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Cell-Free Synthesis and Assembly of Vesicular Stomatitis Virus Nucleocapsids

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The association of newly synthesized vesicular stomatitis virus proteins into nucleocapsid structures was examined in a cell-free system that supports concurrent viral protein synthesis, transcription, and RNA replication. The vesicular stomatitis virus proteins synthesized by this system associated with the newly replicated RNA to form structures that banded in CsCl gradients with marker nucleocapsids. In reactions lacking nucleocapsid templates to program RNA synthesis, the newly synthesized proteins did not associate into nucleocapsid structures. The newly synthesized proteins associated with nucleocapsids were analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate after separation from non-associated proteins by chromatography on Bio-Gel A15M agarose columns. The results of this analysis showed that newly synthesized L, NS, and N proteins associated into nucleocapsids in the in vitro system. In addition, a small amount of newly synthesized M protein was stably bound to the nucleocapsids. The molar ratio of the associated, newly synthesized proteins was 2:350:1,000:10 (L:NS:N:M). More than 90% of the newly synthesized NS protein that associated with nucleocapsids in vitro was of the NS2 subspecies, as assayed by DEAE-cellulose column chromatography. The stability of the association of the newly synthesized proteins with nucleocapsids in the system mimicked that of the association of viral proteins with nucleocapsids from infected cells as measured by salt sensitivity. These data indicate that nucleocapsids were assembled from newly synthesized proteins within our in vitro system and that the molar ratio of assembled proteins was similar to that observed for virion nucleocapsids.

The nucleocapsid of vesicular stomatitis virus (VSV) consists of a negative-stranded RNA genome (molecular weight, 4×10^6) and three virus-specified proteins: (i) N, the major nucleocapsid protein (ii) NS, a phosphoprotein, and (iii) L, a component of the polymerase (8, 19). N is the most abundant protein of the VSV nucleocapsid (1,000 to 2,000 copies per genome) (1, 2, 20), and its association with the genome along with NS and L proteins renders the RNA resistant to RNases. Mellon and Emerson (16) have shown that the L protein requires the phosphoprotein NS to associate with the N protein-coated RNA genome.

The VSV nucleocapsid serves as the template for two distinct RNA synthetic processes which differ in their requirement for protein synthesis. Transcription of the VSV genome to yield leader and the five monocistronic mRNAs can occur in infected cells in the absence of protein synthesis (13, 28). Replication of the genome to produce full-length negative-stranded RNA, however, is a process that is dependent on concurrent protein synthesis (22, 28). It is not known which of the virus-specified protein(s) are required for replication and whether the requirement is for catalytic or stoichiometric amounts of these proteins.

To examine the protein requirement for VSV RNA synthesis, we developed a defined in vitro system which carries out the synthesis of both VSV negative-stranded genome RNA and the five viral mRNAs (6, 7). The system consists of (i) VSV intracellular nucleocapsids to serve as templates for RNA synthesis, (ii) a micrococcal nuclease-treated reticulocyte lysate, and (iii) purified VSV mRNAs to program protein synthesis. The synthesis of VSV negative-stranded, genomic-size RNA in the system is dependent on the level of concurrent protein synthesis. The negative-stranded RNA products synthesized in this in vitro reaction band in CsCl at the same buoyant density as nucleocapsids synthesized in vivo, and they are at least partially resistant to digestion by RNase (7). These two results indicate that the newly synthesized negative-strand-

ed RNA is not present as a naked RNA molecule and suggest that the newly synthesized viral proteins required to obtain negative-stranded RNA synthesis may be able to associate with the RNA to form nucleocapsid structures de novo.

In the work described here, we investigated the possibility that the viral proteins synthesized concurrently with RNA replication in the in vitro system can associate into nucleocapsid structures. The results presented show that the newly synthesized proteins N, NS, and L associate to form structures which have the same stability characteristics and protein components as nucleocapsids synthesized in vivo. We conclude that in this system, VSV nucleocapsid assembly occurs in vitro with newly synthesized proteins.

MATERIALS AND METHODS

Cell culture and virus. Baby hamster kidney cells (BHK-21/13) were maintained in Eagle minimal essential medium containing 5% heat-inactivated calf serum. VSV (Indiana serotype) was propagated in BHK cells as described previously (28). Cells were infected at 2 to 4 PFU/cell, maintained in the presence of 4 μg of actinomycin D per ml, and harvested at 5 to 6 h postinfection for isolation of VSV mRNA or intracellular nucleocapsids.

Components of in vitro replication system. The components of the replication system have been described by Davis and Wertz (7). VSV mRNAs used to program in vitro protein synthesis were recovered from infected cells by centrifugation of cytoplasmic extracts on CsCl gradients (7). The preparation of intracellular nucleocapsids as templates for RNA synthesis has been described (7). Briefly, after disruption of infected cells by Dounce homogenization, cellular debris was pelleted by centrifugation at 10,000 rpm for 10 min at 4°C in a Sorvall SS34 rotor. The supernatant was overlaid onto 3-ml 15 to 30% sucrose gradients in a buffer of 3 mM Tris-hydrochloride (pH 8.1)–3 mM magnesium acetate–66 mM NH_4Cl–14 mM KCl–2 mM dithioerythritol (DTE) and centrifuged in an Beckman SW50.1 rotor at 45,000 rpm for 2 h at 4°C. Nucleocapsid-containing pellets were resuspended in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.7)–2 mM DTE–10% glycerol at a concentration representing 10^6 infected cells per μl. Reactions were carried out in a final volume of 50 μl and contained 70% by volume micrococcal nuclease-treated rabbit reticulocyte lysate (7), approximately 5 μg of VSV mRNAs, and 12% by volume intracellular nucleocapsids. In addition, each reaction contained 50 mM HEPES (pH 7.7), 10 mM creatine phosphate, 1 mM ATP, 0.6 mM each CTP and GTP, 0.1 mM UTP, 0.05 mM each of the 20 amino acids, except for methionine, which was 12.5 μM, 2 mM magnesium acetate, 2 mM DTE, 66 mM NH_4Cl, 14 mM potassium acetate, 2 μg of rabbit liver tRNA, and 50 μCi of [^{35}S]methionine. Reaction mixtures were incubated for 90 min at 30°C. Incorporation of [^{35}S]methionine into protein was assayed by counting radioactivity present in trichloroacetic acid (TCA) precipitates of 2-μl samples removed during incubation. Precipitates were heated to 85°C for 15 min to hydrolyze tRNAs labeled with [^{35}S]methionine.

Salt-detergent treatment and column chromatography of nucleocapsids. Samples for analysis by molecular exclusion column chromatography were prepared in the following manner. Reaction mixtures were diluted to 500 μl with column buffer (10 mM HEPES, 2 mM $MgCl_2$, 2 mM DTE, 66 mM NH_4Cl, 14 mM potassium acetate [pH 7.6]) and adjusted to 0.5% Nonidet P-40 (NP-40). Alternatively, samples were diluted with 250 μl of water and an equal volume of either 2× LSS buffer (0.864 M NaCl, 18.7% glycerol, 1.2 mM DTE, 3.74% Triton X-100) or 2× HSS buffer (1.44 M NaCl, 18.7% glycerol, 1.2 mM DTE, 3.74% Triton X-100) and then incubated at 37°C for 45 min. The samples were applied directly to a Bio-Gel A15M column (0.9 by 60 cm) equilibrated with column buffer. Chromatography was performed with column buffer at a flow rate of 10 ml/h. Fractions (1 ml each) were collected, and 35-μl portions were assayed by counting radioactivity present in TCA precipitates.

Electrophoresis and densitometry. ^{3}H- or ^{35}S-labeled proteins were analyzed by electrophoresis on sodium dodecyl sulfate (SDS)-containing polyacrylamide gels as described by Laemmli and Faure (15). The resolving and stacking gels were of 10% and 3 or 5% acrylamide, respectively. Alternatively, radiolabeled proteins were analyzed by electrophoresis on 10% polyacrylamide gels containing 7 M urea and 0.1% SDS by the method of Kingsford and Emerson (14). After electrophoresis, gels were impregnated with 2,5-diphenyloxazole (PPO), dried, and exposed to preflashed Kodak XAR-5 or Cronex 4 film (27). In some cases, resultant fluorograms were scanned at 632.8 mm to quantitate the intensity of bands with an LKB 2202 Ultrascan densitometer interfaced with an Apple II computer.

CsCl centrifugation. Products of the replication system were analyzed on 20 to 40% (wt/wt) CsCl gradients as described by Davis and Wertz (7). Samples (5 to 10 μl) taken from reaction mixtures were treated with 0.5% NP-40 before being overlaid onto CsCl gradients. The position of the nucleocapsids in the gradients was detected by visual observation. In some cases, fractions were desalted in the following manner. A 5-ml disposable polypropylene tube was bottom punctured and filled to a packed volume of 4 ml with Sepharose 6B over a layer of glass wool. After the application of 0.5 ml of sample to the column, the tube was placed in a 15-ml Corex tube and centrifuged at 100 × *g* in a Sorvall SS34 rotor for 2 min. The sample which eluted into the Corex tube was checked for the absence of salt by refractometry.

NS protein analysis. Reaction mixtures were diluted to a total of 5 ml with 10 mM Tris-hydrochloride (pH 7.2) and centrifuged at 47,500 rpm in a Beckman SW50.1 rotor for 75 min at 4°C to pellet nucleocapsids. The nucleocapsids were resuspended in 1.0 ml of 2× HSS buffer diluted with an equal volume of 10 mM Tris-hydrochloride (pH 7.2) and incubated at 37°C for 45 to 60 min. After dilution of the sample to 5 ml with 10 mM Tris-hydrochloride (pH 7.2), the nucleocapsids were pelleted again. The NS-containing supernatant (nucleocapsid-associated NS) was diluted with 10 mM Tris-hydrochloride (pH 7.2) to a final concentration of 0.1 M NaCl or less and chromatographed on Whatman CF-11 phosphocellulose columns (1.5 by 3.75 cm) as

described by Kingsford and Emerson (14). The flow-through fraction was subsequently analyzed by DEAE-cellulose (Whatman DE52) column chromatography as follows. NS preparations applied to DEAE columns (45 by 0.9 cm) were washed with 20 ml of 0.1 M NaCl in DEAE column buffer (0.2% Triton X-100, 0.19 mM DTE, 10 mM Tris-hydrochloride [pH 7.2]) before eluting with a linear gradient of 0.22 to 0.35 M NaCl in DEAE column buffer at a flow rate of 12 ml/h. The position at which NS1 and NS2 eluted from the column was ascertained by analysis of the chromatographic profile of ^{32}P-labeled NS protein prepared from virions by using HSS buffer.

RESULTS

Analysis of protein products by centrifugation in CsCl. The initial analysis of the products synthesized in the VSV replication system suggested that the negative-stranded RNAs produced in the system were in the form of RNA-protein complexes (7). The RNAs were partially RNase resistant, and they banded in CsCl gradients at a density of 1.3 g/cm^3, the same position as nucleocapsids isolated from infected cells. These data indicated that the negative-stranded RNA synthesized in the system was associated with protein. Thus, we investigated the possibility that the VSV proteins synthesized concurrently with the RNA were associating to form nucleocapsid structures. Specifically, we designed experiments to demonstrate whether or not (i) newly synthesized proteins associated into structures that were stable in CsCl and (ii) these structures cobanded in CsCl gradients with marker nucleocapsids.

The possible association of ^{35}S-labeled proteins into nucleocapsid structures in the in vitro replication system was analyzed initially by centrifugation of products in 20 to 40% CsCl gradients. From 10 to 30% of the total ^{35}S-labeled proteins synthesized in the system banded in the CsCl gradient exactly at the position of the nucleocapsid marker (Fig. 1, top). Previous work has shown that this is also the position at which all negative-stranded VSV RNA synthesized in the system bands in CsCl (7). The VSV-specific proteins synthesized in reactions that lacked nucleocapsid templates (and hence were not synthesizing RNA) did not band in CsCl gradients at this position, but were present predominantly in the top one-half of the gradients (Fig. 1, bottom). These data showed (i) that the proteins synthesized in reactions containing nucleocapsid templates associated into structures that had the same buoyant density as nucleocapsids produced in vivo and (ii) that the VSV proteins synthesized in reactions lacking nucleocapsid templates did not form structures that banded at the position of nucleocapsids.

To test the possibility that the newly synthesized proteins associated with preexisting nucleocapsid templates, proteins were synthesized in a reaction lacking templates; afterwards, templates were added, and the reaction mixture was incubated on ice for 30 min. Analysis of the products by CsCl gradient centrifugation (Fig. 1, bottom) showed that little newly synthesized protein banded at the position of nucleocapsids. This result suggested that the association of ^{35}S-labeled proteins with nucleocapsids in the system did not represent the nonspecific aggregation of newly synthesized proteins with preexisting nucleocapsid templates.

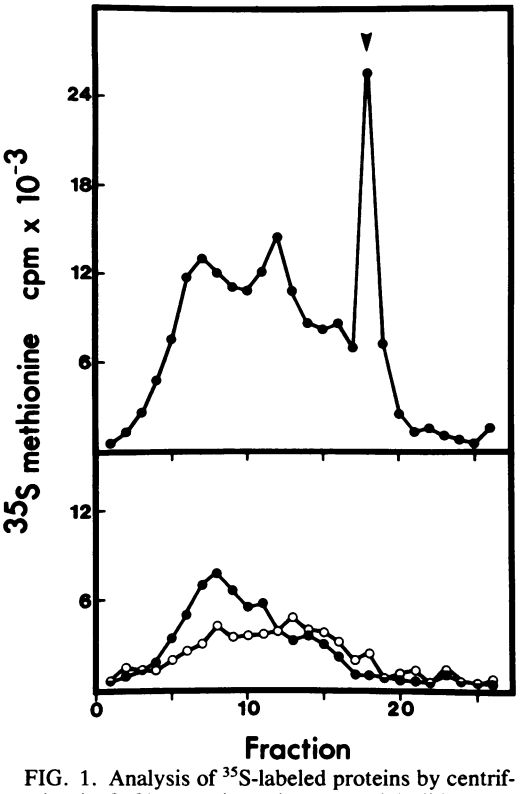

FIG. 1. Analysis of ^{35}S-labeled proteins by centrifugation in CsCl. Reaction mixtures which did (upper panel) or did not (lower panel [●]) contain nucleocapsid templates were incubated for 90 min at 30°C. Samples (7 μl) of each reaction mixture were treated with 0.5% NP-40 and analyzed by centrifugation in gradients of 20 to 40% CsCl (wt/wt) for 16 h at 33,000 rpm in a Beckman SW40 rotor at 4°C (7). After fractionation, portions (35 μl) of each fraction (0.5 ml) were precipitated with TCA and assayed for radioactivity. The bottom of each gradient is to the right, and the position of banded nucleocapsids is denoted by an arrow. In a separate experiment, after incubation for 60 min of a standard reaction mixture lacking intracellular nucleocapsid templates, nucleocapsids were added to the reaction mixture, and incubation was continued for an additional 30 min at 0°C. A sample of the reaction mixture was then treated with NP-40, and the ^{35}S-labeled products were analyzed by CsCl gradient centrifugation (lower panel [○]) as described above.

The total ^{35}S-labeled protein synthesized in the in vitro system and the fraction that banded in the CsCl gradient at the position of nucleocapsids were analyzed by electrophoresis in a 10% polyacrylamide gel (Fig. 2). The results show that although all five of the VSV proteins were synthesized in the in vitro system, only the VSV N protein was observed to band at the position of the marker nucleocapsids. This result is identical to that obtained with nucleocapsids made in vivo, for which it has been shown that the high concentration of salt present in CsCl gradients dissociates the less tightly bound NS and L proteins from the nucleocapsids (18).

In summary, these data demonstrate (i) that newly synthesized N protein is able to assemble into structures that coband with nucleocapsids in CsCl gradients, (ii) that the association of newly synthesized N protein with nucleocapsids in vitro is as stable in high concentrations of salt as the association of N protein with nucleocapsids made in infected cells, (iii) that the newly synthesized proteins by themselves are not able to aggregate into structures that band at the position of marker nucleocapsids, and (iv) that proteins do not associate with preexisting nucleocapsids in the absence of RNA synthesis into structures that are stable to high concentrations of salt. These results, taken together with the finding that newly synthesized negative-stranded RNA is RNase resistant and bands at the density of nucleocapsids in CsCl gradients, indicate that nucleocapsids are being formed de novo in the replication system.

Isolation of nucleocapsids by molecular exclusion chromatography. Having demonstrated that N protein could associate to form stable structures that cobanded with nucleocapsids, we wished to determine whether these structures contained the full complement of nucleocapsid proteins. Since CsCl dissociates all but N protein from nucleocapsids, an alternative method of isolating these structures was developed. The use of column chromatography for analysis of nucleocapsids was advantageous because it did not require the high osmotic pressures or salt concentrations of sucrose or CsCl gradients. For analysis of VSV nucleocapsids, a column containing a matrix of Bio-Gel A15M agarose, which has a molecular exclusion limit of 1.5×10^7 daltons, was used. It was expected that VSV nucleocapsids would elute in the void volume, since their size exceeds 10^8 daltons (based on the number and sizes of proteins estimated to be associated with the VSV nucleocapsid [2] and the size of the genome). In contrast, VSV mRNAs, mRNA-protein complexes, and all of the VSV proteins should be included in the column matrix.

Several experiments were performed to test

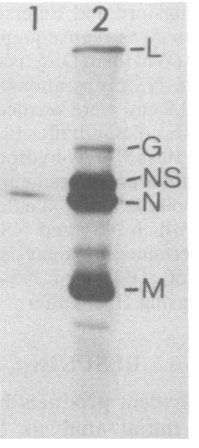

FIG. 2. Electrophoresis of ^{35}S-labeled proteins associated with nucleocapsids. ^{35}S-labeled proteins banding at the position of nucleocapsids in a CsCl gradient (Fig. 1) were desalted and then analyzed by electrophoresis on a 10% polyacrylamide gel containing SDS (15). The gel was impregnated with PPO, dried, and exposed to preflashed Kodak XAR-5 film (27). The fluorogram shows ^{35}S-labeled proteins associated with nucleocapsids (lane 1) versus ^{35}S-labeled proteins applied to the gradient (lane 2).

the predictions that (i) nucleocapsids would elute in the void volume of the column and (ii) no free proteins or mRNA-protein complexes would elute in the void volume, but rather would be included by the column matrix. To test the hypothesis that nucleocapsids would elute in the void volume, a sample of the peak fractions from a CsCl gradient containing ^{35}S-labeled protein associated with nucleocapsids synthesized in vitro was chromatographed on a Bio-Gel A15M column. The profile of material eluting from the column is shown in Fig. 3. The ^{35}S-labeled nucleocapsids eluted as a single peak at the position of the void volume, which contained approximately 100% of the material applied to the column. Similarly, when intracellular nucleocapsids synthesized in vivo in the presence of ^{14}C-labeled amino acids were applied to the column, essentially all radioactive material eluted in the void volume. These data demonstrated that VSV nucleocapsids are not included by the Bio-Gel A15M column, but rather elute entirely in the void volume as a single peak.

To test where free proteins and viral mRNA-protein complexes eluted from the Bio-Gel A15M column, ^{35}S-labeled proteins produced in a reaction mixture to which no nucleocapsid templates had been added were chromatographed on the column. The results are presented in Fig. 3 and show that less than 5% of the ^{35}S-labeled material recovered from the column eluted at the position of the nucleocapsids. Anal-

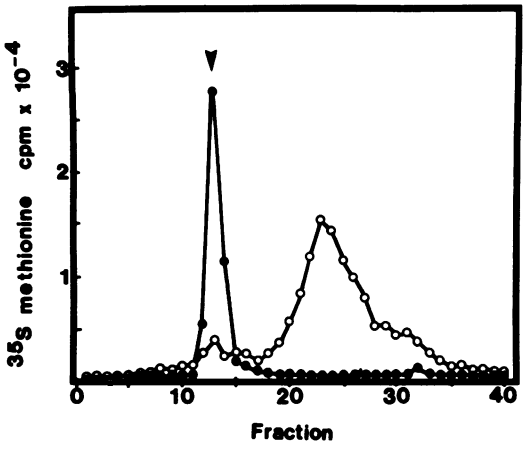

FIG. 3. Isolation of VSV nucleocapsids by molecular exclusion chromatography. CsCl gradient fractions containing ^{35}S-labeled nucleocapsid material were chromatographed on a Bio-Gel A15M agarose column (●). Seven microliters of a reaction mixture containing no nucleocapsid templates was chromatographed on the column (○). Samples (35 μl) of each 1.2-ml column fraction were assayed for radioactivity present in TCA-precipitable material. The position at which marker nucleocapsids eluted is denoted by the arrow.

ysis of the small amount of ^{35}S-labeled protein which eluted in the void volume by polyacrylamide gel electrophoresis indicated that minor amounts of all of the VSV proteins were present (data not shown). These data demonstrate that the Bio-Gel A15M column provides a useful method for the separation of nucleocapsids from non-associated protein. Therefore, this technique was used in subsequent experiments to separate nucleocapsids and their associated proteins from non-associated proteins.

Analysis of ^{35}S-labeled proteins associated with nucleocapsids. ^{35}S-labeled proteins associated with nucleocapsids produced in the replication system were separated from non-associated proteins by molecular exclusion column chromatography as described above. Samples of reaction mixtures in which proteins were labeled with [^{35}S]methionine were treated with 0.5% NP-40 before chromatography on Bio-Gel A15M columns. A representative profile of radioactivity eluting from the column is shown in Fig. 4. This and other similar experiments indicate that 40 to 60% of the ^{35}S-labeled protein recovered from the column eluted in the void volume and therefore was associated with nucleocapsids. Thus, a significant percentage of the total ^{35}S-labeled protein synthesized in vitro associates with nucleocapsids in this replication system.

Experiments were done to test whether the ^{35}S-labeled proteins eluting with the nucleocapsids could be a product of overloading of the Bio-Gel A15M column. Samples of the column fractions containing ^{35}S-labeled nucleocapsid material were passed back through the Bio-Gel A15M column. Only a single peak of ^{35}S-labeled protein was found to elute from the column under these conditions, and this was at the position of the void volume (data not shown). Therefore, the conditions used here for chromatography did not overload the column.

The ^{35}S-labeled proteins associated with nucleocapsids and isolated by molecular exclusion chromatography were analyzed by electrophoresis on 10% polyacrylamide gels containing SDS. A fluorogram of a gel from a representative experiment is shown in Fig. 5. The ^{35}S-labeled VSV proteins L, NS, and N synthesized in the replication system were associated with the nucleocapsids (lane 2). These three proteins are also components of intracellular nucleocapsids synthesized in vivo (25). Thus, these data provide evidence that the viral proteins synthesized in this system assembled into structures containing the same viral proteins as nucleocapsids made in vivo. In addition to NS, N, and L proteins, a small amount of M protein was also associated with the nucleocapsids isolated from our replication system. The quantitation of these proteins is described below.

Analysis of NS protein associated with nucleocapsids in vitro. The NS protein contained in virion nucleocapsids can be dissociated by treatment with high concentrations of salt and detergent and separated into two subspecies, NS1 and NS2, by chromatography on DEAE-cellulose (14). The major NS subspecies present in

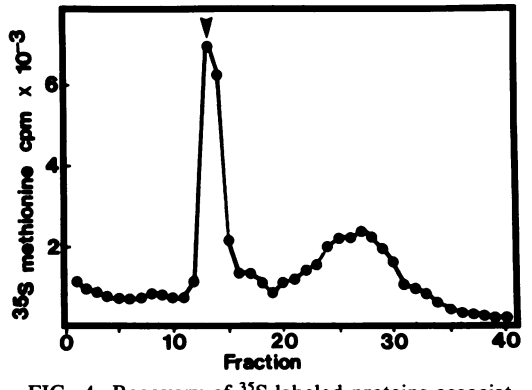

FIG. 4. Recovery of ^{35}S-labeled proteins associated with nucleocapsids by column chromatography. Seven microliters of a 50-μl reaction mixture was treated with 0.5% NP-40 as described in the text and analyzed by chromatography on a Bio-Gel A15M agarose column. Samples (35 μl) of each of the 1.2-ml fractions were assayed for TCA-precipitable radioactivity. The arrow denotes the position at which marker nucleocapsids elute.

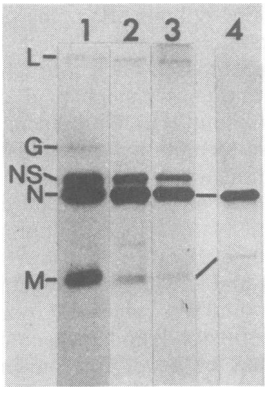

FIG. 5. Identification of ^{35}S-labeled proteins associated with nucleocapsids. Seven microliters of a standard reaction mixture was treated with 0.5% NP-40 (lane 2), 0.43 M NaCl (lane 3), or 0.72 M NaCl (lane 4) and Triton X-100, and nucleocapsids were recovered by chromatography on a Bio-Gel A15M agarose column. Fractions containing ^{35}S-labeled proteins associated with nucleocapsids were analyzed by electrophoresis on 10% polyacrylamide gels containing SDS. Lane 1 is a sample of the unfractionated reaction mixture. The proteins of lane 4 were subjected to electrophoresis on a gel separate from that shown in lanes 1, 2, and 3. The identification of proteins in lane 4 was determined by coelectrophoresis of a sample of the unfractionated reaction mixture in a parallel lane; nucleocapsid-associated M protein comigrated exactly with marker M protein in both gels.

virion nucleocapsids is NS2, shown to be the more active species in supporting VSV RNA synthesis in reconstituted in vitro transcription reactions (14). The composition of the ^{35}S-labeled NS protein, which associates with nucleocapsids in this replication system, was assayed in the following manner. ^{35}S-labeled nucleocapsids from reaction mixtures of the replication system were separated from soluble components by ultracentrifugation, resuspended, incubated in the presence of 0.72 M NaCl and Triton X-100 to release NS proteins, and pelleted again by ultracentrifugation. The NS-containing supernatant was diluted with 10 mM Tris-hydrochloride (pH 7.2) to a final concentration of less than 0.1 M NaCl and chromatographed on a phosphocellulose column. Afterwards, the flow-through fraction which contained NS protein was applied to a DEAE-cellulose column and eluted with an NaCl gradient. The chromatographic profile of a representative experiment is shown in Fig. 6. In this experiment, two peaks of nucleocapsid-associated ^{35}S-labeled NS protein were detected by DEAE-cellulose chromatography. The peaks were identified as NS1 and NS2 protein based on the coelution of the second peak with ^{32}P-labeled NS2 protein dissociated from intact virions. The NS1 protein peak was always much smaller than the NS2 protein peak, representing less than 10% of the amount of NS2 protein present. In summary, these results indicate that the NS2 subspecies, which is the predominant form of NS protein associated with virion nucleocapsids, is produced in vitro and is the major form of NS protein found in association with nucleocapsids isolated from the replication system.

The proteins NS1 and NS2, when isolated from virion nucleocapsids, can each be resolved into two electrophoretically distinct forms, i.e., NS_{top} and NS_{bottom}, on polyacrylamide gels containing urea and SDS (14). To test for the presence of NS_{top} and NS_{bottom} components with nucleocapsids from the replication system, ^{35}S-labeled proteins associated with nucleocapsids were electrophoresed on 10% polyacrylamide gels containing 7 M urea and 0.1% SDS. As seen in Fig. 7, both NS_{top} (NS_T) and NS_{bottom} (NS_B) are present. Since greater than 90% of the NS protein associated with the nucleocapsids from the replication system was NS2, both NS_{top} and NS_{bottom} are derived from the NS2 subspecies.

Stability of the association of ^{35}S-labeled protein with nucleocapsids. The stability of the association of ^{35}S-labeled proteins with nucleocapsids in the replication system was compared to

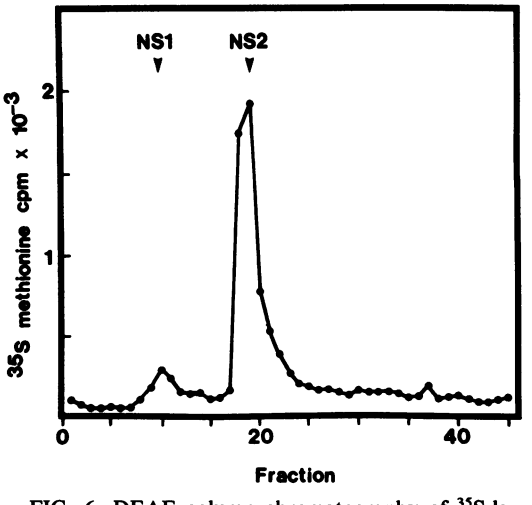

FIG. 6. DEAE column chromatography of ^{35}S-labeled NS protein associated with nucleocapsids. ^{35}S-labeled NS protein was dissociated from nucleocapsids as described in the text. ^{35}S-labeled NS was fractionated by chromatography on a phosphocellulose column, and the flow-through fractions were applied to a DEAE column. NS protein was eluted with a linear gradient of 0.22 to 0.35 M NaCl in DEAE column buffer. The positions of NS1 and NS2 were determined by coelution of the ^{35}S-labeled NS protein with a ^{32}P-labeled NS protein marker obtained from virions.

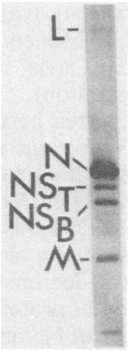

FIG. 7. Electrophoresis of ^{35}S-labeled proteins associated with nucleocapsids on polyacrylamide gels containing urea and SDS. Seven microliters of a reaction mixture was treated with 0.5% NP-40 and chromatographed on a Bio-Gel A15M column. Nucleocapsid-containing fractions were analyzed by electrophoresis on a 10% polyacrylamide gel containing 7 M urea and 0.1% SDS. NS_T, NS_{top}; NS_B, NS_{bottom}.

that of the protein components of virion nucleocapsids. Previous work has shown that certain proteins of vesicular stomatitis virions can be selectively removed by treatment with various concentrations of salt in conjunction with the detergent Triton X-100. Low concentrations of salt remove the viral membrane proteins M and G, but do not remove the N, NS, or L proteins or affect the ability of the nucleocapsid to synthesize RNA (21). Treatment with high concentrations of salt causes the loss of NS and L proteins from the nucleocapsids, and hence these nucleocapsids lose their ability to synthesize RNA (9).

To determine whether the ^{35}S-labeled nucleocapsids produced in the in vitro system had stability characteristics similar to those of virions, these structures were treated with either low (0.43 M) or high (0.72 M) salt concentrations and Triton X-100 for 45 min at 37°C. After incubation, the structures were recovered by chromatography on Bio-Gel A15M columns. The ^{35}S-labeled proteins which remained associated with the nucleocapsids after treatment with low or high salt concentrations were analyzed by electrophoresis on 10% polyacrylamide gels. The data presented in Fig. 5 show that ^{35}S-labeled N, NS, L, and M proteins remained associated with nucleocapsids treated with low concentrations of salt. In contrast, only ^{35}S-labeled N and M proteins remained associated with nucleocapsids subjected to high concentrations of salt. Furthermore, even upon a second treatment with high concentrations of salt, some ^{35}S-labeled M protein remained associated with these nucleocapsids (data not shown). Thus, these data demonstrate that the nucleocapsid proteins L, NS, and N synthesized in the in vitro replication system associate into nucleocapsid structures that have a degree of stability that mimicks that of virion nucleocapsids. In addition, the data presented here show that a small amount of M protein remains stably associated with the nucleocapsids after salt treatment.

Molar ratios of nucleocapsid-associated proteins. The molar ratios of ^{35}S-labeled proteins associated with nucleocapsids were determined by densitometric scanning of fluorograms produced from polyacrylamide gels. The intensities of the bands on the fluorogram representing ^{35}S-labeled N, NS, and M protein were corrected for the number of constituent methionine residues as reported by Rose and Gallione (24) and Gallione et al. (10). Since the number of methionine residues contained within the L protein is not known, the molar ratio of this protein could not be determined accurately by radiolabeling with [^{35}S]methionine. To estimate the number of L proteins that had associated with the nucleocapsids synthesized in vitro, we assumed that 1% (or 19) of the amino acid residues within the L protein were methionine residues.

Table 1 shows the molar ratios of ^{35}S-labeled N, NS, M, and L protein associated with nucleocapsids isolated by column chromatography af-

TABLE 1. Molar ratio of ^{35}S-labeled nucleocapsid proteins relative to labeled N protein[a]

Nucleocapsid treatment	Molar ratio[b]			
	NS	N	M	L[c]
Control (0.5% NP-40)	350	1,000	10	2
Low salt (0.43 M NaCl)	250	1,000	3	2
High salt (0.72 M NaCl)	0	1,000	2	0
Total soluble protein	1,700	1,000[d]	170	8

[a] Nucleocapsids were recovered from the replication system by molecular exclusion column chromatography after treatment with NP-40 or NaCl and Triton X-100. ^{35}S-labeled proteins were identified by electrophoresis on 10% polyacrylamide gels and quantitated by scanning fluorograms produced from the gels.

[b] Ratios were corrected for numbers of methionines contained in each protein (except L) as determined by Rose and Gallione (24) and Gallione et al. (10). The amino-terminal methionine was assumed to be removed from the mature proteins and was not included in the total methionine residues calculated for each viral protein.

[c] L protein was assumed to contain 19 methionine residues.

[d] To facilitate comparison of molar ratios of proteins bound to nucleocapsids with total soluble proteins, all values were adjusted relative to 1,000 N molecules. Of the total N protein synthesized in vitro, 50% or less associated into nucleocapsids.

ter treatment with 0.5% NP-40 or Triton X-100 and either low or high concentrations of salt as compared to the molar ratio of the proteins synthesized by the system. The molar ratios shown here for nucleocapsids before the salt treatment are similar to those reported for virions and indicate de novo assembly of nucleocapsids from newly synthesized proteins. The molar ratios of ^{35}S-labeled protein (N:NS:M:L) associated with nucleocapsids treated under low salt conditions showed that this treatment resulted in the dissociation of a significant amount of NS protein (approximately 100 molecules per 1,000 N protein molecules) that had remained associated with nucleocapsids treated with only the detergent NP-40. From the molar ratio of ^{35}S-labeled protein associated with nucleocapsids treated under high salt conditions, it is evident that for this experiment, the treatment resulted in the disassociation of essentially all NS and L protein from the nucleocapsids. However, it should be noted that in some cases up to 150 ^{35}S-labeled NS molecules per 1,000 N protein molecules remained associated with the nucleocapsids, even after this same treatment. These data also show that even upon high salt treatment of the nucleocapsids from the replication system, a small population of M protein, representing approximately 2 molecules per 1,000 N protein molecules, remained stably bound to the VSV nucleocapsids found in our replication system.

DISCUSSION

We have developed a cell-free system which supports VSV RNA replication as a function of the level of viral protein synthesis (6, 7). The products of RNA replication in this system banded in CsCl and were partially resistant to nuclease digestion, suggesting that the newly synthesized RNAs were associated with protein. For these reasons, we investigated the possibility that viral proteins and RNA synthesized in this system could associate. Results presented in this communication show that VSV proteins synthesized in vitro concurrently with genomic RNA assembled into structures that (i) banded in CsCl gradients or eluted from molecular exclusion columns at the same position as nucleocapsids, (ii) contained the viral proteins L, NS, and N, and (iii) had the same stability as virion nucleocapsids to salt treatment. Together with our previous work (7), these data provide evidence that nucleocapsids are assembled from VSV proteins and genomic RNA synthesized concurrently in vitro. Additional support for de novo nucleocapsid formation comes from experiments in our laboratory which demonstrate that the association of viral proteins into nucleocapsids by the replication system is dependent on VSV RNA synthesis; i.e., newly synthesized proteins do not associate with input nucleocapsid templates in the absence of RNA synthesis (J. T. Patton, N. L. Davis, and G. W. Wertz, manuscript in preparation).

In the studies reported here, we analyzed the association of VSV proteins into nucleocapsids by the use of molecular exclusion column chromatography to separate nucleocapsids from nonassociated proteins. Previous studies by Hill et al. (11) of the assembly of nucleocapsid-like structures in vitro demonstrated that these structures contained N protein but were unable to show the presence of NS and L proteins, both components of intracellular nucleocapsids synthesized in vivo (25). This probably resulted from the use of CsCl gradients to isolate these structures, a method known to dissociate NS and L proteins from nucleocapsids (18). The molecular exclusion technique used here enabled us to isolate intact nucleocapsids produced in vitro without the loss of associated viral proteins. By using this technique, we demonstrated that the newly synthesized VSV proteins N, NS, and L, all necessary components of functional nucleocapsids, did assemble into structures that have the stability characteristics and protein components of virion nucleocapsids. We are currently investigating whether or not these viral proteins synthesized in vitro are functional with respect to enzymatic activity.

The molar ratio of ^{35}S-labeled N, NS, and L proteins making up the nucleocapsids assembled in our replication system was 1,000:350:2 (N:NS:L) (Table 1). Previously, Mellon and Emerson (16) estimated the molar ratio of N to NS to L for intact virions to be 2,000:267:131 based on a molecular weight of NS protein of 40,000. When this ratio is adjusted for the correct molecular weight of NS protein as determined recently from sequence analysis (25,110 [10]), this value becomes 2,000:425:131 or 1,000:217:65. Thus, these data show that more NS molecules are associated with the nucleocapsids assembled in our system than with virions, as reported by Mellon and Emerson (16). This is in agreement with work by Clinton et al. (4) and Bishop and Smith (2), which show that more NS protein is associated with intracellular nucleocapsids synthesized in vivo than with virions. Furthermore, Clinton et al. (4) have shown that a fraction of NS protein contained within virions is dissociated by treatment of these particles with 1% NP-40. Since we routinely treat nucleocapsids synthesized in our replication system with 0.5% NP-40 before column chromatography, it is possible that we have underestimated the relative amount of the NS protein associated with the nucleocapsids synthesized in vitro. These data taken together suggest that some amount of the NS protein

associated with intracellular nucleocapsids may be dissociated during some stage of virion assembly. It is interesting to note that a large pool of soluble NS protein was synthesized by the replication system (Table 1) and that only a small proportion of it associated with nucleocapsids. This is similar to the situation in infected cells, where a large pool of free NS protein is found (12, 26). The function of the pool of NS protein is unknown.

Analysis of the newly synthesized NS protein associated with nucleocapsids showed that it was predominantly of the NS2 subspecies (14). Kingsford and Emerson (14) have shown that NS2 is also the predominant form of NS protein associated with virion nucleocapsids and that it is the more active subspecies of NS protein in promoting viral transcription in vitro. Thus, it is likely that the nucleocapsids assembled in the replication system are capable of binding L protein to produce an enzymatically active template. The nucleocapsids assembled in the in vitro system, however, had fewer molecules of associated L protein than reported for the molar ratio of proteins making up virion nucleocapsids (2). To a large extent, this is due to the small numbers of L protein made by the replication system (Table 1); efficient synthesis of the large L protein (190,000 daltons) in vitro is difficult to achieve.

In addition to the nucleocapsid proteins N, NS, and L, data presented here show the association of a small number of newly synthesized M protein molecules with nucleocapsids. Previous work by Hsu et al. (12) indicates that a small amount of M protein may also be associated with intracellular nucleocapsids from infected cells. These findings are of interest because of recent studies indicating a possible regulatory function for M protein. Results from experiments with VSV class III mutants have demonstrated the involvement of M protein in the regulation of viral RNA synthesis (5). Further studies have shown that high concentrations of purified M protein can partially inhibit RNA synthesis by VSV virion nucleocapsids in vitro and that the level of inhibition decreases with increasing salt concentration in vitro (3, 23, 29). Although greater than 0.2 μg of soluble, non-membrane-associated M protein was synthesized per ml under physiological salt conditions in our replication system, less than 1% of the M protein made associated with nucleocapsids. The molar ratio of N to M found for the nucleocapsids synthesized by the replication system was 1,000:10, in contrast to approximately 1,000:2,000 reported for virions (2). These results show that non-membrane-associated M protein such as that present in our in vitro system does not interact readily with non-membrane-bound nucleocapsids. This may reflect the situation in VSV-infected cells, for which it is known that the plasma membrane rapidly accumulates newly synthesized M protein (17) and is the site of nucleocapsid budding. Thus, the membrane is likely the predominant site in vivo where M protein interacts with the intracellular nucleocapsid. Alternatively, the finding that only a small percentage of the M protein made in our system bound to nucleocapsids could be explained by differences in the conformation or solubility of M protein made in vitro compared to M protein stripped from nucleocapsids, or by the difference between extracellular nucleocapsids, which are prepared with salt and detergent treatment, and the intracellular nucleocapsids used here, which were prepared without such treatments.

In summary, the data reported above show that the concurrent synthesis of VSV negative-stranded RNA and the five viral proteins in a defined cell-free system resulted in the assembly of VSV proteins into structures that have the stability characteristics and protein components of nucleocapsids produced in infected cells. We are currently investigating which of the VSV proteins are responsible for VSV RNA replication.

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