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Increased Activity of Chromatin-bound Ribonucleic Acid Polymerase from Soybean Hypocotyl with Spermidine and High Ionic Strength[1]

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ABSTRACT

Optimal activity of chromatin-bound RNA polymerase from soybeans is obtained with 1 mM Mn^{2+}, but only when high ionic strength or polyamines are included in the medium. Such inclusion does not increase the Mg^{2+} activation of the polymerase, but it does lower the concentration needed for optimum activity from 10 mM to 1 mM. Mg^{2+} activation is inhibited by added Mn^{2+}, and the inhibition is relieved by high ionic strength or spermidine. The RNA polymerase with either cation is almost entirely polymerase I at low and high ionic strength as evidenced by insensitivity to α-amanitin. Treatment of soybean seedlings with 2,4-dichlorophenoxyacetic acid does not change these characteristics; although the activity rises 3- to 4-fold.

It is suggested that chromatin as prepared here may be a selected fraction enriched in polymerase I, which is activated by either Mg^{2+} or Mn^{2+}, and that the Mn^{2+} inhibition of activity is due to a known reaction of Mn^{2+} with DNA which can be relieved by high ionic strength.

The chromatin isolated from soybean hypocotyls previously treated with the synthetic auxin 2,4-D has been shown to have several fold greater RNA polymerase activity than the chromatin isolated from untreated tissue (10, 26–28). Although the characteristics of the chromatin-bound polymerase have been described in some detail, additional information is needed on the response to polyamines and high ionic strength. In animal nuclear and chromatin preparations there is a Mg^{2+}-dependent RNA polymerase I which yields rRNA at low ionic strength and which is insensitive to α-amanitin (14, 15, 17, 25, 35, 36). Additionally, there is an Mn^{2+}-dependent RNA polymerase II yielding mRNA which is active at high ionic strength and is strongly inhibited by as little as 0.1 μg/ml of α-amanitin (14, 15, 17, 21, 25, 33, 35, 36). The polyamine, spermidine, increases the rate of RNA synthesis by polymerases associated with animal chromatin and bacterial DNA (2, 8, 19, 32) and reactivates polymerase reactions that have plateaued (8).

Comparable reports on ionic strength effects with plant preparations are lacking. We report here on experiments which indicate that the Mg^{2+} or Mn^{2+} requiring RNA polymerase bound to soybean chromatin can be stimulated by high ionic strength or spermidine, but that the enzyme remains insensitive to α-amanitin or rifampin.

METHODS AND MATERIALS

Soybean seedlings (*Glycine max*, var. Amsoy) were grown in vermiculite moistened with 0.1 mM $CaCl_2$ in the dark at 28 C. Except as noted in Table IV, all data reported here were obtained with seedlings sprayed with 25 ml of 500 μg/ml 2,4-D solution per 9- × 14-inch tray at 72 hr, and returned to the germinator for an additional 24 hr. Hypocotyls were severed 1 cm below the cotyledons and at the root-shoot transition zone. Control tissue was held in the incubator for 96 hr.

The hypocotyls were weighed, washed in cold distilled water, and homogenized in 0.25 M sucrose, 50 mM tris-HCl (pH 8.0), 1 mM $MgCl_2$, and 10 mM 2-mercaptoethanol for 90 sec at setting No. 6 of a Willems Polytron Model PT20ST (Brinkmann Instruments, Inc.). The homogenate was filtered through four layers of cheesecloth and two layers of Miracloth (Calbiochem). Chromatin was isolated according to Huang and Bonner (13), except that the final centrifugation was through 1.8 M sucrose containing 50 mM tris-HCl (pH 8.0) and 10 mM 2-mercaptoethanol using a SW 27 rotor at 21,000 rpm for 3 hr in a Beckman L2-65B ultracentrifuge. Final suspension was in 50 mM tris-HCl (pH 8.0) and 10 mM 2-mercaptoethanol. This preparation was assayed immediately for RNA polymerase or stored in 50% glycerol solution in liquid nitrogen as suggested by Cherry (9, personal communication). There was no loss in activity of the stored chromatin for periods up to 2 weeks.

RNA polymerase was measured in 0.5-ml volumes containing 12.5 mM tris-HCl (pH 8.0), 2.5 mM 2-mercaptoethanol, 10 mM dithiothreitol, 0.2 μmole each of ATP, GTP, UTP and 0.01 μmole ^{3}H-CTP (1–5 μc), 1 to 3 μg chromatin DNA, and other additions as given in the tables and figures. The reactions were at 37 C for 20 min, except where time was a variable (Figs. 2 and 3) and were stopped by addition of trichloracetic acid and sodium pyrophosphate to final concentration of 5% and 1%, respectively (w/v). The precipitate was collected on Whatman GF/A glass fiber discs, washed five times with 5 ml of 5% trichloracetic acid, and twice with 5 ml of 95% ethanol. After drying, the filters were counted at 36% efficiency in a toluene scintillator containing 4 g/l PPO and 50 mg/l dimethyl POPOP with a Packard Tricarb scintillation counter.

DNA was estimated by the method of Burton (4); ^{3}H-CTP was from New England Nuclear; α-amanitin was a generous gift of T. Wieland (Heidelberg); rifampin was from Calbiochem and actinomycin D was purchased from Sigma. The α-amanitin

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was checked for effectiveness on nuclei isolated from rat uterus and proved to be fully inhibitory toward RNA polymerase II.

RESULTS

Optimal concentrations of Mn^{2+} and Mg^{2+} for RNA polymerase activity are about 1 mM and 10 mM, respectively, at low salt concentrations (Fig. 1). Mg^{2+} is more effective. However, with the addition of 200 mM KCl the Mn^{2+} activity rises 3- to 4-fold, and the optimum concentration for Mg^{2+} drops to 1 mM. In other experiments, it was determined that 200 mM KCl provided the optimum stimulation of activity, and that $(NH_4)_2SO_4$, at the same ionic strength, is about 90% as effective. Occasionally, there was a small increase in activity at 10 mM Mg^{2+} due to KCl (Table II), but most often the results were as in Figure 1. Spermidine at 1 mM produces very similar responses to high ionic strength (Table I).

The time course of RNA synthesis (Fig. 2) shows that high ionic strength does not prevent the commonly observed plateau of activity after 15 to 20 min (26–28). The activity in the presence of spermidine also plateaus at this time (data not shown). Delayed addition of KCl or spermidine to an Mn^{2+}-activated system that had plateaued releases enzyme activity,

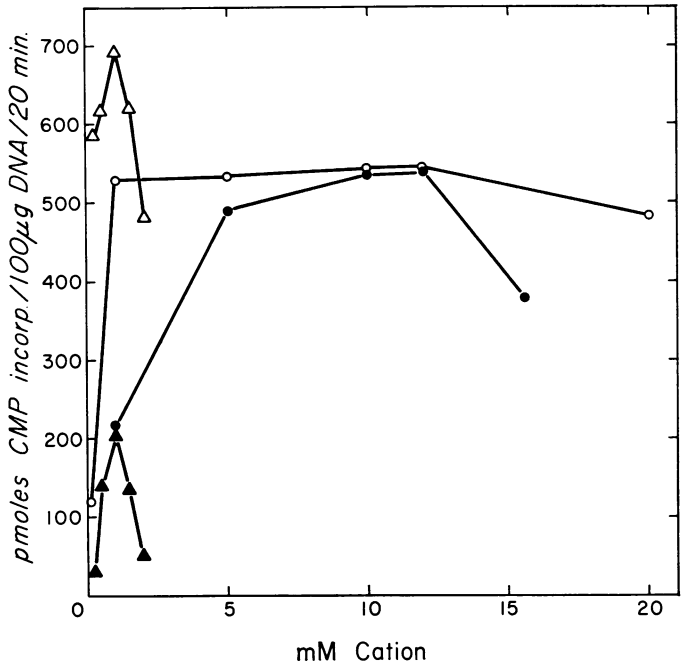

FIG. 1. Effect of Mg^{2+} and Mn^{2+} concentrations on RNA synthesis in the presence of low and high ionic strength. Standard assay conditions were employed with the Mg and Mn concentrations as indicated. High ionic strength assays were run in the presence of 200 mM KCl. Reaction time was 20 min. ●: Mg^{2+} (low ionic strength); ○: Mg^{2+} (high ionic strength); ▲: Mn^{2+} (low ionic strength); △: Mn^{2+} (high ionic strength).

Table I. *Effect of KCl and Spermidine on Mg^{2+}-activated RNA Polymerase*

Additive	10 mM Mg^{2+}	1 mM Mn^{2+}
	pmoles CMP/100 μg DNA·20 min	
None	515	241
200 mM KCl	532	772
1 mM Spermidine	503	715

Table II. *Inhibitory Effect of Mn^{2+} on Mg^{2+}-activated RNA Polymerase*

Mg^{2+} (2.5 mM) + 0.067 mM Mn^{2+} has been used in previous assays of chromatin-bound RNA polymerase from soybeans (27, 28).

Cation	KCl	
	None	200 mM
	pmoles CMP/100 μg DNA·20 min	
10 mM Mg^{2+}	492	540
1 mM Mn^{2+}	110	703
10 mM Mg^{2+} + 1 mM Mn^{2+}	140	324
2.5 mM Mg^{2+} + 0.067 mM Mn^{2+}	194	538

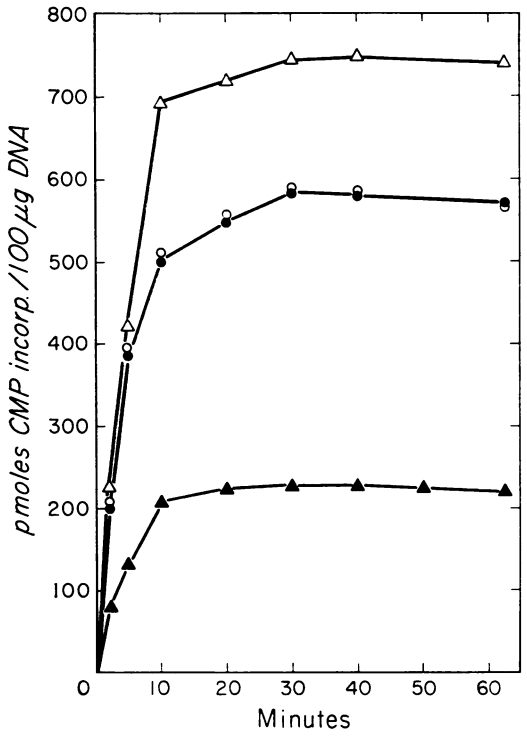

FIG. 2. Time courses for RNA synthesis. Fifty-μl aliquots were removed from the 500-μl reaction mixture and precipitated with 5% TCA and 1% sodium pyrophosphate at the times indicated. ●: 10 mM Mg^{2+}; ○: 10 mM Mg^{2+} + 200 mM KCl; ▲: 1 mM Mn^{2+}; △: 1 mM Mn^{2+} + 200 mM KCl.

thus ostensibly reversing the inhibition, termination, or feedback that produces the plateau (Fig. 3). The plateau cannot then be due to enzyme inactivation, although there is a gradual inactivation shown in the lowered response to successively delayed additions of KCl. Nor can the plateau be a response to the rate of enzyme activity or the amount of polymerization accomplished (Fig. 2). Furthermore, the activity released by delayed addition of KCl or spermidine also tends to come to a plateau (Fig. 3). Apparently, high ionic strength or polyamines must act by releasing an inhibition produced by Mn^{2+}.

It is common in studies of soybean RNA polymerase to use both Mg^{2+} and Mn^{2+} in the reaction medium (10, 26–28). Table II shows that the effects of Mg^{2+} and Mn^{2+} are not additive; indeed, the Mn^{2+} sets the level of the activity obtained, acting in effect as an inhibitor of the Mg^{2+}-activated polymerase. Table

II also reports the promotive effects of ionic strength on Mg^{2+} plus Mn^{2+} activated polymerase at the concentrations of these cations ordinarily used with soybean chromatin (27, 28).

Table III shows that the chromatin-bound RNA polymerase is insensitive to α-amanitin and rifampin, but is equally sensitive to actinomycin D under all conditions tested.

We have confirmed the report of O'Brien *et al.* (27, 28) that 2,4-D treatment increases the RNA polymerase activity of soybean chromatin, usually 3- to 4-fold. High ionic strength does not affect the degree of this stimulation (Table IV). In other experiments not detailed here, we find that the RNA polymerase from control chromatin is also insensitive to α-amanitin.

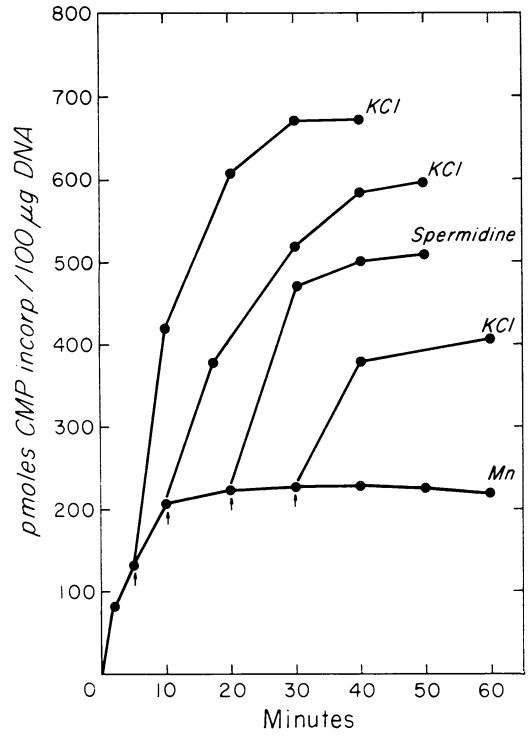

FIG. 3. Influence of 200 mM KCl and 1 mM spermidine on the time course of RNA synthesis in the presence of 1 mM Mn^{2+}. Individual 500-µl volumes containing 1 mM Mn^{2+} were sampled at the times indicated. Arrows indicate where additions were made to the different reaction tubes. Fifty-µl aliquots were precipitated in 5% TCA and 1% sodium pyrophosphate.

Table III. *Failure of α-Amanitin and Rifampin to Inhibit Chromatin-bound RNA Polymerase*

Inhibitors were added initially and the reaction was terminated at 20 min.

Inhibitor	10 mM Mg^{2+}	10 mM Mg^{2+} 200 mM KCl	1 mM Mn^{2+}	1 mM Mn^{2+} 200 mM KCl
µg/ml	% inhibiton			
α-Amanitin				
2	0	1	1	0
20	2	2	1	1
100	10	3	5	2
Rifampin				
100	1	1	2	1
Actinomycin				
0.01	16	28		16
0.1	76	70		74
1	85	86		89
10	93	90		92

Table IV. *Ionic Strength Effects on Chromatin-bound RNA Polymerase from Control and 2,4-D-treated Soybean Seedlings*

Additions	Chromatin from		Increase
	Untreated	2,4-D-treated	
	pmoles CMP/100 µg DNA·20 min		%
10 mM Mg^{2+}	160	525	328
+ 200 mM KCl	172	585	340
1 mM Mn^{2+}	34	118	347
+ 200 mM KCl	200	750	375

DISCUSSION

Considerable success has been obtained recently in isolating RNA polymerases from plants (9, 12, 24, 30, 34). The enzymes tend to be quite labile, but show activity with DNA from a variety of sources. Hardin and Cherry (9) have solubilized the RNA polymerases from soybean chromatin and have shown that maximum activity with denatured calf thymus DNA requires both Mg^{2+} and Mn^{2+} (optimum activity is reported to be 10 mM and 1.25 mM, respectively). Rifampin is ineffective as an inhibitor, but α-amanitin at 10 µg/ml gives inhibition varying from 58% with control polymerase to 21% with polymerase from plants treated with 2,4-D 12 hr previously. Increasing ionic strength with $(NH_4)_2SO_4$ did not promote activity. Horgen and Key (12) have reported solubilization and fractionation of soybean hypocotyl RNA polymerases, with two of the four enzymes being of nuclear origin and insensitive to rifampin. Amanitin inhibits one of the nuclear enzymes (DEAE[2]-polymerase II). DEAE-polymerase I is most active with Mn^{2+} and DEAE-polymerase II is most active with Mg^{2+}.

Studies with chromatin-bound RNA polymerase are still needed, however, to clarify the auxin response for it is still not known to what degree 2,4-D is increasing enzyme level or modifying template readout or both. Quantitatively, the auxin response *in vivo* is largely shown to be in rRNA (7, 18), reflecting activity of the nucleolar enzyme, polymerase I, which is amanitin-insensitive. The chromatin isolated here from either untreated or 2,4-D treated tissue is dominated by an enzyme which is insensitive to α-amanitin, but which is very sensitive to actinomycin D, a property of polymerase I in animal systems (16, 36). (For convenience we will term the soybean enzyme studied here "polymerase I.") Sensitivity or insensitivity of the polymerase to these inhibitors is not affected by ionic strength or spermidine or choice of activating cation (Table III). As shown by Holm (11) only a small fraction of soybean DNA centrifuges down through 1.8 M sucrose; perhaps sucrose density serves to selectively concentrate a fraction of chromatin enriched in bound polymerase of the type described here. Alternatively, solubilization may be required for enzymatic activity of RNA polymerases other than polymerase I in our system.

While our finding that only polymerase I activity is being expressed differs from results with solubilized enzymes (9, 12), it does agree with the results of Bottomley *et al.* (3) who found high concentrations of α-amanitin ineffective in inhibiting

[2] Abbrevation: DEAE: diethylaminoethyl.

Mg^{2+}- or Mn^{2+}-dependent RNA polymerase of spinach and pea nuclei. High ionic strengths were not used, however, so the conditions were not fully comparable. Mn^{2+} plus high ionic strength (or spermidine) are needed to activate the α-amanitin sensitive polymerase II in animal preparations. Our results show that under these conditions the soybean chromatin-bound polymerase activity is still α-amanitin-insensitive, and indicates we are dealing only with polymerase I.

This chromatin-bound polymerase I is optimally activated at high ionic strength with 1 mM Mn^{2+} or 10 mM Mg^{2+}, the latter being 75 to 85% as effective as the former (Fig. 1). That polymerase I can be activated by salt has been reported by Johnson *et al.* (16), who reported a biphasic response in rat liver nuclei. The first stimulation was optimal at 40 mM $(NH_4)_2SO_4$ and was associated with polymerase I; the second stimulation occurred at 400 mM $(NH_4)_2SO_4$ and was associated with polymerase II.

Hypotheses advanced to account for high ionic strength stimulation of RNA polymerase include reinitiation (31), removal of histones (5, 29), removal of an inhibitory RNA product (20), inhibition of nucleases (23), and activation of a second polymerase (35, 36). Another possibility which has not been explored is suggested by the recent reports (1, 6, 22) that Mg^{2+} and Mn^{2+} interact differently with the DNA template. Luck and Zimmer (22) show both cations to stabilize DNA through ionic interactions with phosphate groups, but at low ionic strength Mn^{2+} can in addition react with guanine groups. High ionic strength displaces the Mn^{2+} from the guanine and the secondary effect of Mn^{2+} is lost. Luck and Zimmer suggest that Mn^{2+} reaction with the G:C region of the DNA double helix causes conformational changes which might affect template activity. We believe that the reversal of Mn^{2+} inhibition by high ionic strength or spermidine observed here can be best explained by some such template-releasing mechanism.

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