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Vesicular Stomatitis Virus N and NS Proteins Form Multiple Complexes

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The vesicular stomatitis virus nucleocapsid protein, N, associated specifically with the viral phosphoprotein, NS, in an in vitro system which supported vesicular stomatitis virus RNA replication. Essentially all the N protein was found complexed with NS. In addition, multiple forms of the N-NS complex were detected which differed in their sedimentation properties and ratios of N to NS.

The negative-stranded RNA genome of vesicular stomatitis virus (VSV), the prototype of the rhabdoviruses, encodes five genes. The first step in the expression of these genes is their transcription from the parental nucleocapsid template by its constituent transcriptase. A 47-base leader RNA and five monocistronic mRNAs for the N, NS, M, G, and L proteins are produced (4, 7, 9). An alternative function of the negative-stranded RNA-containing nucleocapsid is the synthesis of full-length positive-stranded RNA to serve as a template for the synthesis of progeny negative-stranded RNAs. The two-step replication process is protein synthesis dependent and produces only encapsidated RNAs. The virus life cycle involves a balance between the two alternate nucleocapsid functions, transcription and replication (2, 13, 14, 16).

In vitro systems have been useful for analyzing the requirements for these two types of RNA synthesis. For example, in a cell-free system for RNA replication, the synthesis of the major nucleocapsid protein, N protein, is sufficient to enable enzymatically active defective interfering particle nucleocapsids to replicate full-length defective interfering particle RNA (9). The availability of functional N protein may be the major factor regulating the balance between replication and transcription.

The simplicity of in vitro systems has been exploited to define minimal requirements for viral RNA synthesis. It is possible that the replication of VSV RNA within the infected cell, in the presence of all the virus gene products and host components, is more complex than the simple interaction of N protein with nascent RNA on replicating nucleocapsid templates. In fact, studies of viral proteins in infected cells have revealed that N protein is specifically associated with the NS phosphoprotein (3, 12). Functional N protein may exist not as a monomeric subunit but as a component of an N-NS complex. This complex may play an important role in the maintenance of N in a functional state and in the regulation of the amount of N protein available to support replication. The experiments reported here were designed to discover the properties of the N-NS complex as it is formed in an in vitro replication system.

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The in vitro system, containing an mRNA-dependent rabbit reticulocyte lysate and hybrid-selected viral mRNAs, was programmed to synthesize viral proteins singly and in combination. The presence of N-NS complexes in the in vitro system was shown by immunoprecipitation of in vitro translation products with monoclonal antibodies specific for N or NS protein. The reactions were constituted as described previously (10), except that nucleocapsid templates were omitted. RNA was isolated from actinomycin D-treated infected baby hamster kidney (BHK) cell cytoplasm, and mRNAs were selected by oligo(dT) chromatography. Individual viral mRNAs were purified by hybridization to filter-bound cDNA copies of viral genes as described previously (10). Equal portions of [^{35}S]methionine-labeled proteins from five separate in vitro translation reactions were immunoprecipitated with either of two anti-N monoclonal antibodies described previously (1) or with an anti-NS monoclonal antibody (A/9/10) which was obtained after immunization of BALB/c mice with purified VSV (Indiana serotype, M-S strain) nucleocapsids. Purified antibodies and in vitro-synthesized proteins were incubated in 0.01 M Tris hydrochloride (pH 7.4)–0.001 M EDTA–0.15 M NaCl at 4°C for 4 to 16 h. Antigen-antibody complexes were precipitated with anti-mouse immunoglobulin G immunobeads (Bio-Rad Laboratories, Richmond, Calif.) at 4°C for 3 to 4 h. The bead-antigen-antibody complexes were washed, and the proteins were dissociated from the beads by boiling in 2% sodium dodecyl sulfate (SDS)–0.1 M 2-mercaptoethanol for analysis by SDS-polyacrylamide gel electrophoresis (8). The results are shown in Fig. 1. Anti-N antibody 1 precipitated N (lane 2) but not NS (lane 3), unless the proteins were both present in the reaction mixture, and then both N and NS were precipitated (lanes 4 and 5). Anti-N antibody 2 only precipitated N protein, even in the presence of NS (lanes 7 to 10). However, the amount of N protein precipitated by this antibody from reaction mixtures containing N and NS proteins was much lower than that precipitated from reactions programmed with the same amount of N mRNA alone. The anti-NS monoclonal antibody precipitated NS (lane 13) and only a trace amount of N protein (lane 12), unless both proteins were present, and then the amount of N in the precipitate was greatly increased (lanes 14 and 15). The above immunoprecipitations were done in the absence of detergent. When 1% Nonidet P-40 and 0.5% deoxycholate were added to the antigen-antibody reaction mixture, only N protein was precipitated by anti-N antibody 1, even when

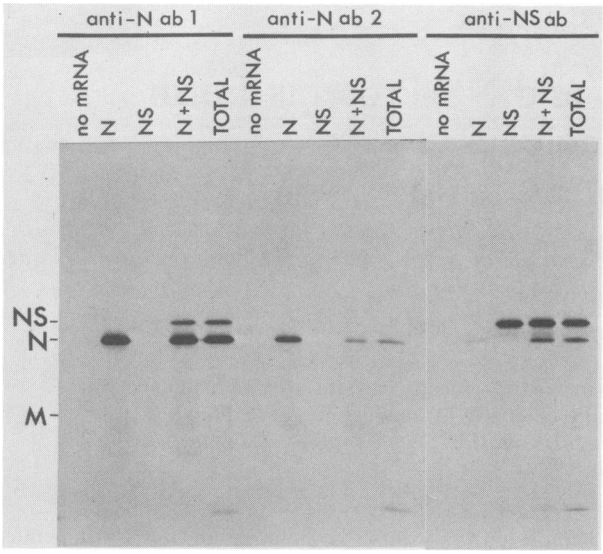

FIG. 1. Immunoprecipitation of in vitro-synthesized viral proteins with monoclonal antibodies. [^{35}S]methionine-labeled proteins were synthesized in vitro in reactions programmed with purified N mRNA, NS mRNA, a mixture of N and NS mRNAs, or total poly(A)$^+$ RNA from VSV-infected cells. A control reaction contained no exogenous mRNA. Equal portions of each reaction mixture were immunoprecipitated by using immunobeads with monoclonal antibody anti-N antibody 1, anti-N antibody 2, or anti-NS antibody. SDS-polyacrylamide gel electrophoresis was performed on the precipitated proteins.

NS was present (Fig. 2, lanes 2 and 5). The more efficient precipitation of N alone in the presence of detergent may have been due to more complete exposure of the epitope under these conditions. (The minor band of NS in lane 2 was due to nonspecific trapping of free NS in the immunoprecipitate or to incomplete dissociation of the N-NS complex.) Taken together, these results show that N and NS proteins made in vitro from either total poly(A)$^+$ viral RNA or individual hybrid-selected mRNAs associate into a detergent-sensitive complex. Anti-N antibody 1 and anti-NS antibody bound to both free and complexed protein (Fig. 1). However, the avidities of an antibody for these two forms may differ. The extreme case is anti-N antibody 2, which showed no binding to the N-NS complex. This antibody was able to react only with the small amount of uncomplexed N protein present in the reactions containing both N and NS proteins. The epitope defined by antibody 2 is also inaccessible in nucleocapsids, since it reacts only with the free, soluble pool of N in intact cells (1).

Rate-velocity sedimentation was used to study the N-NS complexes directly. ^{35}S-labeled proteins synthesized in vitro in reactions programmed by total poly(A)$^+$ infected-cell RNA were centrifuged for 20 h in a glycerol gradient (5 ml; 10 to 30% [wt/wt]) which contained 0.01 M Tris hydrochloride (pH 7.4), 0.001 M EDTA, and 0.15 M NaCl at 234,000 × *g*, conditions which moved hemoglobin to the middle of the gradient. The profile of trichloroacetic acid-precipitable radioactivity is shown in Fig. 3A (●). Another sample was centrifuged in a parallel gradient containing 1% Nonidet P-40 and 0.5% deoxycholate (Fig. 3A, ○). Individual viral proteins were located in the gradient by electrophoresing gradient fractions on SDS-polyacrylamide gels. The autoradiogram of the gel from the gradient without detergent is shown in Fig. 3B. The gel pattern was analyzed by densitometry to quantitate the relative amount of each viral protein in each gradient fraction and to prepare individual sedimentation profiles for the N, NS, and M proteins (Fig. 3D). These results show that N and NS cosediment in the absence of detergent. When N or NS protein was synthesized separately from purified mRNA and centrifuged, it sedimented more slowly than in parallel gradients of reactions programmed with both mRNAs (data not shown). Also, in the detergent-containing gradient the N, NS, and M proteins sedimented as discrete, symmetrical, overlapping peaks in the exact positions that the individual proteins made in reactions programmed with individual purified mRNAs (data not shown). Therefore, the peak of N and NS shown in Fig. 3 appears only when both N and NS proteins are present in the reaction mixture and when detergent is omitted from the gradient. The material in this peak represents the N-NS complexes detected in the immunoprecipitation experiments described above.

The pattern of N and NS proteins is striking in that very little free N or NS is found. Small proportions of N and NS sedimented in fractions 34 to 36 (Fig. 3B). This is consistent with the small amount of N protein precipitated with anti-N antibody 2 from similar reactions. The data shown in Fig. 3 also indicate that more than one type of complex can be formed. At least two types of complex were distinguished which differed in their relative amounts of N and NS (Fig. 3C) and showed different sedimentation rates. Identical reaction mixtures have been shown previously to support RNA replication (5, 10, 15). Therefore, at least one of these N-NS complexes contains N protein which is active in the replication process.

In several experiments, N and NS always cosedimented in fractions 15 to 25. However, the pattern of N-NS complexes appeared to depend on the relative amounts of N and NS proteins synthesized, as shown in the following experiment. Each of three in vitro reaction mixtures contained the same amount of purified N mRNA but differing amounts of purified NS mRNA. The N-NS complexes formed in these

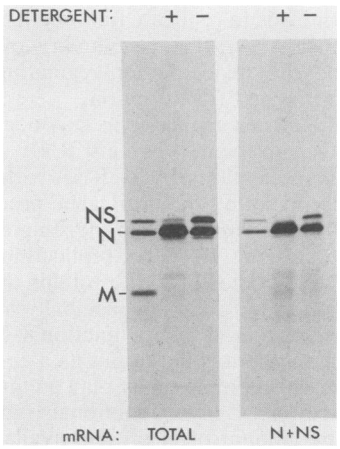

FIG. 2. Immunoprecipitations with an anti-N monoclonal antibody in the presence or absence of detergent. [^{35}S]methionine-labeled proteins were synthesized as described in the legend to Fig. 1 in reactions programmed with total poly(A)$^+$ RNA (TOTAL) or a mixture of purified N and NS mRNAs (N+NS). The total products of each reaction were analyzed by SDS-polyacrylamide gel electrophoresis (lanes 1 and 4), as were the proteins in the immunoprecipitates formed with anti-N antibody 1 in the presence (lanes 2 and 5) or absence (lanes 3 and 6) of 1% Nonidet P-40 and 0.5% deoxycholate.

reactions were analyzed by sedimentation (Fig. 4). All three reactions produced complexes of N and NS. However, in reaction A there remained excess unassociated N protein which sedimented more slowly in the gradient, and in reaction C the excess unassociated NS protein sedimented more slowly. Under conditions of excess N protein (Fig. 4A), two peaks containing NS and N were detected with ratios of NS to N of approximately 0.5 (faster sedimenting) and 1 (slower sedimenting). When the concentration of NS protein synthesized was sufficient to bind all the N protein

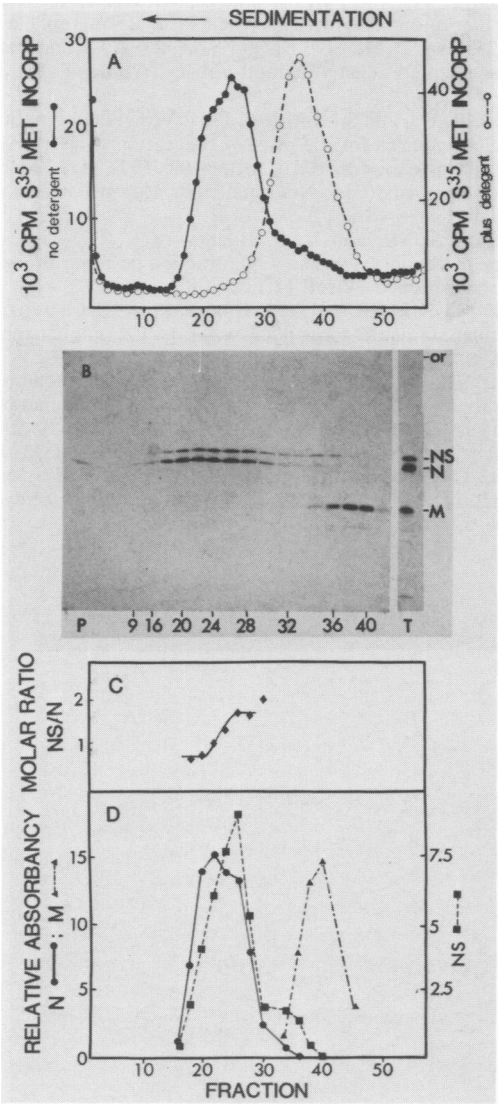

FIG. 3. Sedimentation analysis of VSV proteins synthesized in vitro. Total poly(A)$^+$ RNA from VSV-infected cells was translated in a micrococcal nuclease-treated reticulocyte lysate containing [^{35}S]methionine, and portions of the reaction were centrifuged in parallel glycerol gradients. Trichloroacetic acid-precipitable radioactivity (A) was determined for fractions of gradients with (○) and without (●) detergent. Fractions from the gradient without detergent were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (B). Densitometric analysis of the autoradiogram was used to quantitate each viral protein in each gradient fraction (D). The molar ratio of NS to N in each fraction (C) was calculated by using the relative incorporation of [^{35}S]methionine into NS and N and the number of methionine residues in the two proteins (2, 6).

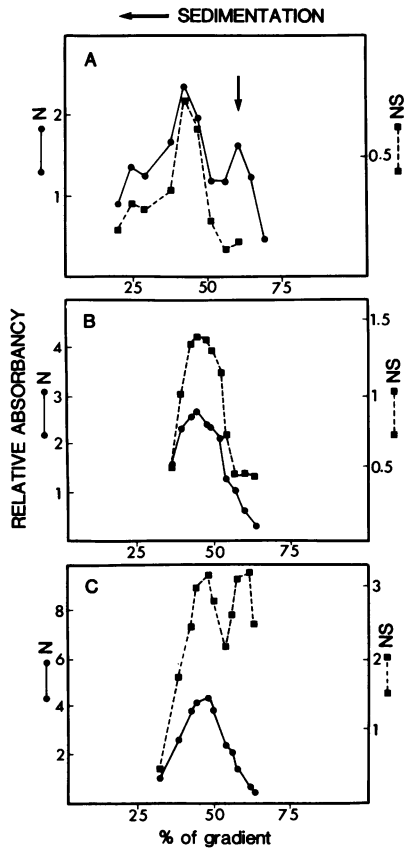

FIG. 4. Sedimentation analysis of N-NS complexes formed with increasing relative amounts of NS. In vitro translation reactions were programmed with a constant amount of purified N mRNA, and the following varying amounts of purified NS mRNA: 0.2 μl (A), 0.5 μl (B), and 1.0 μl (C). ^{35}S-labeled proteins were analyzed as described in the legend to Fig. 3. ●, N protein; ■, NS protein. The arrow in panel A indicates the peak of N protein in a parallel gradient containing a reaction mixture programmed with only purified N mRNA.

made, a single peak with an average NS/N ratio of approximately 1.5 was found (Fig. 4B). This peak was an unresolved mixture of the two overlapping peaks shown in Fig. 3D. Finally, under conditions of excess NS, a complex with an NS/N ratio of approximately 2 was present (Fig. 4C). Different complexes were formed in the presence of different relative amounts of N and NS proteins. This may explain the observed variability in the sedimentation patterns of N-NS complexes obtained with different samples of total poly(A)$^+$ adenylated viral RNA.

Further study of the N-NS complexes is required to determine exactly how many distinct complexes are formed, which of the complexes is active in RNA replication, and whether different complexes contain NS in different phosphorylated states. It has been proposed that the different phosphorylated forms of NS protein may regulate viral RNA synthesis by their association with N protein, rendering it either active or inactive in replication (G. W. Wertz, N. L. Davis, and J. T. Patton, *in* R. R. Wagner, ed., *The Rhabdoviruses*, in press). This model makes several predictions, including the predictions that (i) conditions can be found under which NS protein inhibits replication and (ii) multiple N-NS complexes can be formed, not all of which are active

in replication. Inhibition of replication in vitro by NS protein was demonstrated in reaction mixtures containing abnormally high ratios of NS to N protein (11). Our results are consistent with the second prediction in showing that more than one type of N-NS complex can be formed in an in vitro system for RNA replication.

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