma HeLa cells in MEM containing 5% newborn calf serum and nonessential amino acids (GIBCO/BRL).

Antisera and MAbs. The N-BE polyclonal rabbit antiserum was directed against the denatured, amino-terminal half of the Edmonston strain MV N protein encoded by a *Bam*HI-*Eco*RV DNA fragment (6). To generate the P-BH polyclonal antiserum, the fragment spanning the upstream *Bal*I site to the *Hind*III site (nt 140 to 671) of an Edmonston strain MV P cDNA clone, pcD-P8 (47), was inserted into the pMalc expression vector (New England Biolabs) to produce a maltose-binding protein-P (BH) gene fusion which encodes a protein containing the amino half of the P protein fused with the maltose-binding protein. This gene fusion was transformed into *Escherichia coli* TB1 and induced as described by the manufacturer. The maltose-binding protein-P (BH) fusion protein was affinity purified under nondenaturing conditions from the soluble protein fraction by using an amylose resin as described by the manufacturer (New England Biolabs). The purified protein was used to immunize a female New Zealand White rabbit. Mouse monoclonal antibody (MAb) N46 specific for the Nagahata strain MV N protein was prepared as previously described (2).

Analysis of nucleocapsid-associated MV N protein. CV-1 cells were infected with MV at a multiplicity of infection of 1. When 30% of the cells showed cytopathic effects, they were starved for 1 h in methionine-free MEM (GIBCO/BRL) and labeled for 8 h with 100 μ Ci of [^{35} S]methionine (Express; NEN/Dupont) per 100-mm-diameter plate in methionine-free MEM containing 2% dialyzed calf serum. Cytoplasmic extracts were prepared and fractionated as described previously (7, 20). Fractions (500 μ l) from the interface between the 30 to 40% sucrose containing intracellular nucleocapsids or from the top of the 20% sucrose containing the free proteins were collected. One-fifth of each fraction was mixed with an equal volume of 2 $\times$ RIPA buffer (20 mM Tris [pH 7.2], 300 mM NaCl, 0.2% sodium dodecyl sulfate [SDS], 2% deoxycholate, 2% Triton X-100) and immunoprecipitated as previously described (48) with the N46 MAb or N-BE

* Corresponding author.

antiserum. The proteins were analyzed by electrophoresis on a 10% polyacrylamide-SDS gel (SDS-PAGE) (32).

Denaturation studies of nucleocapsid-associated N protein. Approximately 1/10 of the nucleocapsid fraction from MV-infected cells labeled for 5.5 h with [^{35} S]methionine was treated in one of the following ways: (i) mixing with 4 volumes of RIPA buffer; (ii) mixing with 4 volumes of RIPA buffer, boiling for 3 min, and cooling on ice; (iii) adding dithiothreitol and SDS to final concentrations of 1 mM and 0.5%, respectively, boiling for 3 min, adding 4 volumes of RIPA buffer, and cooling on ice; or (iv) adding 2-mercaptoethanol and SDS to final concentrations of 0.72 M and 0.5%, respectively, boiling for 3 min, adding 4 volumes of RIPA buffer, and cooling on ice. The samples were immunoprecipitated with N46 MAb or N-BE antiserum and analyzed by SDS-PAGE on a 10% polyacrylamide gel.

Construction and expression of N protein deletion mutants in bacteria for epitope mapping. An *EcoRV* fragment spanning nt 46 to 769 from the 5' terminus of the Edmonston strain MV N gene was obtained from the pcD-N7 cDNA clone (46) and ligated in frame with the *trpE* gene in the pATH3 vector (30). This construct, called pATH3-N(EE), encoded the truncated TrpE' peptide fused with the amino half of the N protein from two triplet codons upstream of the initiation Met codon to amino acid 239 (40) (see Fig. 4). The pATH3-N(EEdKK) construct was generated by removing an internal region between the two *KpnI* sites at nt 414 and 558. The released *KpnI* fragment was ligated into the pATH3 vector to generate pATH3-N(KK). The 5'-deletion constructs pATH3-N(H1E), -N(AE), -N(KE), and -N(BE) were generated by removing the sequences upstream of the *BamHI*, *ApaI*, *KpnI*, and *BclI* sites, respectively, from pATH3-N(EE). The 3'-deletion construct pATH3-N(EB) was made by deleting the sequences downstream of the *BclI* site. The pATH3-N(19b) and -N(19d) constructs were made by performing 3' exonuclease III deletions from the *EcoRV* (nt 769) site of pATH3-N(EE). Each construct was sequenced by double-stranded DNA sequencing with oligonucleotide primers specific for the sequences upstream and downstream of the cloning sites (18) to ensure that the constructs had the proper translational reading frame.

The various constructs were transformed into *E. coli* TB1 and induced as previously described (48). For radioimmune precipitation, the bacterial proteins were labeled with 25 μ Ci of [^{35} S]methionine per ml for 30 min. The soluble protein fractions were prepared and immunoprecipitated with either the polyclonal anti-TrpE antiserum (17) or N46 MAb.

For immunoblot (Western blot) analysis, total proteins from the induced bacteria were lysed in protein sample buffer (32), electrophoresed on an SDS-containing 10% polyacrylamide gel, and transferred to nitrocellulose paper. The blots were reacted with a 1:10,000 dilution of either anti-TrpE antiserum or N46 MAb, washed, and visualized by reacting with alkaline phosphatase-conjugated secondary antibodies against rabbit or mouse immunoglobulin G, respectively, as specified by the manufacturer (Promega Biotech).

Expression of MV proteins in human cells by the vaccinia virus-T7 RNA polymerase system. The Edmonston strain MV N and P genes from the pcD-N7 and pcD-P8 cDNA clones (46, 47) were placed under the control of the T7 promoter in the pTZ19RX (2) or pTZ19R vector (Pharmacia), respectively, to generate the pTZ19RX-N7 and pTZ19R-P8 plasmids. The pTZ-EM clone containing the Edmonston strain MV matrix (M) gene was described previously (2). HeLa cells were infected at multiplicity of infection of 3 to 5 with

the recombinant vaccinia virus VTF7-3. The cells were then transfected by the calcium-phosphate method described previously (48) with one of the following combinations: (i) 5 μ g of pTZ19RX-N7 plus 5 μ g of pTZ19R, (ii) 5 μ g of pTZ19R-P8 plus 5 μ g of pTZ19R, (iii) 5 μ g of pTZ19RX-N7 plus 5 μ g of pTZ19R-P8, or (iv) 5 μ g of pTZ19RX-N7 plus 5 μ g of pTZ-EM. At 22 to 24 h posttransfection, the cells were labeled for 6 h with 50 μ Ci of [^{35} S]methionine per 60-mm-diameter plate and lysed in RIPA buffer and the nuclei were pelleted at $12,000 \times g$ for 20 min at 4°C. The proteins were immunoprecipitated with different antibodies and analyzed by SDS-PAGE on a 10% polyacrylamide gel.

Analysis of N-P protein interactions. CV-1 cells were infected with MV at a multiplicity of infection of 2. When 100% of the cells showed cytopathic effects, they were labeled with 100 μ Ci of [^{35} S]methionine per 100-mm-diameter plate for 4 h. Nucleocapsid and free-protein fractions were prepared as described above. The fractions were immunoprecipitated with the N46 MAb or the P-BH antiserum in RIPA or TNT buffer (20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1% Triton X-100). The proteins were analyzed by SDS-PAGE on a 10% polyacrylamide gel.

Pulse-chase analysis of N protein. CV-1 cells were infected with MV at a multiplicity of infection of 4 to 5. When 95 to 100% of the cells showed cytopathic effects, they were starved for 1 h in methionine-free MEM. The cells were pulse-labeled with 100 μ Ci of [^{35} S]methionine for 10 min, washed in MEM containing 100 mM L-methionine, and chased in MEM containing 2% newborn calf serum and 1 mM L-methionine for 0, 30, 120, or 240 min. At each time point, the total cytoplasmic fraction or the nucleocapsid and free-protein fractions were prepared as described above. The fractions were immunoprecipitated with the N46 MAb or N-BE antiserum and analyzed by SDS-PAGE on a 10% polyacrylamide gel.

RESULTS

Nucleocapsid-associated and free N proteins of MV are antigenically distinguishable. We previously noted that the N46 MAb reacted with the N protein associated with the intracellular viral nucleocapsids more efficiently than with the free N protein in MV-infected cells (20). To investigate this phenomenon, we labeled MV-infected cells with [^{35} S]methionine, fractionated the intracellular viral nucleocapsids from the free proteins by sucrose gradient centrifugation, and immunoprecipitated equal portions of these fractions in RIPA buffer with different N-specific antibodies.

A polyclonal antiserum, N-BE, against the denatured amino-terminal half of the N protein of MV reacted more efficiently with the N protein in the free-protein fraction (Fig. 1, lane b) than the N protein in the nucleocapsids (Fig. 1, lane a). This antiserum also detected the smaller degradative products of the N protein in the free-protein fraction (Fig. 1, lane b). In contrast, the N46 MAb against the native MV N protein reacted more efficiently with the N protein in the nucleocapsids (Fig. 1, lane c) than the free N protein (Fig. 1, lane d). These results indicate that the nucleocapsid-associated N protein is antigenically distinguishable from the N protein in the free-protein pool.

Antigenic difference due to conformational change in the N protein. To determine whether the antigenic difference between the two forms of N protein was due to a conformational change, we solubilized the ^{35} S-labeled N protein from gradient-purified viral nucleocapsids in RIPA buffer and

FIG. 1. Antigenically distinguishable forms of the N protein in MV-infected cells. Nucleocapsid (R) and free (F)-protein fractions from ^{35}S -labeled, MV-infected CV-1 cells were immunoprecipitated with the N46 MAb or the N-BE polyclonal antiserum. The proteins were analyzed by SDS-PAGE on a 10% polyacrylamide gel. The location of the N protein is indicated. The numbers on the left indicate the molecular masses (in kilodaltons) of the protein markers (lane M).

tested it with the antibodies under different denaturing conditions.

Under RIPA buffer conditions, the nucleocapsid-associated N protein reacted efficiently with the N46 MAb but inefficiently with the N-BE antiserum (Fig. 2, lanes a and e, respectively). Boiling in the presence or absence of dithiothreitol or 2-mercaptoethanol greatly reduced reactivity of the N protein with the N46 MAb (Fig. 2, lanes b to d) but significantly enhanced reactivity with the N-BE antiserum (Fig. 2, lanes f to h).

FIG. 2. The antigenic difference between the nucleocapsid and soluble N proteins is due to conformational changes. Purified, ^{35}S -labeled, intracellular nucleocapsids were solubilized in RIPA buffer and either boiled at 100°C (+) or not boiled (-) in the presence (+) or absence (-) of 2-mercaptoethanol (B-ME) or dithiothreitol (DTT) and immunoprecipitated with the N46 MAb or the N-BE polyclonal antiserum. The proteins were analyzed by SDS-PAGE on a 10% polyacrylamide gel. The location of the N protein is indicated. The number on the left indicates the location of the 68-kDa protein marker bovine serum albumin (lane M).

These results indicate that the N46 MAb recognizes a conformational epitope on the N protein, whereas the N-BE antiserum recognizes a less folded form of N protein. Therefore, the antigenic difference between the nucleocapsid-associated N protein and the free N protein is due to a conformational difference. The N protein in the nucleocapsid appears to be more highly folded than the free N protein.

Mapping of the N46 MAb epitope. To localize the protein region recognized by the N46 MAb, we generated a series of N gene mutants (see Fig. 4) for the expression of N proteins with different amino, carboxyl, or internal deletions in bacteria (see Materials and Methods). *E. coli* transformed with the pATH3 vector and various constructs was labeled with [^{35}S]methionine. Aliquots of the soluble protein fractions were immunoprecipitated with the anti-TrpE antiserum (Fig. 3A) or the N46 MAb (Fig. 3B). All the constructs expressed the expected TrpE or TrpE-N fusion peptides (Fig. 3A). The electrophoretic mobilities of most of the peptides were in agreement with the calculated molecular sizes (Fig. 3A and 4). However, the N(19d) peptide migrated faster than the N(19b) peptide (Fig. 3A, lanes i and j, respectively) even though the N(19d) peptide contained 21 more amino acid residues [Fig. 4, N(19d) and N(19b)]. Fifty-seven percent of the additional residues are hydrophobic, which may explain the anomalous migration of the N(19d) peptide. Also, the N(KK) peptide and another small fusion protein containing the N(KK) sequence migrated more slowly than expected [Fig. 3A, lanes d and g; Fig. 4, N(KK) and N(KE)]. The anomalous migration of the N(KK) peptide may be due to the high content (25%) of Glu or Asp residues, which may affect binding of SDS to the protein. A high content of acidic residues in the amino region is thought to cause similar anomalous migration in the VSV P protein (4) as well as cellular proteins such as calpastatin (44) and nuclear histone-binding protein N1/N2 (28).

When the same samples were immunoprecipitated with the N46 MAb, only the N(EE) and N(H1E) peptides were detected (Fig. 3B, lanes b and e, respectively). Amino-terminal deletions past Gly-23 (Fig. 3B, lanes f, g, and h), carboxy-terminal deletions past Asp-239 (Fig. 3B, lanes i, j, and k), and the internal deletion which removed amino acid residues 122 to 167 (Fig. 3B, lane c) all abrogated the reactivity of the N protein with the N46 MAb. The N(KK) peptide was also not recognized by the N46 MAb (Fig. 3B, lane d).

Even though the N46 epitope is conformation dependent (Fig. 2), the denatured N protein can partially renature to reform the N46 epitope when it is transferred onto nitrocellulose filter paper in a Western blot analysis (42a). To see whether the N protein fragments can regenerate the N46 epitope after denaturation, we performed Western blot analysis with the deletion mutants. The TrpE and all the mutant N proteins were detected by the anti-TrpE antiserum by Western blot (Fig. 3C), confirming that the proteins were expressed. When the blot was reacted with the N46 MAb, only the N(EE) and N(H1E) peptides were detected (Fig. 3D, lanes b and e, respectively).

These results, which are summarized in Fig. 4, indicate that the amino-terminal half of the N protein between amino acid residues Gly-23 and Asp-239 is both required and sufficient for the formation of the conformational epitope recognized by the N46 MAb. Since the denatured N protein can reform the N46 epitope after the N protein is resolved by electrophoresis, the formation of this epitope does not require other MV proteins or viral genomic RNA. Incidentally, the N protein region recognized by the N46 MAb

FIG. 3. Radioimmune precipitation and Western blot analyses of truncated N proteins. (A and B) One-tenth of the ^{35}S -labeled protein fractions from the induced bacteria was immunoprecipitated with either anti-TrpE antiserum (A) or N46 MAb (B) and analyzed by SDS-PAGE on a 10% polyacrylamide gel. (C and D) Total proteins from the induced bacteria were electrophoresed through an SDS-10% polyacrylamide gel and transferred onto nitrocellulose paper. The blots were reacted with a 1:10,000 dilution of the anti-TrpE antiserum (C) or N46 MAb (D). The blot in panel D contained twice the amount of proteins as the one in panel C. The locations of the protein molecular weight standards (lanes M) are shown on the left.

corresponds precisely to the peptide fragment used to generate the polyclonal N-BE antiserum. Therefore, the N46 MAb recognizes a more folded, native state of the amino-half of the N protein, whereas the N-BE antiserum recognizes a less folded or denatured state of the same protein region.

The N46 form of N protein forms a complex with the P protein. It has been reported recently that the N proteins of Sendai virus (22) and MV (25) interact with their respective P proteins *in vivo*. To determine which form of the N protein interacts with the P protein, we expressed the cloned N and P genes of MV in HeLa cells using the vaccinia virus-T7 RNA polymerase system.

HeLa cells infected with VTF7-3 recombinant vaccinia virus and transfected with the pTZ19RX-N7 plasmid expressed the 61,000- M_r N protein detectable by the N-BE antiserum as well as the N46 MAb (Fig. 5, lanes a and d, respectively). This N clone also expressed a slightly more slowly migrating protein that was more clearly resolved in another gel (Fig. 5, lanes k to n). Nucleotide sequence analysis suggested the more slowly migrating protein was likely due to aberrant translation from an upstream in-frame ATG codon in the cloning vector (data not shown) and should be disregarded. Small amounts of vaccinia virus proteins were nonspecifically precipitated by all antibodies.

The N-BE antiserum efficiently immunoprecipitated the N protein when both the N and P genes were coexpressed in the same cells, as compared with cells transfected with the N

gene alone (Fig. 5, lanes b and a, respectively). Interestingly, the N protein in the same lysate of the cells expressing both the N and P proteins was not immunoprecipitated efficiently by the N46 MAb (Fig. 5, compare lanes e and b). Therefore, the P protein markedly interfered with the immunoprecipitation of the N protein by the N46 MAb but not by the N-BE antiserum.

In cells expressing both the N and P proteins, a protein that comigrated with the P protein was coimmunoprecipitated by the N46 MAb but not the N-BE antiserum under the stringent RIPA buffer conditions (Fig. 5, lanes e and b, respectively). The P protein coimmunoprecipitation was not because the protein cross-reacted fortuitously with the N46 MAb, since N46 did not recognize the P protein expressed alone (Fig. 5, lane f). Expression of the P protein in the doubly and singly transfected cells was confirmed by the P-specific antiserum, P-BH, against the amino-terminal region of the MV P protein (Fig. 5, lanes i and j, respectively). The P-BH antiserum did not coimmunoprecipitate the N protein efficiently under the stringent RIPA buffer conditions (Fig. 5, lane i). However, this antiserum clearly coimmunoprecipitated the N protein under less stringent conditions (see below). Therefore, the N-P protein complexes recognized by the P-BH antiserum may be unstable. Alternatively, a significant fraction of the N protein in the N-P protein complexes in the transfected cells may be unlabeled.

To determine whether coexpression of a different viral protein would have a similar inhibitory effect on immuno-

FIG. 4. Localization of the N46 MAb epitope. The hydropathy profile of the MV N protein was predicted by a GenePro computer program based on a scale developed by Kyte and Doolittle (31). The x axis shows the amino acid number of the N protein. Reactivity of the N protein and mutants (horizontal bars) with the N46 MAb and the observed (Obs.) versus expected (Exp.) electrophoretic mobilities (in M_r , 10^3) are shown. A plus sign indicates that the protein reacted with the N46 MAb in Western blot or immunoprecipitation analysis (Fig. 3) (a minus sign indicates no reaction). The nomenclature denotes the 5' and 3' restriction enzyme recognition sites flanking the N DNA fragments, which encode the amino acid residue numbers of the N protein shown on the right of each peptide. The numbers in parentheses indicate deleted amino acid residues. Abbreviations: E, *EcoRV*; K, *KpnI*; H1, *BamHI*; A, *ApaI*; B, *BclI*.

precipitation of the N protein by the N46 MAb, we coexpressed the N protein together with the M protein of MV. Expression of the N protein was slightly reduced in the presence of the M protein as detected by the N-BE antiserum (Fig. 5, lanes k and l). However, the N protein coexpressed with the M protein was immunoprecipitated by the N46 MAb as efficiently as by the N-BE antiserum (Fig. 5, lanes n and l, respectively). Expression of the M protein was confirmed by immunoprecipitation with an M-specific antiserum (data not shown).

These data suggest that the P protein specifically interacts with the more folded form of N protein and that this interaction interferes with the recognition of the latter by the N46 MAb.

Formation of N-P complexes in MV-infected cells. To investigate whether the N46 form of N protein also complexes with the P protein in MV-infected cells, ^{35}S -labeled proteins

in the nucleocapsid and free-protein fractions from MV-infected cells were immunoprecipitated with the N46 MAb or P-BH antiserum in a Tris buffer (TNT) which contained only the nonionic detergent Triton X-100, to better preserve the ionic protein-protein interactions.

Under these less stringent conditions, the N46 MAb coimmunoprecipitated the N protein and a low level of the P protein in the free-protein fraction (Fig. 6A, lane b). The interaction between the N and P proteins in the free-protein fraction was also detected by the P-BH antiserum (Fig. 6A, lane d). The P-BH antiserum also immunoprecipitated a protein that comigrated with the L protein (Fig. 6A, lane d), indicating the presence of N-P as well as P-L protein complexes in the free protein fraction in MV-infected cells. From the nucleocapsid fraction, both the N46 MAb and P-BH antiserum coimmunoprecipitated the N, P, L, and M proteins (Fig. 6A, lanes a and c, respectively), indicating that

FIG. 5. Synthesis of the N46 form of N protein from the cloned gene and complex formation with the P protein. The N, P, and M proteins were expressed separately or together in HeLa cells by using the vaccinia virus-T7 RNA polymerase system. Total cytoplasmic extracts labeled with [^{35} S]methionine were immunoprecipitated with the N-BE or P-BH polyclonal antiserum or the N46 MAb. The proteins were analyzed by SDS-PAGE on a 10% polyacrylamide gel. Lanes a to j were from the same experiment. Lanes k to n were from a separate experiment, analyzed in the same gel, and autoradiographed for the same time. The locations of the N and P proteins are indicated. The numbers on the left indicate the molecular masses (in kilodaltons) of the protein markers.

the intact nucleocapsids were immunoprecipitated. Most of the labeled P protein was found in the nucleocapsid fraction rather than the free-protein fraction (Fig. 6A, lanes c and d, respectively).

To disrupt the viral nucleocapsids, SDS, Triton X-100, and deoxycholate were added to the same concentrations as in the RIPA buffer to the nucleocapsid and free-protein fractions (Fig. 6B). This treatment reduced coimmunoprecipitation of the proteins which associated through ionic interactions. However, after prolonged exposure of the autoradiogram, the P protein was still detectably coimmunoprecipitated with the N protein by the N46 MAb (Fig. 6B,

FIG. 6. The N46 form of N protein interacts with the P protein in infected cells. Nucleocapsid (R) and free (F)-protein fractions from ^{35} S-labeled, MV-infected CV-1 cells were immunoprecipitated under TNT (A) or RIPA (B) buffer conditions with either the N46 MAb or polyclonal P-BH antiserum. The autoradiogram in panel B was exposed longer to visualize the fainter protein bands. The locations of the N, P, M, and L proteins are indicated. The numbers on the left indicate the molecular masses (in kilodaltons) of the protein markers (lane M).

lanes a and b), and the N protein was still coimmunoprecipitated with the P protein by the P-BH antiserum from the nucleocapsid fraction (Fig. 6B, lanes c and d). Again, most of the P protein was found in the nucleocapsids rather than the free-protein fraction, and the putative L protein was coimmunoprecipitated by the P-BH antiserum (Fig. 6B, lanes c and d).

These results indicate that the N and P proteins form complexes in the free-protein pool and nucleocapsids in MV-infected cells as well as in cells expressing the N and P proteins together. Furthermore, much of the P protein in MV-infected cells is found in the viral nucleocapsids rather than the free-protein pool.

Kinetics of antigenic change and incorporation of the N protein into the nucleocapsids. To gain insight into the relationship between the two forms of N protein and their roles in encapsidation of viral RNA, we examined the synthesis and incorporation of the N protein into the viral nucleocapsids by pulse-chase experiments. MV-infected cells were labeled with [^{35} S]methionine for 10 min and chased with excess unlabeled methionine. Total cytoplasmic extracts were prepared by lysing the cells in RIPA buffer and pelleting the nuclei.

The N-BE antiserum efficiently immunoprecipitated the N protein from the cytoplasmic fraction immediately after the pulse (Fig. 7A, lane a), and the N protein detected by this antiserum decreased during the chase period (Fig. 7A, lanes b to e). In contrast, the N46 MAb did not efficiently immunoprecipitate the N protein immediately after the pulse (Fig. 7B, lane a), but the N protein detected by the N46 MAb increased during the chase period (Fig. 7B, lanes b to e). Therefore, the two forms of N protein detected by the N-BE and N46 antibodies exhibit a precursor-product relationship.

We further fractionated the ^{35} S-labeled MV-infected cell lysates from a pulse-chase experiment into the nucleocapsid and free-protein fractions. Immediately after the pulse, the N-BE antiserum immunoprecipitated the N protein more efficiently from the free-protein fraction than the nucleocapsid fraction (Fig. 7C, lanes b and a, respectively). The N46 MAb initially detected a slightly higher level of the N protein in the free-protein fraction than in the nucleocapsid protein fraction (Fig. 7D, lanes b and a, respectively). During the 30- to 240-min chase period, the N-BE-recognizable form of N protein gradually decreased in the free-protein fraction (Fig. 7C, lanes d, f, and h). This form of N protein did not accumulate in the nucleocapsid fraction (Fig. 7C, lanes c, e, and g). During the same period, the N46-detectable form of N protein increased in the nucleocapsid fraction (Fig. 7D, lanes c, e, and g) but decreased in the free-protein fraction (Fig. 7D, lanes d, f, and h). The reduced amount of the N46-recognizable form of the N protein in the nucleocapsid fraction at the 30-min time point was an experimental error, as further experiments clearly showed the N46-recognizable form of the N protein in the nucleocapsid fraction at this time point (data not shown).

These data indicate that the newly synthesized N protein of MV first appears in the free-protein pool as a relatively unfolded N-BE-recognizable form. It then undergoes a conformational and antigenic change into the mature N46-recognizable form. This latter form of N protein is selectively and quickly incorporated into the viral nucleocapsids.

DISCUSSION

Kinetic studies of the proteins of Sendai virus and VSV showed that the majority of the newly synthesized N protein

FIG. 7. The N protein undergoes posttranslational conformational change preceding encapsidation. MV-infected CV-1 cells were pulse-labeled with [^{35} S]methionine and chased with unlabeled methionine-containing medium. Total cytoplasmic fractions (A and B) and nucleocapsid (R) and free (F)-protein fractions (C and D) were prepared at the times (in minutes) indicated. Equal proportions were immunoprecipitated with the N-BE antiserum (A and C) or the N46 MAb (B and D) and analyzed by SDS-PAGE on a 10% polyacrylamide gel. The location of the N protein is indicated. The number on the left of panels C and D indicates the position of the protein marker bovine serum albumin (68 kDa).

first appears in a soluble-protein pool which supplies N protein for the encapsidation of the viral genomes (24, 27, 29, 39, 41). For MV, the nascent N protein can be chased from the free-protein pool into the cellular plasma membrane, where the nucleocapsids are assembled into virions (16). Using two different MAbs, Arnheiter and coworkers (1) provided direct evidence by immunofluorescence techniques that the N protein species in the soluble-protein pool and the viral nucleocapsids in VSV-infected cells are antigenically distinct. Antigenic differences have also been observed between the free N protein and nucleocapsid-associated N protein in MV-infected cells (20). In this study, we obtained a polyclonal antiserum, N-BE, against the denatured, amino-terminal half of the N protein and a MAb, N46, against a native conformational epitope in the same region (Fig. 3 and 4). Using these antibodies, we show that the N protein of MV undergoes a posttranslational conformational change (Fig. 7). The change from the nascent to the mature form renders most of the sequence-specific epitopes on the MV N protein inaccessible to the N-BE antiserum. At the same time, it creates a conformation-dependent epitope that is recognized by the N46 MAb (Fig. 2).

Buckland and coworkers previously identified three antigenic sites on the N protein of the Halle strain of MV (5). Site I was conserved among different MV isolates and mapped within amino acids 122 to 150. Sites II and III were more variable and localized in the carboxy-terminal region (15). The N46 epitope appears to be distinct from antigenic site I, since the N46 epitope is conformation dependent and the N46 MAb did not recognize the N(KK) peptide (amino acid residues 122 to 167), which included antigenic site I (Fig. 4).

For Sendai virus, Gill and coworkers mapped the epitopes for 11 MAbs against the N protein to the carboxy-terminal or middle region (14). One of these MAbs, called M4, was a potent inhibitor of Sendai viral RNA synthesis *in vitro* (11). The M4 MAb recognized amino acid residues 290 to 295 in a hinge region postulated to allow flexibility in the Sendai virus N protein (14). Our preliminary results suggest that the N46 MAb inhibits RNA synthesis from MV nucleocapsids *in vitro* (42a). Comparison between the predicted amino acid

sequences of the Sendai virus and MV N proteins (36) indicates that the residues recognized by the M4 MAb on the Sendai virus N protein are not conserved and are outside the region recognized by the N46 MAb. These findings suggest that the region in the MV N protein defined by the N46 MAb is not analogous to that mapped by the M4 MAb in the Sendai virus N protein. However, the amino as well as middle regions of the N protein appear to be important for viral RNA synthesis.

The N and P proteins of VSV translated from *in vitro*-synthesized mRNAs formed complexes (10, 34). Likewise, the N and P proteins of MV and Sendai virus expressed from the cloned genes in cultured cells also formed complexes (22, 25). We found that the P protein of MV interfered with the recognition of the N protein by the N46 MAb but that a reduced level of N protein was coimmunoprecipitated with the P protein (Fig. 5). This raises the possibility that the binding sites on the N protein for the N46 MAb and the P protein overlap. This would suggest that the amino-terminal half of the N protein interacts with the P protein. Recently, Ryan and coworkers showed that two MAbs against the carboxy-terminal region of the N protein of human parainfluenza virus 1, which cross-reacted with the Sendai virus N protein, caused the release of the Sendai virus P protein from its respective nucleocapsids. In addition, the MAbs interfered with binding of the P protein to the nucleocapsids (42). The carboxy-terminal 119 amino acid residues of the human parainfluenza virus 1 N protein were important for recognition by these MAbs (42). However, Homann and coworkers used a protein-blotting assay to show that the Sendai virus N protein lacking up to 103 carboxy-terminal amino acid residues still retained 45% P protein-binding activity, whereas N proteins with internal deletions in the amino or middle region of the N protein showed only 2 to 18% P protein-binding activities (21). Together with the observation that MAbs against the amino and middle regions of the N protein inhibited transcription, these data suggest that the amino and middle regions of the N protein are crucial for interaction with the P protein and that this interaction can be influenced by mutations or interactions with antibodies in the amino- or carboxy-terminal region of the N protein.

The N-P protein complexes rather than the N protein alone supported the replication and encapsidation of the VSV and Sendai virus genomes (22, 23, 33, 37, 38). Pulse-chase analysis indicated that the Sendai virus P protein was incorporated into the viral nucleocapsids and virions almost as soon as it was synthesized, whereas the N protein entered the nucleocapsids more slowly (27, 39). In MV-infected cells, much of the labeled P protein was found in the viral nucleocapsids, and the N-P interaction was more readily detected in the nucleocapsids than in the free-protein fraction (Fig. 6). The N protein of VSV expressed from the cloned gene bound nonspecifically to RNA in vitro (35), and the N protein of MV expressed in cultured cells localized in the nuclei (25). Coexpression of the N proteins of these viruses with their respective P proteins prevented nonspecific RNA binding and intranuclear localization of the N proteins (25, 35). Therefore, the P protein clearly influences the properties of the N protein. It has been suggested that the P protein may allow the N protein to specifically recognize the viral RNA (35). Thus far, this putative function of the N-P protein interaction has not been demonstrated (9).

In summary, the present data show that the N protein of MV is synthesized as a relatively unfolded protein that first appears in the free-protein pool. This nascent N protein undergoes conformational maturation to assume a more highly folded form preferentially recognized by the N46 MAb. This conformational change can occur independently in the absence of other viral proteins or viral genomic RNA. The mature form of N protein is quickly incorporated into the intracellular viral nucleocapsids. The P protein preferentially associates with the mature N protein in both the free-protein pool and the nucleocapsids. The amino-terminal half of the N protein recognized by the N46 MAb may overlap with the binding site for the P protein.

ACKNOWLEDGMENTS

We thank Minoru Ayata for providing the N46 MAb, Sharon J. Schultz for providing the N-BE antiserum, and George F. Rohrmann for providing the sequencing primers for the pATH vectors.

This work was supported by research grant VM-60G from the American Cancer Society and by research grant NS31427 and Alzheimer's Disease Center grant AG05136 from the National Institutes of Health.

REFERENCES

1. Arnheiter, H., N. L. Davis, G. Wertz, M. Schubert, and R. A. Lazzarini. 1985. Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. *Cell* 41:259-267.
2. Ayata, M., A. Hirano, and T. C. Wong. 1989. Structural defect linked to nonrandom mutations in the matrix gene of Biken strain subacute sclerosing panencephalitis virus defined by cDNA cloning and expression of chimeric genes. *J. Virol.* 63:1162-1173.
3. Banerjee, A. K. 1987. Transcription and replication of rhabdoviruses. *Microbiol. Rev.* 51:66-87.
4. Barik, S., and A. K. Banerjee. 1991. Cloning and expression of the vesicular stomatitis virus phosphoprotein gene in *Escherichia coli*: analysis of phosphorylation status versus transcriptional activity. *J. Virol.* 65:1719-1726.
5. Buckland, R., P. Giraudon, and F. Wild. 1989. Expression of measles virus nucleoprotein in *Escherichia coli*: use of deletion mutants to locate the antigenic sites. *J. Gen. Virol.* 70:435-441.
6. Castaneda, S. J., and T. C. Wong. 1990. Leader sequence distinguishes between translatable and encapsidated measles virus RNAs. *J. Virol.* 64:222-230.
7. Cattaneo, R., G. Rebmann, K. Bacsko, V. ter Meulen, and M. A. Billeter. 1988. Multiple viral mutations rather than host factors cause defective measles virus gene expression in a subacute sclerosing panencephalitis cell line. *J. Virol.* 62:1388-1397.
8. Curran, J., R. Boeck, and D. Kolakofsky. 1991. The Sendai virus P gene expresses both an essential protein and an inhibitor of RNA synthesis by shuffling modules via mRNA editing. *EMBO J.* 10:3079-3085.
9. Das, T., and A. K. Banerjee. 1992. Role of the phosphoprotein (P) in the encapsidation of presynthesized and de novo synthesized vesicular stomatitis virus RNA by the nucleocapsid protein (N) in vitro. *Cell. Mol. Biol.* 38:17-26.
10. Davis, N. L., H. Arnheiter, and G. W. Wertz. 1986. Vesicular stomatitis virus N and NS proteins form multiple complexes. *J. Virol.* 59:751-754.
11. Deshpande, K. L., and A. Portner. 1984. Structural and functional analysis of Sendai virus nucleocapsid protein NP with monoclonal antibodies. *Virology* 139:32-42.
12. Emerson, S. U. 1987. Transcription of vesicular stomatitis virus, p. 245-269. In R. R. Wagner (ed.), *The rhabdoviruses*. Plenum Press, New York.
13. Fuerst, T. R., E. G. Niles, F. W. Studier, and B. Moss. 1986. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83:8122-8126.
14. Gill, D. S., S. Takai, A. Portner, and D. W. Kingsbury. 1988. Mapping of antigenic domains of Sendai virus nucleocapsid protein expressed in *Escherichia coli*. *J. Virol.* 62:4805-4808.
15. Giraudon, P., M. F. Jacquier, and T. F. Wild. 1988. Antigenic analysis of African measles virus field isolates: identification and localization of one conserved and two variable epitope sites on the NP protein. *Virus Res.* 18:137-152.
16. Gombart, A. F., A. Hirano, and T. C. Wong. 1992. Expression and properties of the V protein in acute measles virus and subacute sclerosing panencephalitis virus strains. *Virus Res.* 25:63-78.
17. Gombart, A. F., M. N. Pearson, G. F. Rohrmann, and G. S. Beaudreau. 1989. A baculovirus polyhedral envelope-associated protein: genetic location, nucleotide sequence and immunocytochemical characterization. *Virology* 169:182-193.
18. Gross, C. H., and G. F. Rohrmann. 1990. Mapping unprocessed epitopes using deletion mutagenesis of gene fusions. *BioTechniques* 8:196-202.
19. Hamaguchi, M., T. Yoshida, K. Nishikawa, H. Naruse, and Y. Nagai. 1983. Transcriptional complex of Newcastle disease virus. I. Both L and P proteins are required to constitute an active complex. *Virology* 128:105-117.
20. Hirano, A., A. H. Wang, A. F. Gombart, and T. C. Wong. 1992. The matrix proteins of neurovirulent subacute sclerosing panencephalitis virus and its acute measles virus progenitor are functionally different. *Proc. Natl. Acad. Sci. USA* 89:8745-8749.
21. Homann, H. E., W. Willenbrink, C. J. Buchholz, and W. J. Neubert. 1991. Sendai virus protein-protein interactions studied by a protein-blotting protein-overlay technique: mapping of domains on NP protein required for binding to P protein. *J. Virol.* 65:1304-1309.
22. Horikami, S. M., J. Curran, D. Kolakofsky, and S. A. Moyer. 1992. Complexes of Sendai virus NP-P and P-L proteins are required for defective interfering particle genome replication in vitro. *J. Virol.* 66:4901-4908.
23. Howard, M., and G. Wertz. 1989. Vesicular stomatitis virus RNA replication: a role for the NS protein. *J. Gen. Virol.* 70:2683-2694.
24. Hsu, C. H., D. W. Kingsbury, and K. G. Murti. 1979. Assembly of vesicular stomatitis virus nucleocapsids in vivo: a kinetic analysis. *J. Virol.* 32:304-313.
25. Huber, M., R. Cattaneo, P. Spielhofer, C. Orvell, E. Norrby, M. Messerli, J.-C. Perriard, and M. A. Billeter. 1991. Measles virus phosphoprotein retains the nucleocapsid protein in the cytoplasm. *Virology* 185:299-308.
26. Kingsbury, D. W. 1990. Paramyxoviridae and their replication, p. 945-962. In B. N. Fields and D. M. Knipe (ed.), *Virology*, vol. 1. Raven Press, New York.

27. **Kingsbury, D. W., C. H. Hsu, and K. G. Murti.** 1978. Intracellular metabolism of Sendai virus nucleocapsids. *Virology* **91**:86–94.
28. **Kleinschmidt, J. A., C. Digwall, G. Maier, and W. W. Franke.** 1986. Molecular characterization of karyophilic, histone-binding protein: cDNA cloning, amino acid sequence and expression of nuclear histone-binding protein N1/N2. *EMBO J.* **5**:3547–3552.
29. **Knipe, D. M., D. Baltimore, and H. F. Lodish.** 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. *J. Virol.* **21**:1128–1139.
30. **Koerner, T. J., J. E. Hill, A. M. Meyers, and A. Tzagoloff.** 1990. High-expression vectors with multiple cloning sites for construction of trpE fusion genes: pATH vectors. *Methods Enzymol.* **194**:477–490.
31. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic characters of a protein. *J. Mol. Biol.* **157**:105–132.
32. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
33. **La Ferla, F. M., and R. W. Peluso.** 1989. The 1:1 N-NS protein complex of vesicular stomatitis virus is essential for efficient genome replication. *J. Virol.* **63**:3852–3857.
34. **Masters, P. S., and A. K. Banerjee.** 1988. Resolution of multiple complexes of phosphoprotein NS with nucleocapsid protein N of vesicular stomatitis virus. *J. Virol.* **62**:2651–2657.
35. **Masters, P. S., and A. K. Banerjee.** 1988. Complex formation with vesicular stomatitis virus phosphoprotein NS prevents binding of nucleocapsid protein N to nonspecific RNA. *J. Virol.* **62**:2658–2664.
36. **Morgan, E. M.** 1988. Evolutionary relationships of paramyxovirus nucleocapsid-associated proteins, p. 163–179. *In* D. W. Kingsbury (ed.), *The paramyxoviruses*. Plenum Press, New York.
37. **Peluso, R. W.** 1988. Kinetic, quantitative, and functional analysis of multiple forms of the vesicular stomatitis virus nucleocapsid protein in infected cells. *J. Virol.* **62**:2799–2807.
38. **Peluso, R. W., and S. A. Moyer.** 1988. Viral proteins required for the in vitro replication of vesicular stomatitis virus defective interfering particle genome RNA. *Virology* **162**:369–376.
39. **Portner, A., and D. W. Kingsbury.** 1976. Regulatory events in the synthesis of Sendai virus polypeptides and their assembly into virions. *Virology* **73**:79–88.
40. **Rozenblatt, S., O. Eizenberg, R. Ben-Lavy, V. Lavie, and W. Bellini.** 1985. Sequence homology within the morbilliviruses. *J. Virol.* **53**:684–690.
41. **Rubio, C., C. Kolakofsky, V. M. Hill, and D. Summers.** 1980. Replication and assembly of VSV nucleocapsids: protein associated with RNPs and the effects of cyclohexamide on replication. *Virology* **61**:270–280.
42. **Ryan, K. W., A. Portner, and K. G. Murti.** 1993. Antibodies to paramyxovirus nucleoproteins define regions important for immunogenicity and nucleocapsid assembly. *Virology* **193**:376–384.
- 42a. **Schultz-Castaneda, S. J.** 1990. Characterization of measles virus plus-sense RNA synthesis. Doctoral thesis. University of Washington, Seattle.
43. **Spehner, D., A. Kirn, and R. Drillien.** 1991. Assembly of nucleocapsidlike structures in animal cells infected with a vaccinia virus recombinant encoding the measles virus nucleoprotein. *J. Virol.* **65**:6296–6300.
44. **Takano, E., M. Maki, H. Mori, M. Hatanaka, T. Marti, K. Titani, P. Kannagi, T. Ooi, and T. Murachi.** 1988. Pig heart calpastatin: identification of repetitive domain structure and anomalous behavior in polyacrylamide gel electrophoresis. *Biochemistry* **27**:1964–1972.
45. **Wong, T. C., M. Ayata, S. Ueda, and A. Hirano.** 1991. Role of biased hypermutation in evolution of subacute sclerosing panencephalitis virus from progenitor acute measles virus. *J. Virol.* **65**:2191–2199.
46. **Wong, T. C., and A. Hirano.** 1986. Functional cDNA library for efficient expression of measles virus specific gene products in primate cells. *J. Virol.* **57**:343–348.
47. **Wong, T. C., and A. Hirano.** 1987. Structure and function of bicistronic RNA encoding the phosphoprotein and matrix protein of measles virus. *J. Virol.* **61**:584–589.
48. **Wong, T. C., G. Wipf, and A. Hirano.** 1987. The measles virus matrix gene and gene product defined by in vitro and in vivo expression. *Virology* **157**:497–508.