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PROTAMINE--A POTENT INHIBITOR OF VESICULAR STOMATITIS VIRUS TRANSCRIPTASE IN VITRO

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SUMMARY

The virion-associated RNA polymerase activity of vesicular stomatitis virus is inhibited by protamine at a concentration as low as 10^{-7}M. The inhibition is reversible, appears to be at the level of initiation and competitive with respect to ATP. Histone IIA and IV are also inhibitory whereas other fractions are not. The endogenous protein kinase activity is significantly inhibited by protamine. Virion-associated RNA or DNA polymerases of several animal viruses are also inhibited by protamine.

INTRODUCTION

The transcribing ribonucleoprotein (RNP) core of vesicular stomatitis virus (VSV) contains a single-stranded genome RNA (molecular weight, 4×10^6) of negative polarity tightly associated with the principal nucleocapsid protein (N), two other minor proteins L and the phosphoprotein NS (1). Five distinct mRNA species are synthesized sequentially by the RNP in vitro (2). Under appropriate reaction conditions the full-length positive strand of the genome RNA, the required intermediate of replication, is also synthesized in vitro (3). This switch from transcription to replication in vitro was achieved by preinitiation of Triton-disrupted VSV with ATP and CTP, reisolation of RNP and then incubation in the presence of the β,γ imido analogue of ATP (AdoPP(NH)P) and the three normal ribonucleoside triphosphates. These results suggested that phosphorylated states of proteins may control transcription in vitro. It has been shown that the phosphorylated NS protein along with L protein and RNP template are necessary components for transcription in vitro (4). Moreover, NS proteins with different degrees of phosphorylation have been shown to be present in the purified virion with differential affinity for the

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RNP (5,6). Purified RNP also contains a protein kinase activity that phosphorylates predominantly the NS protein *in vitro* (7,8). The role of the protein kinase in the multiplication of the virus is presently unclear. In an effort to understand the role of protein kinase activity in VSV and the processes of phosphorylation in general, we have studied the effect of various phosphate acceptor proteins in the *in vitro* transcription process. In this communication we demonstrate that protamine, a low molecular weight (5000 daltons) highly basic arginine-rich polypeptide from trout testis (9) and also a phosphate acceptor protein (10), is a potent inhibitor of the virion-associated RNA polymerase of VSV.

MATERIALS & METHODS

In vitro transcription assay: *In vitro* VSV RNA polymerase reactions (0.2 ml) contained 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 0.05% Triton N101, 1 mM each of ATP, CTP and GTP, 0.1 mM UTP and 10 µc ^{3}H UTP (specific activity = 660 cpm/pmole), α-^{32}P UTP (1.8 Ci/mmole) and 10 to 15 µg of purified VSV (Indiana serotype grown in baby hamster kidney cells - 21, clone 13 adapted to suspension culture) (11). The incubations were at 30°C for desired lengths of time as described in legends. *In vitro* RNA synthesis by purified vaccinia virus (kindly provided by Dr. J. Hurwitz), cytoplasmic polyhedrosis virus (kindly provided by Dr. Y. Furuichi), WSN strain of influenza virus (kindly provided by Dr. R. M. Krug), and reovirus (kindly provided by Dr. A. J. Shatkin) were done under standard conditions as described in references 12, 13, 14 and 15, respectively. Reverse transcriptase activity of Rous sarcoma virus (kindly provided by Dr. A. M. Skalka) was determined using standard assay conditions (16). In each case the amount of purified virus used was approximately 10-15 µg, except for Rous sarcoma virus 100 µg was used. The incubation time was 2 hrs. Protamine, casein, and phosvitin were purchased from Sigma Chemicals and different fractions of histones were purchased from Boehringer Mannheim.

In vitro protein kinase assay: The reaction mixture (0.2 ml) contained 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 5 mM $MgCl_2$, 4 mM dithiothreitol, 50 µM ATP, and γ-^{32}P ATP (final specific activity = 4000 cpm/pmole) and 50 µg of purified RNP. Incubation was at 30°C for 15 min. The phosphorylated proteins were processed according to the method of Moyer and Summers (8) and analyzed by polyacrylamide gel electrophoresis as described (10).

RESULTS

In vitro RNA synthesis by purified RNP was carried out in the presence of various concentrations of protamine. As shown in Fig. 1A, a 50% inhibition of RNA synthesis was achieved at a protamine concentration of 0.45 µg/ml which corresponds to a molarity of approximately 10^{-7}. At concentrations of 1 µg/ml the RNA synthesis was virtually abolished. The inhibitory effect of protamine

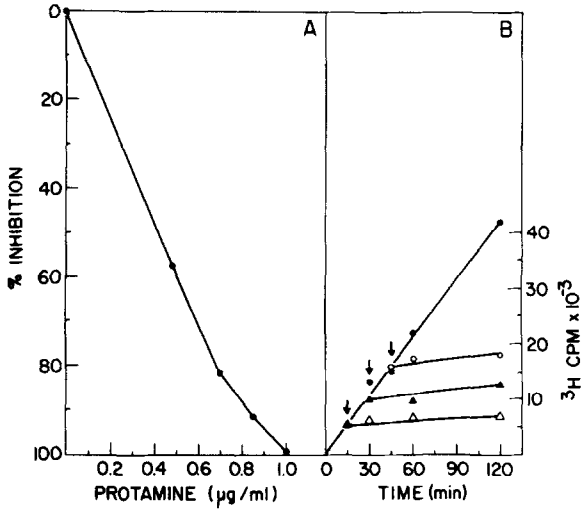
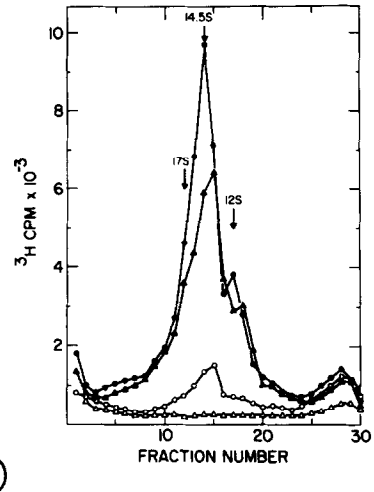


Fig. 1. Inhibition of in vitro transcription by protamine. (A) Standard transcription reaction mixtures were set up as described in Materials and Methods using [^{3}H] UTP as the labeled precursor. Protamine was added at different concentrations and trichloroacetic acid insoluble ^{3}H radioactivity was measured using toluene based scintillation cocktail. (B) In separate standard transcription reaction mixtures 1 µg/ml of protamine was added at different times during RNA synthesis as indicated by arrows. The RNA synthesis was then followed by taking aliquots (20 µl) from the reaction mixtures at different times and assaying for trichloroacetic acid-precipitable radioactivity.

was quite striking when added at different times after the onset of transcription (Fig. 1B). Although the RNA synthesis ceased rapidly after the addition of protamine it can be seen in Fig. 1B that a small but reproducible amount of RNA (approximately 5 to 10%) continued to be synthesized. These kinetic patterns are typical of inhibition at the level of initiation of RNA synthesis where previously initiated RNA chains were completed in the presence of the inhibitor.

We next, determined whether the inhibition of RNA synthesis might have been due to degradation of mRNA by RNase contaminating the inhibitor preparation. Data shown in Fig. 2 clearly indicate that the mRNA products although decreased in amounts remained undegraded and sedimented with characteristic rates. These results indicate that the inhibitory effect of protamine may be at or near the initiation of RNA synthesis since no prematurely terminated RNA products were found in the gradient. In order to determine whether the inhibitory effect of protamine was reversible, the following experiment was performed. RNA synthesis in vitro was



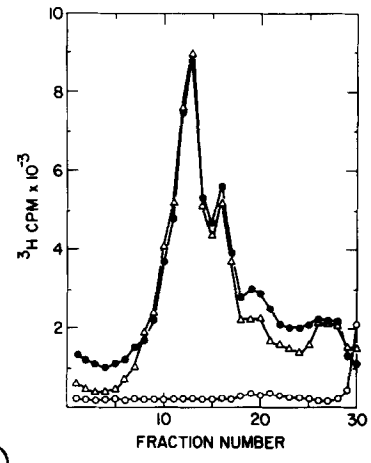


Fig. 2. Velocity sedimentation analyses of VSV mRNA synthesis in the presence of varying concentrations of protamine. Standard transcription reaction mixtures containing different concentrations of protamine were set up using [^{3}H]-UTP as the labeled precursor and incubated for 2 hr at 30°C. The reaction was terminated by addition of SDS and the reaction mixtures were directly layered onto a 15-30% SDS-sucrose gradient. The centrifugation was at 33,000 rpm for 17 hr at 23°C in a SW40 Spinco Rotor. Fractions (40 drops) were collected from the bottom of the tubes and trichloroacetic acid insoluble radioactivity measured. ●—●, no protamine; ▲—▲, 0.5 µg/ml protamine; 0—0, 0.75 µg/ml protamine; Δ—Δ, 1.00 µg/ml protamine. Arrows indicate the sedimentation position of VSV mRNA speices.

Fig. 3. Reversibility of protamine inhibition of VSV RNA synthesis. Two transcription assay mixtures were set up in 0.02 ml in the presence of protamine (1 µg/ml) containing unlabeled precursors and incubated for 15 min. The first sample was diluted to 0.2 ml while maintaining the concentrations of the reaction components including protamine (1 µg/ml). ^{3}H UTP was added and the reaction continued for 45 min (0—0). The second sample was similarly diluted and incubated while reducing protamine concentration at 0.1 µg/ml (Δ—Δ). A third sample (0.02 ml) initially received low protamine (0.1 µg/ml) after 15 min was similarly diluted maintaining protamine concentration at 0.1 µg/ml (▲—▲). RNA synthesized by three samples were individually analyzed by velocity gradient as described in Fig. 2.

carried out in duplicate tubes in the presence of protamine (1 µg/ml) for 15 min. In one of the reaction mixtures the protamine concentration was reduced ten-fold while the concentrations of the rest of the components of reaction remained unchanged. The reaction was continued for an additional 45 min following reduction and the synthesized RNA was analyzed by velocity sedimentation. As shown in Fig. 3, the diluted reaction mixture regained RNA synthetic activity virtually identical to the reaction containing 0.1 µg/ml of protamine present at the beginning of the reaction. These results indicate that the inhibitory effect of protamine is totally reversible.

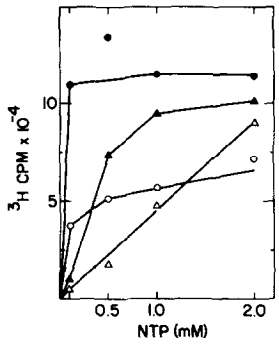


Fig. 4. Synthesis of VSV mRNA in the presence of protamine at varying ATP and GTP concentrations. Standard transcription reaction mixtures were set up as described in the Materials and Methods using [^{3}H] UTP as the labeled precursor excepting that ATP and GTP concentrations were varied between 0.1 mM to 2.0 mM and 0.5 µg/ml of protamine was used. The RNA synthesis after 2 hr incubation were determined by trichloroacetic acid insoluble radioactivity. ATP, ▲—▲; ATP + protamine, △—△; GTP, ●—●; GTP + protamine, ○—○.

The mechanism of inhibition of VSV RNA polymerase by protamine was studied by varying the concentration of ribonucleoside triphosphates in the transcription reaction mixture. As shown in Fig. 4, addition of ATP effectively reversed the inhibitory effect of protamine. In contrast, addition of GTP, had virtually no effect on RNA synthesis over the concentration range of 0.1 to 2 mM. In similar experiments (not shown) it was shown that neither UTP nor CTP had a protective effect similar to ATP. These results suggest that protamine may be a competitive inhibitor with respect to ATP only.

Since protamine is a highly basic protein, it was of interest to study the effect of other basic proteins and phosphate acceptor proteins on VSV RNA polymerase *in vitro*. We also studied the effect of the same proteins on the virion-associated polymerases of several other animal viruses. As shown in Table 1, neutral proteins such as phosvitin and casein have no effect on *in vitro* transcription of VSV. Interestingly, among the basic histone proteins, only IIA and IV were significantly inhibitory. This indicates that the basicity of a protein may not be the absolute criterion for the observed inhibitory effect of protamine. Similar inhibitory effects of protamine and histone IIA on *in vitro* RNA synthesis was observed in vaccinia virus, cytoplasmic polyhedrosis virus, influenza virus,

TABLE 1

Effect of some basic and neutral proteins on virion-associated polymerases of several animal viruses.

Viruses	Concentration Of Proteins Required For 50% Inhibition (μg/ml)						
	Protamine	Histone				Phosvitin	Casein
		I	IIA	IIB	IV		
VSV	0.5	(-)	2	(-)	10	(-)	(-)
Vaccinia Virus	1	(-)	1	(-)	(-)	(-)	(-)
Influenza Virus	1	(-)	10	(-)	(-)	(-)	(-)
Cytoplasmic Polyhedrosis virus	1	(-)	10	(-)	(-)	(-)	(-)
Rous sarcoma virus	5	N.D	N.D	N.D	N.D	N.D	N.D
Reo virus	(-)	(-)	(-)	(-)	(-)	(-)	(-)

The *in vitro* transcription of the viruses were carried out under standard conditions as described in Materials and Methods.

N.D denotes not determined; (-) denotes no effect.

and the reverse transcription of Rous sarcoma virus. The exception was reovirus where no protein had any effect on RNA transcription *in vitro*.

Finally, we studied the effect of protamine on the endogenous protein kinase activity using γ-^{32}P-ATP as the phosphate donor and analyzed the phosphorylated proteins by polyacrylamide gel electrophoresis. As shown in Fig. 5, significant inhibition of phosphorylation (80%) of the NS protein was observed at protamine concentration of 0.5 μg/ml. Phosphorylation of the L protein was inhibited very little, if at all. The appearance of phosphorylated M protein was possibly due to trace contamination of the protein in purified RNP. It is interesting to note that a residual phosphorylation of the NS protein (20%) remained even at 1 μg/ml of protamine at which concentration the *in vitro* transcription was virtually abolished (Fig. 1). Moreover, protamine had no effect on the phosphorylation of the L protein at the various concentrations tested. It was also observed that a second labeled band migrated slightly faster than NS protein (lanes B, C, & D); this protein band has not been characterized.

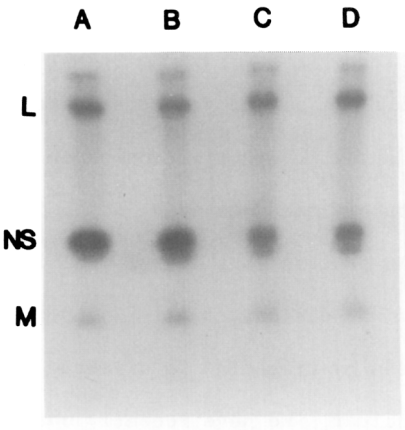


Fig. 5. Effect of protamine on the endogenous protein kinase activity. VSV cores (50 µg) were incubated in the complete reaction mixture (0.2 ml) at varying protamine concentrations with 50 µM ATP and [γ^{32}P] ATP (final specific activity = 4000 cpm/pmole) for 15 min at 30°C. The samples were precipitated with 20% trichloroacetic acid containing 1 mM ATP, 1 mM EDTA. The precipitated proteins were treated with 0.1 N NaOH in the cold for 1 min, reprecipitated with 20% trichloroacetic acid washed, solubilized, and analyzed by 10% polyacrylamide slab gel as described (8). Autoradiogram of the gel was made using Kodak SB5 film. A - without protamine, B - 0.25 µg/ml protamine; C - 0.5 µg/ml protamine; D - 1 µg/ml protamine.

DISCUSSION

In this communication we have shown that protamine, a low molecular weight highly basic protein, can effectively inhibit the viron-associated RNA polymerase activity of purified VSV *in vitro*. A 50% inhibition of RNA synthesis was achieved at a concentration of as low as 10^{-7}M. The inhibition appears to be at the level of initiation of RNA synthesis (Fig. 1). The inhibitory effect can be reversed either by dilution (Fig. 3) or by adding increasing concentrations of ATP in the reaction mixture (Fig. 4). In the latter respect protamine behaves similarly to phosphonoformate and ARA-ATP which have also been shown to be competitive inhibitors of ATP in *in vitro* RNA transcription of VSV (17). Since ATP is the initiating nucleotide for VSV RNA synthesis *in vitro* (18), the results seem to indicate that protamine possibly binds at the initiation site of the RNA polymerase. Since the inhibitory effect is reversible it appears that the nature of the interaction with the viral protein(s) is weak. It is not known which of the viral RNA polymerase proteins (L, NS, or N (1)) binds to protamine.

The NS protein appears to be particularly attractive because it is a phosphoprotein and protamine inhibits the endogenous protein kinase activity that phosphorylates NS protein *in vitro* (Fig. 5). It is not yet clear whether (a) the additional protein band that appears during phosphorylation under inhibitory conditions (Fig. 4) is the lesser phosphorylated component of NS protein, designated as NS1 (19) and (b) the residual NS protein that is not inhibited by protamine is the hyperphosphorylated form of NS, designated as NS2 (19). Whether inhibition of phosphorylation of NS protein by protamine is directly related to the inhibition of transcription is also unclear. We have recently observed that *in vitro* capped mRNA synthesis is more sensitive to inhibition by protamine than the *in vitro* synthesis of the full-length plus strand (3) (data not shown). These results suggest that the primary target of protamine may be the transcriptase and not the replicase. Isolation and characterizaton of the protamine-binding protein, will be important to understand the role of these proteins in viral transcription and replication. It is interesting to note that only histone IIA and IV among the basic histone proteins inhibit VSV transcription (Table 1). Thus, it seems that protamine or these histone fractions may contain some specific regions that interact with VSV proteins. We have also shown protamine inhibits RNA or DNA synthesis *in vitro* by several animal viruses that contain virion-associated RNA or DNA polymerases and in some cases an endogenous protein kinase (Table 1). Further work will show whether this inhibition is mediated by the interaction of protamine with specific viral proteins or by inhibition of the endogenous protein kinase. There is one report, however, that showed histones stimulated polyribonucleotide-directed polydeoxyribonucleotide synthesis by murine leukemia virus (20). Further studies along this line will shed some light on the role of phosphorylation, if any, on transcription/replication of VSV in particular and animal viruses in general.

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