SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF SOYBEAN CHROMATIN-BOUND RNA POLYMERASE

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SUMMARY

A technique has been developed which solubilizes RNA polymerase from soybean chromatin. Dissociation of RNA polymerase from chromatin DNA is achieved by incubation in the presence of 0.5 M $(NH_4)_2SO_4$. Basic proteins and DNA are removed by centrifugation while the inhibitory levels of $(NH_4)_2SO_4$ are removed by Sephadex G-50 chromatography. The polymerase preparation so obtained is dependent on added DNA, actinomycin-D sensitive, rifampin insensitive and partially inhibited by α-amanitin. The polymerase has a Mg^{2+} optimum of 10.0 mM and a Mn^{2+} optimum of 1.25 mM. No differences were noted in template activity of several native DNA's tested. Denatured calf thymus DNA was transcribed more efficiently than the native DNA's examined.

INTRODUCTION

In studying the effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on nucleic acid metabolism in soybeans, we have previously employed a chromatin-bound RNA polymerase system (1, 2). This system has been used to show increases in RNA polymerase activity following auxin treatment, but questions remain as to whether this increased activity is due to changes in the quantity or quality of RNA polymerase or to changes in DNA template availability. These questions coupled with the early reports of multiple RNA polymerases in eucaryotic organisms (3, 4) have lead us to attempt to develop techniques for the solubilization of soybean chromatin-bound RNA polymerases.

MATERIALS AND METHODS

Plant Material: Soybean seed (Glycine max. L. var Wayne) were imbibed overnight in water and sown in moist Vermiculite. The seeds were germinated in the dark in a constant humidity chamber at 29°. After four days of germination the hypocotyls

were harvested and immediately used for chromatin isolation.

Chromatin Isolation and Assay: Chromatin was isolated by the method of Huang and Bonner (5) employing the modifications of O'Brien et al (1) with the exception that 300-1000 g of tissue was employed. Chromatin-bound RNA polymerase assays were performed as described by O'Brien et al (1) as modified by Hardin et al (2).

Chromatin Storage: Isolated chromatin pellets were suspended in 0.01 M Tris-HCl, (pH 8.0), glycerol was added to a final concentration of 50% (v/v) and stored in liquid nitrogen until needed.

Solubilization of RNA Polymerase: Chromatin was incubated at 37° for 30 min in the presence of 0.01 M Tris (pH 8.0), 0.005 M $MgCl_2$, 0.005 M dithiothreitol, 25% glycerol (v/v) and 0.5 M $(NH_4)_2SO_4$. DNA and other insoluble constituents of chromatin were removed by centrifugation at 100,000 g for 1 hour in a Spinco SW39 rotor. The pellets were either resuspended in tris buffer for assay of residual RNA polymerase activity or discarded. The supernatants were chromatographed on a 30 ml Sephadex G-50 column in TGMED [0.05 M Tris (pH 7.9) 25% glycerol (v/v), 0.005 M $MgCl_2$, 0.0001 M EDTA and 0.0005 M dithiothreitol] and the initial absorbance peak at 280 nm was used as DNA-dependent RNA polymerase. The preparations were stored in liquid nitrogen after glycerol was added to a final concentration of 50% (v/v).

Assay of RNA Polymerase: The reaction mixture contained the following in a final volume of 0.2 ml: 20 μmoles Tris-HCl (pH 8.0), 3.0 μmoles Mg^{2+}, 0.125 μmoles Mn^{2+}, 0.1 μmoles each of ATP, CTP and GTP, 0.3 μmoles dithiothreitol, 0.01 μmoles EDTA, 1.25% glycerol, 0.005 μmoles UTP, 7.5 μCi ^{3}H-UTP and 20 μg heat denatured calf thymus DNA or various DNA's as described in appropriate table legends. Reactions were carried out at 37° for either 15 or 30 minutes and killed by sequential addition of 1 ml of 10 mM $Na_4P_2O_7$, 0.5 ml of 5% sodium lauryl sulfate and 3 ml of ice cold 10% trichloroacetic acid (TCA). Acid insoluble precipitates were collected on glass fiber filters (Whatman GF/A) for counting. Alternatively, 0.1 ml of reaction mixture was spotted on Whatman DE81 discs (2.3 cm) which were then processed as described by Blatti et al (6) except for the solubilization step which was omitted.

DNA Isolation: Plant DNA's were isolated from 1 kg of fresh tissue which was lyophilized to dryness and then extracted as described by Bendich and Bolton (7). DNA obtained from this method was further purified by hydroxylapatite chromatography as described by Britten et al (8) to obtain double stranded (native) DNA. Using this technique the following amounts (ug) of native DNA were obtained per 100 g of tissue: pea cotyledons, 64; lily leaves, 168; and soybean hypocotyls, 5.

DNA and Protein Determinations: Protein was determined by the method of Lowry et al (9) and DNA either by the Burton (10) colormetric method or estimated by 260 nm/280 nm ratios (11).

RESULTS

Isolated chromatin and polymerase were very stable under the storage conditions employed with a maximum loss of 10% in activity (Table I). However, as it was noted that activity was lost with repeated freezing and thawing, the preparations for these studies were not refrozen after use.

Results presented in Table II show that very little residual activity remains associated with the DNA pellet after solubilization; the bulk of the RNA polymerase activity was found in the 280 nm absorbance peak from the Sephadex G-50 column. Polymerase activity in the high-speed centrifugation supernatnat was inhibited by the high salt present in the solubilization buffer (results not presented).

Solubilized RNA polymerase required exogenous DNA for activity (Table III). While native calf thymus and various plant DNA's were transcribed with equal efficiency, denatured calf thymus DNA was the most effective template of those compared. Therefore, denatured calf thymus DNA was routinely used in the assay.

Table IV presents data on the metal ion and salt requirements of the enzyme preparation as well as sensitivity to various inhibitors. The enzyme was dependent on the presence of both Mg^{2+} and Mn^{2+} with respective optimum for each being 10 mM and 1.25 mM (data not presented). Low levels of ammonium sulfate

Table I

Storage of Chromatin and Solubilized RNA Polymerase[1/]

Preparation	Initial activity (pmoles ^{3}H-UMP incorporated/15 min)	Storage period	Activity after storage (pmoles ^{3}H-UMP incorporated/15 m
control polymerase[2/]	200	1 week	190
control chromatin[3/]	37	1 month	35
2,4-D chromatin[4/]	24	2 months	23

[1/]Assays were performed as described in Methods. Each value represents an average of four determinations from the same stored preparation. Initial values were determined prior to freezing. Under these conditions the reaction was complete by 15 min. One pmole represents 3000 dpm of ^{3}H-UMP incorporated.

[2/]Activity based on 1 mg of protein.

[3/]An equivalent amount (the same aliquot) of chromatin DNA was added to each assay (for control and 2,4-chromatin).

[4/]Chromatin isolated from soybean plants sprayed with 10^{-3}M 2,4-D 12 hours prior to harvest.

Table II

Solubilization of RNA Polymerase
from Soybean Chromatin

Fraction	Activity[1/] (pmoles ^{3}H-UMP incorporated/15 min)
crude chromatin	1566
chromatin pellet after solubilization	192
solubilized polymerase (280 nm peak from G-50 column)	1689

[1/]Activity values represent 3 mg of protein of the initial crude chromatin preparation

Table III

Efficiency of Various DNA's as Template for Solubilized RNA Polymerase

Source of DNA	pmoles ^{3}H-UMP incorporated/15 min/mg protein
None	60
5 μg native calf thymus	440
20 μg native calf thymus	640
5 μg heat denatured calf thymus	520
20 μg heat denatured calf thymus	780
6.7 μg native soybean	500
6.25 μg native lily	510
12.5 μg native lily	560
6.0 μg native pea	500
12.0 μg native pea	540

(0.05 M or less) had little effect on polymerase activity while higher concentrations (0.1 M or greater) proved inhibitory. The RNA polymerase activity was completely inhibited by actinomycin D (100 μg/ml) and insensitive to rifampin (100 μg/ml). Table V shows that all preparations examined were partially sensitive to α-amanitin (2 μg); the degree of sensitivity being inversely related to the duration of the 2,4-D treatment. The fact that the relative amount of inhibition decreases with time after auxin treatment indicates that the α-amanitin sensitive enzyme (enzyme II) (12) is unaffected while other RNA polymerases increase in activity.

Preliminary experiments on the chromatography of the enzyme on DEAE-Sephadex and DEAE-cellulose indicates multiple peaks (at least three).

DISCUSSION

In order to develop a more control system for studying auxin regulation

Table IV

Effects of Metal Ions, $(NH_4)_2SO_4$ and Inhibitors on RNA Polymerase Activity

System	pmoles ^{3}H-UMP incorporated/ 15 min/mg protein	% inhibition
complete	435	0
-Mn^{2+}	270	38
-Mg^{2+}	230	47
+0.0125 M $(NH_4)_2SO_4$	450	0
+0.025 M $(NH_4)_2SO_4$	425	2
+0.05 M $(NH_4)_2SO_4$	450	0
+0.1 M $(NH_4)_2SO_4$	300	31
+0.2 M $(NH_4)_2SO_4$	310	29
+actinomycin-D (100 µg)	45	90
+rifampin (100 µg)	434	0

Table V

Effect of α-amanitin on Control and 2,4-D[1/] RNA Polymerase

Source of Enzyme	pmoles ^{3}H-UMP incorporated/15 min/mg protein α-amanitin (2 µg) -	+	% inhibition
control plants	228	96	58.0
2 hr 2,4-D plants	250	128	48.8
6 hr 2,4-D plants	348	238	32.0
12 hr 2,4-D plants	616	484	21.0

[1/] 2,4-D polymerase is enzyme solubilized from chromatin isolated from soybeans after treatment with 10^{-3}M 2,4-D *in vivo* at 2, 6, and 12 hrs prior to harvest.

of RNA polymerase, techniques were developed for solubilization of RNA polymerase from chromatin. Our procedure has the advantage of being rapid and efficient in removal of RNA polymerase from soybean chromatin. However, the procedure as described was not suitable for sugar beet chromatin, but a slight modification proved successful (V. L. Dunham, personal communication).

There have been many reports of multiple RNA polymerases from eucaryotic organisms (3, 4, 6). The inhibitor α-amanitin has been shown to specifically inhibit the nucleoplasmic RNA polymerase (enzyme II) (12). Results with α-amanitin and preliminary DEAE-Sephadex columns indicate the presence of multiple RNA polymerases associated with soybean chromatin.

Generally native DNA from plants and calf thymus gave the same activity with the solubilized enzyme preparation, however, denatured calf thymus DNA served as a more efficient template than native DNA.

It appears, therefore, that multiple RNA polymerases are easily solubilized from soybean chromatin in the presence of high salt. Further efforts are being made to resolve these activities by various fractionation procedures. The RNA polymerases prepared by these procedures are currently being used to study possible regulation at this level following 2,4-D treatment of soybean hypocotyls.

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