Kinetic, Quantitative, and Functional Analysis of Multiple Forms of the Vesicular Stomatitis Virus Nucleocapsid Protein in Infected Cells

RICHARD W. PELUSO

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

Received 31 December 1987/Accepted 15 April 1988

Multiple forms of the vesicular stomatitis virus nucleocapsid protein N have been detected in infected cells. One form is complexed with the viral NS protein in a 1:1 molar ratio, and the other forms are distinguished by their more rapid sedimentation rates on glycerol gradients. I performed a series of experiments designed to analyze the relationships between these forms of the N protein. Pulse-chase experiments demonstrate that the N protein is made first as the form which binds to the NS protein, forming a 1-to-1 molar complex, and that with increasing times of chase it is either assembled into nucleocapsids or converted to the two higher sedimenting forms. Using a newly developed quantitative immunoblotting procedure, I have quantitated the three differentially sedimenting species of the N protein and have shown that at later times postinfection (6 to 7 h), the faster-sedimenting forms of the N protein account for as much as 50% of the soluble N protein in the cell. The activity of these forms has been assessed, with only the 1-to-1 molar N-NS complex demonstrating the ability to support the replication and encapsidation of viral genomic RNA. A model for the conversion of the N protein from the active N-NS complex into the other forms of the protein is presented, and the possible function of the N-protein self-complexes is discussed.

The rhabdovirus vesicular stomatitis virus (VSV) possesses a nonsegmented negative-stranded RNA genome in the form of a helical nucleocapsid. Two distinct types of RNA-synthetic events are directed by this structure: transcription and replication (2). Transcription of the genome leads to the synthesis of a short leader RNA followed by the synthesis of five mRNAs, whereas replication leads to the synthesis of full-length copies of the viral genome. Replicating molecules, but not mRNAs, are encapsidated. The nucleocapsid (N) protein of vesicular stomatitis virus plays a crucial role in controlling these processes. This protein associates tightly with replicating genomic RNA to form a nucleocapsid with helical symmetry, rendering the RNA in this structure resistant to degradation by ribonucleases (27). Evidence for multiple forms of the nucleocapsid protein in VSV-infected cells has been presented (23, 24). One form of the protein is complexed with the viral NS protein in a 1:1 molar ratio, and the complex of these two proteins exhibits the ability to support genomic RNA replication *in vitro*. Other forms of the N protein have been detected which appeared to be complexes of N with itself. These N-protein complexes have demonstrated little activity in supporting genomic RNA replication *in vitro*. We performed a series of experiments to determine the relationship between the forms of the N protein, to quantitate the relative levels of each form, and to assess their role in replication of the viral genome. We provide evidence for perhaps three forms of the N protein, distinguished by their rate of sedimentation through glycerol gradients. However, only the complex consisting of one molecule each of N and NS has the ability to support replication of genomic RNA *in vitro*.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of baby hamster kidney (BHK) cells were used for all of the experiments in this paper. The HR strain of the Indiana serotype of VSV

and the MS-T defective-interfering (DI) particle were propagated as previously described (22).

Pulse-chase analysis of soluble proteins. Subconfluent monolayers of BHK cells in 100-mm plastic petri dishes were infected with VSV at a multiplicity of infection of 10. At 4 h postinfection (p.i.) the medium was removed and replaced with fresh medium lacking leucine. This was removed after 15 min and replaced with medium lacking leucine but containing [³H]leucine (200 μ Ci per dish). After a 10-min pulse, one dish of cells was harvested and the others were washed with complete medium containing cycloheximide (100 μ g/ml) to prevent further incorporation of the isotope and incubated in this medium for various periods as indicated below. For harvest, cells were permeabilized with a solution of lysolecithin as described previously (22), scraped into a solution of 0.2 M NH₄Cl, 0.1 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; pH 7.4), 7 mM KCl, and 4.5 mM magnesium acetate, and centrifuged at 800 $\times$ g for 5 min. The supernatant fluid was collected and subjected to centrifugation in a Beckman SW55 rotor at 50,000 rpm for 65 min. The resulting supernatant fluid is designated the soluble protein fraction. Soluble protein was analyzed by centrifugation through 5 to 20% (wt/vol) glycerol gradients (made in scraping salts mix) in an SW41 rotor for 22 h at 32,000 rpm. The gradients were collected from the top into 1-ml fractions. A portion of each fraction was analyzed by immunoprecipitation with an excess of rabbit anti-VSV serum to precipitate all of the viral proteins. The precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Individual protein species were quantitated by excising the appropriate regions from the dried gel with the autoradiogram as a template, dissolving the gel piece in 30% H₂O₂ at 60°C for 12 h, and performing scintillation counting.

Quantitative electroblotting analysis of soluble proteins. Subconfluent monolayers of BHK cells were infected with VSV at 10 PFU per cell. At successive 1-h intervals starting

at 2 h p.i., the cells from one plate were permeabilized and scraped in the salts mix as described above. Soluble proteins were prepared and analyzed by glycerol gradient sedimentation and SDS-PAGE as above, except that samples were not immunoprecipitated prior to gel analysis. The proteins present in each gel were quantitatively transferred to a single sheet of Zetabind (AMF) membrane as described previously (25). The resulting blots were then processed to visualize the viral proteins by using rabbit anti-VSV serum and ^{125}I -labeled protein A as described previously (25). Quantitation was performed by excising the appropriate regions of the blots and measuring radioactivity in a gamma counter.

Activity of glycerol gradient-fractionated soluble proteins in support of RNA replication and encapsidation. Subconfluent monolayers of BHK cells were infected with 10 PFU of VSV per cell. Soluble proteins were prepared at 4 or 7 h p.i. and analyzed by glycerol gradient fractionation as described above. Samples of each gradient fraction were mixed with nucleocapsids from cells that were coinfecting with wild-type VSV and the MS-T defective-interfering particle as described previously (22). All samples were incubated at 30°C for 90 min in a solution containing the following: 0.13 M NH_4Cl , 0.1 M HEPES (pH 8.0), 7 mM KCl, 8 mM magnesium acetate, 1 mM dithiothreitol, 2 mM ATP, 1 mM CTP, 1 mM GTP, 50 μM UTP, 0.1 mM *S*-adenosyl methionine, 1 mM spermidine, 40 U of creatine phosphokinase per ml, 10 mM creatine phosphate, 2 μg of actinomycin D per ml, and 100 μCi of [^3H]UTP (35 Ci/mmol). Each sample was then diluted with an equal volume of water, CaCl_2 was added to 1 mM, and micrococcal nuclease was added to 10 $\mu\text{g}/\text{ml}$ to degrade all nonencapsidated RNA molecules. After incubation at 37°C for 30 min, ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-tetracetic acid (EGTA) was added to 10 mM. The samples were then treated with protease K and SDS and phenol-chloroform extracted, and the RNA was analyzed by electrophoresis on 1.5% agarose-7 M urea gels (22).

Sedimentation analysis of nucleocapsid-derived N protein. Seven confluent flasks of VSV-infected BHK cells were labeled with [^{35}S]methionine (200 μCi per flask) from 2 to 6 h p.i. N protein was isolated from the intracellular nucleocapsid exactly as described by Blumberg et al. (4) and dialyzed against 1 M NaCl-50 mM Tris (pH 7.8)-1 mM EDTA at -3°C. The resulting N protein was then analyzed by glycerol gradient sedimentation in the following ways. One half of the sample was centrifuged for 22 h at 32,000 rpm in an SW41 rotor at 0°C through a 5 to 20% glycerol gradient in the dialysis buffer described above. The other half of the sample was diluted with dialysis buffer lacking NaCl to yield a solution of 0.2 M NaCl, 50 mM Tris (pH 7.8), and 1 mM EDTA and then incubated at 37°C for 30 min to promote the self-assembly of the N protein into disk-shaped structures (3). The resulting protein was analyzed by glycerol gradient sedimentation under identical conditions as the nontreated sample. Samples of each gradient were then analyzed by SDS-PAGE and autoradiography.

RESULTS

Pulse-chase analysis of soluble proteins. Our previous studies have demonstrated that several different forms of the nucleocapsid protein N of VSV were detected when soluble protein extracts of infected cells that were labeled with [^3H]leucine for relatively long periods (1 h) were analyzed by glycerol gradient sedimentation (23, 24). One species of N protein cosedimented with the NS protein near the top of the

gradient, forming a 1-to-1 molar complex; a second heterogeneous species sedimented in the bottom third of the gradient; and a third species was present in the pellet. To establish the relationships among the N-protein forms and to determine whether any postsynthetic processing or maturation processes were responsible for the appearance of multiple N-protein species, I performed a series of short pulse-chase experiments. Cells were labeled with [^3H]leucine for 10 min, and the proteins were analyzed either directly after the pulse or after various periods of chase in the presence of cycloheximide. The results of this experiment are presented in Fig. 1. The data in Fig. 1A demonstrate that the N protein is synthesized as the species that cosediments on glycerol gradients with NS protein in a 1-to-1 complex, i.e., the form of N protein capable of supporting replication of nucleocapsids *in vitro* (23, 24). With increasing times of chase, up to 2 h, there is a dramatic shift in the sedimentation of the N protein to more rapidly sedimenting forms, with two new species present, one in fractions 6 to 12, and another in the pellet fraction (fraction 13). Note that cycloheximide was present in the cultures after the pulse-labeling period to ensure that the changes we observed in the sedimentation properties of the N protein were due to changes in the protein and not to new synthesis. However, essentially identical results have been obtained in experiments in which cycloheximide was replaced with a 1,000-fold excess of unlabeled leucine during the chase periods (data not shown). The amount of N protein in each fraction of each gradient was quantitated by excising the band from the dried gels and counting in a scintillation counter. These results are shown in Fig. 2. These data demonstrate several points. First, approximately 60% of the soluble N protein labeled during the 10-min pulse was still in the soluble fraction after a 2-h chase, indicating that there are pools of N protein in the cell that may be increasing as infection proceeds. The presence of soluble pools of N protein has been reported by others (1, 13, 19, 23, 26, 28). Second, in the pulse-labeled sample, 90% of the N protein sedimented near the top of the gradient, where most of the NS protein also sedimented, but by 2 h of chase, 80% of the N protein that remained in the cell was present as the more rapidly sedimenting forms (fractions 6 to 13). Third, there was a sixfold increase in the faster-sedimenting species of N protein after 2 h of chase relative to the pulse. Fourth, 50% of the N protein in the pulsed sample was converted to more rapidly sedimenting species by 2 h of chase, with the remainder either assembled into nucleocapsids (which were pelleted before the soluble proteins were analyzed) or remaining near the top of the gradients. The percentage of N protein which sedimented in the pellet fraction (fraction 13) reached a plateau at approximately 23% of the total by 30 min of chase, but the percentage in fractions 6 to 12 of the species of N protein exhibiting an intermediate sedimentation rate continued to increase. These results suggest that the N protein is complexed with the NS protein shortly after synthesis and that it can be converted into more rapidly sedimenting N-N complexes as a function of time after synthesis.

Little if any NS protein was detected cosedimenting with the N protein in fractions 7 to 13 of the gradients (Fig. 1). To investigate this more thoroughly, cells were labeled with [^3H]leucine for 5 h beginning at 2 h after infection, rather than pulsed for 10 min, as in the experiment in Fig. 1. When soluble protein preparations from these cells were separated on glycerol gradients and analyzed for the presence of NS protein in the bottom two-thirds of the gradient, small amounts of the NS protein were found to coprecipitate with

FIG. 1. Pulse-chase analysis of differentially sedimenting forms of the VSV N protein present in a soluble form in infected cells. BHK cells were infected with VSV and pulse-labeled with [3 H]leucine for 10 min at 4.25 h p.i. The contents of one plate were harvested after the pulse, and the remaining four plates were washed and incubated further in medium containing cycloheximide (100 μ g/ml) before harvest. Cells were harvested by permeabilizing with lysolecithin, and soluble proteins were prepared and analyzed by sedimentation through 20 to 40% glycerol gradients, which were then fractionated into 13 1-ml fractions. The proteins in each fraction were precipitated with anti-VSV serum and electrophoresed on SDS-polyacrylamide gels. A, pulse; B, 15-min chase; C, 30-min chase; D, 1-h chase; E, 2-h chase. The positions of the viral proteins are indicated. Fraction 1 is the top, and fraction 13 is the bottom (pellet) of each gradient. Marker proteins were analyzed in a separate gradient, with bovine serum albumin (molecular weight 68,000) in fraction 3, β -galactosidase (molecular weight 116,000) in fraction 5, and phosphorylase (B) (molecular weight 185,000) in fraction 10. rRNA markers (18S and 28S) were found in the pellet fractions.

the N protein. Quantitation of these species revealed that the NS protein was greatly reduced in amount relative to the N protein and was present in a ratio of between four and eight N proteins to each NS protein in fractions 8 to 12 (data not shown).

The G protein of VSV was not detected in the soluble fraction of cells after the 10-min pulse, but by 15 min, and certainly by 30 min, of chase two species of G were detected.

One form sedimented in fractions 2 to 4, and the other was found in the pellet fraction. Careful inspection of the gels revealed that the G protein in fractions 2 to 4 exhibited a faster electrophoretic mobility than that of the G protein in the pellet. The G protein near the top of the gradient may be analogous to soluble G protein, a previously described form of the glycoprotein which lacks a membrane anchor domain (7, 15, 16, 20). The failure to detect the accumulation of the

FIG. 2. Quantitative analysis of the N protein during a pulse-chase experiment. The N protein in each fraction of each gradient in the pulse-chase experiment shown in Fig. 1 was quantitated by excising the appropriate region of each gel and counting it in a scintillation counter. (A) The total counts in each band. (B) The percentage of N protein present as the N-NS complex (fractions 1 to 5), as the intermediately sedimenting form (fractions 6 to 12), and in the pellet fraction (fraction 13). Fraction 1 is the top of the gradient.

slowly sedimenting form of the G protein in cells by quantitative electroblotting (see Fig. 3) is consistent with its being shed into the medium.

The NS and L proteins were barely detectable in the soluble fraction of cells after a 10-min pulse, but were detectable by 15 and 60 min of chase, respectively, suggesting that these proteins may enter a soluble pool more slowly than the N protein.

Quantitation of soluble proteins in infected cells. The results that I obtained from the pulse-chase experiments described

above led me to investigate the possibility that levels of the two more rapidly sedimenting forms of the N protein would increase as infection with VSV proceeded, since 60% of the pulse-labeled N protein was still in the soluble fraction of the cell even after 2 h of chase and 80% of it was rapidly sedimenting. I determined the relative levels of the NS protein and each of the N-protein species present in cells as a function of time p.i. by using a recently developed quantitative electroblotting procedure (25). Using this method of analysis, I could avoid complications and artifacts associated with metabolic labeling, such as variability in transport and varying pool sizes of amino acids owing to viral infection. I prepared soluble proteins from VSV-infected cells at successive 1-h intervals starting at 2 h p.i., separated the proteins by glycerol gradient centrifugation, and analyzed the proteins in each fraction by gel electrophoresis and Western immunoblotting. These results are shown in Fig. 3 and 4. At all time points studied, approximately 95% of the NS protein sedimented near the top of the gradients in an increasing pool. Pools of NS protein in cells have been reported by others (6, 8, 10, 13, 17, 18, 29). A very different picture emerged from the analysis of the N protein. At early times p.i. (up to 5 h) the majority of the N protein cosedimented with the NS protein, but at later times (6 to 7 h p.i.) as much as 50% of the N protein in the soluble fraction of the cell sedimented faster as two species (one in fractions 9 to 12 and a second in the pellet fraction [fraction 13]). These data are consistent with the pulse-chase experiments in Fig. 1, which demonstrated a conversion of the slowly sedimenting form of N protein to the other, more rapidly sedimenting forms of N protein. The N protein is apparently stable as a soluble non-nucleocapsid-bound species, as evidenced by its increasing presence in a soluble form as the infection proceeds.

The data obtained from the quantitative electroblotting analysis of soluble VSV protein in cells as a function of time p.i. have been analyzed for relative abundances of total N and NS proteins and for the N protein present as a 1-to-1 N-NS protein complex. These results are shown in Fig. 5, in which the total counts of ^{125}I bound to all forms of the N and NS protein at each time point have been totaled. There is a steady increase in the NS protein level between 3 and 6 h p.i., a slight increase between 6 and 7 h p.i., and an overall eightfold increase from 3 to 7 h p.i. (Fig. 5A). A similar pattern emerges for total N protein, with a sixfold increase from 3 to 6 h p.i. and a slight decrease from 6 to 7 h p.i. This decrease, however, is not always observed, and in this experiment it was due to a decrease in the N protein recovered from the pellet fraction. When extra care is taken to ensure total recovery of the pellet fraction, the soluble N-protein level is slightly higher at 7 h than at 6 h p.i. (data not shown).

A slightly different pattern is seen when the accumulation of the N protein species present as the 1-to-1 N-NS protein complex (fractions 1 to 5) is analyzed (Fig. 5B). Between 3 and 5 h p.i. there is a relatively linear increase in the level of this form of the N protein, with a doubling of the level between 5 and 6 h p.i. and only a slight increase between 6 and 7 h p.i. There is a sixfold increase in the amount of the N-NS complex from 3 to 7 h p.i.

In vitro activity of the N-protein species. Previous reports from our laboratory concerning the ability of the various forms of the N protein to support replication of nucleocapsids demonstrated that all of the activity was associated with the N-NS complex (23, 24). The more rapidly sedimenting forms of the N protein had essentially no activity in this

FIG. 3. Quantitative analysis of soluble viral proteins in VSV-infected cells. BHK cells were infected with VSV, and soluble proteins were prepared from 2 to 7 h p.i. as indicated. Soluble proteins were sedimented on glycerol gradients, the gradients were fractionated, and the proteins in each fraction were analyzed by SDS-PAGE. The proteins in each gel were then quantitatively transferred to Zetabind membranes and reacted with anti-VSV serum followed by incubation with ^{125}I -labeled protein A. Autoradiograms were prepared from each blot. The positions of the viral proteins are indicated on the right side of each blot. Fraction 1 is the top, and fraction 13 is the bottom (pellet) of each gradient.

process. These previous experiments were carried out with proteins present in cells at 4 h after infection (23, 24). In light of the data in Fig. 3 and 4, demonstrating that the abundance of the faster-sedimenting N-protein species is low at 4 h p.i. but high at 7 h p.i., I assessed the differently sedimenting forms of soluble N protein present in cells at 4 and 7 h p.i. for the ability to support replication of nucleocapsids *in vitro*. This result is shown in Fig. 6. At both 4 and 7 h after infection, all of the activity was found with the N-NS protein complex, and not with either of the faster-sedimenting N-protein species, even though these species made up about one-half of the total soluble N protein in the cells at that time. Note that the activity of the N-NS complex was higher at 7 h p.i. than at 4 h p.i., perhaps reflecting its greater concentration at that time.

Sedimentation analysis of nucleocapsid-derived N protein. Several years ago, Blumberg et al. (3) reported that N protein could be isolated from assembled nucleocapsids free from other viral proteins and that this N protein would interact with the leader RNA to form a ribonucleoprotein structure with properties not unlike those of a nucleocapsid. In view of our findings above and elsewhere (23, 24) that the ability to support genomic RNA replication and nucleocapsid assembly is a property of the N-NS protein complex and not of the N protein alone, I investigated the sedimentation properties of the nucleocapsid-derived N protein to determine whether it had properties similar to any of the species of soluble N protein which I found in the cell. These results

are shown in Fig. 7. When conditions reported to prevent self-assembly of the nucleocapsid-derived N protein (high ionic strength, pH 7.8 and 0°C) were used, the result in Fig. 7A was obtained. The N protein was dispersed throughout the gradient, with 43% being in the pellet fraction. When the same preparation of N protein was incubated under conditions which promote its self-assembly into disklike structures (lower ionic strength and 37°C), nearly all (96%) of the N protein was present in the pellet fraction (Fig. 7B), which is not able to support RNA replication *in vitro* (Fig. 6), with the remainder being found near the top of the gradient. The fact that most of the N protein derived from assembled nucleocapsids pelleted through the glycerol gradient when it was warmed in near-physiological salt solution is consistent with my inability to demonstrate that N protein prepared from this source can support the replication of wild-type or DI genomic RNA *in vitro* (unpublished results). Apparently, the small amount of the protein remaining near the top of the gradient is insufficient to support genomic RNA replication.

DISCUSSION

At least three soluble forms of the N protein of VSV, distinguishable by their rates of sedimentation on glycerol gradients, are present in infected cells. One form is complexed with the NS protein in a 1:1 molar ratio, and the other two forms contain much less NS protein or appear to be N-protein self-complexes. The results from the experiments

FIG. 4. Relative levels of the N and NS proteins in a soluble form as a function of time after infection. The radioactivity bound to the N and NS proteins in each lane of each of the blots in Fig. 3 was determined by excising the appropriate areas of the membranes and gamma counting. The results are presented for the N protein (A) and the NS protein (B) from 3 to 7 h p.i.

presented in this communication support the conclusion that the nucleocapsid protein of VSV is synthesized as a relatively slowly sedimenting form capable of binding to the NS protein to form a complex which serves as the substrate for the replication and encapsidation of replicating genomic RNA molecules (Fig. 1, 2, and 6). The more rapidly sedimenting N-protein complexes are unable to support genomic RNA replication *in vitro*. The pulse-chase data presented in Fig. 1 and 2 show that the two more rapidly sedimenting forms of the N protein are derived from the more slowly sedimenting form. Figure 8 diagrams ways in which the various N-protein complexes might be formed. In both schemes, the N protein in the N-NS protein complex can bind to replicating RNA molecules to form a nucleocapsid,

FIG. 5. Relative abundances of total NS, total N, and the N-NS protein complex as a function of time p.i. The data obtained from the quantitative analysis of viral proteins in infected cells (Fig. 3) are plotted as total N and NS (A) or the N protein in the N-NS protein complex (B). For the data in panel A, the total counts of ^{125}I bound to the N or NS protein were summed for each fraction of the gradient at each time point; for panel B, only the counts bound to the N protein in fractions 1 to 5 were totaled.

releasing the NS protein (23, 24). In scheme A, instead of binding to replicating nucleocapsids, the N-NS protein complex dissociates, releasing the NS protein and resulting in the formation of N-N protein complexes exhibiting an intermediate sedimentation rate. The number of N proteins in these complexes is not known, and the diagram is not meant to suggest that they are dimers. The role of the NS protein in these complexes is unclear, and so it is not shown here. These intermediately sedimenting N-protein complexes associate to form the most rapidly sedimenting form of N protein found in the pellet fraction of the gradients. The stoichiometry of N proteins in these complexes is not known. Scheme A therefore predicts a sequential formation of the three forms of N protein. Scheme B, on the other hand, predicts that the formation of each form of the N-protein complexes is independent of the other, but does allow for the possible conversion of the intermediately sedimenting form to the most rapidly sedimenting form of N complexes. We cannot presently distinguish between these two models.

FIG. 6. Activity of fractionated soluble proteins prepared at 4 and 7 h p.i. Soluble proteins were prepared from VSV-infected cells and separated by glycerol gradient centrifugation. Each gradient was fractionated into 13 1-ml fractions. A portion of each fraction was mixed with nucleocapsids from cells coinfecting with VSV and the MS-T DI particle, and replication was allowed to proceed for 90 min in the presence of [3 H]UTP. The micrococcal nuclease-resistant species (encapsidated) were then analyzed by agarose gel electrophoresis. (A) RNA produced by using the 4-h protein sample; (B) RNA produced by using the 7-h protein sample. In both panels the numbers refer to the gradient fractions that were tested, lane A is a control unfractionated reaction, and lane B is a reaction with nucleocapsids and no soluble proteins. The position of the MS-T DI 19S RNA genome and anti-genome is indicated.

We have previously reported that the NS protein in the soluble protein fraction of infected cells can exchange with the NS protein on nucleocapsids (23, 24). Perhaps the NS protein in the complex with the N protein can also exchange with free NS protein, and during this exchange two or more N proteins may associate with each other if they are in high enough concentration. The fact that the more rapidly sedimenting forms of N protein are most abundant at later times after infection, when the total amount of soluble N protein is high, supports this idea.

It has been suggested that the NS protein binds to the N protein to prevent it from self-associating (1, 9, 14, 24). This would suggest that the binding site on the N protein for NS is the same site that is recognized when N binds to itself, or that binding of two or more N proteins to themselves masks the NS-binding site or alters the structure of the N protein in such a way that NS can no longer bind to it to form an equimolar complex. The fact that N-protein complexes exist in a stable form in cells in the presence of a molar excess of NS protein indicates that the formation of N-NS protein complexes from N complexes and free NS is unlikely to occur.

The function(s) of the N-protein self-complexes is unclear at present. We have been unable to detect activity of these

complexes in supporting the replication and encapsidation of viral genomes (Fig. 6) (23, 24). An argument can be made, therefore, that one or both of the more rapidly sedimenting forms of the N protein may recognize and encapsidate leader RNA in the cell, but not the replicating genome. Several lines of evidence indirectly support this notion. First, Blumberg et al. (3) have demonstrated *in vitro* encapsidation of leader RNA by using a preparation of N protein that exhibits sedimentation properties similar to those of the most rapidly sedimenting form of N protein (Fig. 6). Second, leader RNA is encapsidated in infected cells at late times after infection with VSV (5), a time when the level of the N-protein complexes is high. Experiments to test this hypothesis are in progress.

It is of interest that differences in isoelectric point between soluble N protein and nucleocapsid-associated N protein have been reported (12). One major form of N protein was detected in the soluble protein fraction of cells, with two or three additional forms detected in intracellular and viral nucleocapsids. Additionally, a phosphorylated form of N protein was detected only in virions. These results suggest that modifications of the primary structure of N protein are occurring *in vivo*. These modifications may be at least partly responsible for the appearance of the various sedimenting

FIG. 7. Sedimentation analysis of nucleocapsid-derived N protein. [35 S]methionine-labeled N protein was purified from VSV-infected cellular nucleocapsids by using guanidine hydrochloride and cesium chloride gradient centrifugation. The protein preparation was then subjected to sedimentation on glycerol gradients containing 1 M NaCl, 50 mM Tris (pH 7.8), and 1 mM EDTA either directly after purification (A) or after incubation at 37°C for 30 min in 0.2 M NaCl-50 mM Tris-1 mM EDTA (pH 7.8) to promote the formation of disklike N-protein complexes (B). A portion of the proteins in each gradient fraction was analyzed by SDS-PAGE.

FIG. 8. Possible relationships between the three differentially sedimenting forms of the N protein. For details, see the text.

species of N protein which we detect. It will be of interest to analyze each of the forms of N protein for altered biochemical properties that may correlate with the observed difference in sedimentation, the ability to self-associate, and the ability to function as substrate for the encapsidation of replicating genomic RNA molecules.

The experiments reported here lead to the conclusion that the N protein is able to support the replication and encapsidation of replicating DI RNA genomes only if it is complexed with the NS protein in a 1:1 molar ratio. However, Patton et al. (21), using an *in vitro* system programmed with N protein mRNA and DI nucleocapsids, have reported that N protein alone can support this process. An explanation for this discrepancy can be offered. In both systems, the N protein that supports RNA replication sediments slowly on glycerol gradients (11, 21, 23, 24) (Fig. 6). In the report by Patton et al. (21), N protein that was allowed to age *in vitro* before being tested for the ability to support RNA replication demonstrated a much decreased activity. Subsequent glycerol gradient analysis revealed that aging was associated with an increased sedimentation rate of the N protein (11). Our pulse-chase data (Fig. 1 and 2) demonstrate that aging of the N protein occurs *in vivo*, resulting in the conversion of slowly sedimenting active N protein (as an N-NS complex) to more rapidly sedimenting forms unable to support DI genome replication and encapsidation. These experiments suggest one of two things: either the N protein is modified immediately after (or during) its synthesis (either covalently or by binding to the NS protein), and this modification is slowly reversed to allow N to interact with itself or even nonspecifically with cellular RNAs, or the N protein is synthesized in an unmodified form which can associate with NS but not with itself, and a modification to the protein occurs which favors N-N interactions, leading to the appearance of the more rapidly sedimenting forms of N protein. Whichever the case, it appears that to have replicating activity, the N protein must be in a form which is non-self-complexed and in cells the NS protein can bind to N to keep it in this active form. In the coupled transcription-replication

system of Patton et al. (21), N protein that supported DI genome RNA replication was non-self-complexed, and in the experiments reported here and elsewhere (23, 24), the active form of N was shown to be an N-NS protein complex, and not N-N complexes. These experiments point to a crucial role for the NS protein in maintaining a pool of N protein in the cell in a form that can support the encapsidation of replicating genomic RNA molecules, that is, as an equimolar complex of these two proteins. Complexes between N and NS which exhibit stoichiometries other than 1:1 have been described (9, 11; R. W. Peluso, unpublished results), but their relevance to viral genomic RNA replication and encapsidation is uncertain.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant AI 22116 from the National Institute of Allergy and Infectious Diseases.

The excellent assistance of George Rosenberg is acknowledged. I thank Anne Deatly and Frank LaFerla for helpful comments and suggestions on the manuscript.

LITERATURE CITED

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. Banerjee, A. K. 1987. Transcription and replication of rhabdoviruses. *Microbiol. Rev.* 51:66-87.
3. Blumberg, B. M., C. Giorgi, and D. Kolakofsky. 1983. N protein of vesicular stomatitis virus selectively encapsidates leader RNA *in vitro*. *Cell* 32:559-567.
4. Blumberg, B. M., C. Giorgi, K. Rose, and D. Kolakofsky. 1984. Preparation and analysis of the nucleocapsid proteins of vesicular stomatitis virus and Sendai virus, and analysis of the Sendai virus leader-NP gene region. *J. Gen. Virol.* 65:769-779.
5. Blumberg, B. M., and D. Kolakofsky. 1981. Intracellular vesicular stomatitis virus leader RNAs are found in nucleocapsid structures. *J. Virol.* 40:568-576.
6. Cartwright, B. 1973. The distribution of virus proteins in BHK 21 cells infected with vesicular stomatitis virus (Indiana C). *J. Gen. Virol.* 21:407-411.
7. Chatis, P. A., and T. G. Morrison. 1982. Characterization of the soluble glycoprotein released from VSV-infected cells. *J. Virol.* 45:80-90.
8. Clinton, G. M., B. W. Burge, and A. S. Huang. 1978. Effects of phosphorylation and pH on the association of NS protein with vesicular stomatitis virus cores. *J. Virol.* 27:340-346.
9. 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.
10. Emerson, S. U., and Y.-H. Yu. 1975. Both NS and L proteins are required for *in vitro* RNA synthesis by vesicular stomatitis virus. *J. Virol.* 15:1348-1356.
11. Howard, M., N. L. Davis, J. Patton, and G. W. Wertz. 1986. Roles of vesicular stomatitis virus N and NS proteins in viral RNA replication, p. 134-140. *In* B. Mahy and D. Kolakofsky (ed.), *The biology of negative strand viruses*. Elsevier Science Publishing, Inc., New York.
12. Hsu, C.-H., and D. W. Kingsbury. 1980. Vesicular stomatitis virus morphogenesis is accompanied by covalent protein modifications, p. 613-622. *In* B. Fields, R. Jaenisch, and C. F. Fox (ed.), *Animal virus genetics*. Academic Press, Inc., New York.
13. 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.
14. Hudson, L. D., C. Condra, and R. A. Lazzarini. 1986. Cloning and expression of a viral phosphoprotein: structure suggests vesicular stomatitis virus NS may function by mimicking an RNA template. *J. Gen. Virol.* 67:1571-1579.
15. Irving, R. A., and H. P. Ghosh. 1982. Shedding of vesicular stomatitis virus soluble glycoprotein by removal of carboxy-terminal peptide. *J. Virol.* 42:322-325.

16. Kang, C. Y., and L. Prevec. 1970. Proteins of vesicular stomatitis virus. II. Immunological comparisons of viral antigens. *J. Virol.* 6:20-27.
17. Kang, C. Y., and L. Prevec. 1971. Proteins of vesicular stomatitis virus. III. Intracellular and extracellular appearance of virus-specific proteins. *Virology* 46:678-690.
18. Kingsford, L., and S. U. Emerson. 1980. Transcriptional activities of different phosphorylated species of NS protein purified from vesicular stomatitis virions and cytoplasm of infected cells. *J. Virol.* 35:1097-1105.
19. 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.
20. Little, S. P., and A. S. Huang. 1977. Synthesis and distribution of vesicular stomatitis virus polypeptides in the absence of progeny production. *Virology* 81:37-47.
21. Patton, J. T., N. L. Davis, and G. W. Wertz. 1984. N Protein alone satisfies the requirement for protein synthesis during RNA replication of vesicular stomatitis virus. *J. Virol.* 49:303-309.
22. Peluso, R. W., and S. A. Moyer. 1983. Initiation and replication of vesicular stomatitis virus genome RNA in a cell-free system. *Proc. Natl. Acad. Sci. USA* 80:3198-3202.
23. Peluso, R. W., and S. A. Moyer. 1984. Vesicular stomatitis virus proteins required for the *in vitro* replication of defective interfering particle genome RNA, p. 153-160. *In* D. H. L. Bishop and R. W. Compans (ed.), *Nonsegmented negative strand viruses*. Academic Press, Inc., New York.
24. 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.
25. Peluso, R. W., and G. H. Rosenberg. 1987. Quantitative electrotransfer of proteins from sodium dodecyl sulfate polyacrylamide gels onto positively charged nylon membranes. *Anal. Biochem.* 162:389-398.
26. 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 cycloheximide on replication. *Virology* 105:123-135.
27. Soria, M., S. P. Little, and A. S. Huang. 1974. Characterization of vesicular stomatitis virus nucleocapsids. I. Complementary 40S RNA molecules in nucleocapsids. *Virology* 61:270-280.
28. Wagner, R. R., M. P. Kiley, R. M. Snyder, and C. A. Schnaitman. 1972. Cytoplasmic compartmentalization of the protein and RNA species of vesicular stomatitis virus. *J. Virol.* 9:672-683.
29. Wagner, R. R., R. M. Snyder, and S. Yamazaki. 1970. Proteins of vesicular stomatitis virus: kinetics and cellular sites of synthesis. *J. Virol.* 5:548-558.