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RNA polymerase I from higher plants. Evidence for allosteric regulation and interaction with a nuclear phosphatase activity controlled NTP pool

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ABSTRACT

RNA polymerase I was isolated from parsley cells grown in suspension culture and from soybean hypocotyls. Kinetic studies of the enzyme revealed that RNA polymerase I is an allosteric regulated enzyme. The enzyme activity was influenced by nucleoside triphosphates (NTP) and divalent cations. NTP exceeding a 1:1 ratio of these two components acted as allosteric inhibitors, contrary to free divalent cations, which had promotive effects on the RNA polymerase I. Furthermore, isolated nuclei from parsley exhibited a powerful nucleoside triphosphatase (NTPase) activity. Contrary to RNA polymerase I, this enzyme was stimulated by NTP exceeding the 1:1 ratio of NTP and divalent cations. Free divalent cations had an inhibitory effect. Assuming that a causal connection of these two processes does exist, a possible role of this NTPase would be the control of NTP pools in relation to divalent cations and thus regulating RNA synthesis.

INTRODUCTION

It is well known that during the transcriptional process in prokaryotes as well as in eukaryotes nucleoside triphosphates (NTP) are used as precursors of RNA; divalent cations, especially Mg^{2+} and Mn^{2+}, are also necessary for RNA synthesis directed by DNA-dependent RNA polymerases. However, nearly nothing is known about the interaction of these ions and the nucleoside triphosphates in the regulation and control of the activity of RNA polymerases.

In a previous paper we communicated an in vitro system with isolated nuclei from a suspension culture of parsley (1). In this context our primary interest was mainly directed to the role of NTP and divalent cations. In these isolated nuclei RNA synthesis was stimulated, if nucleoside triphosphates and di-

valent cations were present in equimolar concentrations. This stimulation could be partly the result of increasing concentrations of the complex formed between NTP and divalent cations. On the other hand, these nuclei exhibited a very strong nucleoside triphosphatase (NTPase) activity, which may have an influence on RNA synthesis by splitting NTP, thereby lowering the intranuclear nucleoside triphosphate concentration.

In order to study the influence of NTP and divalent cations on RNA synthesis in detail two approaches were made. In one approach the influence of nucleoside triphosphates and divalent cations on the activity of purified RNA polymerase I from higher plant cells was investigated. In the present report we describe kinetic studies with RNA polymerase I isolated from a suspension culture of parsley and from soybean hypocotyls. In the second approach the NTPase activity present in isolated nuclei of parsley was studied with respect to the effect of nucleoside triphosphates and divalent cations on the activity of this hydrolyzing enzyme.

The data presented here may contribute to an understanding of the mechanism of RNA synthesis catalyzed by RNA polymerase I. We are able to demonstrate that RNA polymerase I derived from different higher plant sources shows an allosteric behaviour. The participation of NTPase in the regulation of RNA synthesis in eukaryotic cells is discussed.

MATERIALS AND METHODS

DEAE-cellulose (DE 52) and phosphocellulose (P-11) were purchased from Whatman, [5'-^{3}H]uridine 5'-triphosphate from Amersham Buchler (Braunschweig), unlabeled nucleoside triphosphates and ß-ɣ-methylene AMP (AMPPCP) from Boehringer (Mannheim) and salmon sperm DNA from Serva (Heidelberg).

Plant material

Freely suspended callus cells of Petroselinum crispum were propagated in erlenmeyer flasks or in a fermenter in a medium described previously (3).

Preparation and purification of nuclei

Nuclei were isolated according to a procedure described

previously (1).

Purification of RNA polymerase I from parsley

The enzyme was purified from chromatin isolated at pH 8.0 from 2 kg of cell material. The purification procedure of Guilfoyle et al. (4) was slightly modified. RNA polymerase I could be solubilized in the presence of 0.5 M ammonium sulfate. Further treatments with DEAE-cellulose and phosphocellulose in batches removed the bulk of proteins. These procedures were followed by ion exchange chromatography on phosphocellulose, DEAE-cellulose, and a second phosphocellulose column. The details will be described in a separate paper (5).

RNA polymerase I from soybean

The enzyme was a gift from H.Friedrich (Department of Genetics, Tübingen). It was isolated from 2.4-D treated hypocotyls. Purification was achieved by using Agarose A-1.5m gel filtration, heparin sepharose, and DEAE-cellulose chromatography.

Assay for parsley RNA polymerase I

RNA polymerase activity was assayed essentially by the method of Guilfoyle et al. (4) with several modifications. Unless indicated otherwise, 0.5 ml reaction mixtures contained 50 mM Tris·HCl (pH 8.0), 20 mM $(NH_4)_2SO_4$, 25 % glycerol (v/v), 100 µg/ml of heat denatured salmon sperm DNA, and 0.1 mM [5'-^{3}H] uridine 5'-triphosphate (6 µCi). $MgCl_2$ and $MnCl_2$ concentrations were varied maintaining a 6 : 1 ratio (for special conditions see figure legends). The concentration of unlabeled nucleoside triphosphates (ATP, CTP, GTP) were also varied (see figure legends). The reaction was started by adding 9 µg of enzyme protein (20 µl). The mixture was incubated at 29°C for 30 min. The reaction was terminated by adding 4 ml of 5 % trichloroacetic acid (TCA). The test tubes were kept on ice for 10 min; TCA insoluble material was collected on Whatman GF/C filter discs and washed six times with 5 % TCA (2 ml portions), and twice with 2 ml of cold 96 % ethanol. The dried filter discs were counted for radioactivity in a toluene-PPO-POPOP scintillation mixture.

Assay for soybean RNA polymerase I

The assay conditions were the same as in the case of the

enzyme from parsley cells with the only exception that 50 mM $(NH_4)_2SO_4$ was present. Mg^{2+} and NTP were varied (see figure legends).

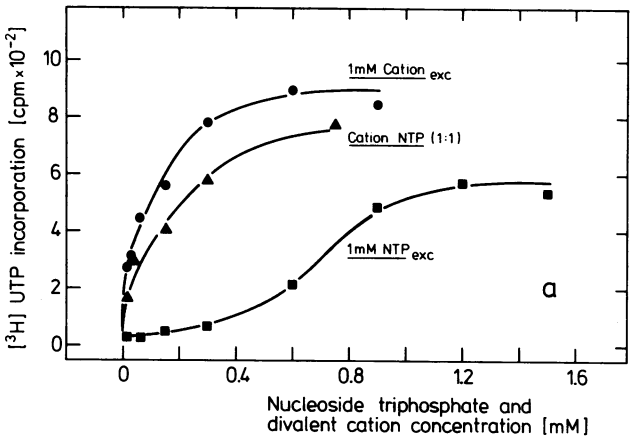
Phosphatase activity of isolated nuclei from parsley

The enzyme activity was measured according to Lin and Morales (6) by determination of P_i released. The reaction was started by adding 20 µl of the substrate solution (as indicated in the figure legends) and 20 µl of nuclei suspension (10^5 nuclei) to 0.96 ml of 50 mM Tris·HCl buffer (pH 7.8). The mixture was incubated at 36°C for 10 min. The reaction was stopped by adding 1 ml molybdovanadate reagent (6). The absorbance of the colour complex, formed by P_i and molybdovanadate was measured after 3 min at 366 nm. The P_i concentrations were calculated from a calibration curve with Na_2HPO_4. Blank values without nuclei were subtracted.

RESULTS

In a seperate paper we demonstrated that RNA synthesis in vitro catalyzed by RNA polymerase I from parsley is not influenced by nucleoside triphosphates and divalent cations given in equimolar concentrations in the range from 4 to 11 mM (5). However, nucleoside triphosphates given in excess of this 1:1 ratio (NTP_{exc}) did inhibit the activity of the enzyme whereas divalent cations in excess ($cation_{exc}$) stimulated RNA polymerase I. To explain this effect, the knowledge of the kinetic behaviour of RNA polymerase I would be absolutely necessary. Therefore, we studied the enzyme kinetics in relation to nucleoside triphosphates and divalent cations, especially Mg^{2+} and Mn^{2+}.

First, we investigated the effect of the substrate concentration on the activity of RNA polymerase I from parsley. Fig. 1a shows a plot of initial velocity vs. substrate concentration. In this experiment the concentrations of unlabeled nucleoside triphosphates (ATP, CTP, GTP in equal amounts) and divalent cations ($MgCl_2$ and $MnCl_2$ in a 6:1 ratio) were increased maintaining a 1:1 ratio of these two groups of ligands. Under such circumstances the enzyme showed a hyperbolic satu-



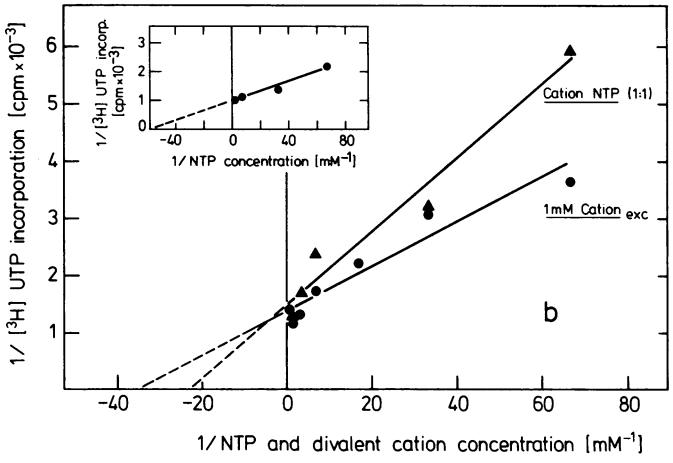


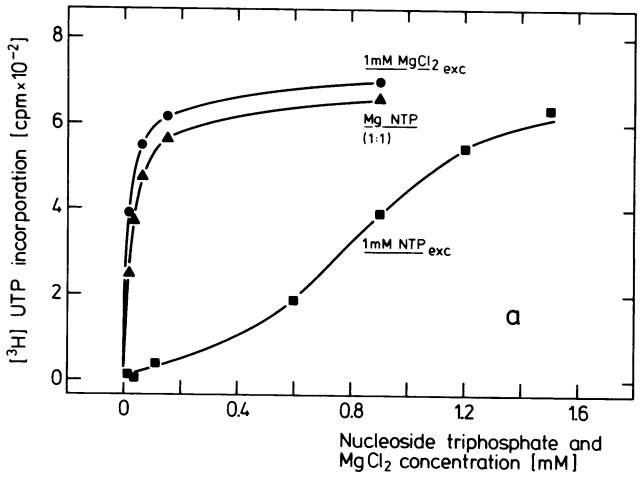
Figure 1a. Effect of increasing substrate concentrations on the initial velocity (V_o) of RNA polymerase I from parsley. NTP (ATP, CTP, GTP in equal amounts) and divalent cations ($MgCl_2$ and $MnCl_2$ in a 6:1 ratio) were increased maintaining a 1:1 ratio. For other constituents of the assay see Materials and Methods. ▲—▲ cations and NTP (1:1), ●—● $cation_{exc}$ (1 mM), ■—■ NTP_{exc} (1 mM).

Figure 1b. Lineweaver-Burk plots of the values in Fig.1a. ▲—▲ cations and NTP (1:1), ●—● $cation_{exc}$ (1 mM). Inset: Effect of increasing concentrations of NTP at high divalent cation concentrations (6 mM $MgCl_2$ and 1 mM $MnCl_2$ were kept constant).

ration curve (Fig. 1a). If divalent cations were given in excess of this 1:1 ratio, a faster increase of the enzyme activity was observed. Lineweaver-Burk plots revealed that RNA polymerase I from parsley obeyed the Michaelis-Menten kinetics with an apparent K_m-value of 0.0442 mM (Fig. 1b), if NTP and divalent cations were increased maintaining a 1:1 ratio. Divalent cations exceeding this 1:1 ratio ($cation_{exc}$) led to lower K_m-values (0.0288 mM), while the maximum initial velocity (V_{max}) remained approximately the same. At a high divalent cation concentration (6 mM $MgCl_2$, 1 mM $MnCl_2$), increasing NTP concentrations also lowered the K_m to 0.0183 mM (see inset of Fig. 2). On the contrary, nucleoside triphosphates (in all 1 mM, ATP, CTP, GTP in equal amounts) given in excess of the 1:1 ratio led to a sigmoidal curve relating initial velocity to substrate concentration (Fig. 1a). This non Michaelis-Menten relationship may signify an allosteric regulated enzyme.

In order to confirm the results with parsley, we repeated these experiments with RNA polymerase I isolated from 2.4-D treated soybean hypocotyls. If nucleoside triphosphates (ATP, CTP, GTP in equal amounts) and $MgCl_2$ were increased maintaining a 1:1 ratio, we likewise observed a Michaelis-Menten kinetics for RNA polymerase I from soybean hypocotyls (Fig. 2a). The apparent K_m-value was 0.024 mM (Fig. 2b). In comparison with RNA polymerase I from parsley, the enzyme derived from soybean had lower K_m-values. If $MgCl_2$ was present in excess in the assay (1 mM), K_m averaged 0.011 mM, whereas V_{max} remained unchanged (Fig. 2a and 2b). As it was the case with RNA polymerase I from parsley, free nucleoside triphosphates (in all 1 mM; ATP, CTP, and GTP in equal amounts) in excess also led to a sigmoid curve, which is characteristic for an allosteric regulated enzyme (Fig. 2a). To support this conclusion, an experiment was carried out in which the effect of nucleoside triphosphate concentrations exceeding the 1:1 ratio on the enzyme activity was tested (Fig. 3). As expected, we received a family of sigmoid curves with different steepness.

Considering these results the conclusions drawn were as follows: 1. The true substrate for the RNA polymerase I under



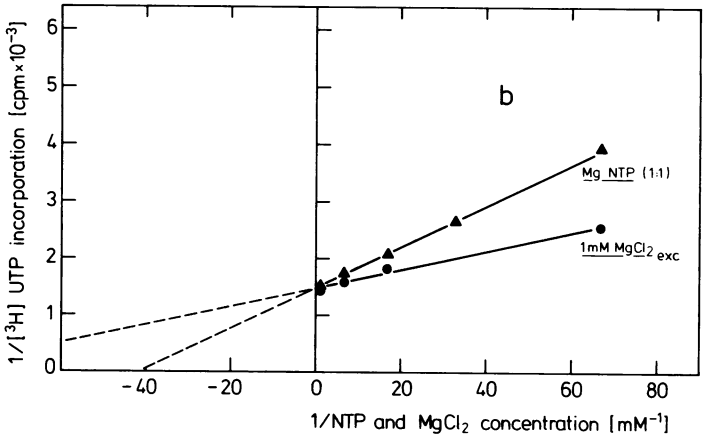


Figure 2a. Effect of increasing substrate concentrations on the initial velocity (V_o) of RNA polymerase I from soybean. NTP (ATP, CTP, GTP in equal amounts) and $MgCl_2$ were increased maintaining a 1:1 ratio. For other constituents of the assay see Materials and Methods. ▲—▲ $MgCl_2$ and NTP (1:1), ●—● $cation_{exc}$ (1 mM $MgCl_2$), ■—■ NTP_{exc} (1 mM).
Figure 2b. Lineweaver-Burk plots of the values in Fig.2a. ▲—▲ $MgCl_2$ and NTP (1:1), ●—● $cation_{exc}$ (1mM $MgCl_2$).

investigation is the complex of NTP and divalent cations.

2. Divalent cations are essential activators of the RNA polymerase I. On the one hand NTP molecules are able to lower the

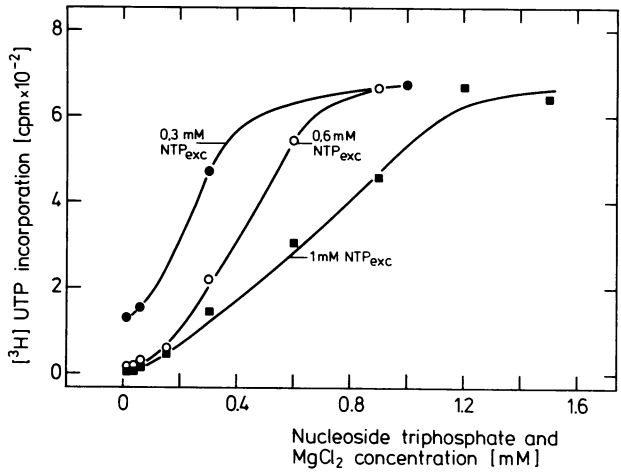


Figure 3. Influence of different NTP concentrations given in excess of the 1:1 ratio of NTP and $MgCl_2$ on the initial velocity (V_0) of RNA polymerase I from soybean. NTP (ATP, CTP, GTP in equal amounts) and $MgCl_2$ were increased maintaining a 1:1 ratio. 0.3, 0.6 and 1.0 mM NTP exceeding this ratio were tested. 0.05 mM [^{3}H]UTP (4 µCi) was present in the assay. For other constituents see Materials and Methods.

effective concentration of these activators by binding them, on the other hand the true substrate for the RNA polymerase I is just the complex between both components. 3. Free NTP represents an allosteric inhibitor, whereas the maximum initial velocity remains nearly unimpaired.

Considering the above conclusion that divalent cations not complexed by NTP are essential activators of RNA polymerase one migt argue that the allosteric effect of NTP added in excess could be due to a decrease of the actual concentration of free Mg-ions. However, such an interpretation would not sufficiently explain the effect of NTP added in excess. Calculated values (7) of not complexed Mg^{2+} showed a hyperbolic characteristic if NTP and Mg^{2+} were increased maintaining equimolarity. Even if 1 mM NTP were present in excess, a hyperbola for calculated free Mg^{2+} was obtained, but with a lower course. After these calculations a Michaelis-Menten kinetics with higher K_m-values should be expected for the polymerase,

but in fact, the enzyme activity showed a sigmoid curve. Therefore, nucleoside triphosphates could not be mere chelating agents for Mg-ions. A comparison of calculated concentrations of free Mg^{2+} and the actual effect of NTP on the enzyme also supports the view that NTP are allosteric inhibitors of RNA polymerase I. If 1 mM NTP was added in excess of equimolar concentrations (0.2 mM) of NTP and $MgCl_2$, the concentration of free Mg^{2+} was calculated to be 30 % of the value without NTP in excess. However, polymerase activity under identical conditions was only 10 %.

In this context an additional experiment with EDTA was carried out (Fig. 4). In this case RNA polymerase I was not active, if NTP and divalent cations did not exceed the EDTA concentration. However, at concentrations of NTP and divalent cations higher than the EDTA concentration, a promotive effect was observed; the activity could be described by a saturation curve. The explanation of this result is the fact that EDTA forms more stable complexes with divalent cations than NTP (8). That means that divalent cations bound by EDTA were no longer available for the formation of the substrate for RNA poly-

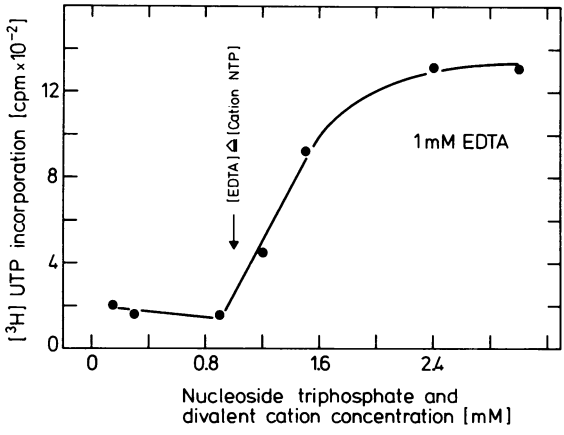


Figure 4. Effect of EDTA on the activity of RNA polymerase I from parsley. 1 mM EDTA was present in the assay. NTP (ATP, CTP, GTP in equal amounts) and divalent cations ($MgCl_2$ and $MnCl_2$ in a 6:1 ratio) were increased maintaining a 1:1 ratio. 0.1 mM [^{3}H]UTP (8 µCi) was present in the assay. For other constituents see Materials and Methods.

merase I.

We also replaced NTP_{exc} by nucleoside diphosphates and orthophosphate. In this experiment we could demonstrate that RNA polymerase I from parsley as well as from soybean obeyed again the hyperbolic Michaelis-Menten relationship with apparent K_m-values of 0.077 (parsley, Fig. 5) and 0.045 mM (soybean, Fig. 6).

Since free NTP caused an allosteric behaviour in contrast to NDP and orthophosphat which did not influence the Michaelis-Menten kinetics, we can strongly support the view that a plausible enzymatic splitting of NTP into NDP and inorganic phosphate (P_i) can partly prevent the inhibitory effect of free NTP on RNA synthesis.

In fact, we were able to detect a very strong NTPase activity in isolated nuclei of parsley (2). We were also able to detect a very potent NTPase activity associated with isolated chromatin from parsley cells thus indicating that the NTPase activity measured in the nuclei was not a contamination by unspecific adsorption of a cytoplasmic enzyme protein bearing NTPase activity.

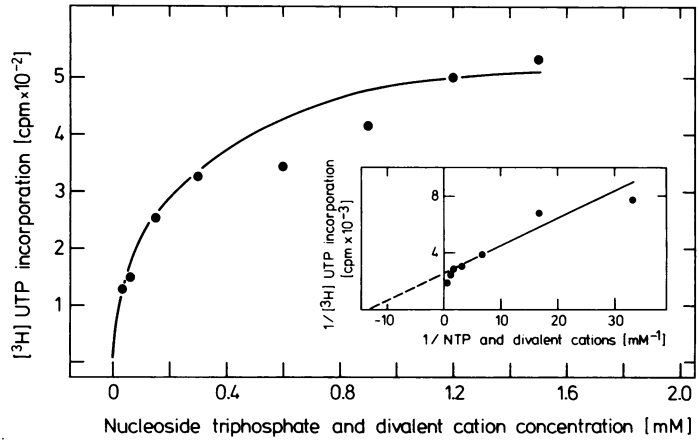


Figure 5. Enzyme kinetics of RNA polymerase I from parsley in dependence on nucleoside diphosphates (NDP) and orthophosphate. Nucleoside triphosphates and divalent cations were increased in a 1:1 ratio (see Fig.1a). 1 mM NDP (ADP, CDP, GDP in equal amounts) plus 1 mM Na_3PO_4 were added in excess of this 1:1 ratio. Inset: Lineweaver-Burk plot.

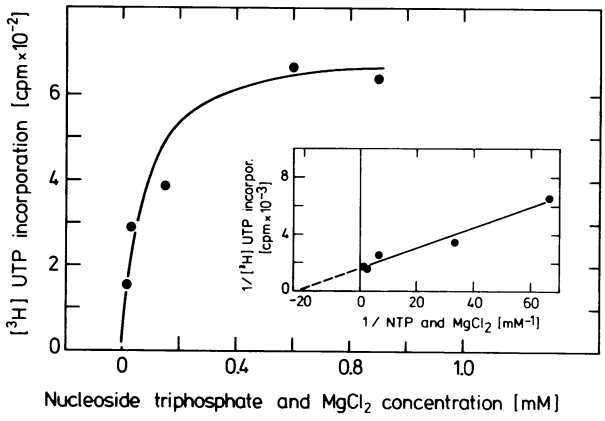


Figure 6. Enzyme kinetics of RNA polymerase I from soybean in dependence on nucleoside diphosphates (NDP) and orthophosphate. Nucleoside triphosphates and $MgCl_2$ were increased maintaining a 1:1 ratio (see Fig.2a). 1 mM NDP (ADP, CDP, GDP in equal amounts) and 1 mM Na_3PO_4 were added in excess of this 1:1 ratio. Inset: Lineweaver-Burk plot.

In a series of experiments the influence of nucleoside triphosphates and divalent cations on the NTPase in isolated nuclei from parsley was investigated. In Fig. 7 an experiment is shown, in which ATP was varied in a range from 1 to 20 mM at different Mg^{2+} concentrations. It is clear from these Lineweaver-Burk plots that with increasing concentrations of $MgCl_2$ the K_m-values of the NTPase were increased, whereas V_{max} remained nearly constant (Table 1). At very low concentrations (0.1 mM $MgCl_2$) a very low K_m was observed, but V_{max} was also lowered indicating that such low concentration of Mg^{2+} had an inhibitory effect on the NTPase.

In a further experiment the influence of free ATP and free Mg^{2+} on the activity of NTPase in isolated nuclei was tested (Fig. 8, and Table 2). In this case ATP and $MgCl_2$ concentrations were increased simultaneously thus maintaining a 1:1 ratio. The Lineweaver-Burk plots revealed the following results: 1. The complex formed between NTP (especially ATP) and divalent cations (especially Mg^{2+}) seemed to be the substrate of this nuclear NTPase in parsley (K_m= 2.57 mM). At concentra-

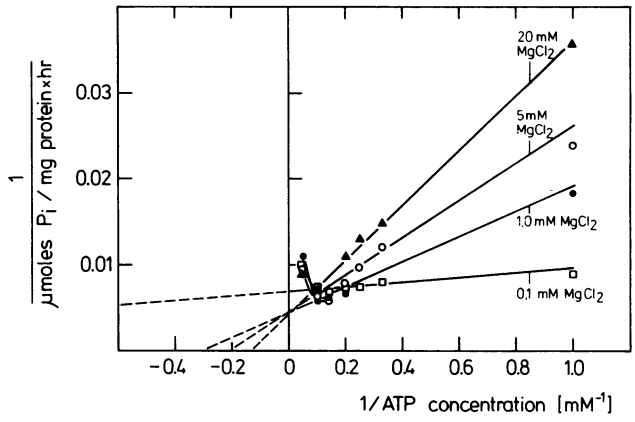


Figure 7. Influence of $MgCl_2$ on the activity of NTPase in isolated nuclei from parsley. Lineweaver-Burk plots are shown. The concentration of ATP was increased in a range from 1 to 20 mM at different concentrations of $MgCl_2$ as indicated in the figure. For incubation conditions and the determination of the enzyme activity see Material and Methods.

tions exceeding 10 mM a substrate inhibition was observed. 2. Free Mg-ions acted as an apparent competitive inhibitor (K_m= 3.53 mM). 3. Free ATP acted as an activator of the NTPase (K_m= 0.0288 mM).

In an experiment in which free ATP was replaced by ß-γ-methylene AMP (AMPPCP) which cannot be hydrolyzed at the ß-γ position by an NTPase but has the same chelating qualities as ATP, a K_m-value of 0.529 mM was determined, likewise indicating a decrease of free Mg^{2+} with their inhibitory effect (Fig. 8, Table 2).

Table 1. Influence of $MgCl_2$ concentration on NTPase activity of isolated nuclei from parsley (see Figure 7).

Mg^{2+} [mM]	K_m [mM]	Velocity [µmoles $P_i \cdot mg^{-1} \cdot hr^{-1}$]
0.1	0.371	147.3
1	3.344	241.5
5	4.97	243.9
20	8.26	255.1

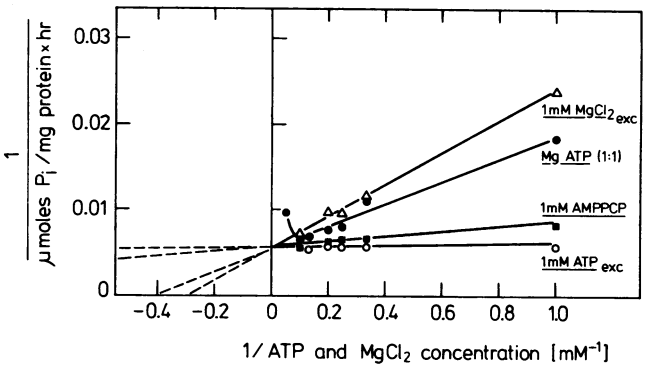


Figure 8. Influence of the complex of Mg^{2+} and ATP on the activity of the NTPase in isolated nuclei from parsley. ATP and $MgCl_2$ were increased maintaining a 1:1 ratio. Lineweaver-Burk plots are shown. ● — ● $MgCl_2$ and ATP (1:1), △ — △ $MgCl_2$ 1 mM in excess of the 1:1 ratio, ■ — ■ 1 mM AMPPCP in excess of the 1:1 ratio, ○ — ○ ATP 1 mM in excess of the 1:1 ratio.

DISCUSSION

Eukaryotic cells contain multiple forms of DNA-dependent RNA polymerases (EC 2.7.7.6) designated as I, II, and III with specific transcriptional properties. In order to control transcription three types of modulation occur within the nuclei: modulation of template availability, modulation of the amount of nuclear RNA polymerase and modulation of the specific ac-

Table 2. Influence of complex concentration on NTPase activity (see Figure 8).

Conditions	K_m [mM]	Velocity [µmoles $P_i \cdot mg^{-1} \cdot hr^{-1}$]
ATP·Mg (1:1)	2.57	191.5
+ 1 mM ATP_{exc}	0.0288	178.3
+ 1 mM Mg^{2+}_{exc}	3.53	189.4
+ 1 mM AMPPCP	0.529	182.5

tivity of the nuclar RNA polymerase. There are several reports on the isolation of protein factors from animal tissues (9) and plant tissue (for review see 10) affecting RNA polymerase activity. However, the influence of nucleoside triphosphates in relation to divalent cations on RNA synthesis has not yet been studied.

In this report we demonstrated that RNA polymerase I isolated from different higher plant cells is an allosteric regulated enzyme. Nucleoside triphosphates not complexed by divalent cations act as allosteric inhibitors, whereas divalent cations are essential activators of RNA polymerase I. However, with nucleoside diphosphates and orthophosphate exceeding the 1:1 ratio of divalent cations and NTPs the Michaelis-Menten kinetics of RNA polymerase I is maintained in contrast to what had been found with free NTP. So we could strongly support the view that a plausible enzymatic splitting of NTP into NDP and P_i can prevent the inhibition of RNA polymerase by free nucleoside triphosphate molecules.

Since the nucleus is the site of a powerful NTPase activity (11) and NTP are the precursors for RNA, Siebert (12) advanced the hypothesis that in animal nuclei the NTPase is engaged in the control of the precursor pools for nucleic acid synthesis. Grummt and Grummt (13) have shown that in mouse Ehrlich ascites cells changes in the absolute pool sizes of purin nucleotides have a direct influence on RNA polymerase I and thus determine the rate of rRNA synthesis. We consistently found that isolated nuclei from parsley and even chromatin from these cells show a powerful NTPase activity (2). From our experiments it seems to be probable that the complex formed by NTP and divalent cations is the true substrate for the nuclear NTPase in parsley. Contrary to what had been found for RNA polymerase, Mg^{2+} act as an apparently competitive inhibitor whereas free ATP acts as an activator for the NTPase.

Considering the results presented here we can summarize that nucleoside triphosphates and divalent cations influence RNA polymerase I and in an opposite way the NTPase in isolated nuclei. One possible function of a phosphatase with NTPase activity in the nucleus could be the control of nuclear nucleo-

side triphosphate pools and thereby regulating the activity of RNA polymerase I by modifying the kinetics from an allosteric to a Michaelis-Menten behaviour.

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Abbreviations: NTP, nucleoside triphosphates; NDP, nucleoside diphosphates; NTP_{exc}, NTP given in excess of equimolar concentrations of divalent cations and nucleoside triphosphates in the assay; $cation_{exc}$, divalent cations given in excess of equimolar concentrations of divalent cations and nucleoside triphosphates in the assay; NTPase, Nucleoside triphosphatase.

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