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Sequence-specific contacts between the RNA polymerase of vesicular stomatitis virus and the leader RNA gene

(nucleocapsid structure/methylation protection/RNA polymerase promoters/protein–nucleic acid interactions)

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ABSTRACT **Methylation-protection studies with nucleocapsids from vesicular stomatitis virus indicate that the viral polymerase (L and NS proteins) contacts the genomic RNA template in the middle of the leader gene, 16–30 nucleotides from the 3′ terminus. The data suggest that the NS protein binds to the sequence**

$$3'\ \text{G-G-U-A-}\overset{20}{\dot{\text{A}}}\text{-U-A-A-U-A-G-U-A-A-}\overset{30}{\dot{\text{U}}}\ 5'$$

and may function as an initiator protein for transcription.

Vesicular stomatitis virus (VSV) contains a negative-strand RNA genome 12,000 bases long that serves as template for the virion-bound RNA polymerase. In the presence of nucleoside triphosphates, detergent-treated virus synthesizes five capped and polyadenylylated RNAs for viral proteins N, NS, M, G, and L and a short uncapped leader RNA (1). Leader RNA synthesis is initiated at the exact 3′ terminus of the genomic RNA, and transcription proceeds sequentially along the VSV genome (2, 3).

Two viral components are required for VSV transcription, the polymerase and the viral template. The RNA polymerase consists of the L protein (M_r, 190,000) and the NS protein (M_r, 32,000) (4). The L protein is probably the catalytic activity of RNA synthesis; the function of the NS protein is not known. The template for transcription is a ribonucleoprotein consisting of genomic RNA and a tightly bound nucleocapsid protein, N. Because transcription starts at the 3′ terminus of VSV RNA, the promoter for the polymerase is presumed to reside in the leader gene. However, the binding sites of the L and NS proteins along the genome have not been determined. Mellon and Emerson (5) have shown by reconstitution of polymerase–template complexes that L protein will only bind to the template in combination with NS protein. NS, on the other hand, will bind alone to purified templates. Thus, the NS protein may direct the binding properties of the L protein to the template.

We have exploited the finding of Gilbert and coworkers (6) that some proteins can alter the susceptibility of base residues to methylation by dimethyl sulfate at their site of binding. Regions of specific protein–nucleic acid contact often show perturbations of the sequence pattern when probed with dimethylsulfate. We report that the N protein of VSV causes no significant perturbations of methylation of guanosine residues for more than 100 nucleotides from the 3′ end of VSV RNA. This finding has allowed us to examine other VSV proteins (L and NS) for their ability to interact at specific sites along the nucleocapsid. We have isolated nucleocapsids from VSV that contain only N, NS, and perhaps some L protein and compared them to nucleocapsids from which L and NS have been removed. We conclude that the VSV polymerase or NS protein alone contacts an A+U-rich sequence in the middle of the leader gene that may be analogous to the Hogness box located near the initiation sites of other eukaryotic genes. We postulate that the NS protein may function as an initiator for transcription by mediating interaction between the L protein and the template.

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MATERIALS AND METHODS

Growth of Cells and Virus. The Mudd–Summers strain of VSV Indiana was propagated in BHK21 cells as described (7, 8). Virus particles were purified by two isopycnic bandings and, in some instances, were subsequently isolated by rate velocity sedimentation in 10–40% sucrose gradients (9).

Isolation of Viral Nucleocapsids. Purified virus particles were lysed in 0.72 M NaCl/1.87% Triton X-100/0.6 mM dithiothreitol/ 9.35% (vol/vol) glycerol/0.05 M Tris·HCl, 7.4 (HSS buffer) for 30–60 min on ice (10). Nucleocapsids containing N and NS proteins were prepared by centrifugation of lysed virus particles for 15 hr at 24,000 rpm in an SW 27 rotor through 15.2% Renografin (in HSS buffer) and onto a cushion of 76% Renografin as described (11). The band at the 15.2%/76% Renografin interface was removed, diluted with 12 ml of HSS buffer, and pelleted for 2 hr at 35,000 rpm in the SW 41 rotor onto 50 μl of glycerol. Alternatively, nucleocapsids from the Renografin interface were subjected to chromatography on Bio-Gel A-5m as described (11). Nucleocapsid pellets were suspended directly in 200 μl of the dimethyl sulfate reaction buffer and analyzed for methylation protection. For further purification, nucleocapsids were layered over a 10-ml linear gradient of 15–47% Renografin in 100 mM NaCl/10 mM Tris, pH 7.4 (NT buffer). After centrifugation for 16 hr at 16,000 rpm in the SW 41 rotor, gradients were fractionated, and the nucleocapsid band was pooled and recovered by pelleting through 20% glycerol in 0.01 M Tris (pH 7.4) onto 50 μl of glycerol. A third cycle of banding in Renografin was performed to ensure complete removal of M, G, L, and NS proteins.

Transcriptase Assays. Isolated nucleocapsids were tested as templates for transcription by the addition of solubilized viral proteins as described (11).

Methylation Protection. Isolated nucleocapsids or phenol-extracted RNAs were suspended in 200 μl of 50 mM sodium cacodylate, pH 7.0/10 mM $MgCl_2$ (dimethyl sulfate buffer) (12). After the addition of 1 μl of dimethyl sulfate, the samples were vortexed and incubated at 30°C for 12 min. Dimethyl sulfate was inactivated by the addition of 75 μl of 1.0 M mercaptoethanol/1.5 M sodium acetate/1 M Tris acetate, pH 7.5/0.1 M EDTA (stop buffer). Methylated nucleocapsids were suspended in 2 mM dithiothreitol/100 mM NaCl/10 mM Tris·HCl, pH 7.4/ 1.5 mM $MgCl_2$/10 mM KCl and pelleted onto 50 μl of glycerol.

Abbreviation: VSV, vesicular stomatitis virus.

Pelleted nucleocapsids were suspended in 300 μl of 0.1 M NaCl/0.05 M Tris, pH 7.4/0.01 M EDTA/ (SET buffer), phenol extracted, and ethanol precipitated. Methylated RNA was labeled at the 3′ end with cytidine 3′,5′-bisphosphate as described (8, 13).

Terminally labeled RNA was either loaded directly onto sequencing gels or subjected to borohydride reduction and β-elimination with aniline according to the method of Peattie (14). After chain scission with aniline, samples were analyzed on urea/polyacrylamide sequencing gels.

Analysis of Nucleocapsid Proteins. Purified virus particles labeled with [^{35}S]methionine or [^{3}H]leucine were incubated in a standard *in vitro* transcription reaction (10) except that nucleoside triphosphates were replaced with [γ-^{32}P]ATP (4000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) in order to label phosphoproteins by the endogenous protein kinase. After incubation of 1 mg of purified VSV with 10 μCi of [γ-^{32}P]ATP in 200 μl for 30 min at 30°C, the samples were diluted to 8 ml with HSS buffer and pelleted through 4 ml of 20% glycerol onto 50 μl of 100% glycerol. Nucleocapsids from the glycerol interface were suspended in HSS buffer and further purified by centrifugation through 15.2%/76% Renografin step gradients as described above. Double-labeled protein samples from each step in the purification were precipitated with 10% (wt/vol) trichloroacetic acid, recovered by centrifugation, and rinsed with 100% ethanol. Dried pellets were suspended in 20 μl of 0.1 M dithiothreitol/2NaDodSO$_4$ 80 mM Tris, pH 8/10% glycerol (loading buffer) and analyzed on 10% polyacrylamide/NaDodSO$_4$ gels in 2.5 M urea by using a stacking gel overlay. Markers were total ^{35}S-labeled VSV proteins.

RESULTS

Methylation of Viral Nucleocapsids. The rationale of the methylation protection method is that the reactivity of base residues to methylation by dimethyl sulfate will be altered when protein is bound. Thus, methylation is either enhanced or diminished in the vicinity of protein binding. Dimethyl sulfate reacts most readily with the N7 position of guanosine and the N3 position of cytosine in RNA. Adenosine and uridine are considerably less reactive. In addition, there is some evidence for the alkylation of phosphate groups in RNA (15). We used two approaches for the study of VSV RNA–protein interactions. In one case, VSV nucleocapsids were labeled at the 3′ end of the RNA with pCp and RNA ligase, probed with dimethyl sulfate, and analyzed according to the chemical RNA sequence determination procedure (14). In the second approach, unlabeled nucleocapsids were treated directly with DMS, the RNA was extracted after inactivation of dimethyl sulfate, and the RNA was terminally labeled. Labeled RNA was then subjected directly to strand scission by β-elimination with aniline. Both approaches yielded similar results, and the RNA in nucleocapsids remained largely nuclease resistant after methylation.

Nucleocapsids were prepared by solubilization in 0.72 M NaCl/1.87% Triton X-100 (in HSS buffer) such that NS and possibly some L proteins were present in addition to N protein. After DMS treatment, RNA labeling, and strand scission with aniline at guanosine residues, fragments were analyzed on RNA sequencing gels. Fig. 1 shows DMS-treated VSV RNA (lane A) and DMS-treated nucleocapsids (lane C). No perturbations of base methylation are evident in the first 12–15 nucleotides at the 3′ end of VSV RNA in nucleocapsids. Starting at the guanosine in position 16 from the 3′ end and extending to position 30, however, there are dramatic alterations in the RNA sequence pattern. Guanosine residues in positions 16 and 26 were protected from methylation, and new bands appear at uridine residues in positions 21, 24, and 30. Methylation of guanosine

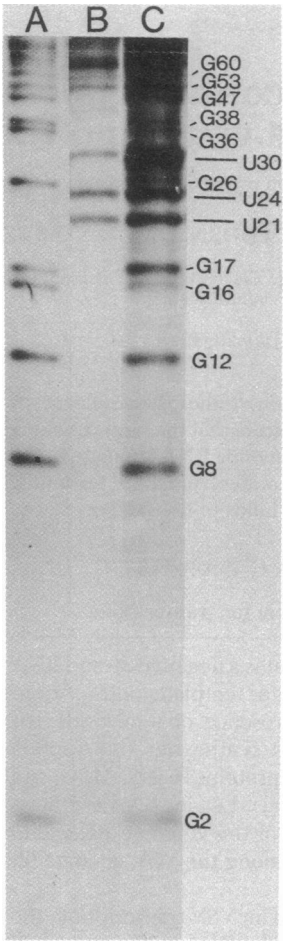

FIG. 1. Base methylation of deproteinized VSV RNA (lane A) and VSV nucleocapsids which contain N, NS, and perhaps L proteins (lanes B and C). After chemical modification, RNA was extracted and labeled at the 3′ end. Lanes A and C were subjected to borohydride reduction and β-elimination with aniline to induce strand scissions at methylated bases. Lane B was not treated with borohydride or aniline.

residues in positions 36, 38, 47, and 53 was otherwise similar to that of VSV RNA. Although variations occurred in relative band intensities from experiment to experiment, the pattern shown in lane C of Fig. 1 is highly reproducible and has been obtained in at least eight separate experiments.

The bands in positions 21, 24, and 30 were not expected because chiefly guanosine and cytosine base residues in RNA are methylated by DMS. Each of these bands results from RNA strand scission at the sequence A-A-U. As shown in Fig. 1, lane B, nucleocapsids treated with dimethyl sulfate but not subjected to borohydride reduction and β-elimination also showed strand scissions at positions 21, 24, and 30 (lane B in Fig. 1). These bands could result from direct chain scission through the formation of phosphotriesters in the phosphate backbone of VSV RNA. Thus, in the presence of N, NS, and L proteins, dimethyl sulfate may alkylate phosphate groups at this site. Some precedent for phosphate alkylation in RNA is available but has not been shown to be affected by the presence of proteins bound to RNA (15). However, we cannot rule out the possibility that uridylate methylations give rise to chain scissions at positions 21, 24, and 30.

The data in Fig. 2 *Upper* were obtained by microdensitometer scans of longer migrations on sequencing gels. The nucleocapsids represented in Fig. 2 *Upper* were additionally purified by banding in 15.2%/76% Renografin gradients and show the same pattern of sequence perturbation as in Fig. 1. The tracings were integrated by computer and the logarithm of the ratios of RNA to nucleocapsids is plotted according to the Gilbert method (6). It is evident that the major perturbations of the dimethyl sulfate reaction that occur in isolated nucleocapsids are in the middle of the leader gene between positions 16 and

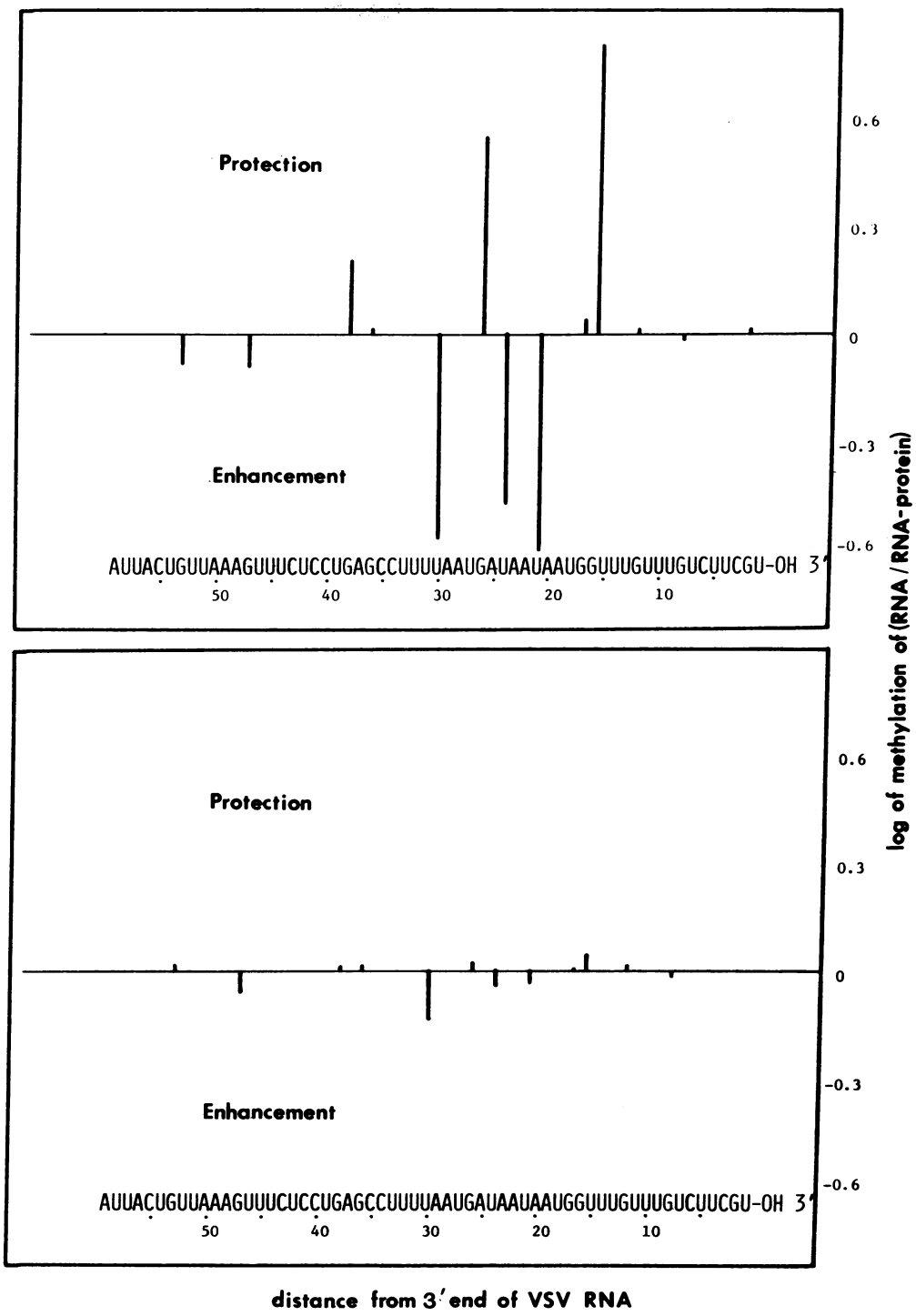

FIG. 2. Computer-plotted ratios of methylation patterns of VSV RNA and VSV nucleocapsids. Densitometer tracings of autoradiograms were integrated and plotted as protection or enhancement of base methylation. (*Upper*) Nucleocapsids after single banding in 15.2%/76% Renografin step gradients. (*Lower*) Nucleocapsids after banding once in Renografin step gradient and twice in 15–47% linear Renografin gradients to remove L and NS proteins.

30 from the 3′ end of VSV RNA. In some experiments, enhancement of guanine-47 was noted but this effect was not readily reproducible.

The results shown in Figs. 1 and 2 *Upper* were obtained with nucleocapsids purified by solubilization in 0.72 M NaCl and 1.87% Triton X-100, but treatment of either intact virus particles or nucleocapsids lysed with 1.87% Triton X-100 alone (no salt) produced similar perturbations of the methylation reaction (data not shown). We conclude that the altered methylation pattern in the middle of the leader gene results from a protein(s) that is bound to the viral RNA during packaging. The protein remains bound to the leader gene after high-salt solubilization and one banding in Renografin step gradients. Because the only detectable proteins on viral nucleocapsids that correlate with the sequence perturbations shown in Figs. 1 and 2 *Upper* are N and NS, we devised methods to remove NS completely from

isolated nucleocapsids, leaving only N protein and RNA. As explained below, loss of the sequence perturbations correlates with the removal of the NS protein (Fig. 2 *Lower*).

Removal of NS Protein from Viral Nucleocapsids. In order to analyze N protein interactions with RNA separately, it was necessary to obtain nucleocapsids free of the polymerase proteins NS and L. Published procedures (11) using HSS buffer solubilization of [^{35}S]methionine- or [^{3}H]leucine-labeled virions followed by pelleting on 15.2%/76% Renografin step gradients produced nucleocapsids that contained only N protein when analyzed by Coomassie blue staining or autoradiography after NaDodSO$_4$/polyacrylamide gel electrophoresis (data not shown). Fig. 3 provides a qualitative estimate of the relative amounts of N protein in relation to total phosphoprotein as nucleocapsids were purified. Viral extracts were incubated at 30°C with [γ-^{32}P]ATP of high specific activity to improve detectability. When the proteins were analyzed on gels, substantial amounts of residual NS (the major phosphoprotein) was detected after solubilization and after Renografin step gradients. Thus, although the majority of the M, G, L, and NS proteins were removed by high-salt solubilization followed by centrifugation through 15.2%/76% Renografin, small amounts of NS still remained on the nucleocapsids. Because the majority of the L and NS proteins on nucleocapsids probably reflects a packaging excess (5), the small amount remaining after HSS buffer stripping might represent specifically bound polymerase and therefore had to be eliminated.

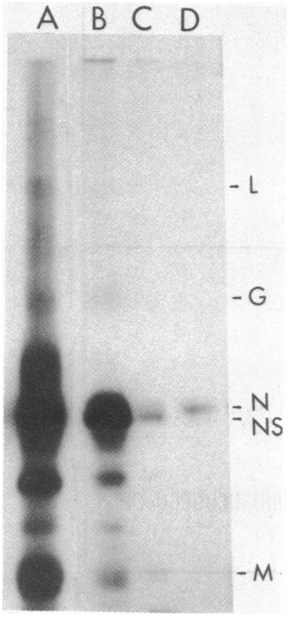

FIG. 3. Polyacrylamide gel analyses of ^{35}S- and ^{32}P-labeled VSV proteins at three stages of nucleocapsid purification. To demonstrate in a qualitative sense the removal of phosphoproteins relative to N protein, a sample at each step was precipitated with 10% trichloroacetic acid. Proteins were recovered by centrifugation, rinsed with 100% ethanol, dried, and loaded on a NaDodSO$_4$/polyacrylamide gel containing 2.5 M urea. Approximate radioactivities in each lane are noted as cpm(^{35}S/^{32}P). Lanes: A, supernatant after [γ-^{32}P]ATP labeling reaction (15,074/15,305); B, nucleocapsid pellet after solubilization in HSS buffer (2300/2597); C, nucleocapsids from *B* after banding at a 15.2%/76% Renografin interface (2100/331); D, nucleocapsids from Renografin step gradient after banding in a linear 15–47% Renografin gradient (4944/152). ^{32}P was determined by Cherenkov radiation; ^{35}S was measured in the ^{14}C channel of a Beckman spectrophotometer and adjusted for approximate radioactive crossover. Fluorography was overnight with Kodak XR-5 film.

Table 1. Double-labeling experiment showing separation of *in vitro* ^{32}P-labeled VSV phosphoproteins from [^{3}H]leucine-labeled viral nucleocapsids by using Renografin

Sample	Radioactivity,* cpm [^{3}H]Leucine	Radioactivity,* cpm [^{32}P]Phosphoprotein	Ratio ^{3}H/^{32}P	Purification Fold	Purification %
Total *in vitro* phosphokinase	251,940	1,136,808	0.222	1	0
HSS nucleocapsid pellet	60,528	282,880	0.214	1	0
Renografin 15.2%/76% step gradient	69,216	184,464	0.375	1.7	40
Renografin 15–47% linear gradient, nucleocapsid peak	50,680	13,660	3.71	16.7	94

* Total trichloroacetic acid-precipitable radioactivity.

When nucleocapsids from Renografin step gradients were banded in 15–47% linear Renografin gradients, the phosphoproteins were removed below detectable levels but the ^{35}S label in N protein remained (Fig. 3, lane D). Additional cycles of banding in Renografin gradients were used to remove G, M, L, and NS proteins completely for methylation protection studies of N protein–RNA interactions. These nucleocapsids remained active templates for transcription and synthesized the same RNA products as detergent-lysed VSV when reconstituted with NS and L proteins (data not shown).

Table 1 shows the purification of nucleocapsids double labeled *in vivo* with [^{3}H]leucine and *in vitro* by the endogenous protein kinase and [γ-^{32}P]ATP. After HSS buffer treatment, the ratio of ^{3}H to ^{32}P did not change. However, after banding at a 15.2%/76% Renografin interface, about 40% of the phosphoprotein was removed in relation to the [^{3}H]leucine-labeled protein. It should be noted that all of the detectable ^{3}H radioactivity is in N protein at this stage of purification (data not shown). Because N protein represents about 30% of the total protein in virus particles, the net removal of phosphoprotein in relation to N protein alone was about 84% after the Renografin step gradient. Depending upon how the interface was collected, however, the extent of purification varied at this step by as much as 3-fold.

By banding double-labeled nucleocapsids in linear 15–47% Renografin gradients, the most significant removal of phosphoprotein was achieved. Sharp peaks of ^{3}H and ^{32}P migrated to separate but overlapping positions (data not shown). Only 6% of the original phosphoprotein comigrated with the ^{3}H-labeled nucleocapsids (Table 1). Thus, in relation to the original N protein content, the net purification was at least 30-fold.

Probing of Nucleocapsids After Removal of Polymerase. VSV nucleocapsids that were banded two or three times in Renografin to remove NS protein showed methylation patterns like those of deproteinized RNA (Fig. 2 *Lower*). Occasionally, an enhanced band appeared at the uridine in position 30 with both Renografin-purified nucleocapsids and RNA deproteinized by a single phenol extraction. After two phenol extractions or methylation at 90°C, however, these extra bands were completely eliminated (Fig. 1, lane A). Thus, the NS protein is probably tightly bound to the RNA and is difficult to remove completely.

We conclude that bases in isolated VSV nucleocapsids that contain only N protein are susceptible to methylation by DMS with a pattern that is like that of VSV RNA. The data of Fig. 2

Lower together with longer gel migrations indicate that the methylation pattern of these nucleocapsids and that of deproteinized RNA are the same for more than 100 nucleotides from the 3′ end. Thus, the N protein does not appear to protect the bases from methylation by dimethyl sulfate by steric hindrance. These results suggest that molecules of N protein are bound to the phosphate backbone of the RNA and do not form specific contacts with bases at the 3′ end of VSV RNA that affect methylation.

DISCUSSION

We have used methylation protection to study sequence-specific protein interactions with VSV RNA. The N protein of VSV renders the RNA resistant to nuclease but does not significantly alter the reactivity of the RNA to methylation by dimethyl sulfate. Thus, this approach allowed us to examine the binding of the RNA polymerase proteins (L and NS) to the nucleocapsid. Each of the VSV proteins can be removed from virus particles by using various combinations of detergent, salt, Renografin, and other reagents. Residual amounts of each protein, however, are difficult to remove by any extraction method. We utilized protein phosphorylation with [γ-^{32}P]ATP of high specific activity to monitor the presence of small amounts of the VSV NS protein. We can isolate nucleocapsids containing predominantly NS and N proteins or nucleocapsids containing only N protein. Although occasionally detected, small amounts of L protein probably did not remain bound to the nucleocapsids isolated on Renografin because aggregated L protein often cosediments with nucleocapsids (unpublished data). We have also obtained similar results by using methylation protection of nucleocapsids reconstituted with purified NS protein (unpublished data).

Transcription of VSV RNA appears to be a linear, sequential process that is initiated *de novo* at the 3′ terminus of the genomic negative strand. Thus, the mechanism by which the VSV polymerase binds to its template and initiates transcription at the 3′ end of the genome probably involves sequence-specific protein–RNA interactions. Approximately 20–25 bases upstream from each of the VSV genes is an A+U-rich sequence that resembles the Pribnow box or Goldberg–Hogness transcription promoter site (16). The most 3′ terminal of these putative promoter sites resides in the middle of the VSV leader gene. In this study, we analyzed protein–RNA interactions in the leader gene and found that, when the NS protein of VSV is present, the reactivity of RNA with dimethyl sulfate, a mild alkylating agent, is altered in the middle of the leader gene. Because the NS protein is required for binding and transcription by the VSV polymerase complex (5, 11), these findings suggest that NS may function as an initiator protein for transcription by recognizing and binding to specific sites on the genome. In this sense, NS is analogous to the initiator factors of mammalian RNA polymerase III described by Roeder and coworkers (17) which bind in the middle of the 5S RNA gene. The transcription products of RNA polymerase III, like the VSV leader, are short, noncapped, nonpolyadenylylated RNAs. In this study, we identified a component of a promoter site of the VSV polymerase at the sequence 3′ G-G-U-A-A-U-A-A-U-A-G-U-A-A-U 5′ in the leader gene. Templates for the start of leader RNA and N mRNA are present symmetrically about 25 nucleotides in both directions from this sequence. We do not know from the present study, however, if this sequence participates in the initiation of transcription of the leader RNA upstream or the N mRNA downstream or both.

A defective interfering particle (DI-LT2) of VSV that contains the leader RNA gene at an internal site 71 nucleotides away from the 3′ end has recently been described (7). Although the complete leader sequence and the start of the N gene are present, neither the leader RNA nor any other mRNA transcript is synthesized *in vitro* by DI-LT2. In spite of the inability of this region to produce transcription products, we have recently found dramatic perturbations of the sequence pattern in the middle of the leader gene of DI-LT2 under conditions such that NS protein remained bound (unpublished data).

The results with DI-LT2 suggest that the NS protein can bind at an internal sequence-specific site along the nucleocapsid and yet fail to initiate transcription. Whether L and NS can together bind to the leader gene of DI-LT2 is not known, however. It is possible that formation of a polymerase complex between NS and L proteins depends upon the position of the leader gene relative to the 3′ terminus. On the other hand, the polymerase complex may form and bind identically at the leader genes of VSV and DI-LT2 but be capable of initiating transcription only when the leader gene is present at the 3′ terminus.

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