JOURNAL OF VIROLOGY, Aug. 1988, p. 2658–2664
0022-538X/88/082658-07$02.00/0

Vol. 62, No. 8

Complex Formation with Vesicular Stomatitis Virus Phosphoprotein NS Prevents Binding of Nucleocapsid Protein N to Nonspecific RNA

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Received 16 February 1988/Accepted 26 April 1988

The interactions between the nucleocapsid protein N and either RNA or the phosphoprotein NS of vesicular stomatitis virus (VSV) were studied by the transcription of N and NS mRNAs from SP6 vectors, followed by translation in a rabbit reticulocyte lysate. Nascent N protein bound tightly to added labeled RNA, as well as to endogenous RNA in the reticulocyte lysate. This binding was demonstrated by three independent techniques. First, labeled N protein and labeled RNA migrated identically as a series of sharp, closely spaced bands in a nondenaturing gel system. Second, translated N protein behaved as a stable ribonucleoprotein complex in CsCl gradients and sedimented to the same density as the authentic N-RNA template of VSV. Third, translated N protein protected a series of labeled RNA fragments from digestion by RNase A. None of the three RNA-binding criteria was satisfied by either translated NS protein or two deletion mutants of N protein or by other components of the reticulocyte lysate. The evidence suggests that the observed binding of RNA by nascent N was not RNA sequence specific, in contrast to the encapsidation process during VSV replication. Moreover, the prior formation of N-NS complexes totally abolished the observed binding of RNA by N. Thus, we propose that NS may be responsible for conferring the sequence specificity of the RNA binding that occurs during VSV genome replication.

The negative-strand RNA genome of the rhabdovirus vesicular stomatitis virus (VSV) is compactly wrapped by the viral nucleocapsid protein N, which protects the phosphodiester backbone of the RNA and yet allows the genome to be transcribed and replicated (1). These latter functions are carried out by the virion RNA-dependent RNA polymerase, an enzyme consisting of the large viral protein L and the phosphoprotein NS (10). The multiplicity of interactions in the four-component VSV nucleocapsid dictates that each of the three protein components be multifunctional. A method which has proven fruitful in unraveling these functions and assigning them to portions of the constituent molecules has entailed the in vitro translation of wild-type or altered mRNAs transcribed from SP6 or T7 transcription vectors. This technique has allowed the assignment of portions of the NS molecule which interact with the N-RNA template or the L protein (9, 11), as well as the definition of NS residues which are phosphorylation targets essential for transcription (6). In the preceding article (14), we used a similar approach to examine the binding of free N to NS and showed that the six complexes formed between these two proteins fell into two distinct classes. In this article, we report the binding of nascent N protein to RNA and the prevention of this binding by prior complex formation with NS protein. The implications of this level of regulation for VSV genome replication are discussed.

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MATERIALS AND METHODS

The N protein SP6 transcription vector pN109, SP6 transcription reactions, in vitro protein synthesis, and nondenaturing polyacrylamide gel electrophoresis (PAGE) were as described in the preceding article (14).

Preparation of RNase T1-fragmented [^{32}P]RNA. Uncapped VSV N gene mRNA, labeled to a high specific activity with [α-^{32}P]CTP or both [α-^{32}P]CTP and [α-^{32}P]UTP (410 Ci/mmol of each), was synthesized with SP6 RNA polymerase as described previously (14) with the following modifications: (i) the cap analog m^{7}GpppGm was omitted, (ii) the final concentration of each unlabeled nucleoside triphosphate was 500 μM, and (iii) the final concentration of each labeled nucleoside triphosphate (included at 1 mCi/200 μl of reaction mixture) was 20 or 25 μM. Following RNA synthesis, the DNA template was digested with 20 U of RNase-free DNase per ml for 15 min at 37°C, and product RNA was twice extracted with phenol-chloroform and then with chloroform and precipitated with ethanol. The RNA pellet was suspended in 100 μl of 0.1 M Tris hydrochloride (pH 7.5)–1 mM EDTA and digested with 400 U of RNase T1 for 60 min at 37°C. The reaction mixture was deproteinized and desalted on a NENsorb column (New England Nuclear Corp.) according to manufacturer instructions, and the dried eluent was then ethanol precipitated twice from 2 M ammonium acetate and finally suspended in 50 μl of H_2O.

CsCl gradients. The formation of complexes with in vitro-synthesized N protein and RNA was assayed by sedimentation through CsCl essentially as described by Davis and Wertz (8). Briefly, 24 μl of rabbit reticulocyte lysate was diluted with 2.5 ml of Nonidet P-40 buffer (3.3 mM Tris hydrochloride [pH 8.6], 3 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40) and layered onto a 1.5-ml cushion of 5% sucrose on top of a preformed 8-ml gradient of 20 to 40% CsCl. After centrifugation at 35,000 rpm with a Beckman

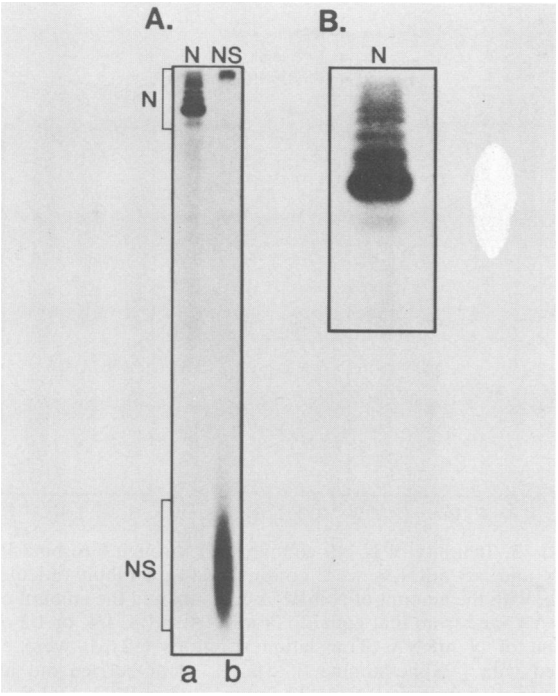

FIG. 1. Fine structure of N protein aggregate in nondenaturing PAGE. (A) N and NS proteins were translated from SP6 mRNAs in a rabbit reticulocyte lysate labeled with [^{35}S]methionine and analyzed by nondenaturing PAGE, as described in the preceding report (14). (B) An expanded view of the top of lane a in panel A.

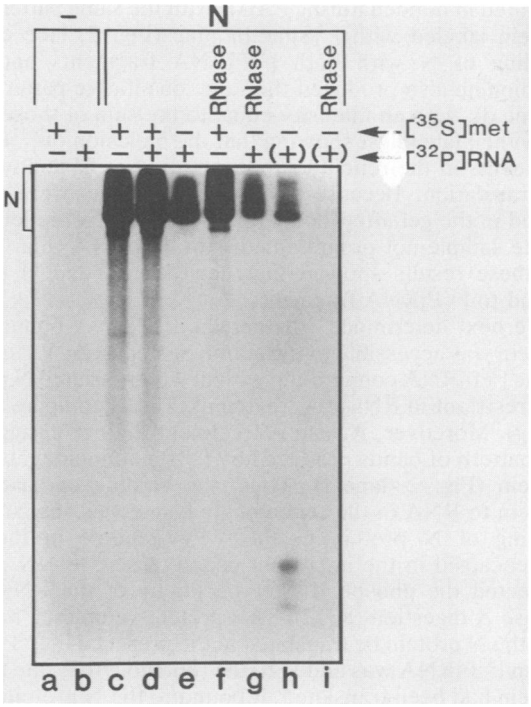

FIG. 2. Binding of newly translated N protein to RNA. N mRNA was translated in a rabbit reticulocyte lysate labeled with [^{35}S] methionine ([^{35}S]met, +) or unlabeled ([^{35}S]met, −). Translation reactions (12 μl) were carried out in the presence (+) or absence (−) of 5.8 ng of RNase T1-fragmented [^{32}P]RNA (3.8×10^8 cpm/μg). For lanes h and i, [^{32}P]RNA was added to the sample after the translation reaction, and samples were incubated for 10 min at 0°C. For lanes f, g, and i, 25 μg of RNase A per ml was added following translation (or following translation and incubation for lane i), and samples were incubated for 30 min at 30°C. Control translation reactions, to which no mRNA was added, were labeled with either [^{35}S]methionine (lane a) or [^{32}P]RNA (lane b). Samples were analyzed by nondenaturing PAGE.

SW40 rotor for 16 h at 4°C, gradient fractions (300 μl) were collected from the top. [^{35}S]methionine-labeled protein or [^{32}P]RNA was determined in a 150-μl sample of each fraction by trichloroacetic acid precipitation, and CsCl density was measured by refractometry.

RNA protection. Rabbit reticulocyte protein synthesis reactions (36 μl), programmed with the indicated mRNAs, were carried out in the presence of RNase T1-fragmented [^{32}P]RNA and then digested with 25 μg of RNase A per ml for 30 min at 30°C. Reactions were terminated by the addition of 10 μg of carrier tRNA, dilution to 200 μl with 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, and addition of sodium dodecyl sulfate to 0.5%. Samples were twice extracted with phenol-chloroform and then with chloroform and twice precipitated with ethanol. [^{32}P]RNA was analyzed by electrophoresis on a 20% polyacrylamide gel containing 8 M urea.

Radioisotopes and enzymes. [α-^{32}P]CTP and [α-^{32}P]UTP were from Amersham Corp. The restriction endonucleases *Bam*HI and *Xho*I were obtained from New England Biolabs, Inc. RNase T1 was from Boehringer Mannheim Biochemicals; RNase-free DNase was from Promega Biotec.

RESULTS

Binding of nascent N protein to RNA. In the preceding report (14), we described the formation of a set of at least six complexes formed between the N and NS proteins of VSV (New Jersey serotype) following cotranslation of N and NS mRNAs in a rabbit reticulocyte lysate. In the nondenaturing PAGE system used to resolve these complexes, N and NS proteins, each translated individually, migrated as shown in Fig. 1A. N protein formed a wide band near the top of the running gel (Fig. 1A, lane a), whereas NS entered far into the gel, forming a streak of probable charge variants, which occasionally resolved into three or four diffuse bands (Fig. 1A, lane b). The fluorogram shown in Fig. 1A was underexposed to demonstrate that the N protein aggregate was actually a collection of many very sharply defined, closely spaced bands, the intensities of which increased as their mobilities increased. This aggregate was more evident in an expanded view of the top of Fig. 1A, lane a (Fig. 1B), as well as in gels electrophoresed for longer periods (data not shown).

The multiplicity and sharpness of the N bands suggested that this protein was actually migrating as a set composed of monomers, dimers, and higher multimers. The multiplicity of N bands also raised the possibility that the cause of N multimerization was the binding of one, two, or more N molecules to small fragments of RNA, which are expected to be present in the reticulocyte lysate as a result of the micrococcal nuclease treatment of endogenous mRNA (16). To test this hypothesis, N protein was synthesized in a rabbit reticulocyte lysate in the presence of a mixture of RNA fragments labeled to a high specific activity with ^{32}P. These fragments were prepared by a total RNase T1 digestion of SP6-synthesized, ^{32}P-labeled N mRNA. N protein synthesized in the presence of [^{32}P]RNA fragments (Fig. 2, lane e)

migrated in nondenaturing PAGE with the same pattern as N protein labeled with [^{35}S]methionine (Fig. 2, lane c). The labeling of N with both [^{32}P]RNA fragments and [^{35}S]methionine also produced the same qualitative pattern (Fig. 2, lane d), with an intensity equal to the sum of those of the individual labelings, showing that the inclusion of [^{32}P]RNA fragments in the reticulocyte lysate did not have any effect on translation. Because [^{32}P]RNA fragments were not retained in the gel after incubation with a control reticulocyte lysate sample not programmed with N mRNA (Fig. 2, lane b), these results showed that newly translated N protein bound to [^{32}P]RNA fragments.

We next determined whether the [^{32}P]RNA bound by N protein was accessible to the action of RNase A. Virtually all of the [^{32}P]RNA comigrating with newly translated N protein was resistant to RNase A treatment (Fig. 2, compare lanes e and g). Moreover, RNase A treatment did not disaggregate the pattern of bands produced by [^{35}S]methionine-labeled N protein (Fig. 2, lane f). Thus, the binding of nascent N protein to RNA in the reticulocyte lysate was similar to the binding of N protein to the VSV genome in the viral nucleocapsid in the important characteristic that N protein protected the phosphodiester backbone of the RNA from RNase A digestion (8, 17). This protective binding required that the N protein be translated in the presence of [^{32}P]RNA. When [^{32}P]RNA was added to the reticulocyte lysate after N protein had been translated, it bound to the N protein bands near the top of the gel, as well as to some faster-migrating components in the translation reaction (Fig. 2, lane h). However, in both cases, all [^{32}P]RNA was removed by RNase A treatment (Fig. 2, lane i), and hence, the RNA must have been nonspecifically associated with N protein. This result reinforced the notion that nascent N protein became irreversibly bound to endogenous (unlabeled) RNA in the reticulocyte lysate during the translation reaction, and thus it was no longer available to bind in a specific manner to [^{32}P]RNA added posttranslationally.

Prevention by NS protein of binding of nascent N protein to RNA. Since NS protein is thought to play a role auxiliary to that of N protein in the replication of VSV genome RNA (7, 12, 18, 19), it was of considerable interest to determine whether the N protein within any of the six N-NS complexes was bound to RNA. To do this, N and NS mRNAs were translated in a rabbit reticulocyte lysate either labeled with [^{35}S]methionine or in the presence of [^{32}P]RNA fragments. Because of the protein concentration dependence of N-NS complex formation (7, 14), this experiment was performed at various ratios of NS mRNA to N mRNA. With [^{35}S]methionine labeling, conditions were chosen which generated predominantly N-NS complexes 1 and 2, complexes 1 through 3, complexes 3 through 6, and complex 6 (Fig. 3A, lanes a, c, e, and g, respectively). However, under all of these conditions, there was no detectable binding of [^{32}P]RNA (Fig. 3A, lanes b, d, f, and h), except to uncomplexed N protein at the top of lanes with lower NS concentrations. Even a fluorogram exposed 48-fold longer than that necessary to detect [^{32}P]RNA binding by uncomplexed N protein revealed no significant [^{32}P]RNA binding by any of the six N-NS complexes (Fig. 3B, lanes b′, d′, f′, and h′). It was clear, then, that the ability of nascent N protein to bind RNA was abolished by both classes of NS binding: that represented by N-NS complexes 1 through 5 and that represented by N-NS complex 6 (14).

Ribonucleoprotein sedimentation in CsCl gradients. In addition to RNase A resistance, another characteristic feature of complexes of VSV N protein and RNA is their stability

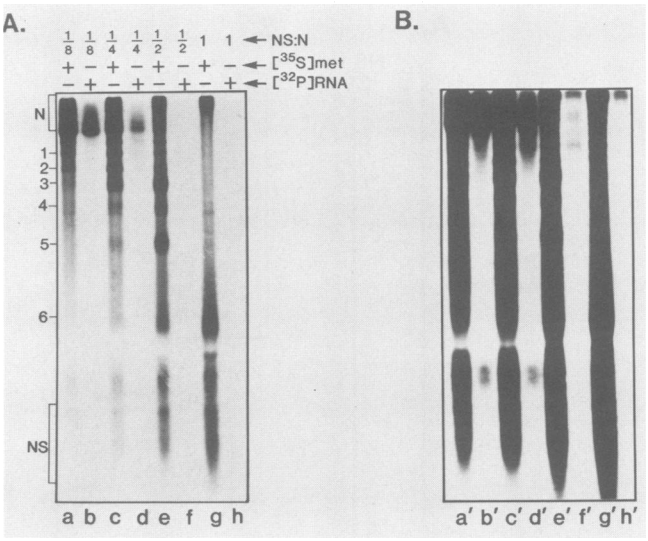

FIG. 3. Inability of N-NS complexes 1 through 6 to bind RNA. (A) N and NS mRNAs were cotranslated in a rabbit reticulocyte lysate, with the amount of N mRNA constant and the amount of NS mRNA varied from that equal to N mRNA to 1/8, 1/4, or 1/2 of the amount of N mRNA. Translation reactions (12 μl) were either labeled with [^{35}S]methionine ([^{35}S]met, +) or carried out in the presence of 5.8 ng of RNase T1-fragmented [^{32}P]RNA (3.8×10^8 cpm/μg) ([^{32}P]RNA, +). Samples were analyzed by nondenaturing PAGE and fluorography was carried out for 8 h. (B) Fluorogram of the gel shown in panel A exposed for 16 days. Lanes a′ to h′ correspond to lanes a to h in panel A.

upon sedimentation through CsCl. This property has provided a standard assay for VSV ribonucleoprotein formation between N protein and leader RNA, genome RNA, or antigenome RNA (3, 4, 8). Thus, we used this same analysis on preformed CsCl gradients to test whether this criterion was met by the complexes formed between nascent N protein and added [^{32}P]RNA fragments and also by the complexes presumed to form between nascent N protein and endogenous RNA fragments in the reticulocyte lysate. Although a considerable amount of labeled translation products or labeled RNA remained nonspecifically aggregated near the top of the CsCl gradients, the results obtained with this analysis were in accord with those obtained with nondenaturing gels. Both [^{35}S]methionine-labeled N protein (Fig. 4A) and N protein translated in the presence of [^{32}P]RNA fragments (Fig. 4D) entered CsCl gradients and sedimented at a density similar to that of authentic N-RNA (Fig. 4G). Because this was a velocity sedimentation experiment, the density values obtained for the peaks of all three samples were lower than those expected for VSV ribonucleoproteins. However, when N-[^{32}P]RNA and virion N-RNA peaks were resedimented in equilibrium CsCl gradients (13), they banded at densities of 1.30 and 1.32 g/ml, respectively (data not shown), in agreement with previously reported values (3, 4).

The formation of CsCl-sedimenting material is a specific attribute of N protein. Neither [^{35}S]methionine-labeled NS protein (Fig. 4B) nor NS protein translated in the presence of [^{32}P]RNA fragments (Fig. 4E) entered appreciably into CsCl gradients. Additionally, when N protein was cotranslated with NS protein (at a ratio at which N-NS complex 6 predominated), it no longer was able to sediment as a ribonucleoprotein (Fig. 4C and F). Thus, CsCl sedimentation analysis of translation reactions of N protein in the absence

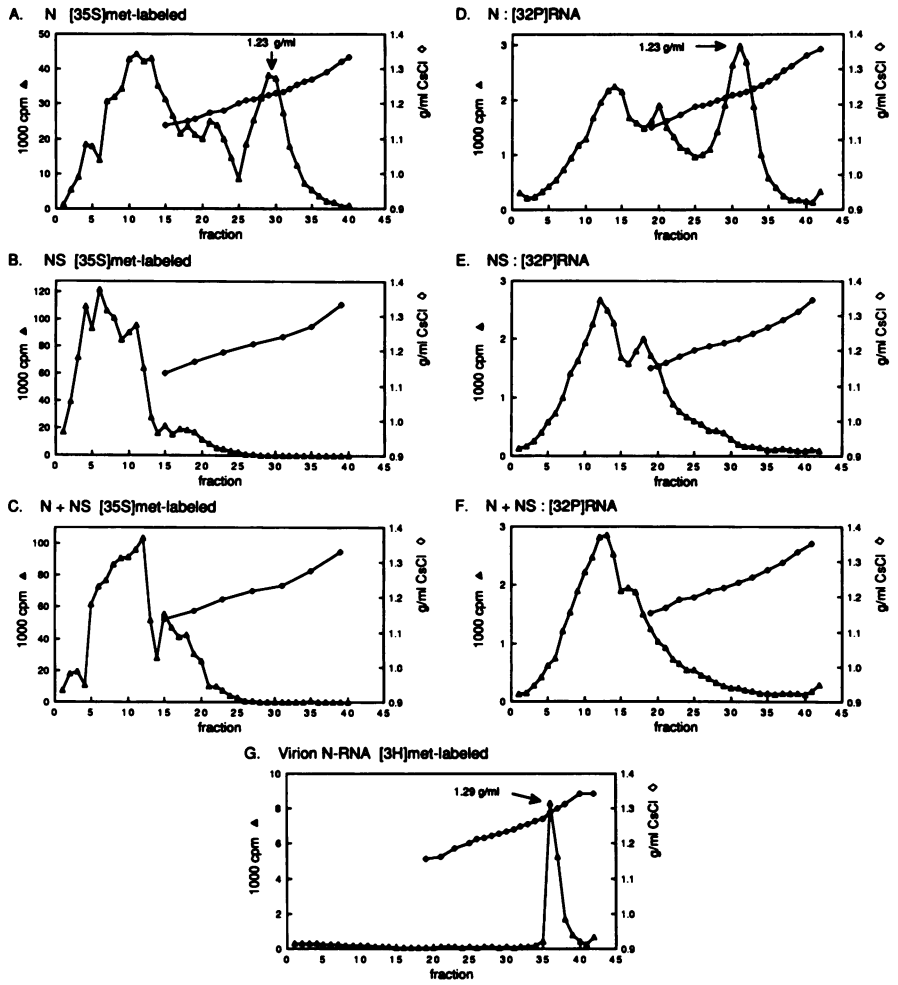

FIG. 4. Sedimentation of N-RNA complexes in CsCl gradients. N mRNA (A and D), NS mRNA (B and E), or both N and NS mRNAs (C and F) were translated in 24 μl of rabbit reticulocyte lysate either labeled with [^{35}S]methionine (A to C) or in the presence of 9.3 ng of RNase T1-fragmented [^{32}P]RNA (3.8×10^8 cpm/μg) (D to F). Reactions were diluted, layered onto preformed 20 to 40% CsCl gradients, and analyzed by sedimentation as described in Materials and Methods. Fractions on the horizontal axes are numbered from the top of each gradient. For each sample, the CsCl gradient began at ca. fraction 15. As a standard, a sample of purified N-RNA from [*methyl*-^{3}H]methionine-labeled VSV (Indiana serotype) (13) was analyzed in the same manner (G). met, Methionine. Arrows indicate the sedimenting N-RNA peaks and their banding densities.

or presence of NS protein was completely in agreement with the analysis of the same reactions by nondenaturing PAGE.

N protein mutants. Two mutants of N protein were generated by manipulation of the SP6 transcription vector pN109 (Fig. 5A), in an attempt to uncouple the RNA-binding and NS-binding properties of N. The first of these mutants, N/*Bam*, had a large deletion near the amino terminus, equal to 24% of the wild-type N molecule. The other mutant, N/*Xho*, had a deletion at the carboxy-terminal 27% of the wild-type N molecule. Both N/*Bam* and N/*Xho* were translated efficiently and were as stable as wild-type N protein in the reticulocyte lysate, as judged by sodium dodecyl sulfate-PAGE (data not shown). These mutant N proteins were translated individually or were cotranslated with NS protein, and their migrations in nondenaturing PAGE were compared with that of wild-type N (Fig. 5B). For both N/*Bam* and N/*Xho* (Fig. 5B, lanes d and f, respectively) translated individually, only a small fraction of the protein entered the running gel, and this protein fraction did so as a diffuse streak. There was no formation of a compact series of bands near the top of the gel as with wild-type N protein (Fig. 5B, lane a). This result suggested that both types of deletion mutation had rendered N protein unable to bind RNA. In addition, the cotranslation of either N mutant with NS protein resulted in no detectable formation of N-NS complexes (Fig. 5B, lanes e and g). Thus, deletion of either end of the N protein molecule abrogated its ability to bind either RNA or NS protein.

The apparent inability of the N protein mutants to bind RNA was confirmed by CsCl sedimentation analysis of N, N/*Bam*, and N/*Xho* proteins translated in the presence of [^{32}P]RNA fragments (Fig. 6). Of these three, only wild-type N protein formed a complex with labeled RNA capable of entering a CsCl gradient (Fig. 6A). The relatively smaller size of the N-[^{32}P]RNA complex in Fig. 6A in contrast to that in Fig. 4D was due to the more than fourfold increase in the amount of [^{32}P]RNA used in this particular experiment. For N/*Bam* (Fig. 6B) and N/*Xho* (Fig. 6C), all [^{32}P]RNA remained at the top of the CsCl gradients. These results, then, reinforced the identification of the aggregate of N protein bands seen near the top of nondenaturing PAGE as a

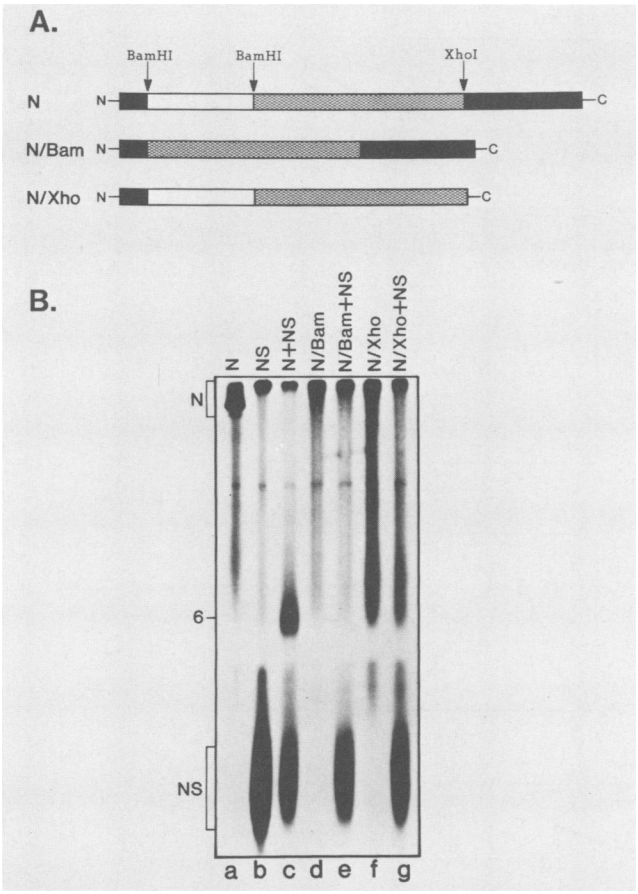

FIG. 5. Inability of mutants of N protein to bind RNA or NS protein. (A) Schematic diagram of N protein and two deletion mutants of N protein, N/*Bam* and N/*Xho*. A deletion was introduced into the N protein SP6 transcription vector, pN109, by removal of the *Bam*HI-*Bam*HI fragment corresponding to nucleotides 80 to 379 of the N mRNA (2). Religation of the remainder of the plasmid resulted in a new vector, pN112, which, when linearized by digestion with *Hin*dIII, could be transcribed into an SP6 mRNA encoding an in-frame deletion mutant of N protein (N/*Bam*) lacking amino acids 23 to 122 of the wild-type N sequence. A second deletion mutant of N protein (N/*Xho*) was obtained by translation of runoff SP6 transcripts of *Xho*I-linerarized pN109. N/*Xho* lacked the carboxy-terminal 112 amino acids of the wild-type N sequence. (B) N, N/*Bam*, N/*Xho*, and NS mRNAs were translated in a rabbit reticulocyte lysate labeled with [^{35}S]methionine either singly (lanes a, b, d, and f) or in N-NS pairs (lanes c, e, and g). Samples were analyzed by nondenaturing PAGE.

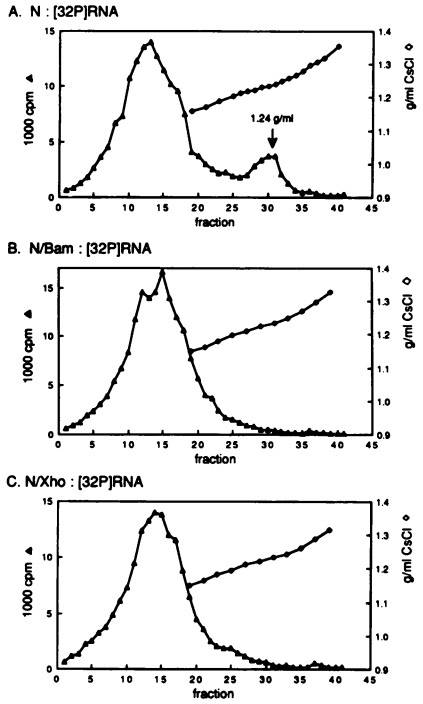

FIG. 6. Sedimentation of N protein mutants in CsCl gradients. N mRNA (A), N/*Bam* mRNA (B), and N/*Xho* mRNA (C) were translated in 24 μl of rabbit reticulocyte lysate in the presence of 39 ng of RNase T1-fragmented [^{32}P]RNA (6.1×10^8 cpm/μg). Reactions were analyzed on CsCl gradients as described in Materials and Methods and in the legend to Fig. 4. The arrow denotes the sedimentation position of N-RNA.

set of N-RNA complexes similar in character to that formed between N protein and VSV genome RNA.

Protection of RNA by newly translated N protein. Further evidence for a specific interaction between nascent N protein and RNA was obtained by examination of the ability of this interaction to protect the RNA in the ribonucleoprotein complex. N protein was translated in a reticulocyte lysate in the presence of [^{32}P]RNA fragments, after which the translation mixture was subjected to RNase A digestion. RNA was then purified and analyzed on 20% polyacrylamide gels containing 8 M urea. Translated N protein (Fig. 7, lane b) protected a number of [^{32}P]RNA species either identical to or derived from those which had been added to the translation reaction (Fig. 7, lane f). Nascent N protein protected only a small fraction of the total [^{32}P]RNA added to the translation reaction, because the labeled RNA was diluted by a large excess of endogenous unlabeled RNA in the reticulocyte lysate. In contrast, cotranslation of both N and NS proteins (Fig. 7, lane c) or translation of either of the mutants, N/*Bam* or N/*Xho* (Fig. 7, lanes d and e, respectively), failed to protect any [^{32}P]RNA species, compared with a control translation reaction to which no mRNA was added (Fig. 7, lane a). In the experiment shown in Fig. 7, the RNA oligomers protected by N protein fell principally in the size range of 18 to 30 nucleotides. However, in experiments in which a more extensive RNase A treatment was used, the size range of protected RNA oligomers decreased to 8 to 18 nucleotides (data not shown). This latter size range, then, may approach the limiting length of RNA protected by one molecule of N protein. The stoichiometry of 1,200 N protein molecules per molecule of VSV genome RNA (11,000 nucleotides) determined by scanning transmission electron microscopy analysis suggests that each molecule of N protein enwraps 9 nucleotides of RNA in the intact virion (22).

DISCUSSION

In the present study, we demonstrated both that newly translated N protein binds to RNA and that the prior formation of N-NS complexes 1 through 6 abolishes the ability of N protein to bind RNA. The binding of RNA by nascent N protein was shown by three independent techniques: (i) comigration of labeled N protein and labeled RNA in nondenaturing PAGE, (ii) sedimentation of labeled N protein or labeled RNA at the same density as VSV nucleocapsids in CsCl gradients, and (iii) N protein-dependent protection of labeled RNA against digestion by RNase A. By

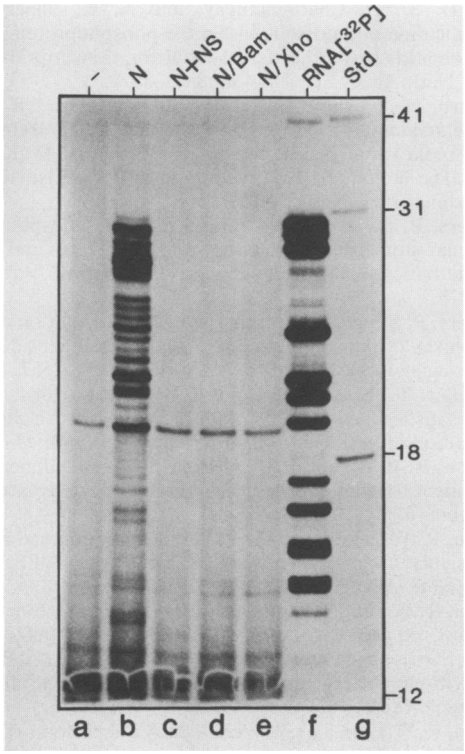

FIG. 7. RNA protection by newly translated N protein. N mRNA, both N and NS mRNAs, N/*Bam* mRNA, or N/*Xho* mRNA (lanes b to d, respectively) was translated in 36 μl of rabbit reticulocyte lysate in the presence of 52 ng of RNase T1-fragmented [^{32}P]RNA (6.1×10^8 cpm/μg). Following translation, samples were treated with 25 μg of RNase A per ml, and [^{32}P]RNA was recovered and analyzed on 20% polyocrylamide gels containing 8 M urea, as described in Materials and Methods. Lane a contained a control reaction in which no mRNA was present during the translation reaction. Lane f contained an amount of RNase T1-fragmented [^{32}P]RNA equivalent to 1.5% the amount used in the reactions in lanes a through e. Lane g contained deoxyoligonucleotides (12-, 18-, 31-, and 41-mers) labeled with ^{32}P at the 5′ end as size standards (Std).

all three of these criteria, tight RNA-binding activity was found to be a specific attribute of N but not of NS, other protein components of the rabbit reticulocyte lysate, or two deletion mutants of N. The mutants N/*Bam* and N/*Xho* were constructed in an attempt to isolate an RNA-binding domain of N, as has been done with certain cellular RNA-binding proteins (5, 20). The bilobed appearance of N protein in VSV nucleocapsids examined by scanning transmission electron microscopy (22) suggested that deletion of either the amino- or carboxy-terminal portion of the molecule might remove a region of N dispensable to the binding of RNA. However, both of our deletion mutants failed to bind either RNA or NS protein. This result may mean that both termini of the N molecule participate in both types of binding or that the extent of each of the deletions was sufficient to impair the overall conformation of N to render it nonfunctional. Clearly, a subtler mutational analysis is required. On the other hand, the two mutants served to reinforce the validity of the three types of RNA-binding assays, making it very unlikely that the observed results with wild-type N were fortuitous.

Most significantly, it was found that the six separable complexes formed between the N and NS proteins (14) did not bind RNA. This result was also confirmed by all three of the RNA-binding criteria mentioned above and is most dramatically demonstrated by comparison of Fig. 3A and B, from which it can be seen that NS binding reduced the affinity of N for RNA by at least 100-fold. Conversely, in the preceding report (14), we showed that N-NS complex formation required the contranslation of the two proteins. When N and NS were separately translated and then mixed, very little complex formation occurred, indicating that at least one of the two proteins underwent an irreversible change shortly following translation. In the light of the results presented here, it is apparent that this irreversible change was the binding of N to RNA. Thus, in the reticulocyte lysate, nascent N protein must partition between two mutually exclusive modes of binding: either to NS protein or to RNA. At least under the in vitro conditions of our experiments, N showed a higher affinity for NS than for the RNA species to which it binds that we have examined.

It is important to note that whereas the binding of N protein to RNA in the reticulocyte lysate had the same physical properties as that to virion N-RNA, it did not seem to be specific for any particular RNA sequence. First, although the majority of the RNA-binding experiments presented here were performed with RNase T1 fragments of a VSV RNA (i.e., the N mRNA), it is unlikely that many of these fragments would individually possess hypothetical nucleation signals for encapsidation. Second, a number of the same RNA-binding experiments conducted with RNase T1-fragmented SP6 RNA which had been transcribed from a template derived from bacteriophage lambda gave the same results (data not shown). Finally, many of the experiments presented here indicate that nascent N protein also bound to endogenous RNA in the rabbit reticulocyte lysate, presumably micrococcal nuclease-fragmented globin mRNA (16).

This latter sequence-nonspecific RNA binding would explain a crucial property of the in vivo replication system for which the source of nascent N protein was a micrococcal nuclease-treated reticulocyte lysate programmed with one or more VSV mRNAs (8, 15). It has been observed that such a coupled system, when programmed solely with N mRNA, requires the continuous synthesis of N protein to sustain the synthesis of genome-length RNA. If N is presynthesized, this system rapidly loses its ability to support replication (8, 15). Our results indicate that this loss of activity, as with the loss of the ability of presynthesized N to form N-NS complexes (14), was probably due to the irreversible binding of N to endogenous RNA in the reticulocyte lysate. On the other hand, in the same system, the cotranslation of NS with N maintains N in a replication-competent state, thus eliminating the requirement for continuous synthesis of N (12). This result may be explained by our finding that the formation of N-NS complexes totally prevented N from carrying out sequence-nonspecific RNA binding. A similar situation prevails in vivo. When N protein is expressed in COS cells in the absence of other viral proteins, it behaves as if it is bound to cellular RNA (21). In contrast, such binding does not normally occur during VSV infection when NS is present.

Our conclusions, then, suggest that NS protein may regulate the direct role that N protein plays in VSV genome RNA replication. In the absence of NS, N appears to bind to any available RNA species, irrespective of sequence. However, when complexed with NS, N no longer binds nonspecific sequences of RNA, but it is clear from the in vitro replication results of other investigators (7, 12, 18, 19) that the N-NS complexes bind the nascent VSV genome or antigenome. This preferential RNA binding may occur either

by recognition of specific RNA sequences that act as encapsidation nucleation signals (3, 4) or by specific interactions with one or more of the other components of the replicating nucleocapsid. The SP6 transcription-rabbit reticulocyte lysate translation system employed in this study and described in the preceding report (14) enable us to examine these possibilities, as well as to define the features of the N molecule which enable it to bind to RNA or to the NS protein.

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