JOURNAL OF VIROLOGY, Jan. 1979, p. 134-142
0022-538X/79/01-0134/09$02.00/0

Vol. 29, No. 1

Role of the Membrane (M) Protein in Endogenous Inhibition of In Vitro Transcription by Vesicular Stomatitis Virus

ANTHONY R. CARROLL AND ROBERT R. WAGNER*

Department of Microbiology, School of Medicine, University of Virginia, Charlottesville, Virginia 22908

Received for publication 30 August 1978

An endogenous transcriptase inhibitor active at high concentrations of vesicular stomatitis (VS) virus was present in trypsinized whole virions but was absent from ribonucleoprotein cores containing only the L, N, and NS proteins. Poly(L-glutamic acid) effectively reversed the transcriptase inhibition. Transcription under noninhibited, inhibited, and poly(L-glutamic acid)-reversed conditions did not appear to greatly affect the nature of the RNA transcription product. The VS virion matrix (M) protein was purified to >98% homogeneity and was found to have an isoelectric point of ~9.0. Purified M protein inhibited transcription by ribonucleoprotein cores, an effect that was partially reversed by poly(L-glutamic acid). Two group III temperature-sensitive (*ts*) mutants of VS virus (*ts*O23 and *ts*G31) with lesions in the M protein exhibited little or no endogenous inhibitor activity compared with two wild-type strains and a group V mutant (*ts*O45) with a lesion in the G protein. The data presented strongly suggest that the virion M protein is responsible for the endogenous inhibition of in vitro RNA synthesis seen at high concentrations of VS virus.

Vesicular stomatitis (VS) virions contain an RNA-dependent RNA polymerase that functions as a transcriptase both in vitro and in vivo (2, 4, 5, 16). This system is capable of synthesizing in vitro at least four of the five viral mRNA's which translate authentic virus proteins (1, 6, 7). It has been suggested that in vivo virus-specific protein synthesis is regulated almost exclusively at the level of transcription (22). However, few data are available on the mechanism of this regulation or other controls that activate or suppress transcription of the VS virus genome.

An observation which may have some bearing on this problem was the report of Perrault and Kingsbury (17) that purified VS virus contains a specific inhibitor of the virion transcriptase. Strong inhibition of RNA synthesis in vitro occurred when VS virions were present at high concentrations (>0.2 mg of virus protein per ml). The inhibitor was considered to be an internal protein and showed some degree of serotype specificity (17). Breindl and Holland (8) subsequently reported that transcribing VS virus ribonucleoprotein (RNP) cores appeared to lack the inhibitory activity found in complete virions.

We have previously reported (10) that certain polyanions, in particular poly(L-glutamic acid), have the ability to reverse the transcriptase inhibitor active at high concentrations of VS virus. Furthermore, some preliminary data suggested that the inhibitor might be the virion membrane (M) protein (10). The data presented below support the hypothesis that the M protein is the endogenous transcriptase inhibitor of VS virus.

MATERIALS AND METHODS

Cell cultures and viruses. The wild-type (*wt*) strain of the Indiana serotype of VS (VS_{Ind}) virus was originally obtained from the U.S. Agriculture Research Center, Beltsville, Md. (23). Temperature-sensitive (*ts*) mutants of VS virus designated *ts*O23(III) and *ts*O45(V) and the wild type from which they were derived (Os.*wt*) were kindly provided by A. Flamand, Faculté des Sciences, Université de Paris-Sud, Orsay, France (14). The *ts* mutant designated *ts*G31(III) was kindly provided by C. R. Pringle, Institute of Virology, Glasgow, Scotland (18).

BHK-21 cells were cultivated as previously described (3). The methods of cultivating and purifying VS virus have been described (12) with the exception that *ts* mutants were cloned and propagated at 31°C.

Chemicals and radiochemicals. Nucleoside triphosphates were purchased from Calbiochem, La Jolla, Calif. Poly(L-glutamic acid), Triton X-100, and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, Mo. Whatman P11 cellulose phosphate was purchased from W. and R. Balston, Ltd., Maidstone, Kent, England. [^{3}H]UTP (12 Ci/mmol) was purchased from Schwarz/Mann, Orangeburg, N.Y.

Polyacrylamide gel electrophoresis. Proteins were analyzed with discontinuous Tris-glycine-buffered sodium dodecyl sulfate slab gels consisting of a 10% resolving gel and a 5% stacking gel as previously described (9). RNAs were extracted and analyzed on 10-cm cylindrical gels containing 2% acrylamide and 0.5% agarose as previously described (24).

RNA polymerase assay. The RNA polymerase

assay was performed as previously described (9, 10). The final reaction mixture consisted of 7.4×10^{-4} M dithiothreitol; 5×10^{-3} M magnesium acetate; 1.44×10^{-1} M NaCl; 0.37% Triton X-100; 5.87% glycerol; 7×10^{-4} M each ATP, CTP, and GTP; and 0.67×10^{-5} M [^{3}H]UTP (230 μCi/μmol) in 3×10^{-2} M Tris-hydrochloride (pH 8.0). Reactions were incubated at 31°C (unless otherwise stated) in stoppered tubes. At the desired time duplicate 100-μl samples were removed from each reaction and placed into 0.6 ml of 67 μM sodium pyrophosphate at 0°C containing 200 μg of yeast carrier RNA. Ice-cold 25% trichloroacetic acid (0.5 ml) was added, and the acid-insoluble RNA was measured by scintillation spectrometry.

Preparation of RNP cores. RNP cores were prepared from purified whole VS virus disrupted at a final concentration of 1 mg/ml in 10 mM Tris (pH 8.0) containing 0.5% Triton X-100, 250 mM NaCl, and 0.2 mg of dithiothreitol per ml at 0°C. The disrupted virus (volume, 2 ml) was immediately layered onto a preformed discontinuous gradient consisting of 0.5 ml of 100% glycerol, 0.5 ml of 50% glycerol in 10 mM Tris (pH 8.0), and 2.5 ml of 10% glycerol in 10 mM Tris (pH 8.0) plus 0.1 M NaCl in a 2- by 0.5-inch (ca. 50.8- by 12.7-mm) cellulose nitrate tube. Centrifugation was performed at 150,000 × *g* for 90 min in an SW50.1 rotor; the RNP cores formed a visible band at the interface of 50 and 100% glycerol.

Purification of VS virus M protein. Purified whole VS virus was disrupted as described for the preparation of RNP cores. Disrupted virus (5 ml) was layered onto 0.5 ml of 50% glycerol in 10 mM Tris (pH 8.0) and 0.1 M NaCl in a 2- by 0.5-inch (ca. 50.8- by 12.7-mm) cellulose nitrate tube and centrifuged at 150,000 × *g* for 90 min in an SW50.1 rotor. The supernatant was dialyzed against 1 liter of phosphocellulose column buffer (10 mM Tris [pH 7.4], 10% glycerol, 0.2% Triton X-100) plus 0.1 M NaCl overnight. The sample was loaded onto a 6-ml P11 phosphocellulose column prepared as previously described (13) and equilibrated in 0.1 M NaCl-column buffer. After the sample was applied, the column was washed with 0.1 M NaCl-column buffer to remove unbound G protein (13). The column was then washed with 0.1 M NaCl-column buffer minus Triton X-100. The Triton X-100 was omitted from the buffer at this point so that the effluent could be monitored by absorbance at 280 nm. The bound protein was removed from the column by increasing the NaCl concentration to 1.0 M. The single peak which was eluted was dialyzed to lower the NaCl concentration to 0.1 M and reapplied to a second phosphocellulose column. This column was eluted with a linear 0.1 to 1.0 M NaCl gradient, and a single peak was observed at approximately 0.4 M NaCl. Polyacrylamide gel analysis showed this material to be >98% M protein (see Fig. 5).

Isoelectric focusing. Isoelectric focusing gels were prepared in plastic tubes (10 by 0.3 cm) and consisted of 4.75% acrylamide, 0.25% *N,N'*-methylenebisacrylamide, 2% Nonidet P-40 (Shell Oil Co., New York, N.Y.), 0.2% *N,N,N',N'*-tetramethylethylenediamine, Ampholines (LKB Instruments, Inc., Rockville, Md.; 1% [pH 9 to 11], 0.4% [pH 7 to 9]), 8 M urea, and 0.03% ammonium persulfate. Samples for isoelectric focusing were suspended in 8 M urea, 2% Nonidet P-40, 10% glycerol, and 1% Ampholines (pH 3.5 to 10). The apparatus used for isoelectric focusing was a model M 137-A purchased from MRA Corp., Clearwater, Fla. The catholyte buffer was 0.4% ethanolamine, and the anolyte buffer was 0.1 M glycine. Samples were applied at the cathode ends of the gels. Focusing was performed at 1 mA per gel until the voltage reached 800 V; this voltage was then maintained for a total running time of 6 h. The gels were stained by the method of Spencer and King (19). The pH gradient of a gel run in parallel with the test gels was determined. The gel was sliced into 0.5-cm pieces, and each piece was soaked for at least 1 h at room temperature in 1 ml of distilled water. The pH was then measured with a Beckman 3500 pH meter.

RESULTS

Localization of endogenous inhibitor within the VS virion. Perrault and Kingsbury (17) originally reported that prior trypsinization of whole VS virions did not destroy the inhibitory activity present in highly concentrated virions with intact glycoprotein spikes. We confirmed this observation and found that trypsinized virions devoid of G protein spikes displayed the same inhibitory profile as that of whole untrypsinized virions (data not shown). It had also been previously reported that transcribing VS virus RNP cores lack the inhibitory activity found in complete virions (8). To confirm this observation and to identify the putative inhibitory protein(s) present in the virion membrane but presumably not in RNP cores, we prepared RNP cores free of membrane components and compared their transcriptase activities and protein composition with those of whole virions in the presence of Triton.

VS_{Ind} virus was disrupted in 250 mM NaCl–0.5% Triton X-100 and centrifuged in a discontinuous glycerol gradient as described in Materials and Methods. The RNP cores were collected, and transcriptase activity was measured as a function of equivalent virion protein concentration. A comparison was made with the transcriptase activities displayed by whole virions at equivalent concentrations. The RNP cores showed a linear increase in transcriptase activity over a considerable concentration range (0.05 to 3.0 mg/ml) (Fig. 1). In sharp contrast, the control reactions, with whole virions, exhibited striking inhibition of transcription, independent of substrate or detergent concentration (8, 10, 17).

Since we had previously demonstrated that poly(L-glutamic acid) was capable of reversing the endogenous transcriptase inhibitor active at high concentrations of VS virus (10), it was of interest to determine the effects of poly(L-glutamic acid) on the transcriptase activity of RNP cores. The transcriptase activities of whole VS_{Ind}

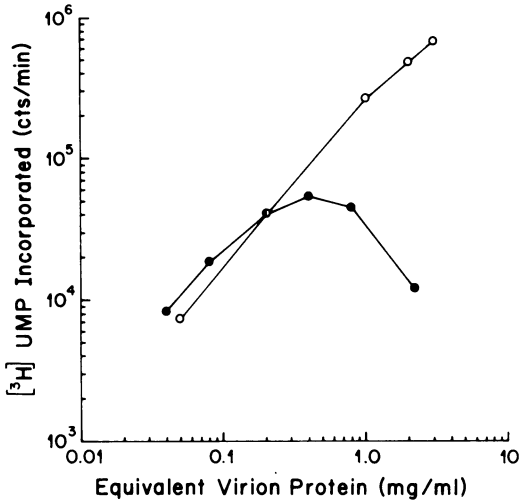

FIG. 1. *Transcriptase activities of VS virus and RNP cores as functions of concentration. The transcriptase activities of purified whole VS_{Ind} virus (wt) (●) and RNP cores (○) isolated from whole virus as described in the text were assayed at various concentrations in a standard in vitro transcription assay (see text). The concentration of RNP cores has been adjusted to an equivalent concentration of whole virus protein, assuming RNP cores to equal 40% of total virus protein (8). Duplicate 0.1-ml reactions were incubated at 31°C for 60 min, and incorporation of [^{3}H]UMP into trichloroacetic acid-insoluble material was determined. Note the data are plotted on a $\log_{10}$ scale on both axes.*

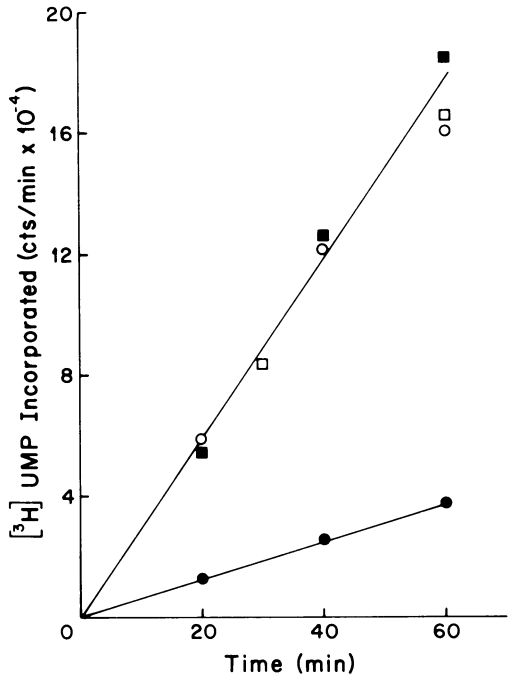

FIG. 2. *Effect of poly(L-glutamic acid) on transcription by whole VS virus and RNP cores. Standard in vitro transcriptase reactions were prepared containing whole virus at 0.8 mg/ml (●), whole virus at 0.8 mg/ml plus poly(L-glutamic acid) at 3 mg/ml (■), RNP cores equivalent to 0.8 mg of whole virus per ml (○), and RNP cores equivalent to 0.8 mg of whole virus per ml plus poly(L-glutamic acid) at 3 mg/ml (□). The reactions were incubated at 31°C; duplicate 0.1-ml samples were removed at 0, 20, 40, and 60 min; and incorporation of [^{3}H]UMP into trichloroacetic acid-insoluble material was determined.*

virus at a protein concentration of 0.8 mg/ml and RNP cores at a concentration equivalent to 0.8 mg of whole virus per ml were measured in the presence and absence of poly(L-glutamic acid).

Figure 2 shows the comparative effects of poly(L-glutamic acid) on the rates and extents of transcription by whole VS virions and their isolated RNP cores. It is apparent from these data that at this concentration (0.8 mg of whole virus per ml) RNP cores were considerably more active than whole virus. Furthermore, the activity of RNP cores was the same as that of whole virus when the endogenous inhibition was reversed by poly(L-glutamic acid). Finally, poly(L-glutamic acid) had no effect on the transcriptase activity of RNP cores.

The proteins present in the RNP cores used in the above experiments were analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. Figure 3 shows the stained pattern of proteins present in whole virus, RNP cores, and the supernatant fraction released by Triton from RNP cores. The RNP cores were found to contain virtually all the N, L and NS proteins, whereas virtually all the G and M proteins were present in the supernatant fraction.

The presence of the endogenous transcriptase inhibitor in trypsinized (spikeless) virus and its absence in RNP cores are consistent with the hypothesis that the M protein is the inhibitor.

Analysis of products synthesized in vitro by virions under inhibited, noninhibited, and poly(L-glutamic acid)-reversed conditions. If the endogenous transcriptase inhibitor of VS virus has any function in regulating transcription, the products synthesized under inhibited and noninhibited conditions may be different. Therefore, the total RNAs synthesized in vitro under various conditions were analyzed by polyacrylamide gel electrophoresis. Standard in vitro reaction mixtures were prepared (see Materials and Methods) containing VS virus at protein concentrations of 0.1 mg/ml (noninhibited), 0.8 mg/ml (inhibited), and 0.8 mg/ml plus poly(L-glutamic acid) (reversed). The reac-

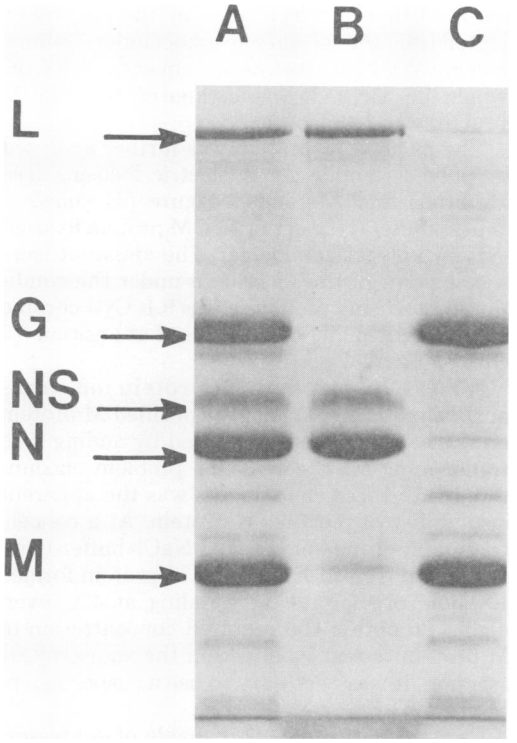

FIG. 3. *Comparative analysis of the proteins in (A) whole VS virions, (B) RNP cores, and (C) solubilized membrane. Samples of the whole virus and RNP cores used in the experiment described in the legend to Fig. 2 and a sample of the supernatant from the RNP core preparation were analyzed by electrophoresis on 10% polyacrylamide slab gels and Coomassie brilliant blue staining as described previously (9). The volumes of the samples were adjusted so that the separate samples were directly comparable.*

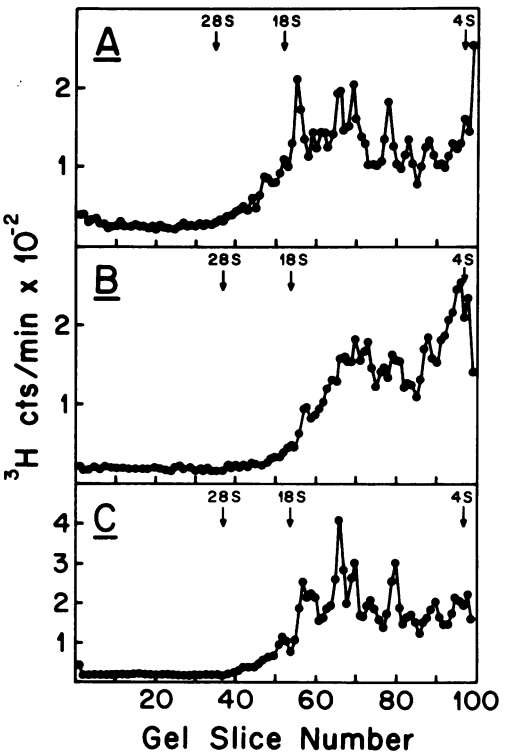

FIG. 4. *Electropherograms of total RNAs synthesized in vitro by (A) uninhibited VS virus, (B) inhibited VS virus, (C) poly(L-glutamic acid)-reversed VS virus. Three separate in vitro reaction mixtures were prepared containing (A) VS virus at 0.1 mg/ml (uninhibited), (B) VS virus at 0.8 mg/ml (inhibited), and (C) VS virus at 0.8 mg/ml plus poly(L-glutamic acid) at 3 mg/ml (reversed). Reactions were incubated at 31°C for 60 min, and the product RNAs were extracted by sodium dodecyl sulfate-phenol-chloroform and subjected to electrophoresis on 2% acrylamide-0.5% agarose gels as described in the text. The totals of trichloroacetic acid-insoluble material extracted from the above reactions were (A) 53,200, (B) 45,800, and (C) 510,000 cpm. Samples were adjusted so that approximately equal counts per minute were applied to each gel. The migration positions of marker rRNA and tRNA are indicated by the arrows.*

tions were incubated at 31°C for 60 min, and the product RNAs were extracted and subjected to electrophoresis on 2% polyacrylamide–0.5% agarose gels as described in Materials and Methods.

The overall patterns of the product RNAs synthesized were similar for all three incubation conditions (Fig. 4). They showed RNAs in the size range 12 to 18S, consistent with the size range of the smaller VS virus mRNA's. The larger, ~28S mRNA was not seen under these conditions. The one notable difference was the larger amount of ~4S material synthesized under inhibited conditions. This could have been the result of degradation or premature termination. This point will be considered in more detail in Discussion. However, it does not appear that the transcriptase inhibitor selectively impeded the synthesis of any specific messenger.

Purification of M protein and its isoelectric point. Preliminary data suggested that the M protein may be the endogenous transcriptase inhibitor present in VS virions (10). The most direct method to test this hypothesis would be to purify the M protein and test its effects on in vitro transcription. A brief description of the purification procedure follows below; a more detailed account appears in Materials and Methods.

Whole VS_{Ind} virus was fractionated into RNP cores and a supernatant fraction by disruption in 0.5% Triton X-100–250 mM NaCl and differential centrifugation. The supernatant fraction,

containing virtually all the G and M proteins (see Fig. 3), was made 0.1 M with NaCl and applied to a phosphocellulose column. The column was washed with buffer containing 0.1 M NaCl and 0.5% Triton X-100 (the G protein does not bind and is washed through [13]). The column was then washed again with 0.1 M NaCl buffer without Triton X-100. The bound protein was eluted from the column with 1 M NaCl, dialyzed against 0.1 M NaCl buffer, and applied to a second phosphocellulose column. The rebound protein was eluted with a 0.1 to 1.0 M NaCl gradient. The M protein eluted at a salt concentration of approximately 0.4 M. A sample of the eluted M protein was analyzed by sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis, and the gel was stained. Figure 5A shows a densitometer trace of the M protein eluted from the second phosphocellulose column compared with a trace of protein extracted from whole VS virus. It was estimated that the M protein was >98% pure.

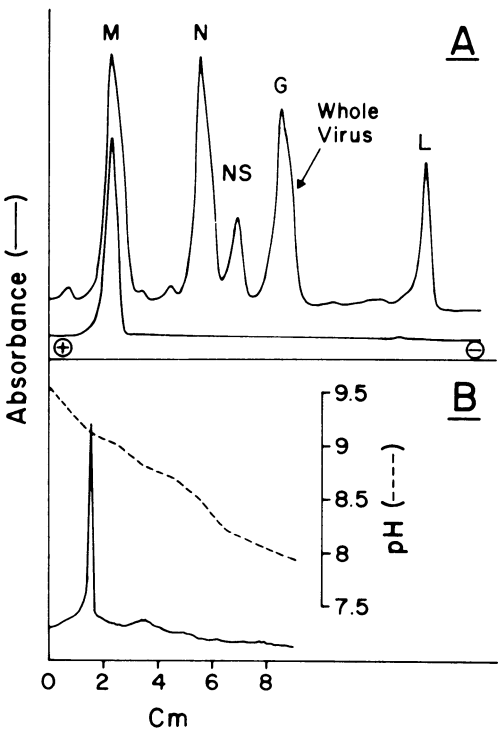

FIG. 5. *Electrophoretic analysis of purified M protein. (A) Purified M protein was analyzed by electrophoresis in a 10% polyacrylamide slab gel; a densitometer trace of the Coomassie brilliant blue staining pattern of whole virus (upper trace) and purified M protein (lower trace) is shown. (B) Purified M protein was analyzed by isoelectric focusing in a 5% polyacrylamide cylindrical gel containing 8 M urea and 2% Nonidet P-40 as described in the text. A densitometer trace of the Coomassie brilliant blue staining pattern is shown. The pH gradient was determined from a parallel gel (see text). Cytochrome c (isoelectric point ≅ 9.4) migrated to position 0.5 cm in a parallel gel.*

The purified M protein was further analyzed by polyacrylamide gel isoelectric focusing (see Materials and Methods). Figure 5B shows a densitometer trace of purified M protein focused in a 5% polyacrylamide gel. The apparent isoelectric point of the M protein under the conditions of focusing used here was 9.1. Cytochrome *c* (isoelectric point ≅ 9.4) focused at position 0.5 cm in a parallel gel.

Effects of purified M protein on transcription. The effects of the purified M protein on transcription were measured by adding it to transcribing RNP cores. One problem encountered with these experiments was the apparent insolubility of purified M protein. At a concentration of ~1 mg/ml in 0.2 M NaCl-buffer (with or without Triton X-100) the M protein formed a visible precipitate on standing at 4°C overnight. To obtain the required concentration of M protein to test its effect on the transcriptase reaction, it was necessary to use a suspension of M protein.

Purified M protein was capable of depressing the in vitro transcriptase activity of RNP cores (Table 1). However, the level of inhibition was

TABLE 1. *Effect of purified M protein on transcription by VS_{Ind} RNP cores*[a]

RNP concn (mg/ml)	M protein concn (mg/ml)	[^{3}H]UMP incorporated (cpm/100 μl per 60 min)
0.08	0	20,100
0.08	0.13	14,200
0.08	0.40	12,200
0.08	0.40 + polyGlu	17,000
0.40	0	103,700
0.40	0.36	58,600
0.40	0.36 + polyGlu	76,300

[a] RNP cores were prepared as described in the text and assayed for transcriptase activity in the presence and absence of purified M protein. One volume of M protein in 0.2 M NaCl-10 mM Tris-hydrochloride (pH 7.4) was added to 2 volumes of RNP cores in 0.72 M NaCl-2% Triton X-100-10% glycerol-5 mM Tris-hydrochloride (pH 8.0) and allowed to stand for 2 min on ice. The reaction was then made up to 10 volumes to provide final concentrations of reactants shown in the text and to concentrations of M protein and RNP cores indicated above. Where appropriate, poly(L-glutamic acid) (polyGlu) was added to the final mixture at 3 mg/ml. Control reactions received an equal volume of buffer. Duplicate 0.1-ml reactions were incubated at 31°C for 60 min, and incorporation of [^{3}H]-UMP into trichloroacetic acid-insoluble material was determined.

far less than that seen in the unfractionated whole virus system. This could have been due to the insolubility of the M protein mentioned above. The inhibition caused by adding purified M protein was partially reversed by the addition of poly(L-glutamic acid), a characteristic of the inhibitor present in whole virions. The purified M protein had a similar effect when low concentrations (0.1 mg/ml) of whole virus were used in place of RNP cores (data not shown).

An unexpected result was that purified M protein (from VS_{Ind}) was also as effective against New Jersey serotype VS of virus as against VS_{Ind} virus (data not shown). The significance of this observation will be discussed later.

Studies using group III *ts* mutants restricted in M protein. In search of data to support our hypothesis that the M protein is the endogenous transcriptase inhibitor of VS virus, we decided to examine the inhibitory characteristics of *ts* mutants with a lesion in the M protein. Preparations were made of *ts*O23 (a group III, or M, mutant), *ts*O45 (a group V, or G, mutant), and the Orsay *wt* (Os.*wt*) strains of VS virus (18); all mutants and the corresponding Os.*wt* were tested at 31 and 39°C for endogenous inhibitory activity of their transcriptases. Both *ts*O45(V) and Os.*wt* exhibited inhibitory characteristics similar to those of the endogenous transcriptase inhibitor previously reported for other strains of VS virus.

Remarkably, the *ts*O23(III) mutant did not exhibit any transcriptase inhibitor at 31°C (permissive) or 39°C (restrictive); this behavior of *ts*O23(III) was dramatically different from that of *ts*O45(V) and Os.*wt*, which were markedly inhibited at 31 and 39°C.

Figure 6 compares the transcriptase activities at 31°C of *ts*O23(III) with those of Os.*wt* and *ts*O45(V) at virus concentrations of 0.1 to 0.8 mg/ml. As noted, when the transcriptase activity of *ts*O23 was measured at 31°C as a function of concentration, it showed an approximately linear increase in activity up to 0.8 of virus protein per ml. In marked contrast, both *ts*O45 and Os.*wt* were severely inhibited above 0.2 mg/ml (Fig. 6A). Furthermore, the transcriptase activity of *ts*O23 was unaffected by poly(L-glutamic acid) up to 0.8 mg of virus protein per ml, whereas Os.*wt* at 0.8 mg/ml was dramatically stimulated by poly(L-glutamic acid).

The effect of the Os.*wt* virus on transcription by *ts*O23(III) was also tested by assaying RNA synthesis of mixtures of *wt* and mutant. Transcriptase reactions were prepared containing Os.*wt* virus (0.4 mg/ml) and *ts*O23 (0.4 mg/ml), both separately and mixed together. The result of mixing the two virus types is shown in Table 2. The mixture of Os.*wt* and *ts*O23 had a much lower transcriptase activity than would have been predicted from the sum of their individual activities. The Os.*wt* appeared to have the ca-

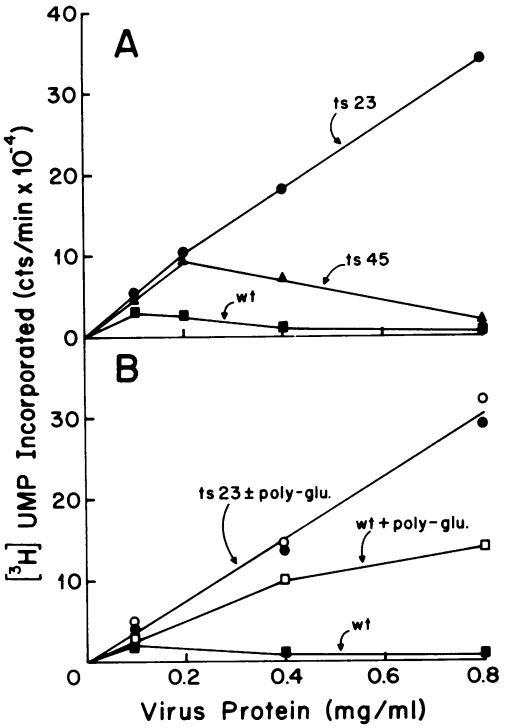

FIG. 6. *Transcriptase activities of tsO23(III), tsO45(V), and Os.wt assayed as functions of concentration at 31°C. Purified preparations of the above VS virus strains were assayed for transcriptase activity at the indicated concentrations of virus protein. Duplicate 0.1-ml reaction mixtures were incubated at 31°C for 60 min, and incorporation of [3H]UMP into trichloroacetic acid-insoluble material was determined. Symbols: (A) ●, tsO23; ▲, tsO45; ■, Os.wt; (B) ●, tsO23; ○, tsO23 plus poly(L-glutamic acid), 3 mg/ml; ■, Os.wt; □, Os.wt plus poly(L-glutamic acid), 3 mg/ml.*

TABLE 2. *Effects of mixing Os.wt and tsO23(III) on transcriptase activity*[a]

Virus and concn (mg/ml)	[3H]UMP incorporated (cpm/100 μl per 60 min)
*ts*O23 (0.4)	136,800
Os.*wt* (0.4)	18,500
Os.*wt* (0.4) + polyGlu	131,300
*ts*O23 (0.4) + Os.*wt* (0.4)	19,200
*ts*O23 (0.4) + Os.*wt* (0.4) + polyGlu	192,100

[a] Duplicate 0.1-ml transcriptase reactions were prepared containing Os.*wt* virus (0.4 mg/ml) and *ts*O23 (0.4 mg/ml), both separately and mixed together. Poly(L-glutamic acid) (polyGlu) at 3 mg/ml was also included in the indicated reactions. After incubation at 31°C for 60 min, the incorporation of [3H]UMP into trichloroacetic acid-insoluble material was determined.

pacity to inhibit both itself and *ts*O23 at these concentrations; hence, the *wt* was dominant over the mutant in this respect. The depressed transcriptase activity of the mixture of Os.*wt* and *ts*O23 could be restored to a considerable extent by the addition of poly(L-glutamic acid).

A second group III mutant, *ts*G31, isolated in Glasgow, was also examined for presence or absence of a transcriptase inhibitor. The transcriptase activity of *ts*G31 compared with that of the corresponding *wt* was measured as a function of concentration ranging from 0.1 to 0.8 mg/ml at both 31 and 39°C (Fig. 7). At 31°C the transcriptase activity of *ts*G31 was inhibited at high concentrations (~0.8 mg/ml) but to a lesser extent than the corresponding *wt*. This finding with *ts*G31(III) differs to some extent from that with *ts*O23(III) (see Fig. 6). At 39°C, although the *wt* was still severely inhibited at concentrations above 0.2 mg/ml, *ts*G31 was not inhibited at concentrations up to 0.8 mg/ml. In fact, the specific activity of *ts*G31 increased with increasing virus concentration. An identical observation was also made when *ts*O23 was assayed at 39°C (data not shown); this augmented transcription of *ts*G31(III) and *ts*O23(III) was perhaps due to increased stabilization of the transcriptase enzyme at higher protein concentrations.

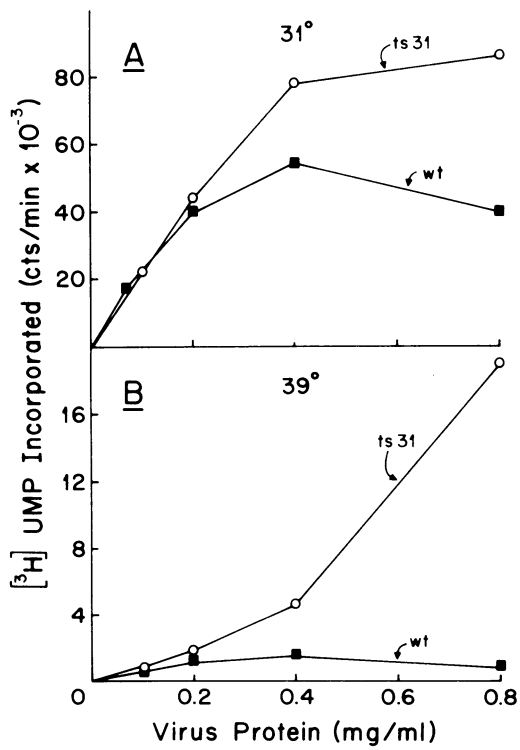

FIG. 7. *Transcriptase activities of tsG31(III)* (○) *and VS$_{Ind}$ wt* (■) *assayed as functions of concentration at both 31 and 39°C. Duplicate 0.1-ml reaction mixtures containing the indicated concentrations of virus protein were incubated for 60 min at either (A) 31°C or (B) 39°C, and incorporation of [^{3}H]UMP in trichloroacetic acid-insoluble material was determined.*

The finding of the altered endogenous inhibition characteristics of group III *ts* mutants with lesions in the M protein strongly supports the hypothesis that the M protein is the endogenous transcriptase inhibitor of VS virus.

DISCUSSION

The nature of the endogenous transcriptase inhibitor present in purified preparations of VS virus has been investigated. We had previously observed that certain polyanions were able to reverse the inhibition seen at high concentrations of VS virus (10). At that time certain other preliminary observations led us to speculate that the endogenous inhibitor was the virion matrix (M) protein. The data presented above support this hypothesis.

The localization of the inhibitor within the virion is consistent with it being the M protein. Trypsinized virions still retain inhibitory activity, whereas membrane-free RNP cores are completely devoid of the inhibitor. The inhibitor does not appear to greatly affect the nature of the RNA synthesized under inhibited conditions; products synthesized under uninhibited and poly(L-glutamic acid)-reversed conditions are almost identical. Although there was somewhat more small-molecular-weight (~4S) RNA synthesized under inhibited conditions, it should be remembered that transcription was depressed by approximately 10-fold. The presence of small RNA could result from either partial degradation or premature termination of transcripts, but neither possibility can account for the high degree of inhibition observed.

Purified M protein was capable of inhibiting transcription by RNP cores. The inhibition by purified M protein was at least partially relieved by the addition of poly(L-glutamic acid), a characteristic of the inhibitor present in whole virions. As previously mentioned (10) and demonstrated here, the M protein is a basic protein with an isoelectric point of approximately 9. This is consistent with reversal of inhibition by certain polyanions. It has been calculated that the molar ratio of M protein to poly(L-glutamic acid) at a level of 50% reversal of inhibition (using VS virus at 0.8 mg/ml) is 1:1.1.

Perrault and Kingsbury (17) originally reported that the endogenous inhibitor of VS virus showed some degree of serotype specificity. We observed, however, that purified M protein from VS$_{Ind}$ virus was about as active against the New

Jersey serotype of VS virus as it was against VS_{Ind}. It is difficult to compare these observations, since Perrault and Kingsbury (17) used UV-inactivated whole virus as a source of their inhibitor. If the viral RNP cores had more affinity for homologous M protein, this might explain the observed results, i.e., reducing the effective concentration of M protein which could interact with the heterologous RNP cores.

We have observed that one group III *ts* mutant (*ts*O23) with a lesion in the M protein has no apparent endogenous inhibitor. A second unrelated group III mutant (*ts*G31) has a limited endogenous inhibitor. These data strongly suggest that the endogenous inhibitor is the M protein.

It may be of some significance that in vivo studies using *ts*O23(III) show that at both permissive and nonpermissive temperatures an increased level of viral RNA synthesis occurs compared with *wt* RNA synthesis under the same conditions (15). A similar situation has been reported for a completely unrelated group III mutant, *ts*G31 (18, 21), which synthesized intracellular RNA at amplified levels at the nonpermissive temperature.

The data presented above strongly suggest that the virion M protein is responsible for the endogenous inhibition of in vitro RNA synthesis seen at high concentrations of VS virus. However, because the inhibitory effect of purified M protein is minimal, probably owing to aggregation of the pure complex, we cannot completely rule out some other unidentified viral component as a transcriptase inhibitor. Maximal transcription by group III *ts* mutant virions serves further to rule out G protein as the endogenous inhibitor.

The role, if any, that this inhibition plays in the in vivo replication of VS virus is unclear at this time. A number of observations indicate that the M protein probably does not play a role in the early stage of the VS virus replication cycle. RNP cores both transcribe as well as virions do in vitro (8) and are infectious (11, 20). In vitro studies show that endogenous inhibition is only demonstrable at high concentrations of virus (8, 10, 17) or M protein. Therefore, in vivo inhibition of transcription by M protein can only occur late in the infectious cycle. The inhibition of RNA synthesis observed in vitro may reflect the association of RNP cores and M protein which must occur during morphogenesis. We are currently attempting to study this system in more detail.

ACKNOWLEDGMENTS

This research was supported by grants AI-11112 from the National Institute of Allergy and Infectious Diseases, BMs-72-02223 from the National Science Foundation, and VC-88 from the American Cancer Society.

LITERATURE CITED

1. **Ball, L. A., and C. N. White.** 1976. Order of transcription of genes of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **73**:442-446.
2. **Baltimore, D., A. S. Huang, and M. Stampfer.** 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Natl. Acad. Sci. U.S.A. **66**:572-576.
3. **Barenholz, Y., N. F. Moore, and R. R. Wagner.** 1976. Enveloped viruses as model membrane systems: microviscosity of vesicular stomatitis virus and host cell membranes. Biochemistry **15**:3563-3570.
4. **Bishop, D. H. L.** 1971. Complete transcription by the transcriptase of vesicular stomatitis virus. J. Virol. **7**:486-490.
5. **Bishop, D. H. L., and P. Roy.** 1971. Properties of the product synthesized by vesicular stomatitis virus particles. J. Mol. Biol. **58**:799-814.
6. **Both, G. W., S. A. Moyer, and A. K. Banerjee.** 1975. Translation and identification of the mRNA species synthesized *in vitro* by the virion-associated RNA polymerase of vesicular stomatitis virus. Proc. Natl. Acad. Sci. U.S.A. **72**:274-278.
7. **Breindl, M., and J. J. Holland.** 1975. Coupled *in vitro* transcription and translation of vesicular stomatitis virus messenger RNA. Proc. Natl. Acad. Sci. U.S.A. **72**:2545-2549.
8. **Breindl, M., and J. J. Holland.** 1976. Studies on the *in vitro* transcription and translation of vesicular stomatitis virus mRNA. Virology **73**:106-118.
9. **Carroll, A. R., and R. R. Wagner.** 1978. Inhibition of transcription by immunoglobulins directed against the ribonucleoprotein of homotypic and heterotypic vesicular stomatitis viruses. J. Virol. **25**:675-684.
10. **Carroll, A. R., and R. R. Wagner.** 1978. Reversal by certain polyanions of an endogenous inhibitor of the vesicular stomatitis virus-associated transcriptase. J. Biol. Chem. **253**:3361-3363.
11. **Cartwright, B., C. J. Smale, and F. Brown.** 1970. Dissection of vesicular stomatitis virus into infective ribonucleoprotein and immunizing components. J. Gen. Virol. **7**:19-32.
12. **Dubovi, E. J., and R. R. Wagner.** 1977. Spatial relationships of the proteins of vesicular stomatitis virus: induction of reversible oligomers by cleavable protein cross-linkers and oxidation. J. Virol. **22**:500-509.
13. **Emerson, S. U., and R. R. Wagner.** 1973. L-protein requirement for *in vitro* RNA synthesis by vesicular stomatitis virus. J. Virol. **12**:1325-1335.
14. **Flamand, A.** 1970. Etude genetique du virus de la stomatite vesiculaire: classement de mutants thermosensibles spontanés en groupes de complementation. J. Gen. Virol. **8**:187-195.
15. **Flamand, A., and D. H. L. Bishop.** 1974. *In vivo* synthesis of RNA by vesicular stomatitis virus and its mutants. J. Mol. Biol. **87**:31-53.
16. **Huang, A. S., and E. K. Manders.** 1972. Ribonucleic acid synthesis of vesicular stomatitis virus. IV. Transcription by standard virus in the presence of defective interfering particles. J. Virol. **9**:909-916.
17. **Perrault, J., and D. T. Kingsbury.** 1974. Inhibitor of vesicular stomatitis virus transcriptase in purified virions. Nature (London) **248**:45-47.
18. **Pringle, C. R.** 1977. Genetics of rhabdoviruses, p. 239-289. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 9. Plenum Publishing Corp., New York.
19. **Spencer, E. M., and T. P. King.** 1971. Isoelectric heterogeneity of bovine plasma albumin. J. Biol. Chem. **246**:201-208.

20. **Szilágyi, J. F., and L. Uryvayev.** 1973. Isolation of an infectious ribonucleoprotein from vesicular stomatitis virus containing an active RNA transcriptase. J. Virol. **11**:279–286.
21. **Unger, J. T., and M. E. Reichmann.** 1973. RNA synthesis in temperature-sensitive mutants of vesicular stomatitis virus. J. Virol. **12**:570–578.
22. **Villarreal, L. P., M. Breindl, and J. J. Holland.** 1976. Determination of molar ratios of vesicular stomatitis virus induced RNA species in BHK-21 cells. Biochemistry **15**:1663–1667.
23. **Wagner, R. R., A. H. Levy, R. M. Snyder, G. A. Ratcliff, Jr., and D. F. Hyatt.** 1963. Biological properties of two plaque variants of vesicular stomatitis virus (Indiana serotype). J. Immunol. **91**:112–122.
24. **Weck, P. K., and R. R. Wagner.** 1978. Inhibition of RNA synthesis in mouse myeloma cells infected with vesicular stomatitis virus. J. Virol. **25**:770–780.