Key words: VSV/NS protein/replication

Vesicular Stomatitis Virus RNA Replication: a Role for the NS Protein

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(Accepted 8 June 1989)

SUMMARY

Synthesis of the vesicular stomatitis virus nucleocapsid (N) protein is required for viral RNA replication. The observation that the N protein forms a rapidly sedimenting species in the absence of other viral proteins and the description of complexes of N protein with NS protein led to the proposal that NS protein binds to N protein to prevent it from self-associating. We tested this model by analysing the physical properties of N protein synthesized alone in an in vitro replication system as compared to N protein synthesized in the presence of the NS protein. These findings were correlated with the ability of the N protein, synthesized under both conditions, to support replication. N protein synthesized at low concentrations in the absence of other viral proteins sedimented at 4S on glycerol gradients and was capable of supporting RNA replication. In contrast, synthesis of increasing concentrations of N protein resulted in formation of a rapidly sedimenting species of N protein which had the physical properties of a protein–protein aggregate and which failed to support RNA replication. Co-synthesis of the NS protein with N protein both prevented the concentration-dependent aggregation of N and restored the ability of high concentrations of N protein to support RNA replication.

INTRODUCTION

Vesicular stomatitis (VSV) is a negative strand RNA virus belonging to the rhabdovirus family. Two RNA synthetic events, transcription and replication, occur from the single-stranded RNA template (Banerjee, 1987). The active template for both RNA synthetic processes is the viral nucleocapsid, which consists of the genomic RNA tightly encapsidated with the nucleocapsid protein, N, and the polymerase complex of the L and NS proteins (Emerson & Yu, 1975). In the absence of viral protein synthesis, the polymerase transcribes a 47 base leader RNA and five monocistronic mRNAs that are capped and polyadenylated (Abraham & Banerjee, 1976; Colonno & Banerjee, 1977). The five mRNAs are translated in the infected cell into five viral proteins and it is in the presence of these viral proteins that the switch from RNA transcription to RNA replication occurs (Wertz et al., 1987). The negative strand virion RNA which first served as a template for the synthesis of mRNAs now serves, in the presence of VSV proteins, as a template for the synthesis of a full-length plus strand RNA. A continuous supply of viral proteins synthesized de novo is required for VSV replication (Wertz & Levine, 1973). It was hypothesized that synthesis of the N protein to encapsidate progeny RNA would modulate the switch from transcription to replication (Leppert et al., 1979; Blumberg et al., 1981). Analysis carried out by several groups of the protein requirement for replication both in vivo and in vitro supported this hypothesis (Patton et al., 1984a, b; Peluso & Moyer, 1984, 1988; Arnheiter et al., 1985).

To determine which viral proteins needed to be synthesized de novo to support genomic RNA synthesis, we developed an in vitro replication system in which the synthesis of individual...
proteins could be programmed in a rabbit reticulocyte lysate by addition of purified individual mRNAs (Davis & Wertz, 1982; Patton et al., 1984a). This system was used to show that de novo synthesis of the N protein alone satisfied the protein requirement for replication (Patton et al., 1984a). When protein and RNA synthesis occurred concomitantly, a linear relationship existed between the amount of N protein synthesized and the amount of genome RNA replicated. Experiments involving microinjection of N-specific monoclonal antibodies into VSV-infected cells confirmed the requirement for the N protein for replication (Arnheiter et al., 1985). In other work the N protein was shown to form a complex with the major phosphoprotein of VSV, the NS protein, in the cytoplasm of VSV-infected cells (Bell et al., 1984; Peluso & Moyer, 1984, 1988). The N protein also forms complexes with the NS protein in the in vitro replication system described above and we distinguished three types of complexes on the basis of molar ratios of N to NS (Davis et al., 1986). Masters & Banerjee (1988a) found that complexes of the N and NS proteins synthesized in vitro could be separated into six forms on the basis of electrophoretic mobility but that these six complexes showed only two molar ratio values. Work by Peluso & Moyer (1988) showed that a complex of N and NS isolated from infected cells could support replication and both Peluso (1988) and Peluso & Moyer (1988) and our group (Howard et al., 1986) showed that under certain conditions N could form a rapidly sedimenting species that did not support replication.

These data led us to speculate that although N protein alone may be sufficient to fulfill the requirement for de novo protein synthesis in replication, other factors may be involved in establishing optimal conditions for replication. The hypothesis that emerged from the work of several groups is that NS protein binds to N protein to prevent it from self-aggregating and thus keeps the N protein available to encapsidate genomic RNA (Peluso & Moyer, 1984, 1988; Davis et al., 1986; Howard et al., 1986). A second role for the NS protein in N-NS complexes has been proposed in which the NS protein prevents N from binding to non-viral RNA and thus confers a specificity on the N protein to encapsidate only VSV-specific RNA (Masters & Banerjee, 1988b).

To test directly the hypothesis that NS interacts with N to prevent it from self-associating and thereby maintains it in a form that supports replication it was necessary to do the following: (i) to determine conditions under which the rapidly sedimenting species of N were generated, (ii) to examine the physical properties of this species of N, (iii) to test whether NS protein could specifically inhibit the formation of the rapidly sedimenting species of N and (iv) to correlate the above data with the ability of N protein to support replication.

Data presented here show that N protein forms at least two different species as a function of N protein concentration. N protein synthesized at low concentrations forms a 4S species that supports replication. N protein synthesized at high concentrations forms a rapidly sedimenting species that does not support replication. Furthermore, co-synthesis of the NS protein with the N protein both prevents the concentration-dependent aggregation of N protein and restores the ability of high concentrations of N protein to support RNA replication. These data support the hypothesis that NS protein interacts with N protein to prevent it from aggregating and thereby to maintain it in a form to support replication.

**METHODS**

**Cell culture and virus.** Monolayers of a continuous line of baby hamster kidney cells (BHK-21/13) were maintained in Eagle's minimal essential medium containing 5% heat-inactivated calf serum. VSV (Indiana serotype) propagated in BHK cells (Wertz & Levine, 1973) was used as a helper virus for the propagation of the defective interfering (DI-T) particle (VSV-DI 0-25, 5' 25% of standard genome). Stocks of the DI-T particle of VSV were originally provided by R. Lazzarini (NINCDS, Bethesda, Md., U.S.A.).

**Isolation of DI nucleocapsids.** A description of the isolation procedure for nucleocapsids has been reported (Wertz, 1983). Briefly, BHK cells infected with VSV helper virus and DI-T particles were washed with HBS buffer (10 mM-HEPES, 10 mM-NaCl, 1 mM-MgCl₂) at 13 h post-infection and scraped off the plates into HBS buffer. The cells were broken open by Dounce homogenization. The intracellular DI nucleocapsids were separated from standard virus nucleocapsids by velocity sedimentation through a 15 to 30% sucrose gradient containing 10 mM-Tris-HCl pH 8.1, 10 mM-magnesium acetate, 66 mM-NH₄Cl, 14 mM-KCl, 2 mM-dithioerythritol (DTE). The DI nucleocapsid band was identified by assaying fractions from a gradient run in parallel that contained radioactively
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The nucleocapsid protein was shown to be the only protein whose de novo synthesis was required to support DI replication in vitro when N protein synthesis and RNA replication occurred simultaneously (Patton et al., 1984a). These studies demonstrated a linear relationship between the total amount of N protein synthesized and the amount of genome RNA synthesized. Under the conditions of concomitant protein and RNA synthesis used in these studies, N protein was continually drawn from the soluble pool to support replication by incorporation into progeny nucleocapsids. Therefore, the pool of free N protein never achieved high concentration. In order to analyse the effect of varying the N protein concentration on the ability of N protein to function in replication we synthesized various amounts of N protein in vitro. 

RESULTS

Effect of N protein concentration on RNA replication levels

In vitro RNA synthesis and protein synthesis. The components of the in vitro replication system have been described previously (Davis & Wertz, 1982). As indicated in the Results section, either 25 μl or 37.5 μl rabbit reticulocyte lysate reactions were programmed with various amounts of either VSV N mRNA or VSV N mRNA and NS mRNA, in the presence of [35S]methionine (400 μCi/ml) and incubated at 30 °C for 1 h. At the end of this time, the 25 μl reactions were adjusted to 25 μl-anisomycin to inhibit further protein synthesis. Intracellular nucleocapsids (1 μl) were added to the 25 μl reactions along with [3H]UTP (1 μCi/ml) and the reactions were incubated at 30 °C for 2 h. In some of the experiments, the N protein or the N and NS proteins synthesized in each reaction were analysed by velocity sedimentation. In these experiments, 37.5 μl lysate reactions were used. After 1 h of protein synthesis at 30 °C, 12.5 μl of the 37.5 μl reaction was layered onto a 4-ml 10 to 30% glycerol gradient (Davis et al., 1986) and centrifuged at 49000 r.p.m. for 18-5 h at 4 °C in a Beckman SW50.1 rotor. These gradients were subsequently fractionated from an 18-gauge needle hole at the bottom of the tube. TCA-precipitable radioactivity was determined for each fraction to locate protein peaks. The remaining 25 μl reaction was adjusted to 25 μl-anisomycin, and DI nucleocapsids and [3H]UTP were added. The reaction was incubated at 30 °C for 2 h and then analysed for RNA synthesis products. 

Caesium chloride equilibrium density gradients. 35S-labelled N protein sedimenting both in the middle (4S) and at the bottom of 10 to 30% glycerol gradients were dialysed separately against 10 mM-Tris--HCl pH 7.5, 1 mM-EDTA, 150 mM-NaCl. Three 100-ml gradients were made containing either the 4S N protein, fast sedimenting N protein, or 3H-labelled intracellular DI nucleocapsids uniformly mixed with a homogeneous solution of caesium chloride (0-51 g/ml w/v). Equivalent amounts (c.p.m.) of the 4S N protein and the fast sedimenting N protein were analysed in separate gradients. The gradients were centrifuged at 30000 r.p.m. at 4 °C for 120 min in a Sorvall TH-641 rotor. The gradients were fractionated, the refractive index of each fraction was determined and used to calculate the corresponding density. The 35S-labelled proteins and the 3H-labelled RNA were located by TCA precipitation and scintillation counting of the fractions. 

Analysis of in vitro products. To assay for protein synthesis, 1 μl was sampled from each reaction and the [35S]methionine-labelled protein products synthesized in vitro were separated by electrophoresis on 10% SDS-polyacrylamide gels (Laemmli, 1970). The polyacrylamide gels were fixed and fluorographed. The relative amounts of protein were determined by densitometric scanning of the fluorograms. The N/NS protein molar ratios were determined by dividing the intensity units ratio of N/NS by a factor of 2.8 to adjust for the different methionine content of the two proteins. To assay for RNA products, the remainder of each reaction was treated with SDS and phenol to deproteinize the RNA. The 3H-labelled RNA was ethanol-precipitated and analysed by electrophoresis on a 1-75% agarose-6 M-urea gel (Wertz & Davis, 1979). The agarose-urea gels were fixed and fluorographed (Laskey, 1980) and the relative amount of RNA synthesized in each reaction was assayed by densitometry of the fluorograms.
Table 1. RNA and N protein synthesis

<table>
<thead>
<tr>
<th>Reaction*</th>
<th>N mRNA (µl)</th>
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<th>RNA:protein ratio</th>
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<td>7</td>
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* Protein synthesis. Micrococcal nuclease-treated rabbit reticulocyte lysate reactions were programmed with increasing concentrations of hybrid-selected N mRNA. 35S-labelled N protein was synthesized during 60 min incubations at 30 °C prior to the addition of anisomycin and DI nucleocapsids (presynthesized N protein). The 35S-labelled N protein products synthesized in each reaction were electrophoresed on a 10% SDS–polyacrylamide gel. RNA synthesis. 35S-labelled RNA products synthesized in each reaction were electrophoresed on a 1-75% agarose–urea gel following deproteinization.
† The level of N protein synthesized in each reaction was determined by densitometric scanning of fluorograms. Exposure times were chosen to give band intensities in the linear range. The values reported are relative intensity units of 35S-labelled N protein. The values obtained from scanning were rounded to three significant figures.
‡ The level of DI RNA synthesis in each reaction was determined as described in †. Both the plus strand and the minus strand of the DI RNA were scanned to determine the level of DI RNA synthesis. The values reported are relative intensity units of 3H-labelled RNA. BD (below detection) was indicated when the level of DI RNA synthesis was too low to be detected by scanning.

*In vitro before adding nucleocapsid templates, and assayed the ability of the presynthesized N to support replication. Increasing amounts of hybrid-selected N mRNA were added to programme protein synthesis in a micrococcal nuclease-treated rabbit reticulocyte lysate, and the reaction mixture was incubated at 30 °C for 1 h before the addition of anisomycin and DI nucleocapsids. Incubations were continued for 2 h. Previous experiments demonstrated that the concentration of anisomycin used in these experiments completely inhibits protein synthesis; thus under these conditions any replication was supported solely by the preformed pool of N protein. At the end of the incubation period, RNA and protein synthesis in each reaction were assayed by electrophoresis in agarose–urea gels or 10% polyacrylamide gels, respectively. The amount of DI RNA synthesis and protein synthesis was quantified by densitometric scanning of fluorograms of dried gels and these data are presented in Table 1. The ability of increasing concentrations of presynthesized N protein to support replication varied. N protein presynthesized in reactions programmed with 1, 2 and 3 µl of hybrid-selected N mRNA supported increasing levels of DI RNA replication (Table 1). However, in reactions that made higher levels of presynthesized N protein, RNA replication decreased. DI genome RNA replication decreased by more than 50% in reaction 7 as compared to its level in reaction 4 despite the synthesis of threefold more N protein in reaction 7 (Table 1).

Sedimentation analysis of N protein

The sedimentation properties of the N protein synthesized under low and high concentrations of mRNA as described above were analysed by velocity sedimentation in glycerol gradients. Increasing concentrations of hybrid-selected N mRNA were added to 37.5 µl translation/repli-
cation reactions. After incubation at 30 °C for 1 h, DI nucleocapsids and anisomycin were added to 25 µl aliquots of the reaction and incubated at 30 °C for 2 h. The remaining 12.5 µl of each reaction was layered onto 15 to 30% glycerol gradients and the proteins were separated by velocity sedimentation.

Fig. 1 shows the sedimentation profile of the N protein synthesized in each replication reaction. These data show that the sedimentation profile of the N protein varied with the total amount of N protein synthesized. At lower concentrations, all the N protein sedimented at approximately 4S. As the concentration of presynthesized N protein increased, N protein no longer banded solely at 4S in the gradient but also sedimented to the bottom of the gradient as a
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![Diagram of Fig. 1: Velocity sedimentation analysis of N protein as a function of concentration. (a) to (e) Sedimentation profiles of presynthesized N protein from micrococcal nuclease-treated rabbit reticulocyte lysates programmed with 1, 3, 5, 7 and 9 μl of hybrid-selected N mRNA respectively. After 60 min incubation, one-third of each reaction was layered onto a 10 to 30% glycerol gradient and the proteins were separated by velocity sedimentation. The distribution of 35S-labelled protein across the gradient was located by TCA precipitation of samples of each fraction. Equivalent volumes of each relevant gradient fraction were then electrophoresed on a 10% polyacrylamide gel, fluorographed and the N protein was quantified by densitometric scanning of the fluorogram. The levels of presynthesized N protein in the gradients were calculated as percentages of the highest level that was achieved (e). The horizontal line in (a) indicates where haemoglobin sedimented in the gradients. Anisomycin, DI nucleocapsids and [3H]UTP were added to the remaining two-thirds of each reaction and incubation was continued for 2 h. 3H-labelled DI RNA synthesized in each reaction was purified by phenol/chloroform extraction and ethanol precipitation. The DI RNA was electrophoresed on a 1.75% agarose–urea gel, fluorographed and quantified by densitometric scanning of the fluorogram. The level of DI RNA synthesis is expressed as a fraction of the highest level that was achieved in each reaction (c). BD (below detection) was indicated when the level of DI RNA synthesis was too low to be detected by scanning.

![Diagram of Fig. 2: Buoyant density analysis of N protein in caesium chloride gradients. N protein synthesized in rabbit reticulocyte lysates was separated into 4S N protein and fast sedimenting N protein by velocity sedimentation in glycerol gradients. The glycerol was removed by dialysis and the two N protein populations were centrifuged to equilibrium in caesium chloride gradients as outlined in Methods. (a) 35S-labelled fast sedimenting N protein; (b) 35S-labelled 4S N protein; (c) 3H-labelled intracellular nucleocapsids. The solid arrowheads mark the gradient fraction of which the density (g/ml) is indicated in the figure.

The level of DI RNA synthesis in each reaction was also quantified. RNA replication correlated with the amount of N protein sedimenting in the 4S region of the gradient. For example, 10 times more N protein was synthesized in the reaction shown in (d) than was synthesized in the reaction represented in (a). However, the majority of the N protein in (d) was found as a fast sedimenting species such that equivalent amounts of N protein sedimented in the middle of the gradient in both (a) and (d). Both these reactions supported approximately equivalent levels of DI RNA replication. These data show that the level of DI RNA replication correlates not with the total amount of N protein made in the system but with the amount of N protein that sediments at 4S.
The formation of the fast sedimenting N protein species was not reversible. A 10-fold dilution of the high $M_r$ N protein species and incubation at 4 °C for 24 h did not change the rapid sedimentation of the N protein.

**Equilibrium density gradient analysis of the N protein**

The two forms of the N protein that differed in their sedimentation properties were analysed by centrifugation to equilibrium on caesium chloride gradients to determine their buoyant densities. VSV DI nucleocapsids were analysed in parallel. Duplicate experiments were carried out in which centrifugation was for 96 or 120 h and identical results were obtained. Reproducibly, the fast sedimenting N protein banded at an equilibrium density of 1.29 g/ml while DI nucleocapsids banded at 1.31 g/ml (Fig. 2). The 4S N protein formed a broader band than the fast sedimenting N protein as would be expected because diffusion is inversely proportional to $M_r$ but the median densities of the two distributions were closely similar. Longer centrifugation times did not alter either profile. A small fraction of the total 4S N protein banded at a buoyant density of DI nucleocapsids (Fig. 2c). Haemoglobin uniformly mixed in a homogeneous solution of caesium chloride was also found to band as a diffuse peak at the top of the gradient similar to the gradient profile of 4S N protein.

**Effect of NS protein on RNA replication**

The preceding experiments demonstrated that when N protein was synthesized in the absence of other viral proteins, it underwent concentration-dependent changes in its ability to support RNA replication and in its sedimentation properties. The possibility that another viral protein might influence these changes in the properties of N protein was tested by introducing other viral mRNAs into the cell-free translation/replication system.

The NS protein is known to form a complex with the N protein in the cytoplasm of infected cells (Bell et al., 1984; Peluso & Moyer, 1984, 1988). Complexes of NS and N protein are also formed in the in vitro replication system (Davis et al., 1986). Therefore, we next tested whether simultaneous presynthesis of the N and NS proteins would affect the level of DI replication in vitro. Fig. 3 shows the results of in vitro replication reactions that were programmed with hybrid-selected N mRNA alone or hybrid-selected N mRNA and increasing concentrations of hybrid-selected NS mRNA. The amount of N and NS protein synthesis and of DI RNA synthesis shown in Fig. 3 were quantified by densitometric scanning and the results are presented in Table 2. Equivalent amounts of N protein were synthesized in each reaction (Fig. 3a lanes 3 to 6 and Table 2). The data in Fig. 3 (lane 2) represent a reaction in which protein and RNA synthesis were simultaneous. When the level of presynthesized NS protein was varied in the presence of presynthesized N protein, the level of RNA synthesis increased as the amount of NS protein synthesis in each reaction increased to an NS to N molar ratio of 0.5 (Fig. 3 lanes 4 to 6). When the molar ratio of NS to N was 0.5 (Fig. 3a lane 5 and Table 2), the level of DI RNA replication was three times higher than that seen with presynthesized N protein alone (Fig. 3 lane 3), and 90% of the level of DI RNA synthesis for that amount of N protein when N protein and RNA synthesis occurred concomitantly (Fig. 3 lane 2 and Table 2). When the NS to N molar ratio was greater than 1 (Table 2 and Fig. 3a lane 6) a decrease in DI RNA synthesis was observed (Fig. 3b lane 6) as has been reported previously (Patton et al., 1984b). Thus an optimal molar ratio of presynthesized NS protein to presynthesized N protein resulted in the rescue of DI replication above the levels seen when N protein was synthesized alone.

**Effect of NS protein on sedimentation properties of N protein**

Since presynthesis of NS protein in addition to N protein affected the level of DI genome RNA replication, we examined the effect of the NS protein on the formation of the fast sedimenting N protein species. In these reactions, the same amount of N protein was presynthesized alone or in the presence of increasing concentrations of NS protein. Densitometric scanning of fluorograms of gels analysing proteins from each gradient fraction quantitatively showed that the same level of N protein was synthesized in each reaction. When N protein was presynthesized alone, 44% of the total N protein sedimented to the bottom of the
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Fig. 3. Effects of NS protein on DI RNA replication. (a) Micrococcal nuclease-treated rabbit reticulocyte lysate reactions were programmed with the same amount of hybrid-selected N mRNA under the following conditions. Lane 2, N protein synthesis and DI RNA synthesis occurred concomitantly; lane 3, N protein was synthesized alone prior to DI RNA synthesis; lanes 4 to 6, N protein and increasing concentrations of NS protein were synthesized prior to DI RNA synthesis. Lane 1 shows the products of a reaction that contained DI nucleocapsids but no mRNA. The VSV NS, N and M proteins are shown as markers. (a) 35S-labelled protein products synthesized during a 1 h incubation at 30 °C. Anisomycin was added to the six reactions and DI nucleocapsids were added to reactions 1, 3, 4, 5 and 6 (lanes 1, 3, 4, 5 and 6) and incubation was continued for 2 h at 30 °C. Reaction 2 (lane 2) was incubated for 1 additional h at 30 °C. (b) 3H-labelled RNA products were purified by phenol/chloroform extraction and ethanol precipitation. The products were electrophoresed on a 1·75% agarose–urea gel. Lanes 1 to 6 correspond to lanes 1 to 6 as described for (a).

Table 2. Effect of NS protein on DI RNA replication

<table>
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<tr>
<th>Reaction*</th>
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<th>NS protein†</th>
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<th>RNA†</th>
<th>RNA : N protein</th>
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* The conditions for the six reactions were as described in the legend to Fig. 3. DI RNA synthesis was supported either by presynthesized N protein alone (reaction 3), by presynthesized N and NS protein (reactions 4, 5, 6) or by concomitantly synthesized N protein alone (reaction 2).

† The levels of 35S-labelled protein and 3H-labelled RNA synthesized in each reaction were quantified by densitometric scanning of fluorograms. Exposure times were chosen to give band intensities in the linear range. The values reported are relative intensity units of 35S-labelled N and NS protein and 3H-labelled RNA. The values reported in the NS : N column are molar ratios calculated from the intensity measurements as described in Methods. Values obtained from scanning were rounded to three significant figures. Both the plus and minus strand of the DI RNA were scanned to determine the level of DI RNA synthesis.
Fig. 4. Velocity sedimentation analysis of N protein: effect of co-translation of NS protein. (a to c) Sedimentation profiles of N protein in a glycerol gradient when synthesized alone (a) or synthesized with increasing amounts of NS protein (b and c). Micrococcal nuclease-treated rabbit reticulocyte lysates were programmed with either hybrid-selected N mRNA or hybrid-selected N mRNA and increasing levels of hybrid-selected NS mRNA. The reactions were incubated for 1 h at 30 °C. One μl was sampled from each reaction and electrophoresed on a 10% polyacrylamide gel which was fluorographed and the molar ratios of the N and NS protein in each reaction were determined by densitometric scanning. The remaining parts of each reaction were layered over a glycerol gradient and the proteins were separated by velocity sedimentation. The 35S-labelled proteins in each gradient were quantified by densitometric scanning of fluorograms. The total amount of N protein synthesized in all three reactions was the same. The shaded boxes represent the sedimentation profile of the N protein. The horizontal line in (a) indicates where haemoglobin sedimented in the gradients.

Gradient (Fig. 4a). When the NS protein was synthesized in addition to N protein at an NS to N molar ratio of 0.1 (Fig. 4b), 42% of the N protein shifted from sedimenting at the bottom of the gradient to banding broadly in the middle of the gradient with the NS protein. This shows that one NS molecule moves four N molecules from the bottom of the gradient to the middle of the gradient. At an NS to N molar ratio of 0.3 (Fig. 4c), all the N protein banded in the middle of the gradient with the NS protein. Our data did not distinguish whether all the N protein banding at 4S is in a complex with the NS protein or whether only a portion of the N protein population is complexed with the NS protein such that the concentration of free N protein was now below the critical concentration for N aggregation. In previous work (Davis et al., 1986), we showed that when the NS to N protein molar ratio differs, the type of NS–N complex that forms also differs. In reactions with an NS to N molar ratio of 0.5 or less, two NS–N complexes have been characterized, one complex with a molar ratio of 0.5 and the other with a molar ratio of 1. Unassociated N protein was also described under these conditions. These results demonstrated that NS protein can specifically prevent the formation of the fast sedimenting species of N protein.

In a separate set of experiments the effect of synthesizing increasing concentrations of M protein, translated together with N protein, on the sedimentation of N protein were examined. The presence of presynthesized VSV M protein had no effect on the fast sedimenting N protein species (data not shown).
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DISCUSSION

It was hypothesized almost 10 years ago that the availability of N protein to encapsidate nascent genome RNA would modulate the switch from RNA transcription to RNA replication (Leppert et al., 1979; Blumberg et al., 1981). Analysis by numerous researchers of the protein requirement for replication both in vitro and in vivo has supported this hypothesis (Patton et al., 1984a; Arnheiter et al., 1985; Peluso & Moyer, 1988). The work of Patton et al. (1984a) further showed that N protein synthesized alone was sufficient to satisfy the requirement for de novo protein synthesis in replication. Subsequent studies, however, have led us and others to question whether N protein alone is optimal for supporting replication or whether other factors may be involved. In particular, the observations by several groups that N protein could exist in a complex with NS protein both in the cell and following translation of the two proteins together in vitro (Bell et al., 1984; Peluso & Moyer, 1984, 1988; Davis et al., 1986; Masters & Banerjee, 1988a) and the observation by our group and by Peluso and Moyer that N protein could form a high Mr species that did not efficiently support RNA replication led to a second hypothesis (Davis et al., 1986; Peluso & Moyer, 1988). This hypothesis states that the formation of a complex between N protein and NS protein might function to prevent self-aggregation of N protein.

Two missing critical pieces of information were required to establish the validity of the second hypothesis: (i) was the high Mr (rapidly sedimenting) form of the N protein an aggregate of N protein or was it perhaps an aggregate of N protein with RNA and (ii) could NS protein specifically prevent the formation of the rapidly sedimenting form of N protein. The work presented in this report directly addresses these questions.

Data presented here have shown the following. N protein synthesized in vitro in the absence of other VSV proteins sediments as either a 4S molecule or as a rapidly sedimenting form, as a function of its concentration. Both the 4S form and the rapidly sedimenting form have average densities of 1.29 g/ml when assayed by CsCl equilibrium gradient centrifugation. This density is characteristic of protein and implies that the rapidly sedimenting form is a protein-protein aggregate rather than a protein-nucleic acid complex. The N protein sedimenting at 4S supports replication; the rapidly sedimenting form of N does not support replication. NS protein synthesized in addition to N protein prevents formation of the rapidly sedimenting form of N protein and maintains it in a form that efficiently supports replication.

Earlier work from our laboratory demonstrated that N protein alone could satisfy the requirement for protein synthesis in replication (Patton et al., 1984a). These experiments were carried out such that RNA and protein synthesis occurred simultaneously. Under these conditions we believe the soluble pool of N never achieves high concentrations because N protein is continuously drawn off to support replication by incorporation into progeny nucleocapsids. By changing the experimental protocol such that protein synthesis was carried out prior to addition of nucleocapsid templates, we were able to establish conditions such that high concentrations of N protein were present in the replication reaction and demonstrate that, as a function of concentration, N protein synthesized alone formed a rapidly sedimenting species that was not functional in supporting replication. The density of this high Mr species of N was 1.29 g/ml, indicating that the rapidly sedimenting species was a protein–protein aggregate. The results presented in Fig. 1 showing that N protein synthesized in the absence of other viral specific proteins forms a high Mr species as a function of increasing concentration and that this form is not functional in supporting replication are consistent with the observation of Sprague et al. (1983) that VSV N protein expressed in COS cells from a simian virus 40 vector forms an N aggregate in the cell and cannot complement temperature-sensitive N protein mutants (J. Sprague, personal communication). The observation of Peluso (1988) and Peluso & Moyer (1988) that a rapidly sedimenting form of N protein found in the infected cell does not support replication whereas a complex of N and NS does, is also consistent with the data reported here. The observation that the rapidly sedimenting form of N has a density of 1.29 g/ml (Fig. 2), consistent with the interpretation that it is a protein aggregate, agrees with the finding of Blumberg et al. (1983) that N protein isolated from nucleocapsids self-assembles and has a
density of 1.28 g/ml. Blumberg et al. (1983) went on to demonstrate that this N could render leader RNA nuclease-resistant. However, neither the rapidly sedimenting form described here nor that described by Peluso (1988) functions stoichiometrically to support replication; neither group has tested its ability to encapsidate leader. Therefore, the function of this form of N protein is presently unknown.

The hypothesis that the NS protein associated with the N protein to prevent it from self-associating and to maintain it in the state to support replication was tested directly in the experiment reported in Table 2 and Fig. 4. N protein was synthesized at concentrations that generated the rapidly sedimenting form of N protein. However, when the same concentration of N was synthesized in the presence of increasing concentrations of NS protein, the rapidly sedimenting species of N protein did not form. Instead, N and NS were found to sediment together at approximately 4S. In previous work Davis et al. (1986) have shown that the N and NS which co-sediment at 4S in these gradients are found in the form of complexes. Depending on the molar ratios of N and NS protein in the in vitro translation system, three types of complexes, one with a molar ratio of N to NS of 2:1, one with a ratio of 1:1 and one with a ratio of 1:2 have been characterized. The precise roles of these different types of complex remain to be elucidated as separation of the complexes in a functional form is extremely difficult. Nevertheless, the data presented show that NS protein prevents aggregation of N protein under conditions of high concentration. In addition, the data presented here show that NS protein co-translated with N protein under conditions of high concentration maintains N protein in a form that efficiently supports replication. Taken together these data define a role for the NS protein in replication.

Data presented in this report and published previously (Patton et al., 1984b) show that the N to NS ratio is critical for establishing conditions that will support RNA replication. Clearly the situation we have examined is one in which N and NS interact such that N does not form a concentration-dependent aggregate and does support RNA replication. We have not eliminated the possibility that a small fraction of the N protein synthesized in our system may also associate with non-specific RNA. Recently, Masters & Banerjee (1988b) proposed that one function of N–NS complexes is to prevent the interaction of N with non-specific RNA so that it can specifically interact with VSV RNA. Their results show that NS can prevent an interaction between N and non-specific RNA; however, only a small fraction of the N protein in their experiment was shown to bind to non-specific RNA. We point out that the weight of evidence presented here is consistent with the characterization of the rapidly sedimenting species of N as predominantly a protein aggregate for the following two reasons. First, N protein bands at a buoyant density of free N protein (1.29 g/ml) not at the density of N protein and RNA-containing nucleocapsids (1.31 g/ml). These density measurements are significantly different, the different species being separated by 10% of the gradient. Second, if non-specific RNA were competing with replicating VSV RNA for N protein in our system, we would not expect low concentrations of N protein to support VSV replication, which it does.

Our data agree with the work of Peluso & Moyer (1988), who observed that a complex of N and NS proteins in the infected cell supports conditions that will support RNA replication whereas a rapidly sedimenting form of N protein does not. They reported the isolation of an NS–N complex in a 1:1 molar ratio that supports replication. In previous work (Davis et al., 1986) we showed that in the in vitro replication system, at a total NS to N protein molar ratio of 0.5, a ratio that supports replication, two such complexes form, a 1:1 and a 1:2. Peluso & Moyer (1988) examined proteins isolated from infected cell extracts under conditions of infection where it was not possible to isolate free N protein in quantities sufficient to analyse its ability to support replication. By use of the in vitro replication system to programme synthesis of individual proteins we have been able to synthesize N in the absence of NS, examine its function, and thereby dissect one functional role for NS interaction with N in complex form. The data presented here confirm earlier work by Patton et al. (1984a) that N protein synthesized alone can support RNA replication and extend the work to show a role for the NS protein in functioning to prevent the concentration-dependent aggregation of N protein and to maintain it in a form capable of supporting RNA replication.
The authors would like to thank John Glass and Drs Andrew Ball, Elliot Lefkowitz, Kevin Anderson and Asit Pattnaik for helpful suggestions. A special thanks to Maria Paulson for typing this manuscript. This work was supported by NIH grants R37 AI 12464 and AI 20181. A preliminary report of part of these experiments has been published (Howard et al., 1986).

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(Received 14 March 1989)