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N Protein Alone Satisfies the Requirement for Protein Synthesis During RNA Replication of Vesicular Stomatitis Virus

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Genomic replication of the negative-strand RNA viruses is dependent upon protein synthesis. To examine the requirement for protein synthesis in replication, we developed an in vitro system that supports the genome replication of defective interfering particles of the negative-strand rhabdovirus vesicular stomatitis virus (VSV), as a function of protein synthesis (Wertz, J. Virol. **46**:513–522, 1983). The system consists of defective interfering nucleocapsid templates and an mRNA-dependent reticulocyte lysate to support protein synthesis. We report here an analysis of the requirement for individual viral proteins in VSV replication. Viral mRNAs purified by hybridization to cDNA clones were used to direct the synthesis of individual proteins in the in vitro system. By this method, it was demonstrated that the synthesis of the VSV nucleocapsid protein, N, alone, resulted in the replication of genome-length RNA by both defective interfering intracellular nucleocapsids and virion-derived nucleocapsids. Neither the viral phosphoprotein, NS, nor the matrix protein, M, supported RNA replication. The amount of RNA replication for a given amount of N protein was the same in reactions in which either all of the VSV proteins or only N protein were synthesized. In addition, RNA replication products synthesized in reactions containing only newly made N protein assembled with the N protein to form nucleocapsids. These results demonstrate that the major nucleocapsid protein (N) can by itself fulfill the requirement for protein synthesis in RNA replication and allow complete replication, i.e., initiation and elongation, as well as encapsidation of genome-length progeny RNA.

Vesicular stomatitis virus (VSV) is one of the simplest of the enveloped, nonsegmented, negative-strand RNA viruses (5). The genome of VSV codes for five proteins that are structural components of the virion. The RNA genome, coated with the major nucleocapsid protein (N) and associated with the phosphoprotein (NS) and the RNA-dependent RNA polymerase (L), forms the viral nucleocapsid. The remaining viral proteins are the matrix protein, M, which is located on the inside surface of the viral membrane, and the glycoprotein, G, which is embedded in the membrane. The viral nucleocapsids are capable of carrying out both RNA replication and transcription. These processes can be distinguished from one another by their requirement for protein synthesis (14, 23). Transcription involves the synthesis of a 46-base-long leader RNA and five monocistronic mRNAs and does not require protein synthesis. In contrast, replication involves the synthesis of genome-length RNA and requires the synthesis of viral proteins.

We have previously described an in vitro system which supports VSV RNA replication, transcription, and nucleocapsid assembly (4, 12). This system, consisting of viral nucleocapsids and a micrococcal nuclease-treated rabbit reticulocyte lysate, replicates VSV RNA when protein synthesis is programmed by the addition of viral mRNA. In the work described in this report, we used nucleocapsids of a VSV defective interfering particle (DI-T) which contain only the 5′ 25% of the standard genome (10, 20) as templates in the in vitro system. This has been done to yield a system in which genome-length RNA replication can be analyzed in the absence of any mRNA synthesis (21). These DI nucleocapsids do not synthesize mRNAs because they lack complete information for all of the structural genes; only a 46-base-long leader RNA is synthesized in the absence of protein synthesis (6, 17, 21). Since no mRNAs are made by the DI nucleocapsids, protein synthesis and, hence, RNA replication in this system can be controlled by the addition of exogenous mRNA. Therefore, by adding individual VSV mRNAs to program the expression of individual viral proteins, it has been possible to investigate the requirement for each of the VSV proteins in RNA replication.

The above studies have been performed using DI nucleocapsids isolated from the cytoplasm of infected cells (intracellular) and DI nucleocapsids prepared from budded DI virions (virion-derived). Intracellular nucleocapsids contain both positive- and negative-strand RNA templates and direct the synthesis of both positive- and negative-strand genome-length RNA in vivo (19). These templates within the infected cell contain the enzymatic activities and the nascent progeny RNAs associated with active replicating complexes (18). Nascent RNAs may remain associated with the purified intracellular nucleocapsids added to the in vitro system to program RNA synthesis. In contrast, virion-derived nucleocapsids contain only negative-strand RNA, the template for the synthesis of genome-length positive-strand RNA. Studies of virion RNA transcription in vitro indicate that there are no nascent RNAs associated with virion-derived nucleocapsids (3). Therefore, whereas synthesis of genome-length RNA by intracellular nucleocapsids in vitro may represent both de novo synthesis and elongation of nascent RNAs, the synthesis of genome-length RNA by virion-derived nucleocapsids represents only de novo synthesis.

We have used both intracellular and virion-derived nucleocapsids in the in vitro replication system in conjunction with purified individual mRNAs to examine the protein synthesis requirement for VSV RNA replication.

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MATERIALS AND METHODS

Cell culture and virus. Monolayers of baby hamster kidney cells (BHK-21/13) were maintained in Eagle minimal essential medium containing 5% heat-inactivated calf serum. VSV (Indiana serotype) propagated in BHK cells (21) was used as helper virus. Stocks of the DI particle of VSV, DI-T (VSV-DI 0.25 (5'), 5' 25% of standard genome), were kindly provided by R. Lazzarini (National Institutes of Health, Bethesda, Md.).

Preparation of purified VSV mRNAs. cDNA clones of VSV N, NS, and M mRNAs (7, 16), gifts of J. Rose (The Salk Institute, San Diego, Calif.), in the *Escherichia coli* plasmid pBR322 were used to select (15) N, NS, and M mRNAs from VSV-infected cell polyadenylated RNA (4). Plasmids were digested with restriction enzyme *Pst*I, boiled for 3 min, adjusted to 10× SSC (1× SSC = 0.15 M NaCl and 0.015 M sodium citrate), and applied to preboiled, 13-mm nitrocellulose filters (10 μg DNA per filter). Each filter was then dried, baked at 80°C for 2 h, boiled for 1 min, dried, cut into eight pieces, and incubated in the presence of approximately 15 μg of polyadenylated infected-cell RNA, 65% formamide (Fluka), 40 mM PIPES [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)] (pH 6.3), and 0.4 M NaCl in a final volume of 100 μl for 3 h at 52°C in a 1.5-ml microfuge tube. Filter sections in each tube were then washed 10 times with 1 ml of 1× SSC containing 0.5% sodium dodecyl sulfate and 3 times with 1 ml of 10 mM Tris (pH 7.4)–2 mM EDTA at 65°C. Bound RNA was eluted by boiling filters in each tube for 2 min with 300 μl of water. The RNA in the supernatant was precipitated in ethanol with 1 μg of rabbit liver tRNA per filter. The precipitate was suspended in 2 μl of water per filter. The purity of the hybrid-selected mRNAs was measured by translating them in an mRNA-dependent rabbit reticulocyte lysate in the presence of [^{35}S]methionine and analyzing the ^{35}S-labeled products by electrophoresis on a 10% polyacrylamide gel (9). Fluorograms of the gels were scanned with a laser densitometer (21). The ratio of expression of the protein encoded by the selected mRNA to that of other viral proteins produced by the same preparation exceeded 1,000:1.

Components of in vitro replication system. A detailed description of the components constituting the replication system has been reported elsewhere (4). Reaction mixtures contained micrococcal nuclease-treated reticulocyte lysate, nucleoside triphosphates, creatine kinase, amino acids, salts, dithioerythritol, purified VSV DI nucleocapsids, 1 μCi of [^{3}H]UTP per μl, and 0.4 μCi of [^{35}S]methionine per μl in a final volume of 25 μl. Intracellular nucleocapsid templates were prepared from BHK cells coinfected for 13 h with VSV (multiplicity of infection = 2) and DI particles at a ratio which gave 99% inhibition of standard virus yield, as described previously (21). Virion-derived nucleocapsids were prepared as follows. DI particles released into the medium of the mixedly infected cultures were purified by velocity sedimentation in a 16 to 30% (wt/wt) glycerol gradient containing RSB (0.01 M Tris-hydrochloride, pH 7.4, 0.01 M NaCl, 1.5 mM $MgCl_2$). Membrane components were dissociated by treatment with 1.8% Triton X-100–0.43 M NaCl–0.6 mM dithioerythritol for 30 min at 30°C. The nucleocapsid component (virion-derived nucleocapsids) was purified by sucrose density gradient centrifugation exactly as described for intracellular nucleocapsids (21). Reactions were programmed for protein synthesis by the addition of either polyadenylated RNA isolated from VSV-infected cells or individual hybrid-selected VSV RNAs as indicated. Reactions were typically incubated for 3 h at 30°C.

Analysis of in vitro products. Samples (1 μl) of each reaction were analyzed by electrophoresis on 10% polyacrylamide gels containing sodium dodecyl sulfate as described by Laemmli (9). ^{35}S-labeled proteins were assayed by fluorography of dried gels. Unless otherwise indicated, the remainder of each reaction, after deproteinization with phenol and sodium dodecyl sulfate, was analyzed for ^{3}H-labeled RNA by electrophoresis on agarose-urea gels (22). Analysis of products by CsCl gradient centrifugation has been described previously (4).

RESULTS

Ability of N, but not NS or M, proteins individually to support VSV RNA replication by intracellular nucleocapsids. The protein requirement for synthesis of genome-length RNA was examined by programming the in vitro system with the individual hybrid-selected N, NS, or M mRNAs to determine whether N, NS, or M proteins, when translated individually in the presence of enzymatically active intracellular nucleocapsids, could promote the synthesis of genome-length RNA. Synthesis of viral RNA and proteins was examined simultaneously in each reaction by double labeling with [^{3}H]UTP and [^{35}S]methionine. Figure 1A shows the ^{3}H-labeled RNA products, and Fig. 1B shows the ^{35}S-labeled protein products of reactions programmed by varying amounts of total VSV mRNA (lanes 2 through 5) or of hybrid-selected N mRNA (lanes 6 through 8). Two additional reactions contained hybrid-selected NS mRNA (lane 9) or hybrid-selected M mRNA (lane 10). Synthesis of N protein alone was sufficient for the synthesis of genome-length RNA (Fig. 1). In addition, neither NS nor M proteins by themselves were able to support the synthesis of genome-length RNA.

By densitometry, the levels of genome synthesis and N protein expression were measured for each reaction containing total mRNA or N mRNA alone and plotted (Fig. 2). The amount of genome synthesis was directly proportional to the amount of N protein synthesis in both sets of reactions. A detailed quantitation of the absolute amounts of RNA and protein synthesized has been reported previously (21). Furthermore, all of the points fell on a single straight line, indicating that the efficiency of RNA replication for a given amount of N protein synthesis in reactions containing either all five VSV mRNAs or only N mRNA was equivalent. This finding suggested that synthesis of the other VSV proteins was not necessary for genome-length RNA synthesis, and that, when produced in the relative amounts shown in Fig. 1A, lane 2, proteins other than N did not affect the level of genome-length RNA synthesis.

In addition to genome-length RNA, faster-migrating RNAs were also made in vitro. The relative amount of these smaller RNAs varied with different nucleocapsid preparations. Since their synthesis depended on a source of N protein (Fig. 1) and they were nuclease resistant (data not shown), they were probably incomplete replicative products. The appearance of discrete bands rather than a continuum of smaller RNAs suggested that there may be sites on the template at which polymerization is attenuated or prematurely terminated.

Examination of the requirement for concurrent N protein synthesis during VSV RNA replication. The ability of N protein synthesized before the addition of nucleocapsid templates (presynthesized N) to support genome-length RNA synthesis was tested. A reaction programmed with N mRNA and containing no nucleocapsid templates was incubated for 45 min, and afterwards, anisomycin, a protein

synthesis inhibitor, was added with nucleocapsid templates. The incubation was continued for an additional 2 h and 15 min. The N protein made in the first 45 min and the RNA products made during the following 2 h and 15 min (Fig. 1A and B, lanes 11) were compared with those made in a 3-h reaction in which RNA and protein were synthesized concurrently (Fig. 1A and B, lanes 6 through 8). The results showed that presynthesized N protein could support DI RNA replication. However, when the level of genome-length

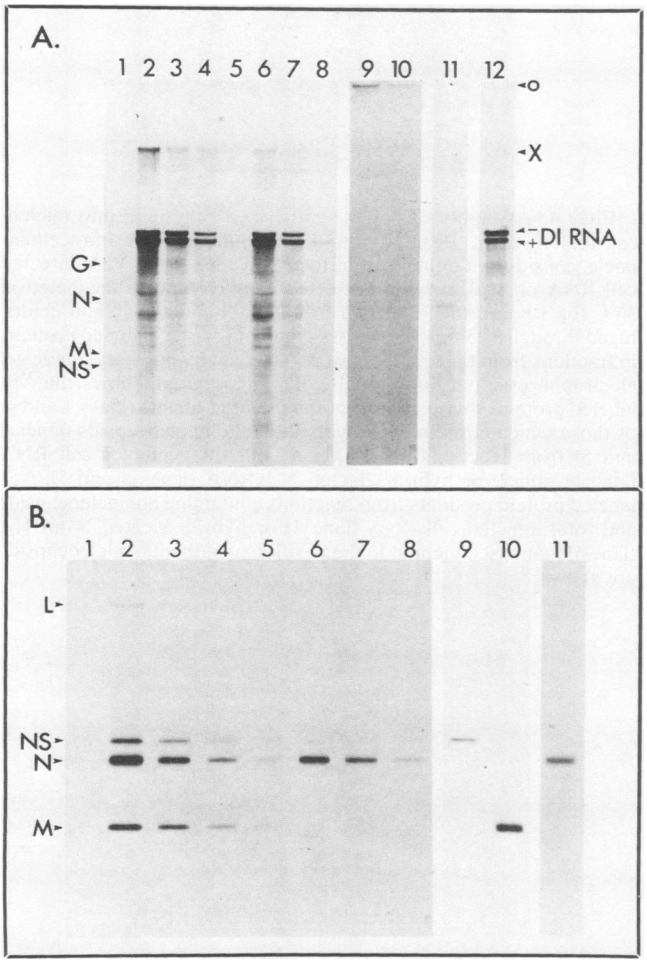

FIG. 1. Synthesis of genome-length RNA and viral proteins in vitro in reactions containing intracellular nucleocapsids and programmed with total VSV mRNA or individual mRNAs. Reactions contained both [^{3}H]UTP and [^{35}S]methionine and were incubated for 3 h at 30°C. Samples (1 μl) were analyzed by polyacrylamide gel electrophoresis for ^{35}S-labeled proteins (B). The remainder after deproteinization was analyzed for ^{3}H-labeled RNAs by electrophoresis on agarose-urea gels (4, 22) (A). RNA and protein products are shown from a reaction containing no added VSV mRNA (lane 1); from reactions containing 0.74 μg (lane 2), 0.185 μg (lane 3), 0.037 μg (lane 4), and 0.007 μg (lane 5) of total polyadenylated VSV mRNA; from reactions containing 5 μl (lane 6), 1.25 μl (lane 7), and 0.3 μl (lane 8) of hybrid-selected N mRNA; from a reaction containing 4 μl of hybrid-selected NS mRNA (lane 9); and from a reaction containing 4 μl of hybrid-selected M mRNA (lane 10). Products in lane 11 are from a reaction in which 4 μl of hybrid-selected N mRNA was translated for 45 min and then nucleocapsids and anisomycin were added for 2 h and 15 min. RNA purified from DI intracellular nucleocapsids labeled in vivo with [^{3}H]uridine is shown in lane 12. The positions of ^{3}H-labeled VSV mRNAs run in a parallel lane are indicated. The full-length RNA product (X) derived from a larger DI particle was also detected.

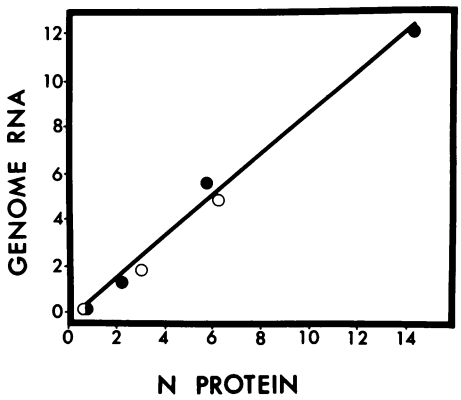

FIG. 2. Correlation of genome-length RNA synthesis and N protein expression. Fluorograms shown in Fig. 1 were analyzed by densitometry. Exposure times were chosen to give band intensities in the linear range (21). The relative absorbance units of ^{35}S-labeled N protein for a given reaction are plotted versus the total amount of genome-length RNA (plus and minus strands) synthesized in that reaction. Results are shown from reactions to which varying amounts of total VSV mRNA (●) or hybrid-selected N mRNA (○) were added. The line has been fitted with a correlation coefficient of 0.995 to the results from reactions containing total VSV mRNA, using a least-squares analysis.

RNA synthesis and the level of presynthesized N protein were measured, it was observed that the amount of genome-length RNA synthesized per unit of N protein in reactions containing only presynthesized N protein was not more than 25% of that expected based on results of reactions in which N protein and RNA were synthesized simultaneously.

Ability of N protein, alone, to support RNA replication by nucleocapsids from budded DI particles. We used the in vitro replication system to determine whether DI nucleocapsids purified from budded virions, i.e., virion-derived nucleocapsids, in contrast to intracellular nucleocapsids, required the synthesis of a viral protein(s) in addition to N protein to produce genome-length RNA. DI virion-derived nucleocapsids were purified away from membrane components (see above) to give templates consisting of negative-strand RNA in association with N, NS, and L proteins (data not shown). Reactions contained the reticulocyte lysate, extracellular nucleocapsids, [^{3}H]UTP, [^{35}S]methionine, and either total VSV mRNA or hybrid-selected N mRNA. Analysis of the RNA products showed that in the absence of added VSV mRNA, no genome-length RNA was made (Fig. 3, lane 4). When protein synthesis was programmed by adding total VSV mRNA, genome-length positive-strand RNA was synthesized from the negative-strand RNA template (Fig. 3, lane 5). The inhibition of protein synthesis by anisomycin completely blocked genome-length RNA synthesis (Fig. 3, lane 7). The addition of N mRNA alone resulted in the synthesis of genome-length positive-strand RNA (Fig. 3, lane 6). Thus, N protein alone fulfilled the protein synthesis requirement for RNA replication.

Quantitation of the levels of RNA replication (Fig. 3) and N protein synthesis (data not shown) in the reactions programmed with total VSV mRNA or N mRNA alone showed that equal amounts of N protein and equal amounts of genome-length RNA were made in the two reactions. Thus, no detectable stimulation of genome-length RNA synthesis occurred when viral proteins in addition to N were synthesized. In summary, VSV DI nucleocapsids, whether purified from budded DI virions or from infected cells, required only a source of N protein to synthesize genome-length RNA.

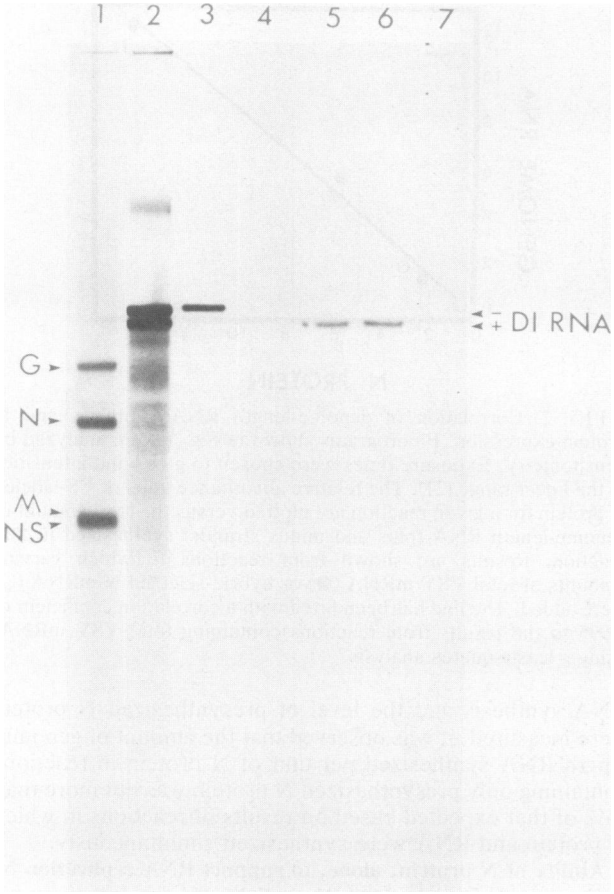

FIG. 3. Synthesis of genome-length RNA in vitro directed by nucleocapsids prepared from budded DI virions. Virion-derived nucleocapsids were used as templates for RNA synthesis in the in vitro replication system described in Fig. 1. ^{3}H-labeled RNA products, separated by agarose-urea gel electrophoresis, are shown from reactions containing no mRNA (lane 4), total VSV mRNA (lane 5), hybrid-selected N mRNA (lane 6), or total VSV mRNA plus 25 μM anisomycin (lane 7). Markers labeled with [^{3}H]uridine in vivo are total polyadenylated VSV mRNA (lane 1), genome RNA from purified intracellular nucleocapsids (lane 2), and genome RNA from virion-derived nucleocapsids purified in parallel with unlabeled template nucleocapsids (lane 3).

Production of nucleocapsids in the presence of only N protein and newly replicated RNA. In infected cells, newly replicated VSV RNA rapidly assembles with viral proteins to form nucleocapsids. These nucleocapsids render the RNA resistant to degradation by RNase (19) and band at a specific density in CsCl (1.30 g/cm^{3}). We determined whether N protein alone was sufficient for nucleocapsid assembly or whether other VSV proteins might be required for this process. To assay for nucleocapsid assembly in reactions programmed with only N mRNA, replication products were tested for (i) the presence of newly synthesized N protein, (ii) nuclease resistance, and (iii) the ability to band in CsCl at the density of authentic nucleocapsids.

(i) Intracellular nucleocapsids were isolated from reactions incubated with either total VSV mRNA or N mRNA alone by velocity sedimentation in sucrose gradients. ^{35}S-labeled protein products which remained at the top of the gradient and those which sedimented with nucleocapsids were analyzed by polyacrylamide gel electrophoresis (Fig.

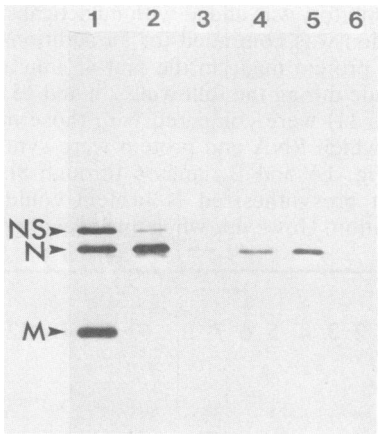

FIG. 4. Assembly of newly synthesized N protein into nucleocapsids in vitro. Reaction mixtures containing DI intracellular nucleocapsids and either 1 μl of total polyadenylated VSV-infected cell RNA or 3 μl of hybrid-selected N mRNA were incubated at 30°C for 3 h and then adjusted to 0.05% Nonidet P-40 and centrifuged through a 15 to 30% sucrose gradient (21). ^{35}S-labeled proteins in fractions from the gradients were analyzed by polyacrylamide gel electrophoresis and fluorography. The fluorograph shows the ^{35}S-labeled proteins that remained at the top of gradients (lanes 1 and 4) or those which cosedimented with authentic nucleocapsids (lanes 2 and 5) from reactions programmed with total infected-cell RNA (lanes 1 and 2) or hybrid-selected N mRNA (lanes 4 and 5). ^{35}S-labeled protein products from reactions containing no nucleocapsids and total infected-cell RNA (lane 3) or hybrid-selected N mRNA (lane 6) that cosedimented to the position of authentic nucleocapsids are shown.

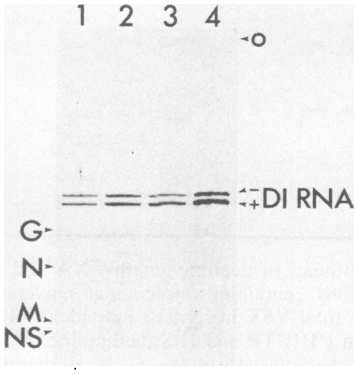

FIG. 5. Nuclease resistance of RNA products produced in reactions containing intracellular nucleocapsids programmed with hybrid-selected N mRNA. Reaction mixtures containing DI nucleocapsids and either 2 μl of total polyadenylated RNA (lanes 1 and 2) or 4 μl of hybrid-selected N mRNA (lanes 3 and 4) were incubated at 30°C for 3 h. ^{3}H-labeled polyadenylated RNA isolated from VSV-infected cells was then added to serve as an internal control for the nuclease treatment, and the reactions were divided into two equivalent fractions. $CaCl_2$ was added to one fraction to give an additional 3.6 mM Ca^{2+}, micrococcal nuclease was added to 20 μg/ml, and the reaction was incubated for 15 min at 20°C (lanes 1 and 3). The second fraction was incubated likewise but in the absence of added $CaCl_2$ and micrococcal nuclease. Afterwards, both halves were deproteinized and the RNA products were analyzed as described for Fig. 1.

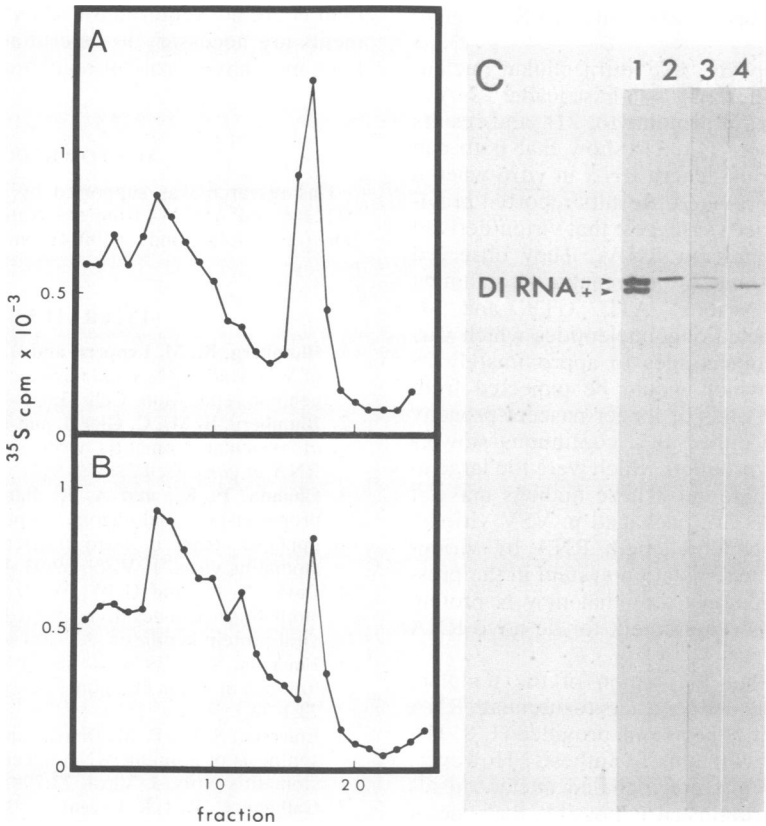

FIG. 6. Equilibrium density gradient centrifugation of RNA and protein products made in the presence of N mRNA alone. In vitro reaction mixtures programmed with N mRNA alone and containing DI intracellular nucleocapsids (A) or virion-derived nucleocapsids (B) were banded in CsCl as described previously (4). Gradient fractions were assayed for ^{35}S-labeled protein by trichloroacetic acid precipitation. Radiolabeled products contained in the peak at nucleocapsid density (fractions 16 through 18) were concentrated by pelleting and extracted with phenol. (C) The purified, CsCl-banded, ^{3}H-labeled RNAs made in reactions containing intracellular nucleocapsids (lane 3) or virion-derived nucleocapsids (lane 4) and N mRNA alone were analyzed by agarose-urea gel electrophoresis. Genome RNA markers were from DI intracellular (lane 1) or virion-derived (lane 2) nucleocapsids labeled with [^{3}H]uridine in vivo.

4). Nucleocapsids recovered from a reaction programmed with total VSV mRNA contained ^{35}S-labeled N and NS proteins (Fig. 4, lanes 1 and 2) as we previously reported for standard VSV (12). L protein was not synthesized in detectable amounts in this experiment. Results obtained from the reaction programmed with N mRNA alone (Fig. 4, lanes 4 and 5) showed that newly synthesized N protein assembled into nucleocapsids in the absence of any other newly synthesized viral proteins.

(ii) Genome-length RNAs synthesized in a reaction containing intracellular nucleocapsids were treated with micrococcal nuclease in the reaction mixture after the addition of Ca^{2+} and ^{3}H-labeled purified mRNAs as internal controls for nuclease activity. Genome-length RNA made in the reaction with N mRNA alone, like that made in the control reaction with total VSV mRNA (see also reference 21), was resistant to digestion under conditions in which mRNA in the same preparation was completely digested (Fig. 5).

(iii) Additionally, reaction mixtures programmed with only N mRNA and with either DI intracellular or virion-derived nucleocapsids were assayed for the presence of products which banded at the position of nucleocapsids in CsCl gradients. A large proportion of the newly synthesized N protein banded at the position of authentic nucleocapsids (Fig. 6A and B). Neither free N protein nor RNA banded at this position. ^{3}H-labeled RNA isolated from the peaks at nucleocapsid density included negative- and positive-strand genome-length RNA or positive-strand genome-length RNA for reactions containing intracellular or extracellular nucleocapsids, respectively (Fig. 6C, lanes 3 and 4). An identical pattern of CsCl-banded RNA products was obtained from reactions programmed with total VSV mRNA (data not shown). Therefore, we conclude that the assembly of stable, nuclease-resistant nucleocapsids is solely dependent upon N protein.

DISCUSSION

We have used hybrid-selected VSV mRNAs in an in vitro replication system to determine which of the viral proteins are required for the synthesis and encapsidation of VSV genome-length RNA. The synthesis of N protein alone by the reticulocyte lysate in reactions containing either intracellular or virion-derived nucleocapsid templates supported the synthesis of DI genome-length RNAs. In our reactions, N protein alone was as efficient in supporting VSV replication as the same amount of N protein in reactions containing all five viral proteins. In addition, N protein, by itself, was able to associate with genome-length RNA to yield stable, nuclease-resistant, nucleocapsid-like structures. The results presented demonstrate that the major nucleocapsid protein, N, alone both satisfies the viral protein requirement for VSV

RNA replication and promotes the assembly of VSV nucleocapsids.

Virion-derived nucleocapsids, like intracellular nucleocapsids, are capable of synthesizing a 46-base leader RNA in vitro without a source of viral proteins (6, 21), and results presented here and elsewhere (13, 21) show that both can direct the synthesis of genome-length RNA in vitro when a source of viral proteins is provided. Results reported previously by Chanda and Banerjee (3) suggest that virion-derived nucleocapsids contain no nascent RNAs. They observed that "incomplete" in vitro transcription reactions containing detergent-disrupted VSV virions, ATP, GTP, and [α-^{32}P]CTP produced radiolabeled oligonucleotides which varied in length from a few nucleotides to approximately 28 bases. Longer products, which would be expected from limited elongation at the 3′ ends of longer nascent progeny RNAs, were not detected either as a continuous slower-migrating background or as products which were too large to enter the 20% polyacrylamide gel. These findings make it unlikely that nascent RNAs are packaged in VSV virions. Hence, the synthesis of genome-length RNA by virion-derived nucleocapsids in our replication system in the presence of N protein alone demonstrates that new N protein fulfills the protein synthesis requirement for de novo RNA replication.

It has been proposed that the region of the template immediately following the leader gene acts to attenuate RNA synthesis unless a source of N protein is provided (1, 8, 11). Our results are consistent with this hypothesis. However, since the in vitro reaction mixture included nucleocapsids which carried NS and L proteins, it is possible that one of these proteins might perform a unique function at the postulated attenuation site. In any case, we have proven that a source of newly synthesized viral protein other than N is not necessary to obtain read-through of any proposed attenuation site.

The data presented here have also shown that VSV does not require the concurrent synthesis of N protein for RNA replication. The reason for the decreased efficiency of N protein synthesized in reactions before the addition of nucleocapsid templates in supporting RNA replication is unknown. It is possible that "premade" N protein self-assembles (2) in our system, thereby reducing the concentration of N protein available to drive replication. Alternatively, N protein may undergo post-translational modification such that it is capable of promoting RNA replication for only a short period of time after it is synthesized.

Previously, Blumberg et al. (2) used reconstitution experiments to demonstrate that N protein was able to assemble with leader RNA into nucleocapsid-like structures in the absence of other viral proteins. Our results indicate that N protein, by itself, is also able to assemble with genome-length RNA into structures that are indistinguishable from VSV nucleocapsids. We cannot rule out the possibility that small amounts of viral proteins in addition to N are necessary for nucleocapsid assembly and that they are being provided to the in vitro system by the nucleocapsid templates added into the reactions to direct RNA synthesis.

In summary, we have used an in vitro system to define the requirement for the synthesis of proteins in VSV RNA replication. Our results show that nucleocapsid templates which contain the enzyme components L and NS require only a source of new N protein to synthesize genome-length RNA. Thus, VSV RNA replication does not require the synthesis of a new core polymerase, L, or phosphoprotein, NS. Although these data show that new viral proteins other than N are not required for RNA replication, further experiments are necessary to determine whether other VSV proteins may have a role in regulation of this process.

ACKNOWLEDGMENTS

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