Assembly and Transcription of Synthetic Vesicular Stomatitis Virus Nucleocapsids

SUE A. MOYER,^{1*} SHERIN SMALLWOOD-KENTRO,¹ ABRAHAM HADDAD,² AND LUDVIK PREVEC²

Department of Immunology and Medical Microbiology, University of Florida College of Medicine, Gainesville, Florida 32610,¹ and Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada²

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The functional template for transcription of vesicular stomatitis virus (VSV) RNA is a ribonucleoprotein particle (nucleocapsid) consisting of the negative-strand sense genomic RNA completely encapsidated by the viral nucleocapsid (N) protein. As an approach to create nucleocapsids in vitro, we demonstrate here the specific encapsidation by purified N protein of in vitro-synthesized RNA sequences representing the 5' end of both the negative- and positive-strand VSV genome-length RNAs. As few as 19 nucleotides from the 5'-end of positive-strand RNA allowed maximal encapsidation, although the 5' terminal 10 nucleotides would allow partial (50%) encapsidation. Sequences downstream of the binding site can be of any origin. Specific encapsidation of VSV sequences was dependent on the presence of uninfected cell cytoplasmic extracts or poly(A). The synthetic nucleocapsids have the properties of RNase resistance and a buoyant density typical of wild-type VSV nucleocapsids. We have encapsidated a synthetic virionlike RNA species which contained just the terminal sequences of the virion RNA: the N encapsidation signal from the 5' end and the leader gene from the 3' end. This assembled nucleocapsid could function in vitro as a transcription template for the VSV RNA polymerase.

Vesicular stomatitis virus (VSV) is a cytocidal, membrane-enveloped virus which has served as the prototype of the rhabdovirus group. Comprehensive reviews (5, 35) have recently covered many aspects of the biology and biochemistry of VSV. The virion contains a single-stranded RNA genome of the negative-strand sense whose gene order, 3'-leader-N-NS-M-G-L-5', and sequence have been determined (reviewed in reference 31). The virion contains a single helical nucleocapsid in which the genome RNA is tightly bound to about 1,300 molecules of the N protein (47 kDa), rendering the RNA nuclease resistant. The nucleocapsid has associated with it the subunits of the viral RNA polymerase, the L (241 kDa) and NS (30 kDa) proteins, and is surrounded by a lipid bilayer membrane containing the M (26 kDa) and G (57 kDa) proteins (31, 34). During RNA replication, the negative-strand genome RNA serves as the template for the synthesis of the complementary positive-strand RNA, which in turn is utilized for the synthesis of progeny RNA. The unique feature of negative-strand viruses is that RNA replication requires ongoing protein synthesis, with the synthesis of both the positive- and negative-strand genome RNAs coupled to their encapsidation by the viral N protein.

A model for replication has been proposed whereby the binding of N protein to nascent leader RNA may act as an antiterminator to allow the RNA polymerase to read through the signals for mRNA processing at the intergenic boundaries. This readthrough would permit synthesis of the fully encapsidated, genome-length RNA instead of the monocistronic mRNAs (2, 5, 9). In support of this model, Blumberg and co-workers (6, 8) have shown that 75% of the leader RNA in the infected cell is encapsidated by N protein and that purified N protein selectively encapsidates purified leader RNA in vitro with 5'-to-3' polarity. A comparison of sequences at the ends of the RNAs of many rhabdoviruses

suggests that the initial binding site for N protein within the leader RNA resides in the 5'-terminal 14 to 18 nucleotides containing the conserved 5'-end-most ACGAA pentanucleotide, bases 13 to 18 (AAACCA), and five A residues at positions 1, 4, 7, 10, and 13 from the 5' end of the leader RNA (17, 27). From the stoichiometry of RNA and protein within the nucleocapsid, it has been calculated that one N polypeptide binds for each nine nucleotides of virion RNA (34).

We have previously developed cell-free systems for in vitro transcription and replication of the wild-type and defective interfering genome RNAs of VSV and Sendai virus (10, 29). We have used these in vitro systems to characterize viral as well as host proteins that are required for negative-strand RNA replication and encapsidation (3, 25, 30). A major block in the field of negative-strand RNA viruses, however, has been the absence of a direct test for regulatory (*cis*) sequences required for RNA synthesis. This is a consequence of the fact that any synthetic RNA must first be encapsidated by the N protein to create a nucleocapsid substrate which is the only template recognized by the viral polymerase. Recently several laboratories have developed methods for encapsidation of different negative-strand viral RNAs (4, 19, 23). We report here the procedure for the specific encapsidation of synthetic VSV RNA by purified VSV N protein, which we have used to characterize some basic parameters of the assembly reaction. In addition, we have synthesized and encapsidated a model negative-strand virionlike RNA and showed that this synthetic nucleocapsid could function in vitro as a transcription template for the VSV RNA polymerase.

MATERIALS AND METHODS

Cells and virus. The Indiana (Ind) (HR strain) and New Jersey (NJ) (Ogden) serotypes of VSV were grown in baby hamster kidney cells and purified as described previously (24, 26).

* Corresponding author.

FIG. 1. Construction of plasmids having VSV terminal sequences located downstream of an SP6 polymerase initiation site with or without intervening non-VSV sequences. The starting material for VSV sequences (pJS77) as well as the details of construction are as described in the figure or in Materials and Methods. The SP6 promoter is indicated by the large arrow, VSV sequences are an open box, and vector sequences are the solid line. Plasmids p1, p60, and psPL6 all have some non-VSV sequences (as indicated) in the region between the SP6 transcription start and the authentic VSV sequences. Plasmid psPL6 is missing the first nucleotide of VSV sequence (I). Plasmid p45 and all derivatives of p45 have the authentic terminal A residue of VSV at the +1 position of the SP6 transcript. Restriction sites of importance: *Bam*HI (B), *Bgl*II (Bg), *Hind*III (H), *Pst*I (P), *Sph*I (S), *Dde*I (D). dNTPs, Deoxynucleoside triphosphates.

Preparation of VSV plasmid constructs. The strategy for preparation of the VSV plasmids is outlined in Fig. 1. Plasmid pJS77 (33), which contains a cDNA copy of 360-bp beginning at the 3' end of VSV(Ind) genome RNA bounded by a poly(G) sequence that was added during the cloning procedure, was the source of a *Pst*I-to-*Bgl*II fragment that contains the poly(G) sequences and all of the authentic VSV terminal nucleotides to the *Bgl*II site within the N gene of VSV. This fragment was cloned into the *Pst*I-to-*Bam*HI site of pSP64 (Promega Corp.) to give plasmid p1. To reduce the number of nucleotides between the SP6 polymerase transcription start site and the VSV sequences, we inserted a buffer sequence into the *Hind*III site, cut it with *Pst*I, and

digested it with exonuclease Bal31 for various lengths of time. A *Hind*III linker was ligated onto the truncated end, and the *Hind*III sites were joined by digestion and ligation. Plasmid p60 was selected by sequencing from some of the products. This plasmid was itself cut at *Hind*III and treated with Bal31 for short times. After religation, we obtained plasmid psPL6, which, though missing the terminal VSV nucleotide, had only four bases between the transcription start site and VSV sequences. By using oligonucleotide site-directed mutagenesis in M13 as described by Zoller and Smith (36), the *Sph*I-to-*Bam*HI fragment from p60 was used to create p45, in which transcription would initiate directly at the VSV sequence. The mutagenized region was sequenced to confirm authenticity and reinserted into a pUC vector by using the *Sph*I-to-*Bam*HI sites. *Bam*HI digestion of p45 followed by treatment for various times with Bal31 and religation of a *Bam*HI linker resulted in a family of plasmids with the *Bam*HI site positioned different distances from the transcription start site, as determined by sequence analysis.

Sequences of transcripts. The RNA transcripts used in this study are listed by number in Fig. 3, with the relative number of VSV and non-VSV nucleotides indicated. Transcripts 1 through 6 have authentic VSV positive-strand 5' ends and are from the family of p45 derivatives (Fig. 1) cut at their different *Bam*HI sites. The 3'-terminal non-VSV sequence of each of these transcripts is CCG derived from the linearized *Bam*HI linker. RNA transcript 7 is from p45 cut at the authentic *Bam*HI site 129 nucleotides into the VSV sequences. Transcripts 8 and 9 have 19 nucleotides of authentic 5' VSV sequence followed by 9 nucleotides (CCGGAU CCG) or 33 nucleotides (CCGGAUCCAAUUGUUAUCCG CUCACAAUUGGGG) from plasmid DNA, respectively. Transcripts 14, 15, and 16 are from *Dde*I digests of psPL6, p60, and p1 (Fig. 1), respectively, with non-VSV sequences 5' of VSV sequences. The non-VSV transcript A has the sequence GAAUACAAGCUUGGGCUGCAGCCGCCG ACCUCCGAAGGUCGUUACCGUUAACCGCC; the non-VSV sequence B is a direct transcript of the linker region of pSP64 cut with *Eco*RI.

In vitro synthesis of VSV RNAs. Unlabeled or [α - 32 P]UTP-labeled 5' VSV(Ind) RNAs were synthesized from the various SP64-VSV plasmids linearized with *Bam*HI, using SP6 polymerase (Promega Biotec) as described in the manufacturer's protocol except that 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4) was substituted for Tris buffer. In addition, other VSV RNAs [10 to 13 and NJ(77)] were synthesized in vitro with T7 RNA polymerase from a synthetic partially double-stranded DNA template as described by Milligan et al. (22). Oligonucleotides were synthesized by the DNA Core of the Interdisciplinary Center for Biotechnology and Research of the University of Florida. In a 20- μ l reaction mixture, 15 to 30 pmol of each of the synthetic DNAs (the top strand of the T7 promoter [18-mer] and the bottom strand containing the bottom strand of the T7 promoter) and the DNA of choice were incubated with 100 μ Ci of [α - 32 P]UTP (ICN) and purified T7 RNA polymerase (150 U/ μ l) at 37°C for 2 h. When unlabeled RNA was prepared, the radioisotope was omitted and UTP was raised to 1 mM. The RNA products were isolated by phenol-chloroform extraction and in some cases further purified by electrophoresis on an 8% acrylamide-8 M urea gel, eluted, and ethanol precipitated.

Purification of VSV N protein and RNA polymerase. The N protein of purified VSV of both the Indiana and New Jersey serotypes was isolated by a modification of the methods of Emerson and Wagner (15) and Blumberg et al. (7). Purified

VSV (0.5 mg/ml) was incubated in low-salt solubilizer (10 mM HEPES [pH 7.4], 5% [vol/vol] glycerol, 1.85% Triton X-100, 0.6 mM dithiothreitol [DTT], 0.3 M NH_4Cl , 1% lysolecithin) for 2 h at 4°C. The virus suspension (2.5 ml) was layered over a 2-ml cushion of 30% (vol/vol) glycerol and spun at 50,000 rpm for 90 min at 4°C in the SW55 rotor. The nucleocapsid pellet was resuspended in 1 ml of high-salt solubilizer (10 mM HEPES [pH 7.4], 1 M NH_4Cl , 5% [vol/vol] glycerol, 0.1% Triton X-100, 0.6 mM DTT) and incubated at 4°C for 30 min. The suspension was layered over a 3.5-ml 30% (vol/vol) glycerol cushion and spun at 50,000 rpm for 90 min at 4°C in the SW55 rotor. The supernatant fluid contained the viral RNA polymerase (L and NS proteins), which was stored at -70°C. The RNA-N pellet was resuspended in 1 ml of high-salt solubilizer and purified by banding on a 20 to 40% (wt/wt) CsCl gradient at 36,000 rpm for 17 h at 4°C in the SW41 rotor. The RNA-N band was diluted, pelleted, and spun on a 20 to 40% (wt/wt) CsCl gradient containing 3 M guanidinium HCl at 36,000 rpm for 17 h at 4°C in the SW41 rotor, which dissociated the nucleocapsid. The fractions containing the N protein were pooled, dialyzed in buffer containing 0.1 M HEPES (pH 7.4), 1 M NH_4Cl , and 1 mM DTT and stored at 4°C. The typical yield was about 600 μg of N protein from 4 mg of VSV.

In vitro encapsidation of RNA and transcription of synthetic nucleocapsids. For the encapsidation reaction, ^{32}P -labeled RNAs were collected, resuspended in H_2O , boiled for 1 min, and quickly chilled. Samples were incubated for 30 min at 37 or 45°C with N protein as indicated in the text in 50- μl reaction mixtures containing 50 mM HEPES (pH 7.4), 0.5 M NH_4Cl , RNasin (1 U/ μl), and 0.1% Nonidet P-40 in the presence or absence of ~50 to 100 μg of uninfected cell soluble protein (USP). Optimal encapsidation occurred when the concentration of the stock of N protein was less than 300 $\mu\text{g}/\text{ml}$. At concentrations above this level, the N protein was apparently aggregated and inactive even in the 1 M NH_4Cl storage buffer. For the preparation of USP, a cytoplasmic extract of uninfected cells was prepared in 0.1 M HEPES (pH 7.4)–0.2 M NH_4Cl –1 mM DTT (30); after centrifugation at 50,000 rpm for 75 min at 4°C in the SW55 rotor, the supernatant fluid (i.e., the USP) was removed and stored at -70°C. The amount of USP added was determined empirically for each preparation of USP such that the encapsidation of the non-VSV RNA with N protein in the presence of USP was less than 20% of that with N protein alone. After encapsidation, the samples were digested with micrococcal nuclease (10 $\mu\text{g}/\text{ml}$) for 30 min at 30°C. The nuclease-resistant RNA was purified by phenol-chloroform extraction, ethanol precipitated, and analyzed by electrophoresis on an 8% acrylamide–8 M urea gel. Alternatively, encapsidated labeled RNA was analyzed by banding on a 20 to 40% (wt/wt) CsCl gradient at 50,000 rpm for 64 h at 4°C in the SW55 rotor as described previously (8).

To test for transcription of a synthetic VSV virion-like nucleocapsid, unlabeled RNA [NJ(77)] (~50 ng) was encapsidated with purified N(NJ) protein (~80 μg) in a 250- μl reaction mixture as described above. The sample was dialyzed against buffer containing 10 mM HEPES (pH 8.0), 10 mM NH_4Cl , 0.2 mM DTT, and 5% (vol/vol) glycerol. Samples (80 μl) of the nucleocapsids were incubated at 30°C for 3 h with VSV RNA polymerase (12 μg) in a reaction mixture (500 μl) containing 50 mM HEPES (pH 8), 0.1 M NH_4Cl , 4 mM DTT, 5 mM MgCl_2 , 1 mM each ATP, GTP, and CTP, 50 μM UTP, 70 μCi of [α - ^{32}P]UTP, 1.8% (vol/vol) glycerol, and 0.01% Triton X-100. As a control, stripped wild-type VSV (NJ) RNA-N template purified twice by CsCl centrifugation

was transcribed under identical conditions. The product RNA was isolated and either analyzed directly or hybridized in neutral trichloroacetate (12) with a synthetic DNA oligomer (47 nucleotides) complementary to VSV(NJ) positive-strand leader RNA. In the latter case, RNase A- and T_1 -resistant RNA was purified and analyzed by electrophoresis on an 8% acrylamide–8 M urea gel.

RESULTS

Specific encapsidation of positive-strand VSV RNA. As a source of RNA transcripts containing VSV sequences, DNA encoding the 3'-terminal sequences of VSV(Ind) genome RNA was cloned into an SP64 plasmid vector and modified as described in Materials and Methods and Fig. 1. One plasmid, p45 (Fig. 1), was constructed such that transcription with SP6 polymerase from the SP6 promoter initiated exactly with VSV sequences. From plasmid p45 we also constructed a series of plasmids in which the downstream *Bam*HI site was positioned different distances from the transcription start site. These constructs yielded SP6 transcription product RNAs containing different lengths of positive-strand VSV RNA beginning with an authentic 5' end. The 3' end of each RNA contains 3 extra nucleotides derived from transcription of the *Bam*HI linker site used to linearize the plasmid for runoff transcription (see Fig. 3). While transcription by SP6 polymerase from p45 and its derivative plasmids was not as efficient as it was from plasmids that contain the usual G residue at the +1 position, we showed by primer extension that the majority of transcripts began at the A residue that marks the authentic VSV terminal nucleotide (data not shown).

Initially we attempted to encapsidate these synthetic positive-strand VSV RNAs transcribed from the p45 plasmids by the addition of the soluble protein fraction of VSV-infected cells (VSV-SP) which we had shown earlier supported cell-free RNA replication (29). This extract had been reported by Mirakhur and Peluso (23) to encapsidate genome RNA purified from a VSV defective interfering particle. However, we could detect no encapsidation under a variety of conditions either with this fraction alone or by the de novo synthesis of the synthetic VSV RNA in the presence of VSV-SP (data not shown).

A procedure that did allow encapsidation was adapted from the methodology of Blumberg et al. (6, 7), who demonstrated that positive-strand leader RNA isolated from VSV-infected cells could be encapsidated by purified N protein in vitro. We found that this modified procedure allowed specific encapsidation of positive-strand VSV RNAs varying in length. For example, radiolabeled synthetic positive-strand VSV(Ind) RNAs of 41 (no. 1) and 81 (no. 6) nucleotides incubated with USP alone in the absence of purified N(Ind) protein remained sensitive to nuclease digestion (Fig. 2, lane USP of each panel). This observation suggested that USP alone did not contain proteins that could protect RNA. Addition of purified N(Ind) protein alone to these two samples encapsidated the synthetic VSV RNAs, since the full-length molecules were resistant to nuclease digestion (Fig. 2, #1 and 6, lanes N). Surprisingly, two non-VSV, plasmid-derived RNAs, A and B, were also protected from nuclease digestion and hence were apparently encapsidated by N protein alone (Fig. 2, lane N). We found that addition of USP together with the VSV N protein was necessary for specific encapsidation of viral RNAs and discrimination from non-VSV RNA, since under these conditions the VSV RNAs (1 and 6) were still encapsidated,

FIG. 2. In vitro encapsidation of synthetic positive-strand VSV(Ind) RNAs by VSV N(Ind) protein. Samples of each radiolabeled synthetic VSV(Ind) RNA (1, 6, 8, and 10) and non-VSV RNAs A and B (see Fig. 3 and Materials and Methods for a description) were incubated as follows: lanes USP, with USP (50 µg) alone; lanes N, in the presence of N(Ind) protein (4 µg) alone; lanes N+USP, in the presence of both N and USP for 30 min at 37°C. The samples were digested with micrococcal nuclease, and the resistant RNA was isolated and analyzed by electrophoresis on an 8% polyacrylamide-8 M urea gel. XC indicates the xylene cyanol dye.

while the two non-VSV RNAs (A and B) were not (<10%) (Fig. 2, lanes N+USP).

The specificity of the encapsidation for VSV RNA was confirmed because in a mixture of the VSV (no. 1) and non-VSV (A) RNAs in the presence of USP and N protein, only the VSV RNA was nuclease resistant (Fig. 2, Mixture). We have further shown that the USP must be added to the non-VSV RNA simultaneously with N protein to prevent nonspecific binding, since its addition following the reaction did not remove bound N protein. Titration experiments showed that increasing amounts of N protein up to 1.2 µg gave a linear increase in specific encapsidation of VSV RNA 1 up to 100%, after which the reaction became saturated (data not shown). From the specific activity of the RNA, we calculated that the N protein at 1.2 µg in the reaction was in a 1,000-fold excess over that required to encapsidate RNA 1 with one N protein per nine nucleotides. All subsequent experiments were performed under conditions in which N protein was greater than 4 µg per reaction.

On the basis of results just described, a series of overlapping VSV 5' positive-strand RNAs was synthesized and analyzed for specific encapsidation (N protein in the presence of USP) (Fig. 3). VSV RNAs ranging from 41 to 47 nucleotides (1 to 3) could be encapsidated consistent with the previously described encapsidation of authentic positive-strand leader RNA (6). Moreover, positive-strand VSV RNAs of 55 to 81 nucleotides (4 to 6) which now include the leader-N mRNA junction and various amounts of the downstream N mRNA were also specifically encapsidated with N protein. With a longer VSV RNA of 129 nucleotides (no. 7), however, full-length encapsidation did not occur even with a 10-fold excess of N protein in either the presence or absence of USP. In this case, an RNA of ~90 nucleotides (instead of full-length product) was protected, suggesting that encapsidation initiated and continued to nucleotide 90. In another experiment (not shown), an RNA of 172 nucleotides with 92 and 80 bases from the 5' and 3' ends of virion RNA, respectively, was encapsidated in vitro, suggesting that nucleotides 90 to 129 in RNA 7 have some feature which

prevented encapsidation. In any event, different RNAs longer than 90 nucleotides have been encapsidated.

An alternative method to confirm encapsidation in vitro is to measure the ability of the products to band as a ribonu-

Length of RNA				Specific Encapsulation	
A. (+) Leader-like RNAs					
#	Total	VSV	Non-VSV		
1.	41	38	3	5' ——— x 3'	+
2.	44	41	3	5' ——— x 3'	+
3.	47	44	3	5' ——— x 3'	+
4.	55	52	3	5' ——— x 3'	+
5.	63	60	3	5' ——— x 3'	+
6.	81	78	3	5' ——— x 3'	+
7.	129	129	0	5' ——— 3'	-
8.	28	19	9	5' —xxx 3'	+
9.	52	19	33	5' —xxxxxxxxx 3'	+
10.	27	10	17	5' —xxxxxx 3'	±
B. Other VSV RNAs					
5' Non-VSV					
11.	49	48	1	(-) Leader RNA	+
12.	56	55	1	Complement of (-) Leader RNA	-

FIG. 3. Synthetic VSV RNAs tested for encapsidation by the VSV N protein. The SP64-VSV plasmids described in Fig. 1 were transcribed by SP6 polymerase in the presence of [α - 32 P]UTP, and the radiolabeled VSV RNA products were tested for encapsidation by N(Ind) (4 to 8 µg) in the presence or absence of USP as described in the legend to Fig. 2. The line indicates the relative length of the VSV sequence with the correct 5' terminus, and the x's indicate the lengths of non-VSV sequences at the 3' end. The full-length RNAs were cut out of the gel and counted, and the percent specific encapsidation was determined as [counts per minute of RNA (N+USP)/counts per minute of RNA (N)] $\times$ 100. Symbols: +, greater than 50%; $\pm$, 20 to 50%; -, less than 20%, as determined in three separate experiments.

FIG. 4. Analysis of synthetic nucleocapsids by CsCl gradient centrifugation. Samples of ^{32}P -labeled RNA 3 (350,000 cpm) were incubated in a 100- μl reaction mixture with 8.7 μg of N(Ind) protein in either the absence (◆) or presence (□) of 224 μg of USP for 30 min at 37°C. The samples were analyzed by centrifugation on a 20 to 40% (wt/wt) CsCl gradient as described in Materials and Methods. Samples (0.25 ml) were collected and counted directly by Cerenkov radiation. The sedimentation position (fraction 12) of the wild-type nucleocapsid is indicated by the arrow, as determined on a parallel gradient. Sedimentation is from left to right.

cleoprotein particle in CsCl. As an example, synthetic VSV RNA 3 (Fig. 3) incubated with N protein in both the absence and presence of USP yielded nucleocapsids that banded at the same density as the wild-type virus nucleocapsid (Fig. 4). These data suggested that stable, tight binding of N protein occurred regardless of the presence or absence of USP. In similar experiments, the non-VSV RNA A (Fig. 2) could be encapsidated into a CsCl-stable nucleocapsid with N protein alone, although no encapsidation occurred when USP was also added (data not shown).

In vitro encapsidation of other VSV RNAs. Utilizing the specific encapsidation assay, we next tested the minimum length of VSV RNA sequence that was required to initiate specific N protein binding. Encapsidation of full-length RNA occurred efficiently with only the 5'-terminal 19 nucleotides of positive-strand RNA, even when non-VSV sequences of 9 or 33 nucleotides were present at the 3' end of the RNA (Fig. 3, no. 8 and 9). In addition, the terminal 20 nucleotides with a single G added at the 5' terminus was also efficiently encapsidated (see Fig. 6, #13). An RNA (no. 10) with only the 5'-terminal 10 nucleotides of the positive-strand RNA and 17 non-VSV nucleotides added at the 3' end also allowed some full-length encapsidation, although the efficiency dropped to 50% of that seen with transcript 8 (Fig. 2 and 3). Since one N protein molecule was calculated to bind about 9 nucleotides of VSV RNA (34), these data appeared to define the minimal acceptable encapsidation sequence (10 nucleotides). The data also showed that sequences to the 3' end of the encapsidation site can be of any origin.

To further test the specificity of the encapsidation reaction, an RNA was synthesized corresponding to negative-strand leader RNA, which should possess a site for N protein encapsidation that is utilized *in vivo* for formation of negative-strand nucleocapsids. A second RNA, corresponding to the complement of negative-strand leader RNA, which should not have an encapsidation site, was also prepared. Each RNA contained a single non-VSV 5' G that was added to facilitate its synthesis by the T7 polymerase but did not

FIG. 5. In vitro encapsidation of VSV(Ind) negative-strand leader RNA by N protein. Samples of the purified synthetic ^{32}P -labeled RNAs (negative-strand leader RNA and its complement) were incubated as follows: lane USP, with USP (50 μg) alone; lane N, in the presence of N(Ind) protein (4 μg) alone; lane N+USP, in the presence of both N and USP for 30 min at 37°C. Nuclease-resistant RNAs were analyzed as described in the legend to Fig. 2. XC indicates the xylene cyanol dye.

interfere with specific encapsidation of the VSV RNA (see Fig. 6, #13). Encapsidation experiments showed that the negative-strand leader-like RNA was encapsidated by N protein both in the absence and in the presence of USP with good efficiency; however, USP abolished (>90%) the N protein binding to the complement of negative-strand leader RNA (Fig. 3 and 5), as expected. Thus, both known VSV encapsidation signals were selectively encapsidated *in vitro* in the presence of N protein and USP, while another VSV sequence was not.

We have also found that the required VSV N protein encapsidation signal need not be at the exact 5' end of the RNA. Using the method of Milligan et al. (22), we synthesized an RNA (no. 13) containing the 5'-terminal 20 nucleotides of positive-strand VSV RNA with an additional G at the 5' terminus. This RNA was efficiently (78%) encapsidated by N protein (Fig. 6, #13) in the presence of USP, as were the other previous bona fide terminal VSV RNAs discussed above. In other constructs (Materials and Methods), non-VSV sequences (4, 19, and 35 nucleotides) were placed preceding the 5' end of the VSV sequence (Fig. 6, #14, 15, and 16, respectively). In each case, the RNA transcripts were still specifically encapsidated, although the encapsidation efficiency of the latter two with longer 5' non-VSV sequences dropped to 50%. In addition, because of a deletion in the preparation of the construct for transcript 14, the RNA is missing the 5'-terminal A residue of VSV; however, this deletion and the addition of the four non-VSV nucleotides still gave good encapsidation. The extra non-VSV sequences in these RNAs were nested sets of the 5' end of the non-VSV RNA B which was not encapsidated (Fig. 2), implying that these sequences do not themselves contain a specific N binding site. Since full-length products were obtained, these data suggested that N binding initiated within the internal VSV sequence of RNAs 14 to 16, followed by subsequent encapsidation with N protein in both the 3' and 5' directions.

FIG. 6. Effect of 5' non-VSV sequences on encapsidation. Labeled RNAs with various lengths of non-VSV sequences at the 5' end of the VSV(Ind) 5'-terminal RNA were synthesized as described in Materials and Methods. The line and x's indicate the relative lengths of VSV and non-VSV sequences, respectively. Samples of each were incubated as follows: lanes USP, with USP (70 μ g) alone; lanes N, in the presence of N(Ind) protein (5 μ g); lanes N+USP, in the presence of both N and USP for 30 min at 37°C. Nuclease-resistant RNAs were analyzed as for Fig. 2. XC indicates the xylene cyanol dye.

Nature of the specific encapsidation. To investigate how the presence of the cellular soluble protein fraction might mediate the specificity of encapsidation, we asked whether specific components such as bovine serum albumin (BSA), tubulin, or tRNA would alter the encapsidation reaction. Tubulin was previously shown to stimulate VSV RNA synthesis (25). None of the components had any effect on in vitro encapsidation by N protein alone and could not substitute for USP (Table 1).

There are previous reports (1, 18, 32) that N protein also binds to VSV mRNAs in the infected cell, which was attributed in some cases to binding to the mRNA poly(A) tail. We found that the addition of poly(A) did, in fact, compete preferentially for the binding of N protein to the

TABLE 1. Effects of various components on encapsidation of VSV and non-VSV RNAs

Additions for nuclease-resistant RNA ^a	Non-VSV A		VSV #3	
	cpm	%	cpm	%
None	9,455	100	5,210	100
USP (70 μ g)	125	1.3	79	1.5
Tubulin (70 μ g)	117	1.2	56	1.1
N (1.5 μ g)	9,500	100	5,244	100
N + USP (70 μ g)	967	10.2	3,359	64.5
N + BSA (70 μ g)	7,038	74.4	5,651	108
N + tubulin (70 μ g)	8,982	95.0	5,356	103
N + tRNA (70 μ g)	9,543	101	5,319	102

^a ³²P-labeled RNAs were purified by gel electrophoresis, and samples were incubated with the indicated additions at 37°C for 30 min, micrococcal nuclease treated (except for the first sample), and phenol-CCl₃ extracted. Nuclease-resistant RNA was quantitated by Cerenkov counting after separation by polyacrylamide-urea gel electrophoresis as described in the legend to Fig. 2.

FIG. 7. Effect of poly(A) on specific encapsidation of RNAs. Samples of ³²P-labeled non-VSV RNA A (◆) or VSV RNA 3 (□) were incubated with N(Ind) protein (4 μ g) in the presence of increasing concentrations of poly(A) (~100,000 Da; Sigma). The micrococcal nuclease-resistant RNAs were separated by polyacrylamide-urea gel electrophoresis and quantitated by Cerenkov counting. The data represent the average values of three experiments and are presented as the percent resistant RNA relative to the control reaction with N protein alone in the absence of poly(A).

non-VSV RNA (Fig. 7). In the presence of low levels of poly(A), the amount of encapsidation of the non-VSV RNA was considerably less than that of the VSV RNA, providing a differential between the two RNAs. If high enough amounts of poly(A) were added, however, encapsidation of VSV RNAs could also be inhibited. The data suggest that specificity is mediated by competition by poly(A), which binds N protein less well than VSV RNA but better than non-VSV sequences. We have synthesized ³²P-poly(A)₂₀ in vitro and find that it can be encapsidated by N protein as the above data suggested, but at a level of only 30% of the level of encapsidation of positive-strand VSV RNA (no. 3) in the presence of USP. The poly(A)₂₀, of course, contains the five-times-repeated A residue motif but lacks the other required viral sequences.

Encapsidation and transcription of a synthetic, virionlike VSV nucleocapsid. To study the sequences in the virion RNA which might regulate transcription of the VSV nucleocapsid, we synthesized, by the procedure of Milligan et al. (22), a small prototype virion RNA (Fig. 8A). We chose to use VSV(NJ) RNA instead of VSV(Ind), with which the preceding studies were performed. VSV(NJ) had been the serotype of choice for recent transcription studies (11, 16) because it had excellent transcriptase activity and, in our hands, 20-fold-better in vitro replicative activity than VSV(Ind) (24). Since overall size was a limitation in the in vitro encapsidation reactions, the model RNA (Fig. 8A), designated NJ(77), contained 77 bases, with 26 bases from the exact 5' end of virion RNA (the encapsidation site) and 51 bases from the exact 3' end of virion RNA (the positive-strand leader gene) (including the transcription initiation site) (27). The RNA contained one extra G residue at the 5' end of the VSV sequence added to facilitate T7 transcription. We showed above that an extra G residue did not affect encapsidation (Fig. 6).

We first tested whether NJ(77) RNA was specifically encapsidated by purified VSV(NJ) N protein. With a mixture of NJ(77) RNA and the non-VSV RNA B, both were degraded in the encapsidation assay in the absence of N protein (Fig. 8B, lane USP). Both RNAs were encapsidated

FIG. 8. In vitro encapsidation of NJ(77) RNA by N(NJ) protein. (A) Schematic diagram of NJ(77) RNA. The boxes indicate the VSV(NJ) leader gene and the putative sites for encapsidation and transcription, including the intergenic nontranscribed AAAA, the putative NS protein binding site, and the initiation site at the 3' end (27). (B) Gel analysis. Samples of a mixture of purified ^{32}P -labeled VSV NJ(77) RNA (upper band) and ^{32}P -labeled non-VSV RNA B (lower band) were incubated as follows: lane N, in the presence of N(NJ) protein (6 μg) alone; lane N+USP, in the presence of both N(NJ) (6 μg) and USP (50 μg); lane USP, with USP (50 μg) alone for 30 min at 45°C. Lane M shows the sample without encapsidation or nuclease digestion. Nuclease-resistant RNAs were analyzed as described for Fig. 2. XC indicates the xylene cyanol dye.

nonspecifically by N(NJ) protein in the absence of USP (lane N). The N(NJ) protein did encapsidate NJ(77) RNA, but not the non-VSV RNA in the presence of USP (lane N+USP). The overall methodology for specific encapsidation is thus applicable to VSV of both serotypes.

The most critical proof of the correct assembly of the NJ(77) nucleocapsid was to test directly its capacity to be transcribed in vitro by added VSV RNA polymerase (L and NS proteins). When the synthetic NJ(77) nucleocapsids encapsidated in the specific reaction were incubated with the VSV(NJ) RNA polymerase, we detected an RNA product about the length of the nucleocapsid template (Fig. 9, lanes a and g). The amount of this product increased with increasing amounts of the NJ(77) nucleocapsids, and no product RNA was synthesized in the absence of either the NJ(77) nucleocapsids or the viral polymerase (data not shown). A transcription product of identical size was obtained with NJ(77) nucleocapsids encapsidated with N(NJ) protein in the absence of USP, showing that the encapsidation observed in a nonspecific reaction (Fig. 8) also yields a functional nucleocapsid (data not shown). As controls for the specificity of transcription, less than 3% of the product was synthesized from unencapsidated NJ(77) RNA (Fig. 9, lane b), and no RNA was synthesized from a VSV positive-strand RNA, no. 14 (Fig. 3), either in its encapsidated form or as RNA (Fig. 9,

FIG. 9. Transcription of a synthetic VSV(NJ) nucleocapsid template. Purified unlabeled VSV RNAs [NJ(77) and no. 14; 1 μg of each] were encapsidated with purified VSV(NJ) N protein (31 μg) and USP (50 μg) in a 250- μl reaction mixture. The non-VSV RNA A (1 μg) was encapsidated with N protein in the absence of USP. Samples (80 μl) of the nucleocapsids or an equivalent amount of unencapsidated RNAs (~ 0.3 μg) were transcribed by purified VSV(NJ) RNA polymerase in the presence of [α - ^{32}P]UTP, and the total products were analyzed from NJ(77) nucleocapsid (lane a), NJ(77) RNA (lane b), no. 14 nucleocapsid (lane c), no. 14 RNA (lane d), non-VSV A nucleocapsid (lane e), and non-VSV A RNA (lane f). In a second experiment, the NJ(77) nucleocapsid was transcribed and either the total product (lane g) or product selected by hybridization to a deoxynucleotide (47 bases) complementary to positive-strand leader RNA (lane h) was analyzed and compared with the hybridized product from transcription of wild-type RNA-N(NJ) (lane i) as a marker. XC indicates the xylene cyanol dye.

lanes c and d). RNA 14 contained a specific encapsidation signal and formed an authentic nucleocapsid, but it did not possess a transcription initiation signal and was not transcribed. Likewise, the non-VSV RNA A could be encapsidated in the nonspecific reaction, but neither the encapsidated form nor the RNA itself served as a template for transcription (Fig. 9, lanes e and f). In a second experiment, the product of transcription of the NJ(77) nucleocapsid was selected by hybridization to a deoxyoligonucleotide probe (47 nucleotides) that was the complement of positive-strand leader RNA; the RNase-resistant product RNA is shown in Fig. 9, lane h. The largest hybridized product was 47 nucleotides, identical to the size of the hybridized product synthesized in a reaction with purified wild-type VSV(NJ) nucleocapsids (Fig. 9, lane i). These data suggested that in vitro encapsidation produced synthetic VSV nucleocapsids which could, in fact, be specifically transcribed by the viral RNA polymerase and that the product was of the correct positive-strand sense and contained the proper 5' 47 nucleotides.

DISCUSSION

We have modified the methodology of Blumberg et al. (6, 7) to develop an assay for the specific encapsidation of synthetic VSV RNAs containing the putative encapsidation signal for the initial binding of N protein required for

encapsidation. The 5' ends of both negative- and positive-strand leaderlike VSV RNAs can be specifically encapsidated by purified VSV N protein, whereas other VSV RNAs cannot (Fig. 2 and 3). In these reactions, a significant molar excess (1,000-fold) of N protein is required to drive the encapsidation. A key feature of the assay was that poly(A) or USP was required to make the assay specific for the VSV encapsidation signal, since in the absence of these components the VSV RNAs as well as several non-VSV RNAs also were bound by N protein in a nuclease-resistant form (Fig. 2). We have shown that poly(A) will bind N protein weakly, but apparently enough to compete for N binding to completely heterologous RNA but not with VSV RNAs containing an encapsidation signal. Blumberg et al. (6) first reported that N protein would bind authentic VSV positive-strand leader RNA isolated from infected cells and suggested that the 5'-terminal 14 nucleotides possessed the binding site. Our results support the importance of the 5'-terminal sequences and suggest that the N protein binding site may be as few as 10 nucleotides, although 19 nucleotides gave optimal encapsidation (Fig. 2 and 3). It is not clear why the earlier study failed to obtain significant nonspecific encapsidation of all small RNA fragments as we observe here; however, it is possible that the preparation of low-molecular-weight RNA from infected cells may have had some RNA species which provided the discrimination.

The data presented here also suggest that, at least for the *in vitro* reaction, the VSV encapsidation site need not be at the exact 5' end of the RNA. The addition of 1, 4, 19, and 35 non-VSV nucleotides upstream still allowed encapsidation of full-length RNA, although the efficiency dropped somewhat for the latter two (Fig. 6). Thus encapsidation with VSV N may proceed in both the 3' and 5' directions from the encapsidation signal. In the case of the longest RNA (no. 16) used in these experiments, about four molecules of N protein may be added 5' of the encapsidation signal.

We have not been able to define in detail a unique sequence for the encapsidation site in these reactions. An RNA (no. 14) that was missing the normal VSV 5'-terminal A was encapsidated very well (Fig. 6), and poly(A) was encapsidated to some extent. Another control plasmid-derived RNA that was A+U rich (5'-GAAUACAAGCUUG AUAAUAUUGGUUC-3') and (if the 5' G is not counted) contained A residues at positions 1, 4, 7, 13, 16, and 19 but had other sequence differences from VSV (underlined) was also specifically encapsidated (data not shown). This pattern of A residues lacks only the A at position 10 in the VSV consensus sequence but contains extra alternating A's at positions 16 and 19.

Despite the fact that the *in vitro* encapsidation system used in these experiments must be considered artificial in that N protein is required in excess and is kept in soluble form only in high-salt solution, it is significant that we can demonstrate functional and relatively specific encapsidation *in vitro*. N protein readily forms nonfunctional aggregates in the test tube or in the cell cytoplasm (6, 28, 30, 33). From studies of the association of N and NS protein (13, 20, 21, 28, 30), it seems likely that the phosphoprotein NS normally plays a role in maintaining the N protein in an unaggregated state until it associates with VSV RNA. We tested whether the addition of NS (or other viral) protein in the soluble protein fraction of infected cells would have an effect on *in vitro* encapsidation but found none.

We found that either USP or poly(A) was necessary for specific encapsidation. Poly(A) sequences within the USP fraction may be responsible for the effect of USP, but this

has not yet been determined. While any model of the mechanism of the functional role of poly(A) in our *in vitro* system must remain highly speculative, one possibility may be that it simulates the role played by NS in the infected cell. Since poly(A) has the repeat A sequence thought to be important in the encapsidation signal, it is possible that individual N protein molecules associate with poly(A) stretches. As long as the N protein on the poly(A) remains unassociated with adjacent N molecules, this complex may represent a source of nonaggregated N protein. The ways in which such a complex might enhance specific associations are, however, presently unknown.

The most crucial test for the authenticity of a synthetic nucleocapsid created by *in vitro* encapsidation is whether it can serve as the template for transcription with the VSV RNA polymerase. Since transcription in VSV is known to initiate only at the 3' end of virion RNA (14), we synthesized a virionlike negative-strand RNA containing just the ends of the VSV(NJ) virion RNA (Fig. 8). This RNA could be specifically encapsidated with N(NJ) protein and, in fact, was transcribed into a template length positive-strand RNA by the viral polymerase (Fig. 9). Initiation appeared to occur at the 3' end of the nucleocapsid RNA, since the product selected by the leader probe was the same size as leader RNA synthesized from the wild-type virus nucleocapsid. Neither the unencapsidated negative-strand NJ(77) RNA alone or other RNAs or nucleocapsids lacking the 3' end of virion RNA were transcribed, demonstrating the specificity of transcription for an encapsidated RNA containing the 3' end of virion RNA. The method of encapsidation, however, did not seem to matter, since we got identical transcription from the NJ(77) nucleocapsid encapsidated in either the specific or nonspecific reaction.

Transcription of the NJ(77) nucleocapsid yielded a product the apparent length of the template, but no leader-length RNA (Fig. 9), as is synthesized by virus. The synthetic template has the leader gene fused directly to the 5' 26 nucleotides of the genome RNA and thus lacks the normal N gene sequences downstream of the leader gene. The absence of leader RNA synthesis from this template suggests that the distal N gene sequences may be required for proper termination during leader RNA synthesis. The ability to create completely synthetic nucleocapsids with biological activity should in the future allow the dissection of the conserved *cis* sequences that appear by sequence analysis to be important for the synthesis of leader RNA and the synthesis and processing of mRNAs, including initiation, capping, methylation, and polyadenylation. Ultimately, one would like to extend this analysis to the RNA signals required for *in vitro* RNA replication and encapsidation.

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