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Purified matrix protein of vesicular stomatitis virus blocks viral transcription *in vitro*

(RNA synthesis/electron microscopy)

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ABSTRACT **One of the major structural proteins of vesicular stomatitis virus is a small, nonglycosylated, matrix protein which associates with the nucleocapsid core during final stages of morphogenesis and budding. Biochemical and genetic studies suggested that the matrix protein regulates RNA synthesis both *in vitro* and *in vivo*. We have purified biologically active matrix protein from the virus and have directly shown that it significantly inhibits RNA synthesis *in vitro* mediated by the virion-associated RNA polymerase at low ionic strength (0.02 M). The inhibition was >80% when the ratio of matrix protein to the major nucleocapsid protein in the transcribing complex was 2:1 (wt/wt). The inhibition was found to be at the level of RNA chain elongation and not at the initiation step. Electron microscopic studies revealed that inhibition of transcription by matrix protein was accompanied by a profound structural change of the transcribing nucleocapsid from an extended structure to a highly compact form. At higher ionic strength (0.12 M), the matrix protein failed to interact with the nucleocapsid. The matrix protein appears to be involved in condensing the nucleocapsid and blocking transcription during maturation of the virus particle.**

Vesicular stomatitis virus (VSV) is a bullet-shaped membrane-maturing virus (50 × 175 nm) belonging to the rhabdovirus group (1). The single-stranded genome RNA of negative polarity is tightly associated with the nucleocapsid protein N. With two other minor proteins, L and NS, the resulting ribonucleoprotein particle (RNP) becomes highly condensed during final stages of maturation and is enclosed within the membrane envelope in a helically wound form (2). Glycoprotein G and matrix protein M are located outside and beneath the lipid bilayer, respectively. The structural integrity of the condensed RNP (50 × 175 nm) (3–5) is lost when the virus is disrupted with a nonionic detergent. The RNP is released in an extended ribbon-like coiled structure with dimensions of 20 × 700 nm (3, 5). The purified RNP is transcriptionally active and synthesizes five mRNAs *in vitro* when incubated in the presence of four ribonucleoside triphosphate precursors (6). Recently it has been shown that removal of only the protein G by treatment with detergent in the absence of salt maintains the intact condensed structure of the RNP (7, 8). These results suggested that protein M probably is involved in the maintenance of the ordered structure of the RNP and its association with the RNP depends on the ionic strength of the disruption medium.

In vitro transcription studies generally have been carried out with detergent-disrupted virions in the presence of an optimal salt concentration (0.1–0.14 M NaCl) (9). At a lower salt concentration the transcription reaction is significantly inhibited (10). It appears that protein M interacts with the RNP structure at low ionic strength (7, 8) and may play a role in the transcription process (10). Moreover, genetic and biochemical studies (10–14) have also suggested that RNA synthesis by VSV is modulated by protein M.

In this communication, we present direct evidence that purified protein M strongly inhibits RNA synthesis *in vitro* at low ionic strength. Furthermore, under the inhibitory condition, protein M profoundly alters the structure of the RNP.

MATERIALS AND METHODS

Purification of VSV. VSV (Indiana serotype) was grown in baby hamster kidney cells (BHK-21, clone 13) adapted to suspension culture and purified as described (15).

Isolation of Various Components of Purified VSV. (*a*) To isolate RNP (N–RNA–L–NS complex), purified virus (500 μg/ml) was disrupted in 10 mM Tris·HCl, pH 8.0/5% (vol/vol) glycerol/0.4 M NaCl/1.85% Triton X-100/0.6 mM dithiothreitol (final concentrations). RNP was sedimented by centrifugation, in an SW 60 rotor at 45,000 rpm for 2 hr, through 30% (vol/vol) glycerol onto a 100% glycerol cushion.

(*b*) To isolate a mixture of proteins G and M the supernatant obtained from the top of the centrifuge tube in *a* was dialyzed against 10 mM Tris·HCl, pH 8.0/20% glycerol/0.2 M NaCl/1 mM dithiothreitol (buffer A) and then concentrated on dry Sephadex G-200 beads.

(*c*) To isolate N–RNA complex, purified virus (250 μg/ml) was disrupted in the same buffer as described above, except that the final NaCl concentration was 0.8 M (16). The complex was purified by centrifugation through 15% (wt/vol) Renografin solution and collected on a 76% Renografin pad. The N–RNA complex was again treated with buffer containing 0.8 M NaCl and purified by pelleting on a glycerol pad (100%) as described for RNP above.

(*d*) To isolate a mixture of proteins L and NS, RNP was purified as in *a* and treated with buffer containing 0.8 M NaCl (final concentration) without Triton X-100. The complex was centrifuged through 30% glycerol as described above, and the released proteins L and NS were recovered from the top of the centrifuge tube, dialyzed against buffer A, and concentrated on dry Sephadex G-200 beads.

Purification of Protein M. Purified VSV (500 μg/ml) was mixed with [^{3}H]methionine-labeled VSV (700,000 cpm), disrupted with buffer containing 0.4 M NaCl (final concentration), and centrifuged through 30% glycerol as described above. The supernatant fraction from the top of the centrifuge tube was collected and dialyzed against 10 mM KP_i buffer (pH 7.4) containing 10% glycerol, 0.2% Triton X-100, and 1 mM dithiothreitol. The fraction was loaded onto a hydroxyapatite column (10 × 0.7 cm) equilibrated with the same buffer. The proteins were eluted with a 0.01–0.6 M linear gradient of KP_i. Fractions (0.5

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Abbreviations: VSV, vesicular stomatitis virus; RNP, ribonucleoprotein particle.

ml) were collected and 15-μl aliquots were assayed for radioactivity to locate the protein peaks. Pooled fractions of proteins M and G were further purified by rechromatography on hydroxyapatite column. The final pooled fractions were dialyzed against buffer A and concentrated on dry Sephadex G-200. Concentrations of purified proteins G and M were 300 μg/ml. The proteins in each fraction were analyzed by electrophoresis on 10% $NaDodSO_4$/polyacrylamide gels as described (6).

Electron Microscopy. Samples were obtained directly from the transcription reaction mixture and prepared for electron microscopy by using the benzyldimethylalkylammonium chloride method essentially as described by Vollenweider *et al.* (17). The samples were adsorbed onto Parlodian-coated grids, rotary shadowed with platinum/palladium, and observed in a Philips 201 electron microscope, at 60 kV beam voltage and 20,000-fold magnification.

RESULTS

Effect of Salt on Various Transcribing Particles of VSV. It is well established that the virion-associated RNA polymerase activity of VSV is active *in vitro* when purified virus particles are disrupted with nonionic detergent (9) or melittin (18). Under these conditions, RNA synthesis occurred in the presence of all viral protein components. RNA synthesis also occurred after removal of proteins G and M by isolating the RNP from detergent-disrupted virus (9). RNP can be further separated into N–RNA complex and a soluble mixture of proteins L and NS by high-salt treatment. Reconstitution of RNA synthesis occurred when these two fractions were combined (15). A dramatic difference in ionic strength requirement was observed when these transcribing particles were prepared by these different procedures. α-^{32}P-Labeled *in vitro* products analyzed by electrophoresis on 20% polyacrylamide gel yielded larger mRNA species at the origin and smaller RNA products, including the 9S RNA (19), leader RNA (20), and promoter proximal sequences (21), in the gel (Fig. 1). The RNA species seen immediately above the dye marker are small 5′-proximal oligonucleotides of N–mRNA of chain length 11–14 bases (22). Synthesis by detergent-disrupted virus was greatly decreased (>90%) when the concentration of NaCl was lowered from 0.12 M (lane B) to 0.02 M (lane A). In contrast, RNA synthesis by purified RNP was virtually unaffected at these concentrations of NaCl (lanes C and D). Similarly, the effect of NaCl was seen when transcription was carried out with RNP combined with the low-salt supernatant (containing proteins G and M) (lanes E and F). However, the salt had no effect on RNA synthesis when high-salt core and low-salt/high-salt supernatant (containing only proteins L and NS) were mixed (lanes G and H). These results clearly indicate that the virion-associated RNA polymerase activity was unaffected by NaCl concentration within the range 0.02–0.12 M (lanes D and H). Inhibition of RNA synthesis at low ionic strength was seen only when the transcribing RNP was associated with proteins G and M (lanes A and E). These findings confirm previous results (10) and suggest that interaction of either of these proteins with RNP was prevented when the NaCl concentration of the reaction mixture was increased (lanes B and F).

Effect of Purified Proteins G and M on Transcription *in Vitro*. In order to demonstrate directly whether protein G or M was involved in the inhibitory process, a low-salt fraction (containing proteins G and M) was prepared from purified virus and the proteins were separated by hydroxyapatite column chromatography. ^{3}H-Labeled purified virus was included with the fraction to monitor the protein peaks. Two peaks of radioactivity were clearly separated by a linear elution gradient of KP_i (Fig. 2). The proteins in peaks A and B were pooled and further purified by adsorption and elution from hydroxyapatite column. The proteins in each peak were analyzed by polyacrylamide gel electrophoresis and found to be essentially pure protein G (peak A) and protein M (peak B). The proteins in each fraction were studied for their effects on *in vitro* transcription

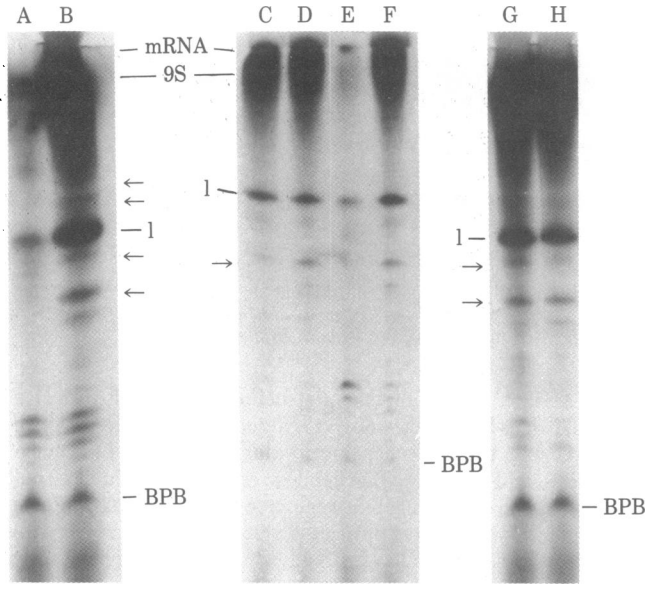

FIG. 1. RNA synthesis by various transcribing particles of VSV at 0.02 and 0.12 M NaCl. The reaction mixture (0.2 ml) contained 50 mM Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.05 mM CTP, 1 mM ATP, GTP, and UTP, 20 μCi (1 Ci = 3.7×10^{10} becquerels) of [α-^{32}P]CTP (410 Ci/mmol), and transcribing particles at salt concentrations indicated below. The reactions were terminated after 2 hr at 30°C by the addition of $NaDodSO_4$ to 0.5% (final concentration). The RNA products were purified as described by Colonno and Banerjee (20) and analyzed by electrophoresis at 600 V for 15 hr on a 20% polyacrylamide slab gel (30 × 14 × 0.15 cm) containing 8 M urea. RNA bands were located by autoradiography on Kodak XR-5 film. The lanes contained: purified VSV (10 μg) in 0.05% Triton N-101 in 0.02 M NaCl (A) and 0.12 M NaCl (B); RNP (3 μg) in 0.02 M NaCl (C) and 0.12 M NaCl (D); RNP (3 μg) and a mixture of proteins G and M (6 μg) in 0.02 M NaCl (E) and 0.12 M NaCl (F); N–RNA complex (3 μg) and a mixture of proteins L and NS (3 μg) in 0.02 M NaCl (G) and 0.12 M NaCl (H). Migration positions of mRNA, 9S RNA, leader RNA (1), and bromophenol blue dye (BPB) are indicated.

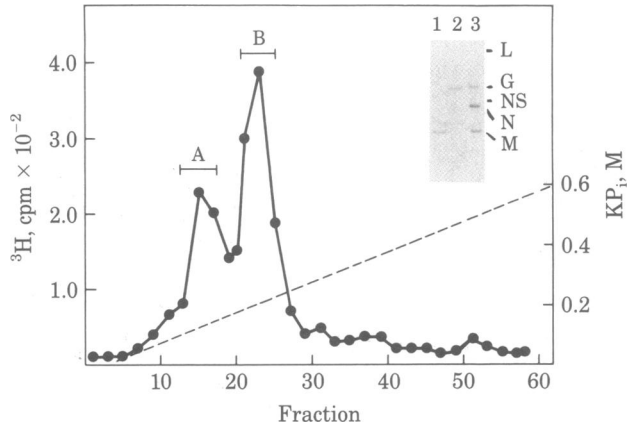

FIG. 2. Purification of proteins G and M by hydroxyapatite column chromatography. A mixture of proteins G and M was isolated from purified VSV and chromatographed. (*Inset*) Repurified proteins in peak A and peak B were analyzed by electrophoresis in 10% $NaDodSO_4$/polyacrylamide gel and stained with Coomassie blue R 250. 1, 2, and 3 represent proteins from peak B, peak A, and purified virus, respectively. L, G, NS, and M represent viral structural proteins.

reaction. Addition of protein M strongly inhibited RNA synthesis *in vitro* at 0.02 M NaCl, whereas a normal level of transcription occurred in the presence of 0.12 M NaCl (Fig. 3). The inhibition was >80% at a mass ratio of approximately 1:2 (RNP/protein M) and maximal inhibitory effect (90%) was obtained with RNP/protein M 1:5 (Table 1). In contrast, addition of protein G to the transcribing complex had virtually no effect on RNA synthesis. These results clearly indicate that the matrix protein specifically blocks transcription at low ionic strength, presumably by its interaction with the RNP.

Effect of Protein M on Initiation and Chain Elongation of RNA. Using detergent-disrupted virus and incomplete transcription conditions (i.e., absence of one or two ribonucleoside triphosphates), we previously showed that triphosphorylated oligonucleotides representing the 5′ ends of leader RNA (pppA-C) and mRNAs (pppA-A-C-A) were synthesized *in vitro* (23). By using this reaction, initiation events occurring at multiple sites on the genome RNA template can be monitored. In order to study whether inhibition of RNA synthesis by protein M was at the level of RNA chain initiation or at the elongation step, partial reactions with ATP and [α-^{32}P]CTP were carried at 0.02 and 0.12 M NaCl. The triphosphorylated oligonucleotides were dephosphorylated to achieve better resolution (23) on analysis by polyacrylamide gel electrophoresis. At low salt concentration there was at least a 4-fold increase in the leader RNA initiation (A-C synthesis) compared to mRNA initiations (A-A-C-A synthesis) which remained virtually unaltered (Fig. 4). These results indicate that interaction of protein M with the RNP was not at the initiation site of the RNA synthesis but possibly was on the N–RNA template such that it prevented extension of RNA chains. It is interesting to note that increase of salt concentration to 0.12 M inhibited A-A-C-related initiations (lane B) in partial reactions. Whether or not protein M plays a role in this differential initiation process is unclear.

Electron Microscopic Studies of Protein M–RNP Interactions. The above biochemical evidence indicated that, at low ionic strength, purified protein M interacted with the transcribing RNP such that RNA synthesis was profoundly affected. Because it has been suggested that protein M alters the RNP structure under certain conditions (7, 8) we first investigated the effect of the addition of high-salt supernatant (containing

Table 1. Effect of purified proteins M and G on transcription by RNP

Additions		Acid-insoluble radioactivity, cpm × 10^{-5}	
Protein M, μg	Protein G, μg	At 0.02 M NaCl	At 0.12 M NaCl
None	None	1.62	1.65
1.0	None	0.93	1.58
2.0	None	0.56	1.6
3.0	None	0.45	1.64
6.0	None	0.31	1.61
15.0	None	0.17	1.62
None	1.5	1.57	1.62
None	3.0	1.63	1.65
None	6.0	1.81	1.8
None	9.0	1.8	1.82

Transcription reactions were carried out as in Fig. 1, with purified RNP (3 μg) at 0.02 and 0.12 M NaCl. Proteins M and G were added as indicated. After incubation for 2 hr at 30°C, cold trichloroacetic acid-insoluble radioactivity in each tube was determined.

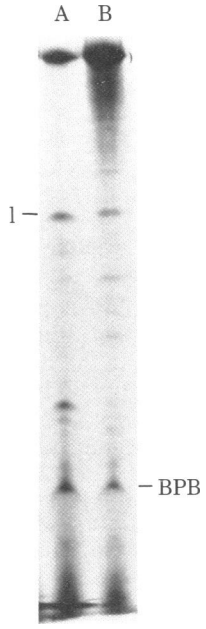

FIG. 3. Effect of purified protein M on RNA synthesis by RNP. Transcription reaction mixtures (0.2 ml) were as in Fig. 1 and included RNP (3 μg) and purified protein M (6 μg) at 0.02 M NaCl (lane A) and 0.12 M NaCl (lane B). The RNA products were analyzed as in Fig. 1. l and BPB, migration positions of leader RNA and bromophenol blue dye, respectively.

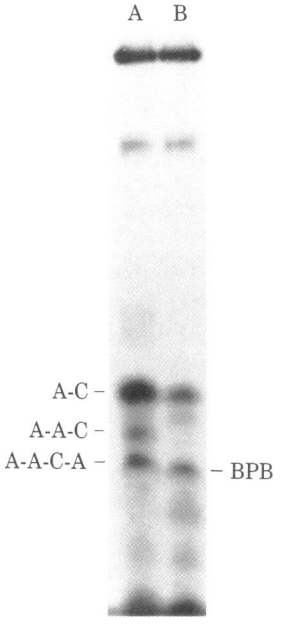

FIG. 4. Initiation of leader RNA and mRNA during interaction of protein M with N–RNA complexes. A partial transcription reaction was carried out with N–RNA complex (3 μg) and supernatant fraction containing proteins L, NS, G, and M (20 μg) in standard reaction mixture in the presence of 0.02 or 0.12 M NaCl, as in Fig. 1 except that 1 mM ATP, 30 μM CTP, and 50 μCi of [α-^{32}P]CTP were added as the ribonucleotide precursors. After incubation at 30°C for 2 hr, the RNA products were extracted with phenol, treated with calf intestinal alkaline phosphatase, and analyzed by electrophoresis in a 20% polyacrylamide gel as described by Chanda and Banerjee (23). Lanes: A, 0.02 M NaCl; B, 0.12 M NaCl. A-C, A-A-C, and A-A-C-A are the compositions of the synthesized oligonucleotides (23). The supernatant used in this experiment was prepared by treating purified virus with disruption buffer containing 0.8 M NaCl (final concentration). The released proteins were recovered from the top of the centrifuge tube after the N–RNA complex was centrifuged through 30% glycerol and dialyzed and concentrated.

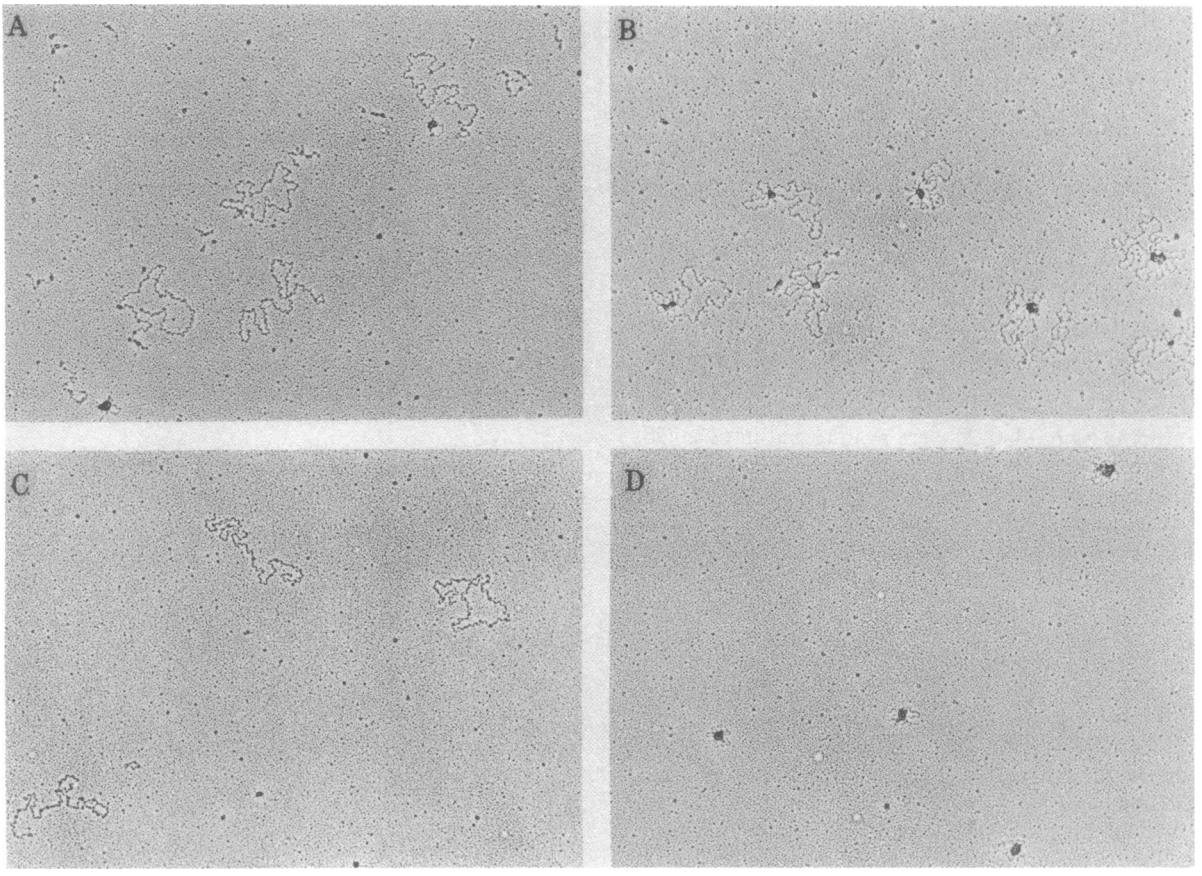

FIG. 5. Electron microscopy of nucleocapsid in the presence of protein M at 0.02 and 0.12 M NaCl. Transcription with N–RNA complex and the supernatant fraction containing proteins L, NS, G, and N was carried out at 30°C for 2 hr in the presence of 0.02 or 0.12 M NaCl as in Fig. 4. An aliquot of the reaction mixture was prepared for electron microscopy as described (17) at the desired NaCl concentrations. Structures of the nucleocapsid are shown at 0.12 M NaCl (*A*) and at 0.02 M NaCl (*B*). Purified protein M (6 μg) was added to the N–RNA complex (3 μg) and the resulting structures were visualized at 0.12 M NaCl (*C*) and 0.02 M NaCl (*D*). Scale: 2.75 cm represents 0.5 μm.

proteins L, NS, G, and M) on the N–RNA complex at 0.02 M and 0.12 M ("normal") salt transcription conditions. A dramatic structural change of the RNA occurred in the presence of the supernatant fraction at low salt compared to the same structure at normal salt (Fig. 5 *A* and *B*). Under normal salt conditions, the RNP had a typical extended ribbon-like beaded structure observed previously (3, 5). Addition of the supernatant fraction did not alter this structure. In contrast, in low salt, the long beaded structure was converted into a condensed form. Thus, the change of structure of N–RNA complex from extended form to condensed form virtually blocked the transcription process *in vitro* when proteins L, NS, G, and M were added.

We next studied the effect of purified protein M added directly to the N–RNA complex, in the presence of two salt concentrations. Purified protein M profoundly changed the structure of N–RNA complex at low salt (Fig. 5*D*). In fact, the change was more dramatic than observed with the transcription using N–RNA complex and high-salt supernatant (Fig. 5*B*). More than 80% of the particles scored contained this highly condensed structure. When purified protein G was used in place of protein M under similar conditions, the structure of the nucleocapsid was identical to that shown in Fig. 5 *A* and *C* at two salt concentrations (data not shown). These results directly demonstrate that purified protein M retains its biological function and specifically interacts with the nucleocapsid to change its conformation *in vitro* and consequently regulate the virion-associated RNA polymerase activity.

DISCUSSION

Results of biochemical and genetic studies suggest that the major nonglycosylated matrix protein (protein M) of VSV plays a role in the morphogenesis and replication of the virus (7, 8, 10, 13). Protein M appears to be involved in the budding process (24, 25) and in the regulation of RNA synthesis in infected cells (11, 12). By disrupting and fractionating virus under different ionic conditions, Wilson and Lenard (10) showed that crude virus fractions containing proteins G and M inhibited RNA synthesis *in vitro* only at low ionic strength. Also, nucleocapsid (RNP) retaining protein M was transcriptionally inactive at low ionic strength. In contrast, when purified protein M was added in large excess (2- to 5-fold) in the transcription reaction, the level of inhibition was significantly lower (30–40%) than that observed with whole viruses (13). Consequently it was not clear whether inhibition of transcription was mediated by protein M or if protein G also played a role in this process. Structural studies initiated by Newcomb and Brown (7, 8) suggested that protein M possibly was the determinant factor in the formation of the condensed structure of RNP.

In the present studies we purified the viral protein M to homogeneity by chromatography on a hydroxyapatite column (Fig. 2). The protein fraction was virtually free from contamination by protein G. The protein thus obtained was stable and biologically active as shown by its inhibitory effect on the *in vitro* transcription reaction at low ionic strength (Fig. 3). The inhibition was extensive (>90%) (Table 1) and mimicked inhibition of transcription observed with disrupted (Fig. 1, lane A)

or reconstituted virus (Fig. 1, lane E). Protein G was inactive in this reaction (Table 1). These results directly support the conclusion that only protein M interacts with the nucleocapsid at low ionic strength, resulting in virtual shut-off of transcription. Recently Pinney and Emerson (22) showed that purified protein M failed to inhibit total RNA synthesis *in vitro* at low ionic strength but regulated synthesis of some small 5′ proximal oligonucleotides of N–mRNA (chain length, 11–14 bases). Although we observed some change in small oligonucleotides synthesis, the overall inhibition of total RNA synthesis was quite profound (Fig. 3, lane A). It is not clear whether the failure to observe significant inhibition of RNA synthesis by protein M in previous studies (13, 22) was due to the method of purification of the protein or the conditions of RNA synthesis *in vitro*.

Purification of protein M has enabled us to determine the stoichiometry of protein M–nucleocapsid interaction. It appears that, at a protein N-to-protein M ratio of approximately 1:1 (wt/wt) in RNP, >70% inhibition was achieved (Table 1). Because the molecular weight of protein M (26,000) is approximately half that of protein N (50,000) it can be concluded that the two molecules of protein M and the one molecule of protein N can cause extensive inhibition of transcription. It has been reported that the number of molecules of proteins N and M in purified virus is approximately 2,300 and 4,700, respectively (1). Thus, it seems that purified virus particles contain similar number of protein M relative to protein N required for the observed condensation of RNP and inhibition of transcription. Inhibition of transcription of RNP by protein M at a mass ratio of 1:1 is more than 90% when crude preparations of low-salt supernatant (containing proteins G and M) were used (Fig. 1, lane E). The decrease of inhibitory activity of purified protein M [70% at 1:1 ratio (Table 1)] is possibly due to loss of native structure of the protein during purification process.

The inhibition of RNA synthesis was clearly at the level of RNA chain elongation and not at the initiation site (Fig. 4). Thus, it appears that low ionic strength favors binding of protein M to the RNP such that movement of the RNA polymerase enzyme is restricted (22). Increase of salt concentration dissociated protein M (this removes the block) and favored RNA chain elongation, release, and reinitiation. Because the intracellular ionic concentration is >0.1 M (26), the interaction of protein M with the RNP possibly occurs at the membrane prior to or at the time of the budding process. This interaction causes RNP to cease RNA synthesis prior to maturation. From the predicated sequence of VSV structural proteins, protein M is the most basic (27). Moreover, the amino-terminal sequence of protein M is highly basic (27). Whether this basic domain plays a role in the interaction with the genomic RNA in the nucleocapsid is unclear.

Finally, we have demonstrated directly that purified protein M interacts with RNP *in vitro* during transcription at low ionic strength and changes its structure to a compact form (Fig. 5). This observation is similar to that made recently by Newcomb *et al.* (8), who dissociated and reconstituted the skeleton structure by using purified virus. In our studies we have not been able to reconstitute the native helical structure of the RNP (8) after protein M interaction. This may be due to the inherent deficiency of our purified system or we have not maintained the precise reassembly condition *in vitro*. Heggeness *et al.* (28) observed that, in the absence of protein M, the virus nucleocapsid is loosely coiled and fully extended at low ionic strength (0.01 M), whereas at higher salt concentration (1 M) the RNP is tightly coiled with the appearance of a rigid rod. Conformational change of RNP during packaging has also been seen in virus-infected cells (29). Thus, it seems that the viral RNP is highly flexible and plastic and changes its conformation in response to the change in microenvironment. Ability to reassemble purified protein M and nucleocapsid should give us an opportunity to study in detail the mode of interaction of matrix protein with the virus nucleocapsid *in vitro*.

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