**In vitro** assembly of a functional nucleocapsid from the negative-stranded genome RNA of a defective interfering particle of vesicular stomatitis virus

(RNA genomes/transfection/encapsidation)

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**ABSTRACT** The template for transcription and replication of negative-stranded RNA viruses is a ribonucleoprotein structure, the nucleocapsid. We have developed a system that supports assembly of the negative-stranded RNA genome of a defective interfering (DI) particle of vesicular stomatitis virus (VSV) into a nucleocapsid in vitro. This system uses extracts from wild-type VSV-infected cells as a source of proteins to encapsidate the RNA. In vitro assembled nucleocapsids were compared to in vivo-derived nucleocapsids by the following characteristics: nuclease resistance of the encapsidated RNA, CsCl density banding of labeled RNA in a position coincident with nucleocapsids, correct sedimentation rate in sucrose gradients, the presence of the nucleocapsid protein on the nucleocapsids, and the infectivity of the in vitro assembled nucleocapsids. We conclude that the system we present is capable of assembling the isolated genome of a rhabdovirus DI particle into nucleocapsids indistinguishable from those produced during the course of intracellular DI replication.

Detailed genetic analysis of viruses with negative-stranded RNA genomes has not been possible since the genome RNA is not infectious or biologically active. Rather, the template for transcription and replication of negative-stranded RNA viruses is a nucleocapsid structure composed of the RNA genome and one or more viral proteins. For the rhabdovirus vesicular stomatitis virus (VSV), the template consists of the RNA and the nucleocapsid protein N. The RNA in this structure is resistant to RNase degradation, and the nucleocapsid bands in CsCl gradients. Two viral proteins NS and L form the enzymatic activity, which transcribes and replicates the viral nucleocapsid template (1).

Plasmids containing bacterial or phage promoters are available that allow production of viral RNA molecules from cDNA clones in vitro. In fact, several infectious positive-stranded RNA genomes have been produced by using this type of plasmid (2-18). To apply this technology to a study of controlling sequences in the transcription and replication of a virus with a noninfectious negative-stranded RNA genome, systems must be developed that will assemble such RNA molecules into the template for transcription and replication, the nucleocapsid.

We report the development of such a system by using a defective interfering (DI) particle of VSV. This particle contains a genome of 2208 bases with complementary ends (19). The particle is able to grow in cells infected with a wild-type helper virus and therefore contains information necessary for its genome to be replicated and assembled into a nucleocapsid structure. This DI particle also exhibits limited transcriptional activity, producing a 46-base leader RNA (20). The nucleocapsids that are assembled in vitro possess the following properties of nucleocapsids that are not shared by the genomic RNA alone: (i) the RNA is resistant to RNase degradation, (ii) the nucleocapsids band at the appropriate density in CsCl, (iii) the nucleocapsids display the correct sedimentation rate in sucrose gradients, (iv) the viral nucleocapsid protein is found on the nucleocapsids, and (v) progeny DI particles are produced when the in vitro assembled nucleocapsids are introduced into cells infected with a helper virus. This system reports in vitro assembly of a biologically active nucleocapsid from the isolated genome of a negative-stranded RNA virus and will allow for precise determination of cis-acting regulatory signals for transcription and nucleocapsid assembly of VSV.

**MATERIALS AND METHODS**

Growth, Labeling, and Purification of VSV and the Mudd-Summers (MS-T) DI Particle of VSV. Monolayer cultures of baby hamster kidney (BHK) cells were used for all of the experiments reported here. The HR strain of VSV (plaque purified) and the MS-T DI particle were propagated and purified as described (21, 22). For the production of DI particles with radiolabeled genomes, BHK cells were cultured for 1 day in medium lacking phosphate and then infected with VSV and an amount of MS-T that inhibits >90% of the wild-type virus production. Actinomycin D (5 µg/ml) was added, followed in 0.5 hr by H232P04 (125 µCi per 107 cells; 1 Ci = 37 GBq). DI particles were purified from the culture fluid after 22-24 hr.

**Purification of RNA from MS-T DI Particles.** Purified DI particles were incubated in 0.1 M NaCl/0.05 M sodium acetate, pH 5.1/0.01 M EDTA/0.5% sodium dodecyl sulfate (SDS)/heparin (50 units/ml)/proteinase K (500 µg/ml) for 1-2 hr at 37°C. The RNA was repeatedly extracted with phenol/chloroform (1:1) and chloroform alone and was recovered by ethanol precipitation.

**Preparation of Cell Extracts and Encapsidation of DI Genome RNA.** Cytoplasmic extracts of wild-type VSV-infected BHK cell extracts were prepared 4 hr postinfection by permeabilization with lysolecithin (21). Permeabilized cells were scraped from culture dishes in a reaction mixture consisting of 0.1 M Hepes, pH 8.0/0.1 M NH4Cl/7 mM KCl/8 mM Mg acetate/1 mM dithiothreitol/1 mM spermidine. Nuclei were removed by centrifugation at 800 x g for 5 min, and the resulting cytoplasmic extract was used for encapsidation of added DI RNA. Encapsidation reaction mixtures (250-300 µl) contained cytoplasmic extract from 107 cells. Yeast tRNA was added to 1 mg/ml as a competitive

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Abbreviations: VSV, vesicular stomatitis virus; MS-T, Mudd-Summers defective interfering particle of VSV; DI, defective interfering.

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substrate for contaminating RNase. Purified MS-T RNA in water was heated for 2 min at 98°C–100°C and quenched prior to its addition to the reaction mixture.

In experiments in which soluble proteins were tested for their ability to encapsidate DI RNA genomes, the cytoplasmic extracts were centrifuged at 55,000 rpm for 72 min in an SW55 rotor. The resulting supernatant fluid is designated the soluble protein fraction (21). For the experiments in which radiolabeled infected cell proteins were used to encapsidate DI genomic RNA, VSV-infected cells were labeled with [3H]leucine (100 μCi/ml) from 2.5–4 hr postinfection.

Analysis of Encapsidated RNAs. Products of the encapsidation reactions were treated with micrococcal nuclease to digest nonencapsidated RNAs, and the nuclease-resistant labeled RNAs were analyzed by electrophoresis on agarose gels as described in the legend. CsCl gradient analysis of encapsidation reaction mixtures was performed as described (21). Sucrose gradient analysis of encapsidation reaction mixtures (after micrococcal nuclease treatment) was performed as described (22). The infectivity of in vitro assembled DI nucleocapsids was assayed by DEAE-dextran treatment of cells (23, 24). Confluent monolayers of BHK cells in 100-mm dishes were washed with minimal essential medium (MEM) containing DEAE-dextran (200 μg/ml). The cells were then incubated with 2 ml of this medium for 1 hr at 37°C. The medium was aspirated, and the products of encapsidation reactions were added to the cells. Plaque-purified wild-type VSV (10 plaque-forming units per cell) was added to each reaction mixture, and the resulting RNA species were analyzed by electrophoresis on agarose gel, with the results shown in Fig. 1. The assembly of the purified RNA genome of a DI particle of VSV into a nucleocapsid in vitro was assessed by several criteria. The first was the development of resistance to degradation by micrococcal nuclease. RNA was extracted from purified DI particles that had been metabolically labeled with 32P. The RNA was denatured and added to extracts of wild-type VSV or mock-infected cells prepared by permeabilization. After incubation at 30°C for 90 min, half of the reaction mixture was treated with micrococcal nuclease, and the RNA from the other half was extracted without nuclease digestion. The resulting RNA species were then analyzed by agarose gel electrophoresis, with the results shown in Fig. 1. This experiment demonstrates the generation of nucleocapsid resistance when DI genomic RNA was added to extracts of VSV-infected cells (first lane), but not when added to extracts of uninfected cells (third lane). Equivalent amounts of added RNA were recovered from each reaction mixture in the absence of nuclease treatment (second and fourth lanes). Using the autoradiogram as a template, we excised the regions of this gel containing the intact 19S DI genome RNA and determined the amount of radioactivity by scintillation counting. These results demonstrated that 53% of the RNA that was added to the infected cell extract was recovered as intact RNA after incubation with micrococcal nuclease (first lane vs. fifth lane), while no intact RNA was recovered from nuclease-treated uninfected cell extracts (third lane vs. fifth lane). The radioactivity migrating faster than intact 19S RNA represents breakdown products. Note that some of these fragments are nuclease resistant, suggesting that they may have been encapsidated.

Resistance to RNases is only one of several properties that distinguish nucleocapsids from the genome RNA of VSV or its DI particles. A second property is buoyant density in CsCl. Nucleocapsids band in CsCl at a density of 1.33 g/ml, while nonencapsidated RNA exhibits a much higher density (25). We analyzed MS-T DI genomic RNA added to unin-
ected and VSV-infected cell extracts for the formation of nucleocapsids by centrifugation on preformed 20–40% CsCl gradients, conditions used previously to analyze nucleocapsids (22). The results in Fig. 2 clearly demonstrate that DI RNA added to extracts of wild-type virus-infected cells bands on the gradient in a position identical to that occupied by nucleocapsids produced in vivo. Extracts of uninfected cells were unable to support this process.

The sedimentation rate of the MS-T DI RNA genome is 19S, and that of the MS-T nucleocapsid is 80S. We therefore analyzed DI nucleocapsids assembled in vitro for their sedimentation rate on sucrose gradients, comparing it to that exhibited by in vivo-synthesized MS-T DI nucleocapsids. As shown in Fig. 3, nucleocapsids assembled in vitro in extracts of wild-type VSV-infected cells exhibit a sedimentation profile indistinguishable from that observed with in vivo assembled DI nucleocapsids. Extracts of uninfected cells were unable to support the assembly of such structures.

As a further test for authenticity of the in vitro assembled DI nucleocapsids, we examined the nucleocapsids for the presence of viral proteins. Extracts were prepared from [3H]leucine-labeled VSV-infected or mock-infected cells. DI genomic RNA was added, and the nucleocapsids assembled in these extracts were sedimented on sucrose gradients. Wild-type VSV plus MS-T coinfected cell extracts were sedimented on an identical gradient to serve as a marker for the position of the nucleocapsids. The fractions of the gradient that contained the DI nucleocapsids (see Fig. 3) were pooled, the nucleocapsids were pelleted, and the proteins were analyzed by SDS/PAGE (Fig. 4). The major protein found on the in vitro assembled DI nucleocapsids was the N protein, the structural unit of the nucleocapsid. Prolonged exposure of the gel revealed a faint trace of the NS protein present in the samples in lanes 1 and 2 (data not shown). No viral proteins were present in this region of the gradient when extracts from uninfected cells were used (lane 3).

The data in Figs. 1–4 provide evidence that the structures that are being assembled in vitro from purified MS-T RNA share properties with authentic nucleocapsids. We further tested the authenticity of these nucleocapsids by asking if they could function as templates for the production of progeny DI particles if they were introduced into cells and provided with a helper wild-type virus. DEAE-dextran was used to facilitate the uptake of nucleocapsids into uninfected cells. DEAE-dextran-treated cells were incubated with in vitro nucleocapsid assembly reactions and then infected with plaque-purified wild-type VSV to provide helper functions. [3H]Uridine was then added to label the genome of the progeny wild-type and DI particles. The labeled particles released from the cells were analyzed by sedimentation on sucrose gradients, and the RNA in the particles was analyzed by agarose gel electrophoresis (Fig. 5).

Fig. 5A shows a graph of a sucrose gradient analysis of progeny VSV and DI particles from control experiments in which cells were infected with either VSV alone or VSV plus MS-T. The positions of the two types of virus particles are indicated. Fig. 5B shows the results from a sucrose gradient analysis of particles produced from cells that had been treated with in vitro encapsidation reaction mixtures from uninfected cell extracts or VSV-infected cell extracts. Only extracts of VSV-infected cells were able to support the assembly of DI nucleocapsids that could direct the production of DI particles when introduced into cells provided with a wild-type helper virus. Sucrose gradient analysis of progeny from cells treated with encapsidation reaction mixtures derived from soluble protein preparations of VSV-infected cells failed to reveal the presence of DI particles (data not shown), a result enforced by the results in Fig. 5C.

Analysis of the RNA present in progeny particles from cells that were treated with in vitro encapsidation reaction mixtures is shown in Fig. 5C. Lanes 1 and 2 are controls, showing the positions of the 42S wild-type VSV genome RNA and the 19S MS-T DI particle genome RNA. The doublet in lane 2 represents the plus and minus strands of 19S RNA, both of which are often packaged by DI particles and separate under these conditions of
DISCUSSION

Our goal was to develop a system that would support the assembly of the isolated negative-stranded RNA genome of a DI particle of VSV into a functional nucleocapsid in vitro. The ability to accomplish this will allow us to apply the techniques of molecular genetics to the study of regulatory sequences in this group of viruses, since it is the nucleocapsid, rather than the RNA alone, that is the template for both transcription and replication. We chose the MS-T DI particle of VSV as a source of genome RNA in these studies because of its relatively small size and the fact that it retains the information needed to be transcribed, replicated, and assembled into a nucleocapsid by the viral proteins provided in trans by a wild-type helper VS virion. Hence, it is a useful system for the identification of cis-acting regulatory signals. We used a variation of our previously described in vitro genomic RNA replicating system derived from VSV-infected cells as the source of the proteins needed to assemble the RNA into a nucleocapsid. The data we present demonstrate that nucleocapsids assembled in vitro possess all of the characteristics of those produced in infected cells, including the functional ability of being able to replicate when introduced into cells in the presence of a helper virus.

Soluble protein fractions from VSV-infected cells were apparently unable to support assembly of nucleocapsids in vitro. This was surprising since we have previously shown this fraction to be active in supporting the replication and assembly of both wild-type and DI particle nucleocapsids in vitro from infected cells and also to support the initiation of RNA genome replication, even after being fractionated (21, 29, 30). It is possible that initiation of nucleocapsid assembly in vitro from isolated 19S MS-T RNA is a much more difficult step relative to encapsidation of nascent genomes during synthesis. We have recently observed that incubation of extracts on ice leads to a decreased ability to encapsidate RNA. Since the preparation of soluble proteins involves a centrifugation step for more than an hour, this could explain the failure of these fractions to encapsidate isolated genome RNAs, suggesting that a factor(s) essential for nucleocapsid assembly is labile.

We were able to detect the production of DI particles from in vitro assembled nucleocapsids in a single passage in cell culture. This is in contrast to the findings with the positive-stranded RNA of Sindbis virus DI particles, in which several passages of culture fluid were necessary before DI particles were detected (2). This could be due to more efficient transfection of nucleocapsids (for MS-T) relative to naked RNA (for Sindbis DI), relatively efficient encapsidation of the MS-T particle RNA in vitro, or a higher yield for MS-T. The efficiency of encapsidation of MS-T genome RNA (as measured by nuclease resistance) varied from between 10% and 55%. We have been unable to define all factors that influence this value, although we suspect the presence of nucleases to have a large influence. It is also possible that base pairing between the complementary ends of the MS-T DI genomic RNA would prevent encapsidation and hence might influence this parameter.

The precise mechanism that VSV uses to produce replicative nucleocapsids is not known. However, two general models are possible. Replicating RNA molecules can be encapsidated as nascent chains, or they can be encapsidated after their synthesis and release from the template. Although the available evidence strongly suggests that encapsidation occurs on nascent genomes (1), our demonstration that full-length RNAs are encapsidated in vitro does not rule out the latter possibility. The leader RNA of VSV is found as a nucleocapsid at late times of infection (31, 32). The data presented here support the hypothesis that these structures could arise as the result of encapsidation of free leader RNA.

This system, in conjunction with CDNA cloning into the appropriate transcription plasmid, could prove useful for development of an expression vector/vaccine system for the introduction of genes into cells in the form of chimeric nucleocapsids of VSV or one of its DI particles. Since VSV
from cells treated with profile from control infections released from analyzed MS-T mixtures from mock-infected RNA VSV-infected cells; a of wild-type virus, making on Institutes of Health Grant 12.


The graphic is described in the text.

Fig. 5. Infectivity of in vitro assembled nucleocapsids. Unlabeled RNA from purified MS-T DI particles was added to encapsidation reaction mixtures from mock-infected or VSV-infected cell extracts. The products of these reactions were added to DEAE-dextran-treated BHK cells. Plaque-purified wild-type VSV was then added as helper, followed by addition of actinomycin D and [3H]uridine. After 22 hr, the culture fluid was analyzed for the presence of MS-T DI particles by sucrose gradient analysis (A and B) or agarose gel electrophoresis (C). (A) Sucrose gradient profile from control infections with VSV alone (c) or VSV plus MS-T coinfection (o) to mark the sedimentation position of wild-type VSV and the MS-T DI particle, respectively. (B) Results obtained from DEAE-dextran-treated cells exposed to DI genome RNA encapsidation reaction mixtures from uninfected cell extracts (o) or wild-type VSV-infected cell extracts (c). (C) Agarose gel analysis of the RNA present in particles released from reaction mixtures described above. Lanes: 3 and 2, RNAs from the control reaction mixtures in A; 3–6, RNAs in particles released from cells treated with encapsidation reaction mixtures. Lane 3 is from cells exposed to in vitro encapsidation reaction mixture derived from VSV-infected cells; lane 4 is from uninfected cells; lane 5 is from the soluble protein fraction of VSV-infected cells; lane 6 is from 19S DI genome RNA added directly to cells without encapsidation in vitro. The positions of the 42S VSV genome and the 19S MS-T DI genome are indicated. We thank Dr. Anne Deatly and Frank La Ferla for their helpful comments on this manuscript. This work was supported by National Institutes of Health Grant AI-22116 (R.W.P.).