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INHIBITION OF VESICULAR STOMATITIS VIRUS TRANSCRIPTASE COMPLEX BY THE VIRION ENVELOPE M PROTEIN

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SUMMARY. An inhibition of the RNA-dependent RNA polymerase associated with Vesicular Stomatitis Virus occurred when the reaction was stimulated by whole virions and not by enzymatically active ribonucleocapsid (RNP) cores. The matrix (M) protein and the glycoprotein (G), both components of the virion envelope, were purified and added separately to the polymerase reaction stimulated by RNP cores. The G protein fraction had no effect on the reaction, except in some experiments ; in that case, the inhibition was demonstrated to be the consequence of a NTPase activity. The M protein fraction never showed such an activity, but induced a strong inhibition of the polymerase reaction. The data presented suggest that the M protein is the transcriptase inhibitor present in the whole virions.

Previous studies on ts[1] mutants of VSV have shown an enhancement of the transcription rate of viral mRNA in cells infected by mutants synthesizing an altered matrix (M) protein, which is one of the virion envelope proteins (1). Since the report of Perrault and Kingsbury (2), the presence, within the envelope, of a viral component inhibiting the in vitro viral transcription has been documented (3, 4). These observations suggested a possible effect of the M protein on the transcriptase complex activity of VSV measured in vitro. Moreover, Marx et al. (5) have demonstrated that both M protein and glycoproteins from the envelope of another negative strand virus, Sendaï virus, had an inhibitory effect on the in vitro transcriptase reaction. We report below the effects of the addition of purified M protein and glycoprotein (G), the second protein of the virion envelope, on the polymerase activity of VSV-RNP cores.

MATERIALS AND METHODS

Viral material : The wild type strain of VSV (Indiana serotype) was grown on chick embryo cells as previously described (6). Fresh stocks were purified by centrifuging to equilibrium (Spinco rotor SW 27, overnight at 5° C, 25,000 rpm) in a preformed glycerol (30-0%)-potassium tartrate (0-50%) gradient containing Tris 10 mM, NaCl 1 M, EDTA 1mM, pH 7.6. RNP cores were extracted from purified virions according to Breindl and Holland (3) and used immedia-

[1] *Abbreviations used : ts, temperature sensitive ; VSV, Vesicular Stomatitis Virus ; RNP, ribonucleocapsid ; SDS, sodium dodecyl sulfate ; NTP, nucleoside triphosphates ; NTPase, nucleoside triphosphates phosphohydrolase.*

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tely. Viral envelope proteins were extracted following a modified procedure of Mac Sharry et al. (7). Purified virions at a concentration of ≃ 0.5-1.0 mg of protein per ml in 0.01 M Tris buffer pH 7.6 were treated with Triton X-100 at a final concentration of 0.5%. The solubilized proteins were separated over a 20% glycerol cushion from the insoluble viral proteins by centrifugation (Spinco rotor SW 65, 1 h at 5° C, 45,000 rpm). The supernatant fluid was decanted : it constituted the G protein fraction. The pellet was resuspended in Tris 0.01 M, NaCl 1 M, pH 7.6, at a protein concentration of ≃ 0.5-1.0 mg/ml and was treated with Triton X-100 at a final concentration of 0.5%. The supernatant fluid separated by the same centrifugation step as above, was decanted and thoroughly dialyzed against 0.01 M Tris buffer, pH 7.6 at 4° C. The material which precipitated during dialysis was collected by low speed centrifugation (Spinco rotor SW 65, 20 min at 5° C, 10,000 rpm) and resuspended in Tris buffer 0.01 M : this was the M protein fraction.

In vitro polymerase assay : The *in vitro* polymerase reaction mixture consisted of 50 mM Tris-HCl, pH 8.4, 100 mM Na acetate, 100 mM K acetate, 8 mM Mg acetate, 5 mM dithiothreitol, 1.2 mM each of ATP, GTP and CTP, 0.12 mM of UTP and 0.004 mM $[^3H]$ UTP (12 Ci/mmol). Triton X-100 was added to a final concentration of 0.02%, when entire virions were to be tested. Viral material was present at a concentration of 0.250 mg/ml of whole virion proteins (or equivalent to for RNP cores), and the temperature of incubation was 30° C. The reaction was followed by measuring the amount of radioactivity in TCA insoluble material, each aliquot being in duplicate and each cpm value being corrected for the value measured at time 0 (≃ 500 cpm in 25 µl).

ATPase assay : The release of free ^{32}Pi from $[\gamma\text{-}^{32}P]$ ATP was measured by incubating the viral material and this nucleoside triphosphate (3,000 Ci/mmol) under the same conditions as described for the RNA polymerase assay, except for the absence of other unlabeled nucleoside triphosphates. Aliquots were removed at intervals during the course of incubation at 30° C and analyzed by ascending chromatography on 3MM Whatman paper. The paper was developed with a mixture of n-butanol, pyridine and ammonia 0.880 (40:20:40, v/v) at 28° C for 18 h. The ATP and free Pi were located by autoradiography of the chromatogram, then the ^{32}P radioactivity of the corresponding spots was determined by Cherenkov counting.

RESULTS AND DISCUSSION

The existence of an endogenous transcriptase inhibitor in the VS virion was first suggested by the observation of an unexpected virion concentration dependence of the yield of RNA synthesized *in vitro* (2). The inhibition of the reaction which occurred when the concentration of whole virion was increased above a determined value, was not observed when RNP cores were used in the same concentration range (3). This striking difference between whole virion and RNP core activities was also observed in our experiments (Fig. 1). Controls were done which ruled out some trivial effect relevant to the presence of Triton X-100 only in the virion assay and not in the RNP cocktail (data not shown). But, in addition, it appeared that in many experiments the shapes of the kinetic curves differed from one another. Fig. 1 illustrates such an example : whereas RNA synthesis stimulated by RNP cores was always linear, a divergence from linearity appeared as soon as the concentration of whole virions reached a precise range. The more concentrated the virions were, the more rapidly the curve bent to a plateau. This suggested a possible li-

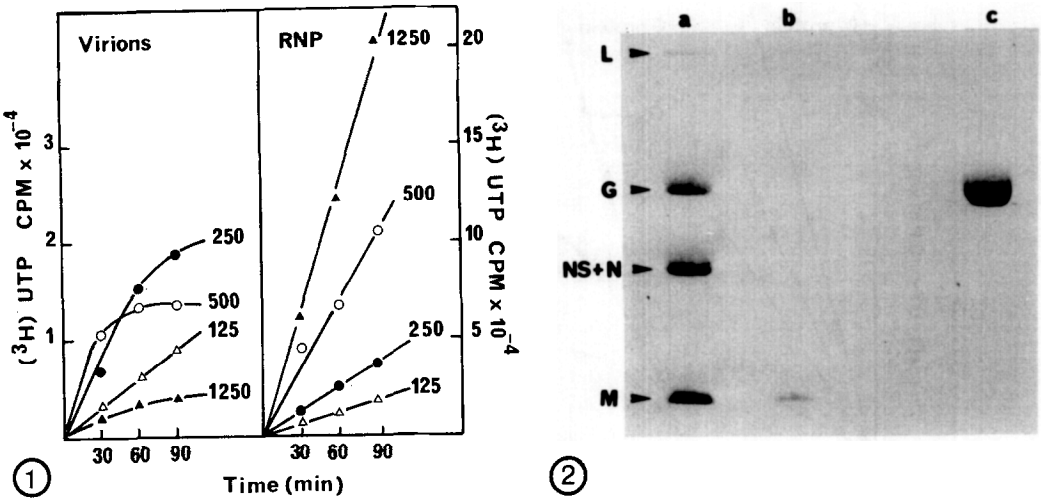


Fig. 1. Effect of viral protein concentrations on the in vitro RNA synthesis stimulated by whole VS virions or RNP cores. In vitro transcription reaction mixture (described in Materials and Methods) were prepared with virions or RNP cores at concentrations equivalent to 125 µg/ml, 250 µg/ml, 500 µg/ml and 1.25 mg/ml of whole virion proteins. The reactions were incubated in stoppered tubes at 30° C, and duplicate aliquots were removed from each reaction at 30, 60 and 90 min. Acid insoluble RNA was measured by scintillation spectrometry.

Fig. 2. Comparative analysis of the proteins in whole virions (a), M protein fraction (b) and G protein fraction (c). M and G protein fractions, extracted as described in Materials and Methods, were samples of materials used in experiments illustrated by Fig. 3. Protein samples were precipitated overnight at 4° C by the addition of 2 volumes of ethanol and the pellets were dissolved in a solution of 62.5 mM Tris-HCl pH 6.8 with 1 M urea, 1% SDS and 1% mercaptoethanol. After boiling for 3 min the samples were subjected to electrophoresis on discontinuous Tris-glycine-buffered SDS-slab gel consisting of 10% resolving gel and 3% stacking gel according to Laemmli's procedure (12). Electrophoresis was from top to bottom. The protein bands were stained with Coomassie blue.

mitation of substrates. In view of the linear curves observed for the RNP cores under the same conditions, the only available explanation seemed to be the disappearance of NTP occurring when the virions have kept their membrane components. It is known that VSV possesses a NTPase activity (8), the location of which might be within the viral envelope, as is the case for most viruses which bud out from the host cell surface. Its presence would be critical for the demonstration of an inhibitor of the RNA polymerase reaction. We therefore paid attention to it in the following experiments conducted to test the effect, on the polymerase reaction, of the G protein and M protein fractions isolated from virions as described in Materials and Methods. A sample of each fraction was analyzed by SDS-polyacrylamide slab gel electrophoresis and the gel was stained (Fig. 2). The densitometer

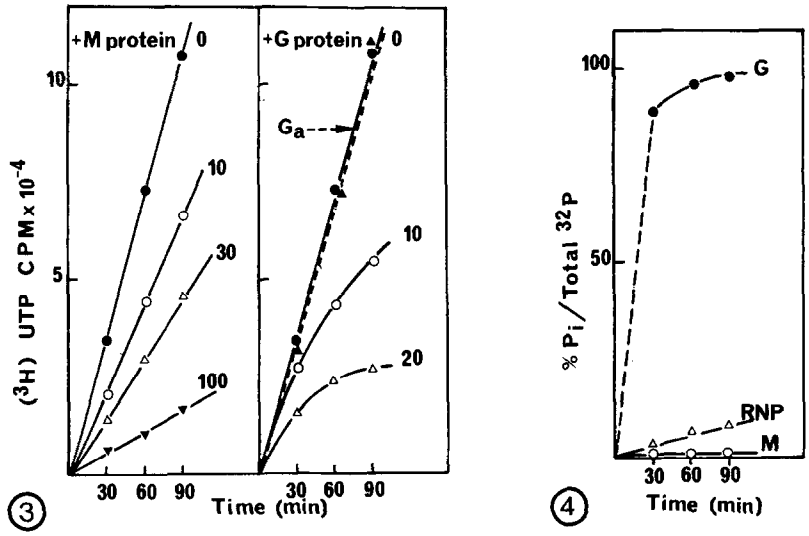


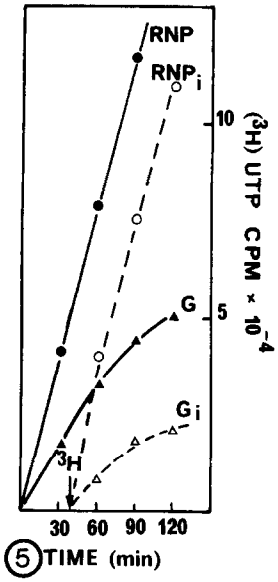
Fig. 3. Effect of the addition of G or M protein fraction on the in vitro RNA synthesis by VSV-RNP cores. Identical 200 µl reaction mixtures were each prepared with fresh RNP cores equivalent to 50 µg of whole virion proteins. One sample served as control and did not receive M or G protein fraction (curve 0). Three samples were supplemented with the M protein fraction (respectively 10, 30 and 100 µg per assay) and 2 samples with the G protein fraction (10 and 20 µg per assay). Incorporation of [^{3}H] UTP into acid precipitable RNA was determined as described in Fig. 1. The dotted curve (Ga) was obtained in another experiment in which the G protein fraction (25 µg) did not exhibit any effect on the reaction.

Fig. 4. ATPase activity in different protein fractions extracted from VS virions. Three equivalent (200 µl) reaction mixtures were prepared on the basis of the RNA polymerase reaction, but containing [γ-^{32}P] ATP and no other nucleoside triphosphate. One sample contained VSV-RNP (equivalent to 50 µg of whole virion proteins), the second contained 20 µg of the G protein fraction and the third 20 µg of the M protein fraction. During incubation at 30° C aliquots were removed at intervals and subjected to paper chromatography and autoradiography as described in Materials and Methods. The percentage of [^{32}P] phosphate over the totality of label recovered was recorded.

traces corresponding to the electropherogram (data not shown) enabled us to estimate that the M protein was $\geqslant$ 95% pure and the G protein $\geqslant$ 98% pure. Fig. 3 illustrates the results of their addition to RNP cores in the polymerase reaction. Concerning the addition of the M protein fraction, the results were always unequivocally clear-cut : the RNA polymerase reaction was depressed and the degree of inhibition followed the increase of the M protein concentration. Moreover, the kinetic curves were strictly linear for the period of time examined. On the other hand, various results were obtained with the G protein fraction : either no effect on the polymerase reaction was observed, or various degree of inhibition occurred. Fig. 3 illustrates an experiment in which the G protein fraction exhibited a strong

inhibitory effect ; it can also be noticed that the curves seem to reach a plateau. On the basis of the observations reported above, we looked for the possible presence of a NTPase activity in the M or G protein fraction, which would account for an only apparent inhibitory effect on the polymerase complex. Only the ATPase activity was checked, since the results of Roy and Bishop (8) argued for a unique enzyme bearing the four NTPase activities which acted by splitting only the γ-Pi. We followed the release of free ^{32}Pi from $[\gamma\text{-}^{32}P]ATP$ in the polymerase reaction mixture containing RNP cores with and without each of the viral envelope proteins. One can see in Fig. 4 that the NTPase activity was virtually absent from the M protein fraction and, in contrast, especially high in the G protein fraction. However, we noticed, on repeated experiments, various levels of activity in the G protein fraction, even an absence. If the degradation of NTP was responsible for the inhibitory effect of the G protein fraction, then pre-incubation at 30° C of the G protein fraction in the polymerase reaction cocktail would lead to a decreased amount of RNA synthesized by comparison with the synthesis measured when $[^3H]$ UTP was added at time O of the incubation. Fig. 5 shows the results of an experiment in which a 40 min-incubation was performed prior to the addition of $[^3H]$ UTP. In the case of the RNP cores alone, the slope of the curve was identical with or without pre-incubation. On the contrary, in a polymerase reaction mixture pre-incubated in the presence of the G protein fraction, a diminution of the RNA synthesis was observed by comparison with the not pre-incubated companion sample. We therefore concluded that the effect observed after addition of the G protein fraction to the RNP polymerase complex was rather an artifactual *in vitro* effect related to the presence of a NTPase activity in the fraction. In contrast, all data argued for a direct effect of the M protein fraction on the RNP polymerase complex leading to an attenuation of its activity. Complementary data for this assumption are given in Fig. 6. First, neither the addition to the RNP cores of a protein unrelated to the VSV proteins, i.e. bovine serum albumine, nor the addition of M protein heated at 100° C for 2 min, modified the kinetics of the polymerase reaction. Secondly, the inhibition obtained with the M protein fraction did not appear to be linked to a ribonuclease, because purified VSV mRNAs extracted from wild type infected HeLa cells were not degraded when added to a polymerase assay in the presence of the M protein fraction.

In conclusion, a restriction of the *in vitro* VSV polymerase activity was observed in the presence of the M protein fraction. The absence of detectable enzymic activities such as NTPase or RNAse in this fraction suggested that the M protein acted directly on the polymerase complex and attenuated



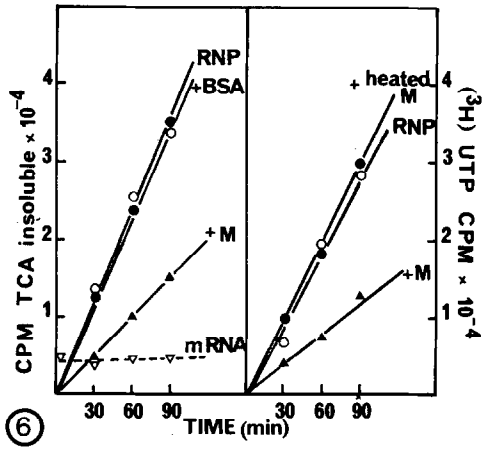


Fig. 5. Effect of a pre-incubation of the G protein fraction on the RNA polymerase assay. Four polymerase reaction mixtures (200 µl) were each prepared with RNP equivalent to 50 µg of whole virion proteins. To two samples, 20 µg of the G protein fraction were added. One sample with only RNP (RNP$_i$) and one sample with G (G$_i$) were incubated at 30° C for 40 min prior to the addition of [^{3}H] UTP (+). The two other reaction samples (RNP and G) were incubated immediately after the addition of the radioactive precursor as usual.

Fig. 6. Control experiments of the in vitro RNA polymerase reaction. Reaction mixtures (200 µl) were each prepared with VSV-RNP equivalent to 50 µg of whole virion proteins. One sample did not receive [^{3}H] UTP but 25,000 cpm of [^{3}H] uridine labeled purified mRNA isolated from HeLa cells infected with VSV wild type : aliquots (40 µl) removed at intervals, were TCA precipitated to follow a possible degradation of the radioactive RNA. Other samples served to determine the incorporation of [^{3}H] UTP as usual. Two samples were independent controls (RNP), another received 30 µg of fresh M protein fraction (+ M), another received 30 µg of the M protein fraction boiled for 2 min prior to its addition (+ heated M), the last one received 30 µg of bovine serum albumin (+ BSA).

its efficiency. This is supported by our observations on the in vivo RNA syntheses induced by ts mutants (1) the ts defect of which has been demonstrated to be linked to the M protein (9, 11). Moreover, the good agreement between the in vitro and in vivo results suggest that the M protein would act in the cytoplasm of VSV infected cells in a similar way as in the in vitro reaction. The M protein appears thus far as the best candidate for the inhibition of the VSV transcriptase reaction detected by various authors (2, 4). However, the NTPase activity which was present in the VSV envelope and which was solubilized together with the G protein may also occasionally hamper the in vitro polymerase reaction. Since we always

observed a good correlation between the absence of NTPase acticity in the G protein fraction and the absence of effect of this fraction on the polymerase reaction, we conclude that the G protein by itself does not inhibit the *in vitro* polymerase reaction. Since the procedure of preparation of glycoproteins from Sendaï virus was essentially similar to ours, it might be that the strong inhibitory effect of this fraction on the polymerase reaction previously reported (5), was related to a NTPase activity. In our case, the NTPase activity was not regularly detected, and when it was present, it showed various degrees of activity : it might therefore conceivably not be a true intrinsic virion component, but rather be a cellular contaminant packed during the budding process of virions. However, as such an activity has scarcely been noticed by authors working on the polymerase reaction, we suppose that the cell strain used for growing our virus stocks, or our purification procedure, favors the presence of the activity. The important point remains that it is now demonstrated that a virion envelope protein, thought up to now to be only a structural VSV protein, might also play a role at the level of the transcriptase complex *in vitro* as well as *in vivo*. The effect of the M protein on the nature of the RNAs synthesized *in vitro* is currently under investigation. This aspect of the work, which deals with the mechanism of the inhibition, will be reported elsewhere.

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Note added in proof : During the preparation of the manuscript we learnt results of A. Carroll and R. Wagner (in press) which are in good agreement with ours.

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