Characterization of an Endogenous Transcription Inhibitor from *Physarum polycephalum*

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A substance has been purified from isolated nuclei of *Physarum polycephalum* by equilibrium and velocity gradient centrifugations, ion exchange chromatography and gel filtration which has a high molecular weight, can be labeled *in vivo* with ^{32}P, is heat stable and resistant to amylases, proteases, nucleases and phosphodiesterase but is sensitive to phosphatases or hydrolysis. This material consists of phospate and glycerol. It selectively inhibits *in vitro* transcription of RNA polymerases, predominantly the homologous enzyme A by binding to the enzyme. In the presence of this inhibitor of transcription a stable RNA polymerase-template complex cannot be formed. Binding to and inactivation of RNA polymerase is reversible at high ionic strength.

Introduction

It is known that microorganisms respond to limitations in their nutrients by the reduction of synthesis rates and contents of macromolecules, especially of stable RNA. This phenomenon of stringent control [1] can be correlated with an elevated level of organic polyphosphates in bacteria [2]. There is evidence that guanosine tetraphosphate interferes with the transcription of ribosomal RNA [3]. Higher cells activate a pleiotypic program as a consequence of environmental changes employing complex reactions with hormones and cyclic nucleotides [4].

Lower eucaryotes, like the slime mold *Physarum*, might provide simpler systems to analyse transcription control of RNA classes [5]. One possible model involves selective inhibitors of the multiple RNA polymerases present in eucaryotic cells. Starvation induces the encystment of microplasmodia of *Physarum* [6]. During this differentiation process (spherulation) a marked decrease of rRNA synthesis and accumulation has been observed [7–9] together with a drastic reduction of the corresponding RNA polymerase A activity [10].

Previously a polyphosphate-like material had been detected in *Physarum* [11]. A strong negative correlation of polyphosphate level with RNA synthesis had been known for some time [12] and has recently been confirmed for *Physarum* [13].

In this paper we demonstrate an inhibitor of RNA synthesis in nuclei from *Physarum*. This substance has been purified and characterized. After binding to RNA polymerase it prevents *in vitro* transcription by inhibiting the attachment of the enzyme to its template.

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Materials and Methods

Cultures and labeling of Physarum

Physarum was grown axenically on semidefined medium as described previously [14]. Cultures were kept as a shaken suspension of microplasmodia. Macroplasmodia were produced by fusion of microplasmodia on a filter paper [15]. Fusion time was 90 min and was followed by the addition of growth medium. Macroplasmodia were cultured at the surface of filterpapers kept in petridishes in the dark at 26 °C. In some experiments microplasmodia were incubated for the last 20 h of a three day growth period in 100 μCi [^{32}P]orthophosphate/ml growth medium.

Preparation and assay of RNA polymerases

RNA polymerases were isolated from homogenates of plasmodia, separated into an amatoxin insensitive enzyme (RNA polymerase A or I) and a sensitive enzyme (RNA polymerase B or II) on DEAE Sephadex A25 and further purified by DNA cellulose

chromatography and glycerol gradient centrifugation as described previously [16]. In the experiments described in this paper RNA polymerase A (approximately 85% pure on SDS polyacrylamide gels) at 380 units/mg protein was used (1 enzyme unit = 1 nmole UMP incorporation/per 2 min at 30 °C on denatured calf thymus DNA). Some experiments were performed with RNA polymerase B (410 units/mg protein) or *E. coli* RNA polymerase (200 – 400 units/mg protein). The enzyme activity was assayed in a total volume of 100 µl (containing 50 mM Tris HCl, pH 7.5, 0.5 mM dithioerythreitol, 15 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mM each of ATP, GTP, CTP, 0.05 mM UTP, 10 µCi of [^{3}H]UTP (24 Ci/mmol) over 15 min at 30 °C with 6 µg native calf thymus DNA. RNA polymerase A from mouse Ascites cells was a generous gift from K. Schäfer (Konstanz).

Preparation of the factor

The factor was coextracted with DNA. Its inhibitory effect was determined by adding 10 µl of the factor-preparation to the above described assay with RNA polymerase A. Nuclei from microplasmodia or macroplasmodia were prepared as published [17]. DNA was extracted from isolated nuclei after lysis with sodium dodecyl sulfate and lauryl sarcosinate (each 2.5%). DNA was purified with phenol-chloroform, precipitated with ethanol, spooled on a glass rod and dissolved in 15 mM NaCl and 1.5 mM Na-citrate. The DNA preparation was treated with RNases (A, 2.5 units/ml and T_1, 50 units/ml) for 2 h at 35 °C followed by proteinase K (20 units/ml, 35 °C) over night and a second phenol extraction and alcohol precipitation [18].

The factor was separated from DNA by a centrifugation in a cesium chloride gradient and glycerol gradient and chromatography on DEAE Sephadex followed by gel filtration on Sephadex G25.

Preparation of slime

Extracellular slime [19] was obtained by centrifugation from the growth medium after sedimenting the plasmodia. The slime was dialysed against water and precipitated with alcohol and further purified by equilibrium and/or velocity gradient centrifugations. Its polysaccharide content was determined by the anthrone method with glucose as standard [20].

DNA filter binding assay

In these assays DNA, RNA polymerase and factor were incubated in binding buffer (10 mM Tris HCl, pH 8, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM mercaptoethanol, 200 µg/ml bovine serum albumin, 50 mM NaCl) for 10 min at 23 °C. The mixture was filtered through millipore filters (2 ml/min) and washed 2 times with the binding buffer [21]. Blank values with DNA alone ranged from 3 – 5% of the input DNA, and a linear binding of DNA to the filter was obtained with increasing amounts of *Physarum* RNA polymerase A or *E. coli* RNA polymerase. T_7 DNA at 6000 cpm/µg was kindly provided by R. Knippers, Konstanz.

Gradient centrifugation

CsCl gradients were adjusted to 1.7 g/ml at 22 °C and centrifuged for 60 h at 22 °C at 35 000 rpm in a Ti 50 rotor of a Beckman ultracentrifuge. Linear glycerol or sucrose gradients (10 – 30%) were centrifuged at 40 000 rpm at 15 °C for 22 h in a SW 41 rotor.

Polyphosphate determination

Polyphosphate was purchased from Merck-Schuchardt and the high molecular weight fraction of this material (obtained in the void volume of the eluate from a Sephadex G25 chromatography column) was used in the RNA polymerase assays and for quantitation of polyphosphate as acid labile phosphate [6]. Phosphate was determined by a standard procedure [22].

Detection methods for carbohydrates

An elementary analysis of 2 mg of the purified factor by combustion was performed by the Microanalytical Laboratory of the University Stuttgart.

Paper chromatography

The descending method of paper chromatography was employed on 3 MM paper from Whatman with the following solvent system: ethylacetate, propanol and water (8 : 2 : 4 by volume, upper phase).

Thin layer chromatography

Ascending chromatography was performed on cellulose DC-plates from Schleicher & Schüll with a *n*-butanol-pyridine-water (6 : 4 : 3 by volume) solvent system or on silica gel glass plates from

Merck with a propanol-water (7 : 1 by volume) solvent system.

Carbohydrates used for comparison:

Glycerol, erythritol, ribose, deoxy-ribose, xylose, arabinose, sorbitol, dulcitol, mannitol, glucose, galactose, mannose, fructose, gluconic acid, glucaric acid, sedoheptulose, saccharose, trehalose, lactose, lactulose, maltose and raffinose.

Detection reagents for carbohydrates (listed in 23)

Aniline phosphate, anthrone reagent, bromphenol-blue boric acid, *p*-aminodimethyl-anilin-stannous chloride, Fleur's reagent, β-indolylacetic acid-trichloroacetic acid, naphthoresorcinol, nitraniline-periodic acid, orcinol-trichloroacetic acid, phloroglucinol, Seliwanoff's reagent, sodium nitroprusside-sodium hydroxide, urea acid, vanillin-perchloric acid.

Standard detection reagent was periodate-anisidine for paper chromatography and cellulose thin layer chromatography and 50% sulfuric acid (10′ 150°) for silica gel thin layer chromatography.

Nuclear magnetic resonance spectroscopy

Proton magnetic resonance spectra (^{1}H-NMR) were recorded with a Bruker HX90 spectrometer operating in the Fourier transform mode.

Chemicals

The following enzymes were obtained from Boehringer and tested as recommended by the manufacturer:

Trypsin (Enzygel), proteinase K, α- and β-amylases, DNAase I, RNAases A and T_1, snake venom phosphodiesterase, acid phosphatase and alkaline phosphatase (Enzygel), *E. coli* RNA polymerase.

Results

Demonstration of a factor in isolated nuclei inhibiting in vitro RNA synthesis

An inhibitory substance was detected in DNA preparations obtained from isolated nuclei from fully grown cultures of *Physarum*. We observed very low template activity of crude *Physarum* DNA (prepared by phenol extraction, alcohol precipitation and proteinase digestion) when it was assayed with either *Physarum* RNA polymerase A or B or *E. coli* RNA polymerase (Table I). No increase in template activity occurred at DNA concentrations of 5 – 150 μg in the enzyme assay. Control experiments have not revealed substantial RNase activity (less than 10% of added radioactive RNA had become acid soluble after 30 min at 30 °C) of the DNA preparation of *Physarum*. However, the template activity of commercial calf thymus DNA with all three enzymes was significantly inhibited after the addition of increasing amounts of crude *Physarum* DNA.

Purification of the same DNA template by CsCl gradient centrifugation changed *Physarum* DNA to as good template as calf thymus DNA, while the latter DNA was not altered in its template activity (Table I). In comparing *in vitro* RNA synthesis by the three enzymes with crude or purified *Physarum* DNA, we consistently found that RNA polymerase A from *Physarum* became relatively more active than the other enzymes (Table I).

In order to locate the inhibiting factor, each fraction of the CsCl gradient was tested for its effect on the RNA polymerase assay. It was found that the fractions towards the bottom of the gradient contained the inhibitory substance. Since the isolation of the inhibitor from isolated nuclei of *Physarum* did not prove its nuclear location *in vivo* the following three control experiments were done: We have

Enzyme	Source of template [a]				Relative Template activity (pure/crude)	
	Calf thymus		*Physarum* nuclei			
	Commercial	Purified [b]	Crude	Purified [b]	Calf thymus DNA	*Physarum* DNA
Physarum RNA-pol. A	6400	6250	300	5100	1	16
Physarum RNA-pol. B	4600	4500	360	3900	1	10
E. coli RNA-pol.	26 000	26 800	800	9000	1	11

Table I. Template activity of *Physarum* DNA and calf thymus DNA.

[a] Template activity cpm UMP incorporation after 15 min of incubating the complete assay mixture at 30 °C.
[b] By CsCl centrifugation.

previously used the separation of cytosol by centrifugation of unbroken macroplasmodia as a sensitive and convincing test for the location of RNA polymerases A and B in nuclei [10]. The same procedure indicated that the cytosol was free of the inhibiting factor.

The amount of inhibitor, based on the degree of inhibition of RNA polymerase A from *Physarum*, obtained from the total homogenate of half a culture – or from nuclei prepared from the other half – was approximately equal.

In mixing experiments the purified inhibitor (50 000 cpm, ^{32}P, prepared from 5 cultures of *Physarum*) was added to a crude homogenate from half a culture or to isolated nuclei from the other half. Less than 10% of the added radioactivity was found in either purified or repurified nuclear preparation. From these observations we concluded that the inhibitor did not absorb on the nuclei during the isolation procedure.

Purification of the inhibitor

In the following analysis a culture of *Physarum* was incubated in [^{32}P]orthophosphate for 20 h. Nuclei were isolated and the crude DNA preparation was extracted with phenol and chloroform, precipitated with alcohol, treated with nucleases in a dialysis bag and reextracted by phenol and chloroform before CsCl centrifugation. The inhibiting fractions were concentrated towards the bottom of the gradient (Fig. 1 a). The degree of inhibition parallels the amount of acid labile phosphate and ^{32}P labeled material in the gradient. The inhibitor was also separated from polysaccharide consistently detected in nuclear preparations from *Physarum* which sediments between DNA and the inhibiting factor (Fig. 1 a). Further purification of the factor was obtained on glycerol gradients (Fig. 1 b). The inhibitor could be located as a symmetric peak on the gradient by its effect on *in vitro* transcription or by ^{32}P label. From the sedimentation of hemoglobin as marker molecule in parallel gradients a value of approximately 4S could be assigned to the inhibitor.

The inhibitor was not adsorbed on phosphocellulose. However, it was quantitatively retained on DEAE Sephadex and eluted as a sharp peak at 0.5 M NH_4Cl (Fig. 1 c). The factor appeared in the void volume after gelfiltrations on Sephadex G25 and 50 but not on Sephadex G100. Since the inhibitory effect on *in vitro* RNA synthesis in all steps of purification ran parallel with the content of acid labile phosphate, we have used this parameter in the quantitative studies described below.

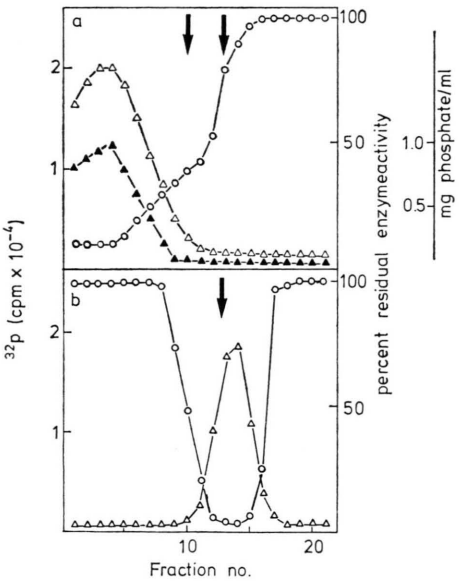

Fig. 1. a. Localization of an inhibitor of *in vitro* transcription after CsCl gradient centrifugations of nuclear extracts from *Physarum*. Microplasmodia were incubated in 100 μCi/^{32}P orthophosphate/ml of growth medium for 20 h. DNA was extracted from isolated nuclei. The preparation was treated with nucleases and centrifuged on a CsCl gradient adjusted to 1.7 g/ml for 40 h at 35 000 rpm and 15 °C. Inhibition of *in vitro* transcription was tested by adding aliquots of fractions to a complete assay with RNA polymerase A (3 u/mg, 100%=47 000 cpm) (–○–○–). ^{32}P was determined by scintillation counting (–△–△–) and acid labile phosphate was measured as described in Materials and Methods (–▲–▲–). The right arrow indicates the position of DNA in a separate gradient, the left arrow the position of the nuclear polysaccharide determined by the anthrone reaction. b. Glycerol gradient centrifugation of the factor. Fractions 1–4 of Fig. 1 a were pooled, dialysed, lyophilized and applied to a linear 10–30% glycerol gradient. The gradient was fractionated from the bottom after centrifugation at 40 000 rpm for 22 h at 15 °C. ^{32}P labeled material and inhibition of *in vitro* transcription were determined as described in Fig. 1 a (–△–△–) ^{32}P; (–○–○–) enzyme activity. Hemoglobin was added as marker (arrow).

Inhibition of RNA polymerases by the endogenous factor

The factor from *Physarum* specifically interferes with transcription since enzymes employed in purification procedures (trypsin, DNAase, RNAase and amylases) and DNA polymerase of *Physarum* (W. Schiebel, München, personal communication) or mouse Ascites cells (B. Otto, Konstanz, personal communication) were not inactivated in the presence

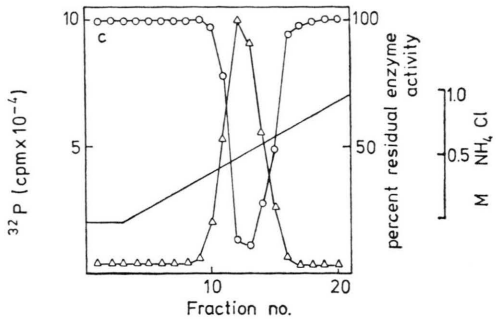

Fig. 1. c. Chromatography of the factor on DEAE Sephadex. The material in fractions 12–15 of Fig. 1 b was pooled and absorbed on DEAE Sephadex A25 and eluted by a linear gradient of ammonium chloride. The factor was located by ^{32}P labeled material (–△–△–), maximum inhibition of *in vitro* transcription was found in fraction 12 and 13. (–○–○–) enzyme activity.

of the factor at concentrations (0.1 – 1 mM phosphate), which completely inactivate RNA polymerases.

In the following experiments the purified inhibitor was incubated in increasing amounts with each of the three RNA polymerases (A and B from *Physarum* and *E. coli* enzyme) of equal activity (based on UMP incorporation with native commercial calf thymus DNA). We found that RNA polymerase A from *Physarum* was significantly more inhibited at lower concentrations of the inhibitor (Fig. 2). This enzyme was almost completely inactive at 4 μM phosphate. These results were confirmed for crude and highly purified enzyme preparations and were also obtained in the presence of 100 μg/ml BSA or

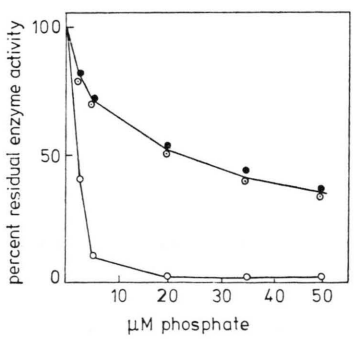

Fig. 2. Preferential inhibition of *in vitro* transcription by RNA polymerase A from *Physarum*. Inhibition of RNA synthesis on double stranded calf thymus DNA (yield after 10′ incubation at 30 °C) by an increasing concentration of the inhibitor (μM acid labile phosphate) RNA polymerase A (–○–○–), RNA polymerase B or RNA polymerase A from mouse Ascites (–⊙–⊙–), and *E. coli* RNA polymerase (–●–●–), 0.1 unit of each enzyme/assay.

human γ-globulin or in the presence of an RNA polymerase A preparation which had been inactivated by storage without glycerol. The predominant inhibition of RNA polymerase A from *Physarum* was still observed when enzyme A and B were mixed after purification and the mixture was then tested with the inhibitor in the presence or absence of amatoxin. These control experiments indicated a preferential inhibition of RNA polymerase A of *Physarum* by the endogenous factor excluding any influence by contaminating proteins. We then asked whether this effect was specific for the homologous enzyme and determined the inhibition of an animal RNA polymerase A. Enzyme A from mouse Ascites cells was only inhibited to the same low degree as RNA polymerase B from *Physarum* or *E. coli* polymerase (see Fig. 2).

Since we based the degree of inhibition of RNA synthesis *in vitro* on the phosphorus content of the factor from *Physarum* nuclei we tested the effects of orthophosphate, pyrophosphate and inorganic polyphosphate (M.W. >25 000) on the *in vitro* polymerase assay. We found that 500 – 2000 times more of these compounds had to be used to obtain 50% inhibition of UMP incorporation as compared with the phosphate content of the endogenous factor. No comparable inhibition of *Physarum* RNA polymerase A seen in Fig. 2 was revealed with any of these substances. Therefore, the factor isolated from *Physarum* did not inhibit RNA polymerase activity in an unspecific manner due to its phosphate content alone.

Since RNA molecules, resistant to RNase treatment, would cosediment on CsCl gradients, the factor was purified after labeling of plasmodia separately with each of the 4 ribonucleosides. After glycerol gradient analysis the inhibitor could be clearly separated from the contaminating tritiated material (Fig. 3 a).

To ask whether the inhibitor could represent a high molecular weight poly ADP ribose polymer we treated the inhibitor with phosphodiesterase; it retained more than 90% of its inhibitory effect excluding poly ADP ribose as a major component (Table II). In contrast to treatment with proteases, amylases and nucleases we obtained complete inactivation by phosphatases or alkaline and acid hydrolysis (Table II). The radioactive profile of the treated inhibitor was analysed by glycerol gradient centrifugation. Proteinase, amylase or heating at

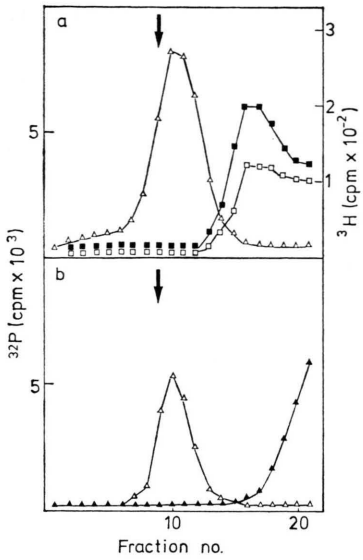

Fig. 3. a. Glycerol gradient centrifugation of the factor after *in vivo* incorporation of [^{3}H]ribonucleotides in microplasmodia. Aliquots of a microplasmodial culture were incubated for 10 h in 10μCi/ml growth medium either with [^{3}H]uridine, [^{3}H]cytidine, [^{3}H]guanosine, or [^{3}H]adenosine. The factor was prepared from isolated nuclei, treated with nucleases, centrifuged on CsCl gradients as described in Fig. 1 a and run on glycerol gradients (as described in Fig. 1 b). [^{3}H]labeled material was located by scintillation counting of aliquots of each fraction. (−□−□−) [^{3}H]-uridine, (−■−■−) [^{3}H]adenosine; the [^{3}H]label after incubation in guanosine and cytidine was indistinguishable from that of adenosine. In parallel gradients ^{32}P-labeled factor was centrifuged (−△−△−) and in all gradients hemoglobin marker was present (arrow). b. Effect of various treatments on the sedimentation behaviour of the factor. The ^{32}P-labeled factor was treated as described in Fig. 1 b. (−△−△−) control and after digestion with proteinase K, $\alpha+\beta$ amylase or heating (100 °C for 10 min). (−▲−▲−) after digestion with acid or alkaline phosphatase or hydrolysis in alkali or acid.

100 °C had no effect on the sedimentation of the inhibitor (Fig. 3 b), in contrast to phosphatase or hydrolysis. Since fully grown cultures of *Physarum* contain large amounts of an extracellular slime, a galactose polymer [19], control experiments were performed with purified slime. It could be seen that the inhibitor was not a component of slime.

Other control experiments with increasing Mg^{2+} and/or Mn^{2+} concentrations have indicated that the inhibition by the endogenous factor is not due to chelating divalent cations from the enzyme assay.

Binding of the inhibitor from Physarum to RNA polymerase

The inhibitor prepared from isolated nuclei sedimented as a distinct peak on glycerol gradients as could be seen from the degree of inhibition of the *in vitro* RNA synthesis (Fig. 4 a). RNA polymerase sedimented as another peak and could be located by assaying each fraction of a separate gradient for enzyme activity (Fig. 4 b). A mixture of purified RNA polymerase A from *Physarum* with the inhibitor, carefully adjusted to a clearly detectable degree of inhibition (60 – 80% of a control assay) revealed a low yield of active RNA polymerase in the gradient (Fig. 4 c) as predicted from the inhibition of the mixed preparation before centrifugation. The same result was obtained when equal enzyme units of partially or highly purified polymerase preparations were used or bovine serum albumin at 100 μg/ml was added to the enzyme prior to centrifugation. When the same sample as used in Fig. 4 c was centrifuged in the presence of salt (above 0.8 M NH_4Cl) the RNA polymerase has been recovered at full activity (Fig. 4 d). These results suggest that the inhibitor binds to the RNA polymerase and that

Table II. Partial characterization of the factor of *Physarum*.

Enzyme	Treatment	Inactivation
trypsin (bound to enzygel)	0.3 units/ml	none [a]
proteinase K	20 units/ml	none
α-amylase	100 units/ml	none
β-amylase	100 units/ml	none
DNAase I	100 units/ml 20 mM $MgCl_2$	none
RNAase	RNAase A 2.5 units/ml plus RNAase T_1 50 units/ml	none
phosphodiesterase	5 units/ml	none
alkaline phosphatase	free: 40 units/ml bound to Enzygel: 3 units/ml	complete
acid phosphatase	6 units/ml	complete
	100 °C, 10 min	none
	0.1 M NaOH, 37 °C. 24 h	complete
	0.5 N HCl, 80 °C, 1 h	complete
	1 N H_2SO_4, 100 °C, 15 min	complete

[a] Inhibitor concentration was adjusted to 80% inhibition of *in vitro* RNA synthesis (20% residual RNA polymerase activity). The inhibitor was treated with free or matrix bound enzymes for 5 hours at 30 °C. All enzymes were active under the conditions used as ascertained by degradation of appropriate substrates. After the treatments the inhibitor was reisolated by CsCl gradients. No inactivation (none) of the inhibitor after the treatment was scored when the residual RNA polymerase activity was still 20 – 30% of a control without inhibitor. Complete inactivation of the inhibitor means that the results were within 90 – 100% of the RNA polymerase activity in control assays without inhibitor.

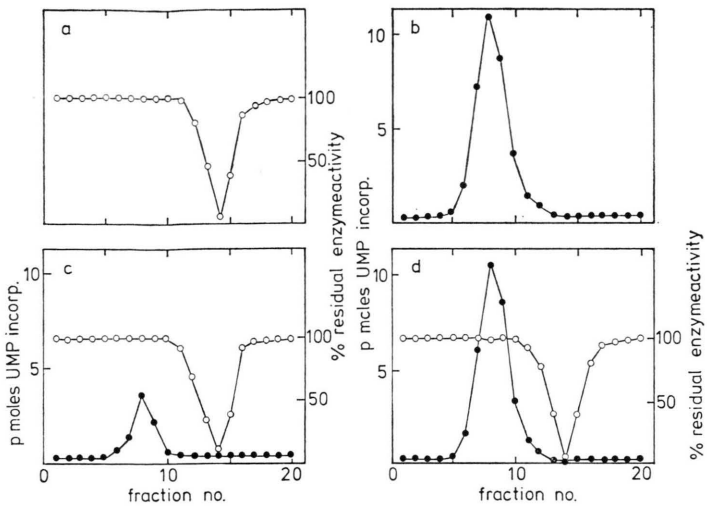

Fig. 4. Reactivation of RNA polymerase inhibited *in vitro* by the endogenous factor from *Physarum*. The factor and RNA polymerase were centrifuged, alone or as a mixture on linear glycerol gradients (10–40% glycerol in 50 mM Tris-HCl, pH 8, 20 h at 0 °C at 40 000 rpm) gradients were fractionated from the bottom. Each fraction was tested either for inhibition of a complete *in vitro* RNA synthesizing system (○–○) or for RNA polymerase activity (●–●).
a) Factor (no salt or with 1 M NH_4Cl);
b) RNA polymerase (no salt or with 1 M NH_4Cl);
c) mixture of factor and enzyme, adjusted to 75% inhibition, no salt;
d) like c) but with 1 M NH_4Cl.

binding and inhibition of *in vitro* transcription is reversible at high ionic strength.

In separate experiments *in vivo* ^{32}P labeled inhibitor together with each of the templates (native or denatured calf thymus DNA, native nucleolar *Physarum* DNA, poly d(AT) or poly d(GC) were centrifuged in glycerol gradients under various conditions (not shown). In no instance had ^{32}P labeled inhibitor bound to native or synthetic templates. These results indicate that the inhibitor, isolated from *Physarum* nuclei, interacts specifically with RNA polymerases but not with DNA.

The inhibitor from Physarum prevents binding of RNA polymerase to template

In these experiments RNA polymerase assays were adjusted to 50% inhibition by adding the assay mixture and the inhibitor (at 1 μM phosphate) simultaneously to the enzyme. However, there was no inhibition when DNA and enzyme were preincubated (Table III, line b), whereas complete inhibition was observed when the inhibitor and the enzyme were incubated before the start of the reaction (Table III, line c). These results were not affected by a wide range of NTP concentrations. By increasing the amount of template in the assay without preincubation (6–60 μg native calf thymus DNA/assay, constant concentration of inhibitor, 1 μM phosphate) a smaller degree of inhibition (20% *versus* 60%) was observed indicating that inhibitor and template competed for RNA polymerase molecules.

A complete blockade of transcription (Table III, line c) might suggest that initiation and elongation of transcription were equally affected. However, if the inhibitor was added to an RNA polymerase assay after it had been started for 5 min, there was no immediate cessation of transcription and UMP incorporation continued for about 10 min (Fig. 5). The kinetics of inhibition by the endogenous compound was indistinguishable from that detected with heparin or in the presence of high salt (0.5 M NH_4Cl) (Fig. 5).

Preincubation (5 min, 0 °C)	Start of reaction with NTP, Mn^{2+}, Mg^{2+} and ...	RNA polym. activity [a] (percent)	DNA-RNA polym. complex [b] (percent)
a) DNA+RNA polym.	–	100	100
b) DNA+RNA polym.	factor	95	92
c) factor+RNA polym.	DNA	5	3

Table III. Effects of preincubations with the factor on the *in vitro* transcription of native DNA by RNA polymerase A from *Physarum* or on the DNA-RNA polymerase complex formation.

[a] The RNA polymerase activity was determined as cpm UMP incorporation within 5 min after the start of reaction (100% = 4×10^4 cpm); duplicate values were within 10%.
[b] DNA-RNA polymerase complexes were measured in a filter binding assay where 100% corresponded to binding of 7200 ± 200 cpm of native [^{3}H]labeled T_7 DNA by RNA polymerase A to millipore filters; input was 8800 ± 100 cpm DNA (1.5 μg DNA). Blank values of 80–120 cpm obtained without RNA polymerase but in the presence of factor have been substracted.

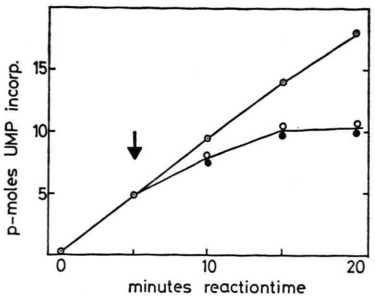

Fig. 5. Effect of the factor on the kinetics of UMP incorporation by RNA polymerase A. UMP incorporation was monitored at 5 min intervals, control (⊙—⊙); in a parallel experiment either the factor (10 μM phosphate) (○—○) or heparin (100 μg/ml) (●—●) were added at 5 min of the reaction time (arrow).

In other experiments the inhibitor was added to isolated nuclei or nucleoli from *Physarum* where only the elongation of RNA chains, which were initiated *in vivo*, occurred *in vitro* [24]. The inhibitor had no effect even if it was added at a hundred fold higher concentration than that which would completely block *in vitro* transcription.

From these experiments it seemed that the inhibitor, once bound to RNA polymerase, prevented initiation of transcription. One step prior to this process is the binding of RNA polymerase to the template. A filter binding assay was employed to ask whether RNA polymerase, treated with the inhibitor, can still bind to DNA. First, the range of

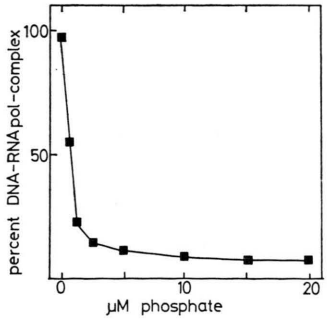

Fig. 6. Effect of the factor from *Physarum* on the formation of an RNA polymerase-DNA complex. A constant amount of RNA polymerase A (0.1 unit/assay) was incubated with increasing concentrations of the factor (based on its acid labile phosphate content, μM) for 5 min at 0 °C and then [^{3}H]-labeled DNA was added. The mixture was filtered after 10 min at 25 °C through millipore filters and bound DNA was determined by scintillation counting. In controls without factor 6000 ± 300 cpm of the input DNA (8500 ± 50 cpm, native phage T_7 DNA) were complexed with RNA polymerase (100%). Without RNA polymerase 3—5% of the input DNA was bound to the millipore filters in the absence or presence of the factor.

linear binding reaction of a constant amount of DNA with increasing concentrations of RNA polymerase A from *Physarum* was determined. Then samples of enzyme, at a concentration which would bind 75% of the DNA, were preincubated with increasing concentrations of the inhibitor (Fig. 6) (for 10 min at 0 °C or 30 °C). We observed that the binding of RNA polymerase to DNA was drastically inhibited when the enzyme had been treated with the endogenous compound at any of the two temperatures. In control experiments nucleoside triphosphates were added during the incubation of enzyme with inhibitor. The results were the same as those seen in Fig. 6 confirming a 50% inhibition of binding by 1 μM phosphate concentration of the endogenous compound.

In other control experiments (not shown) a coincidence of inhibition of enzyme activity and loss of binding of enzyme to DNA was observed. Furthermore, enzyme bound to DNA during a preincubation period was resistant to the factor (Table III, line b).

Partial characterization of the inhibitor

From the above listed data the purified factor was nondialysable, sedimented with 4S and contained phosphate. It behaves like a homogeneous substance. A partial analysis of the purified inhibitor yielded 25% carbon, 5% hydrogen, 23% residue after combustion (which can mainly be accounted for by phosphate) and oxygen, but no significant amount of nitrogen.

Furthermore, acid labile phosphate of the factor has directly been determined to make up 11% of the dry weight. Since three forth of the total phosphate, is acid labile as determined by ashing the sample [11], the factor contains about 14% phosphate.

In an attempt to identify the carbohydrate component of our factor, we treated it first with carrier-bound phosphatase, and than with 16 different detection reagents for carbohydrates (see Materials and Methods).

On comparing the reactions with those of 22 test substances the unknown carbohydrate behaved as a polyol.

Paper chromatography and TLC of the untreated factor confirmed its high molecular weight and/or high charge, since it did not migrate from the point of application. Only after phosphatase treatment did the carbohydrate fraction move as a single spot with

polyol character and comprising 90% of the carbohydrate material (as determined with H_2SO_4 or by the periodate – anisine reaction). Its R_f-value on paper chromatographs was 0.24 as compared to: glucose = 0.04, ribose = 0.14, glycerol = 0.24, deoxyribose = 0.26.

By Cellulose TLC an R_f value of 0.51 was obtained as compared to: glucose = 0.32, ribose = 0.44, glycerol = 0.51, deoxyribose = 0.55.

The migration behaviour of glycerol and the carbohydrate component of the factor from *Physarum* was identical for all the solvent systems used in this analysis.

The following control experiment shows that the glycerol-like component of the factor is only released after hydrolysis. The purified factor was applied to a paper chromatograph – where it did not move – was then eluted with 0.05 N HCl, hydrolyzed for 24 h at 100 °C under vacuum, lyophylized and chromatographed on silica gel TLC. Over 70% of the carbohydrate of the factor was recovered and moved as a single spot, again with the R_f value of glycerol. This control, along with ion exchange chromatography, extensive dialysis, and gelfiltration on Sephadex G25, rules out a contamination of the factor by free glycerol.

To further substantiate that glycerol is the carbohydrate component of the factor, its NMR spectrum was compared to that of glycerol and glycero-3-phosphate. The spectrum of untreated (Fig. 7 A) and of the phosphatase treated factor (Fig. 7 B) is very similar to that of glycerol (Fig. 7 D). After phosphatase digestion the signal at 5.25 ppm is decreased whereas the signal at 5.03 ppm is increased. However, there was no close similarity between the spectra of untreated factor (Fig. 7 A) and of glycero-3-phosphate (Fig. 7 C). Therefore, the factor is not made up of glycerophosphate units alone. More information on the linkage of phosphate and glycerol of the transcription inhibitor of *Physarum* might be expected from the spectrum of glycero-1,3-diphosphate, a substance which has not been available to us.

Discussion

DNA extracted from *Physarum* nuclei was an active template for the *in vitro* RNA polymerase assay only after purification by equilibrium density centrifugation. An inhibiting substance was located near the bottom of the CsCl gradient and could be further purified by velocity gradient centrifugation, chromatography on DEAE Sephadex A25 and gelfiltration.

This material could be labeled with [^{32}P]orthophosphate *in vivo*. Radioactivity and inhibition of *in vitro* RNA synthesis copurified in all steps of factor preparation. Furthermore, the degree of inhibition and amount of ^{32}P label were strictly correlated. The phosphate groups were essential for the inhibitory activity of the factor as deduced from the sensitivity towards phosphatases. The small and unselective effect of authentic polyphosphate on the RNA polymerases and the sensitivity to alkaline hydrolysis indicate that the inhibitor from *Physarum* is not an inorganic polyphosphate.

The purified factor is of high molecular weight according to gel filtration and its 4S value on velocity gradients. It does not contain nucleosides or polypeptides nor significant amounts if nitrogen. The isolation procedure makes it unlikely that lipids are present. Furthermore, resistance to phosphodiesterase argues against a large ADP-ribose polymer [25]. The factor consists of acid labile and acid stable phosphate in relative proportions of 3 : 1 and a single carbohydrate component which can be released by a phosphatase treatment and has been tentatively identified as glycerol by its chromatographic behaviour and its NMR spectrum.

Several ways of a linkage of glycerol and phosphate exist: an alternating chain of the two molecules with possible branching points at the phosphate groups, a backbone of polyphosphate with glycerol attached by ester bonds or a combination of the two possibilities.

Since the NMR-spectrum of the factor is different from glycero-3-phosphate the second alternative is less likely. The available data favour a structure of the first or third alternative, although the exact configuration of the transcription inhibitor from *Physarum* is not yet known. Our results indicate that the endogenous inhibitor cannot intercalate into DNA like actinomycin D [26] since it has no affinity to any of the templates tested. This inhibitor does not affect the elongation of RNA chains as does streptolygidin [27] or amatoxin [28] nor does it interfere with the substrate binding of free enzyme like Rif AF/013 [29] or compete for ATP during transcription like exotoxin [30]. The endogenous factor from *Physarum* acts like heparin, an assumed initiation inhibitor [31], although the occurance of a true

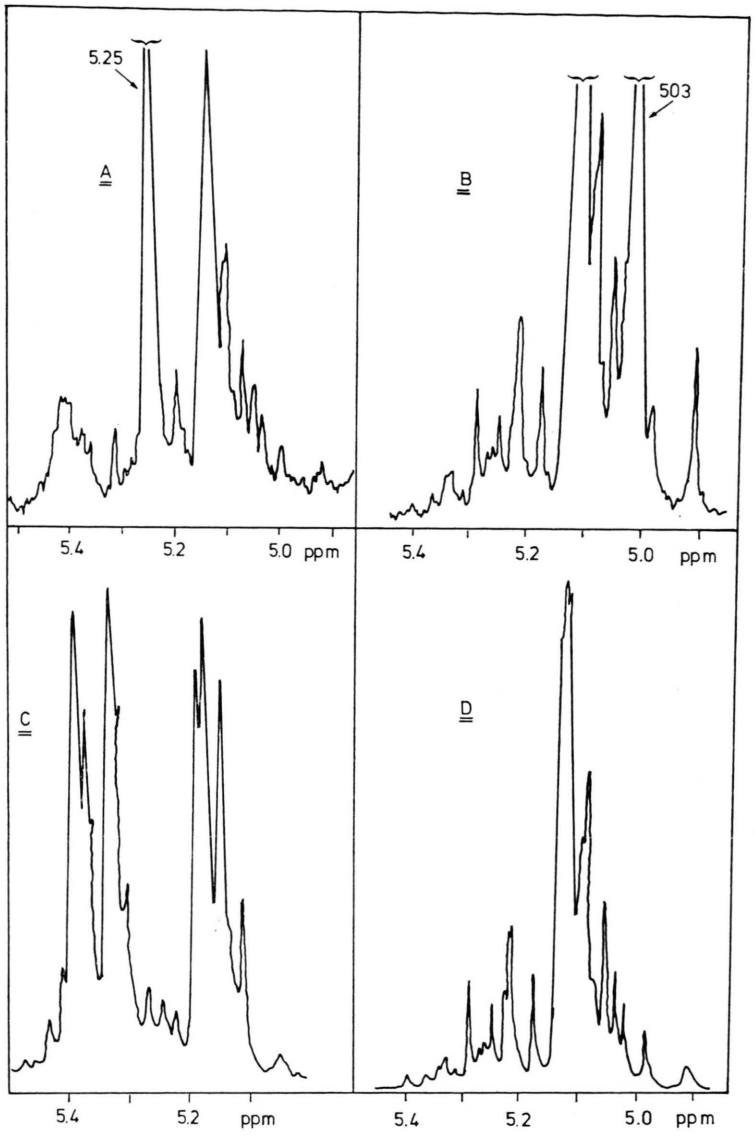

Fig. 7. NMR spectra of the factor. Samples of each compound tested were prepared dissolving 2–4 mg in 0.3 ml 99.8% D_2O. The spectra representing a composite of 70 scans were run at room temperature. Chemical shifts were measured in parts per million (ppm) using an internal capillary tube with tetramethylsilane as standard. The absorption peak of the solvent (H_2O) appeared at 6.27 ppm.
A) Untreated factor;
B) factor treated with "Enzygel"-bound alkaline phosphatase (see Table II);
C) glycero-3-phosphate;
D) glycerol.

heparin resistant complex in an *in vitro* assay-system has been questioned for eucaryotic RNA polymerases [32].

The inhibition cannot be explained by chelating divalent cations like an endogenous factor in rats [33] and is fully reversible at high ionic strength.

The inhibitor, labeled *in vivo* with ^{32}P, binds to RNA polymerase under conditions where other proteins like albumin, glutamate dehydrogenase and nuclear proteins including histones, do not bind (ref. [13] and unpublished results).

Furthermore, the factor has a specific effect on RNA polymerases, not on DNA polymerases, and inhibits all preparations of RNA polymerase A from *Physarum* at lower concentrations as compared to RNA polymerase B from *Physarum*, RNA polymerase A from mouse or *E. coli* RNA polymerase.

The inhibitor prevents formation of a RNA polymerase-DNA complex. The effective inhibition of the formation of this complex and of transcription only after preincubation of inhibitor with enzyme, and the protection of polymerase molecules bound to DNA, clearly indicate that the binding of enzyme to template is blocked.

Endogenous polyphosphates affecting *in vitro* transcription have been described in some other

lower eucaryotes and bacteria. Polyphosphate was isolated from yeast DNA preparations and shown to inhibit *in vitro* initiation of transcription [34]. Diguanosine tetraphosphate obtained from a water mold inhibited homologous RNA polymerase 10 times more efficiently than heterologous RNA polymerases [35]. However, the mold enzymes were resistant to guanosine tetraphosphate [36], a substance that has long been known to be involved in stringent control of bacteria [1] and shown to interfere with transcription of ribosomal genes [3].

We assume that the endogenous inhibitor, prepared from isolated nuclei of *Physarum* may play a role in the regulation of *in vivo* transcription. Coordinate fluctuations of the inhibitor, RNA polymerase A and ribosomal RNA *in vivo* [6, 7, 9, 10 and 13] have been described in the life cycle of *Physarum*. The competition of endogenous inhibitor and template for RNA polymerase A could be considered an effective transcription control of ribosomal cistrons. Recent demonstration of the inhibitor in isolated nucleoli from starving cultures in concentrations which are able to inactivate most extractable RNA polymerase A from these cultures, of a tighter binding of the inhibitor to RNA polymerase A as compared to polymerase B or *E. coli* enzyme, of reactivation *in vitro* of inactive RNA polymerase A isolated from differentiating plasmodia [13] and disappearance of ^{32}P labeled inhibitor after refeeding *in vivo* [37] support this hypothesis.

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