Isolation and Properties of Nuclei from Control and Auxin-treated Soybean Hypocotyl[1]

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ABSTRACT

A quick procedure for the isolation of nuclei with good yield from soybean hypocotyl (*Glycine max* var. Wayne) was developed. The isolated nuclei appeared to retain their structural integrity. They were typically ellipsoidal with minima and maxima diameter of about 6 and 8 to 10 micrometers. While the nuclei were similar in size, the nucleoli were significantly larger in nuclei from auxin-treated tissue. The DNA content per nucleus was 4 ± 1 picograms for both untreated and auxin-treated tissues. The DNA:RNA:protein ratio of isolated nuclei in untreated and auxin-treated tissues was 1:3.1:11 and 1:5.4:21.7, respectively. The purified nuclei were active in RNA synthesis; the level of RNA polymerase II activity expressed in the nuclei from untreated tissue was 50 to 60% higher than RNA polymerase I. The nuclei from auxin-treated tissues contained about 2.5 times as much RNA polymerase I activity as nuclei from untreated tissue. The purified nuclei from both untreated and auxin-treated tissues were also active in the incorporation of ^{3}H-TTP into DNA.

Studies on the mechanism of plant hormone action have received much attention in recent years. Hormones are involved in the regulation of many different physiological processes; some lines of evidence indicate that in several cases this regulation may involve the control of the transcription or translation process (17). Because it is generally difficult to isolate plant nuclei in sufficient yield for extensive biochemical work, the isolation of plant chromatin has been used routinely in the study of the influence of auxin on the transcription of RNA (11, 24). Recent studies with soybean chromatin isolated by conventional methodology (13) show that this chromatin possesses practically all of the RNA polymerase I activity of the tissue but little or no RNA polymerase II activity; most of the RNA polymerase II is present in the high speed supernatant fractions (20). The routine use of this heavy chromatin fraction in studies on hormone enhancement or regulation of RNA synthesis precludes any observations on the influence of the hormone on RNA polymerase II-mediated RNA syn-

thesis. Isolated nuclei thus are essential to the study of *in vitro* RNA synthesis if conditions relating to the *in vivo* state are to be approached. Isolated nuclei from other systems have been used to great advantage in the study of several important problems: *e.g.* the study of the transcription of defined RNA species (15, 23, 25, 30, 34), the intranuclear metabolism and release of RNA (26), the post-transcriptional additions of poly(A) to HnRNA (16, 32, 33), the mechanisms involved in DNA replication (6, 9, 10, 22), the modification of chromatin proteins and their role in gene expression (18, 28, 35, 39), the preparation of high mol wt DNA (1), and enzyme localization (8, 29). We have pursued the isolation of nuclei from soybean hypocotyl with the objective of isolating large quantities of "intact," biologically active nuclei to be used in studies on the auxin regulation of RNA synthesis. In this report, we describe a procedure for isolating nuclei from control and 2,4-D-treated soybean hypocotyl. The attendant RNA and DNA synthetic activities, gross morphology, and macromolecular composition are described.

MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* var. Wayne) which had been treated with 10% Clorox were germinated in moist vermiculite at 28 C. After 72 hr of germination those seedlings designated as "treated" were sprayed to run-off with a 2.5×10^{-3} M (pH 6) solution of 2,4-D. Mature hypocotyl tissue from control and treated seedlings was harvested after an additional 24 hr (*i.e.* at 96 hr).

Solutions. Buffer A contains 25 mM MES-NaOH buffer (pH 6), 20 mM KCl, 20 mM $MgCl_2$, 0.6 M sucrose, 40% glycerol, 1% monothioglycerol, and 10 mM 2-mercaptoethanol. Buffer B contains buffer A and 0.02% Triton X-100. Buffer C contains 25 mM MES-NaOH buffer (pH 6), 20 mM KCl, 20 mM $MgCl_2$, 30% glycerol, 1% monothioglycerol, and 10 mM 2-mercaptoethanol.

Preparation of Crude Nuclei. Crude nuclei were isolated by a method modified from that of Stern (36). Tissue (150 g) was homogenized in 300 ml of buffer A for 2 min at a setting of 5 with a Willems polytron PT-20-ST Brinkmann Instruments Inc.). Homogenization and all subsequent procedures were conducted at 0 to 4 C. The homogenate was filtered through six layers of cheesecloth and one layer of Miracloth. The filtrate was centrifuged at 2500g for 20 min. The pellet was suspended in buffer B and recentrifuged at 2500g for 20 min.

Sucrose Gradient Purification of Nuclei. The washed nuclear pellet was suspended in buffer A and layered over 25 ml of buffer C containing 1.2 M sucrose. Centrifugation was at 23K rpm for 10 min at 2 C in a SW-27 rotor (Beckman L2-65 B ultracentrifuge). The nuclear pellet was resuspended in 1 M sucrose containing buffer C and layered over a discontinuous

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sucrose gradient containing 1.2, 1.4, and 1.8 M sucrose in buffer C (10 ml-10 ml-11 ml). The gradients were centrifuged at 4K rpm for 25 min in a SW-27 rotor. The purified nuclei were collected from the 1.2 M and 1.4 M zones by pipet. Some of the nuclear preparation was stained with methyl green-pyronin Y (37) for microscopic observation to ascertain the degree of intactness of the nuclei.

RNA Polymerase Assay. The RNA polymerase activities of the nuclei preparation were assayed at 28 C in a 0.25 ml reaction mixture containing 50 mM tris-HCl (pH 8), 10 mM dithiothreitol, 5 mM $MgCl_2$, 20% glycerol, 0.4 mM each of ATP, GTP, and CTP, and 0.02 mM ^{3}H-UTP (1 μCi; 1 pmole = 134 cpm). Other additions are described in table and figure legends.

Solubilization of RNA Polymerases. RNA polymerases I and II were solubilized from isolated nuclei as described earlier (7). The solubilized proteins were fractionated on DEAE-cellulose as described by Roeder and Rutter (29).

^{3}H-TTP Incorporation. DNA synthetic activity of the isolated nuclei was measured at 28 C in a 0.25 ml reaction mixture containing 50 mM tris-HCl (pH 8), 20% glycerol, 5 mM $MgCl_2$, 2 mM ATP, 75 mM $(NH_4)_2SO_4$, 0.4 mM each of dGTP, dATP, and dCTP and 0.01 mM ^{3}H-dTTP (2 μCi; 1 pmole = 540 cpm). The reaction was terminated by addition of 2 ml of 10% trichloroacetic acid containing 8 mM sodium pyrophosphate. The precipitate was collected on GF/C glass fiber disks, washed four times with 3 ml of 5% trichloroacetic acid and twice with 4 ml of 95% ethanol. The filters were dried under heat lamps and counted in a liquid scintillation spectrometer. The RNA and DNA polymerase activities were expressed on a DNA basis or nuclei number as noted in tables and figures.

Composition Analysis of Nuclei. The extraction of DNA and RNA from the isolated nuclei was accomplished using the method of Howell (12). DNA was determined using the diphenylamine assay (3) with calf thymus DNA as standard. RNA content was estimated by the orcinol method (31) using yeast RNA as standard. Protein was determined by the Lowry Folin-phenol method (21) using a BSA standard.

RESULTS

Purification of Nuclei. When the suspension of crude nuclei was fractionated on discontinuous sucrose gradients (layers of 1.2, 1.4, and 1.8 M), most of the nuclei from untreated (control) tissue were present in the 1.2 M sucrose band while the nuclei from auxin-treated tissue banded primarily in the 1.4 M zone. These observations were made using light and phase-contrast microscopy on preparations both before and after methyl green-pyronin Y staining. The nuclei in these bands were not appreciably contaminated with membranous material; however, some starch grains were present in all preparations (Fig. 1). The auxin treatment did not significantly alter the size of nuclei (about 6 $\times$ 8 to 10 μm in size in both treated and untreated tissue). On the other hand, the size of the nucleolus was increased significantly by auxin treatment (the diameter was 1.5 to 2.5 μm for untreated and 4 to 6 μm for treated nucleoli) (Fig. 1). While the nucleoli were larger in auxin nuclei, no multinucleolate nuclei were observed in the present work as had been reported for in situ observations (2, 4, 5). About 25% of the nuclei were recovered in the purified state based on the DNA content of the tissue and the amount of DNA present in the preparations of nuclei. The nuclei were isolated from mature tissue (fully elongated cells) and were nonuniform in shape and size. This presented a problem in obtaining totally pure, intact nuclei. Some nuclei were ruptured during purification but the majority (greater than 80%) appeared fully intact.

Composition of Isolated Nuclei. The relative DNA, RNA, and protein contents of purified nuclei isolated from untreated and auxin-treated tissue are given in Table I. The relative DNA:RNA:protein values of about 1:3:11 for nuclei from untreated soybean hypocotyl are significantly different from values (1:0.3:3.3) reported for pea (38). Similar data to those for pea (38) were obtained when pea nuclei were analyzed by us (1:0.6:4). The relative values for pea and soybean differ primarily in that the amount of DNA (4 $\pm$ 1 pg) is only about 30% of that of a pea nucleus. The amount of DNA per
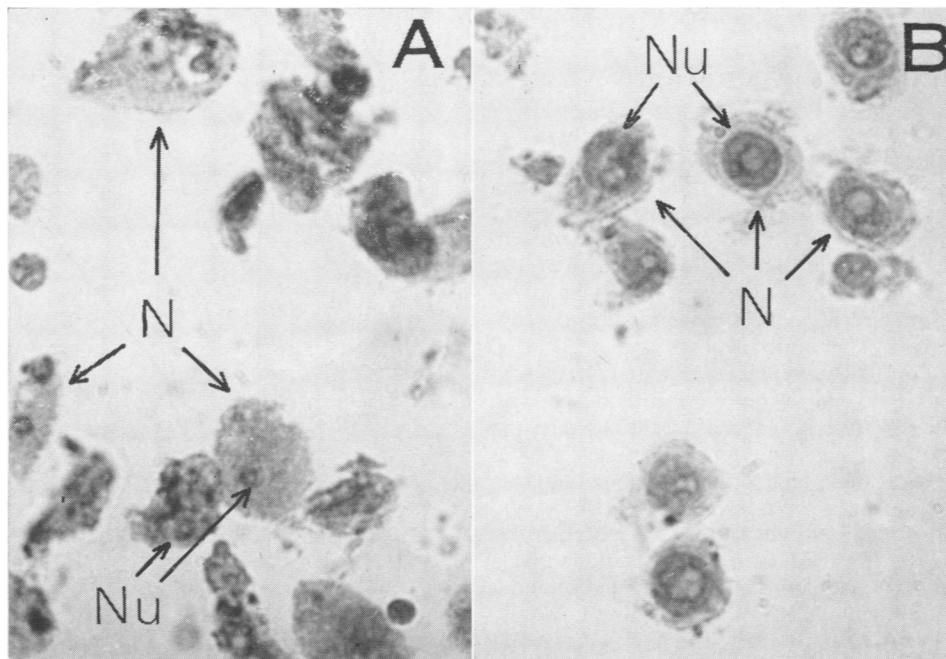

Fig. 1. Photomicrographs of purified nuclei from untreated (A) and auxin-treated soybean hypocotyl (B). Stained with methyl green-pyronin Y N: nucleus; Nu: nucleolus $\times$ 2100. The lighter structure within the nucleolus (B) appears to be a nucleolar vacuole.

Table I. *Chemical Composition of Soybean Nuclei Isolated from Untreated and Auxin-treated Soybean Hypocotyl*

The data are an average of five experiments. The amount of DNA per isolated nucleus from either untreated or auxin-treated was 4 ± 1 pg.

Nuclei Source	DNA	RNA	Protein
		μg	
Untreated	100	310 ± 20	1110 ± 150
Treated	100	540 ± 50	2170 ± 210

Table II. *RNA Polymerase Activities of Nuclei Isolated from Untreated and Auxin-treated Soybean Hypocotyl*

In these experiments cpm [^{3}H]-UMP incorporated per 3.6 × 10^5 nuclei was measured at 28 C for 20 min (134 cpm = 1 pmole).

Nuclei Source	(NH$_4$)$_2$SO$_4$	α-Amanitin		Polymerase I[1]	Polymerase II[2]
		−	+		
	mM				
Untreated	50	2310	1620		
	200	3340	760	1620	2580
Auxin-treated	50	4810	4060		
	200	5970	2790	4060	3180

[1] Polymerase I activity was based on 4 μg/ml α-amanitin insensitivity at 50 mM (NH$_4$)$_2$SO$_4$.

[2] Polymerase II activity was determined as the difference of incorporation in the absence and in the presence of 4 μg/ml α-amanitin at 200 mM (NH$_4$)$_2$SO$_4$.

nucleus was similar for untreated and auxin-treated tissue. However, the nuclei from auxin-treated tissue contained almost twice as much RNA and protein as the control nuclei. The enlarged nucleoli might suggest that this large increase in RNA and protein reflects primarily an increase in ribonucleoprotein particles associated with rRNA synthesis and processing in the nucleoli.

RNA Synthetic Activity. The purified nuclei were very active in RNA synthesis (Table II, Fig. 2). A linear relationship existed between nuclei concentration in the assay and RNA synthetic activity (Fig. 3). The relative activities of RNA polymerase I and II were assessed by varying the (NH$_4$)$_2$SO$_4$ and α-amanitin concentrations in the assay; RNA polymerase I is optimally active at 50 mM (NH$_4$)$_2$SO$_4$ and is insensitive to α-amanitin while RNA polymerase II is optimally active at 200 mM (NH$_4$)$_2$SO$_4$ and is inhibited by low concentrations of α-amanitin (see Fig. 4 for selective action of α-amanitin on soybean RNA polymerases). The ionic strength optima and α-amanitin sensitivities for RNA polymerases I and II in soybean nuclei are similar to those reported previously with animal nuclei (14, 27, 42). Based on this differential assay, the level of RNA polymerase II activity expressed in nuclei from untreated tissue was 50 to 60% higher than RNA polymerase I activity. These relative levels of nuclei-directed RNA synthetic activity were also obtained when the RNA polymerases were solubilized from the purified nuclei and fractionated on DEAE-cellulose (Fig. 4). Nuclei from auxin-treated tissue contained about 2.5 times as much RNA polymerase I activity as control nuclei (Table II); in contrast, RNA polymerase II activity was increased by auxin treatment by an average of only about 25% over a series of experiments.

Incorporation of ^{3}H-dTTP by Isolated Nuclei. The purified nuclei from control and auxin-treated tissue were active in the incorporation of ^{3}H-dTTP into DNA (Table III). The activity was partially dependent upon the presence of the other three dNTPs. Ribonucleoside triphosphates would not replace the dNTPs in supporting the incorporation of ^{3}H-dTTP. The product of the reaction was sensitive to DNase. The incorporation of ^{3}H-dTTP by nuclei from auxin-treated tissue was similar to that obtained with control nuclei.
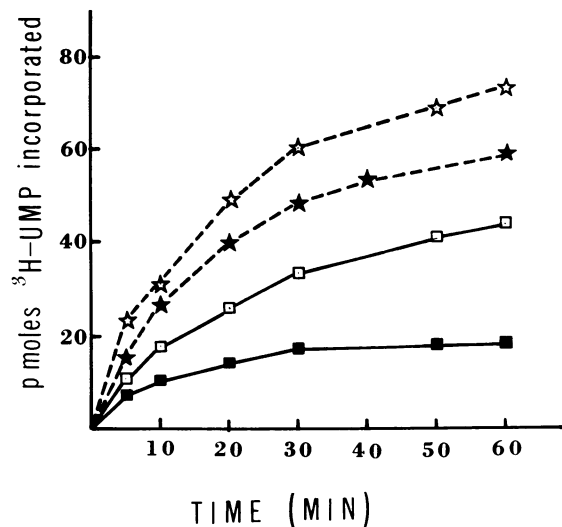

FIG. 2. Kinetics of ^{3}H-UTP incorporation into RNA by isolated nuclei from untreated and auxin-treated soybean hypocotyl. The amount of nuclear material used in each reaction mixture was 1.75 μg of DNA. ■, Nuclei (untreated) assayed at 50 mM (NH$_4$)$_2$SO$_4$; □, nuclei (untreated) assayed at 200 mM (NH$_4$)$_2$SO$_4$; ★, nuclei (auxin-treated) assayed at 50 mM (NH$_4$)$_2$SO$_4$; ☆, nuclei (auxin-treated) assayed at 200 mM (NH$_4$)$_2$SO$_4$.
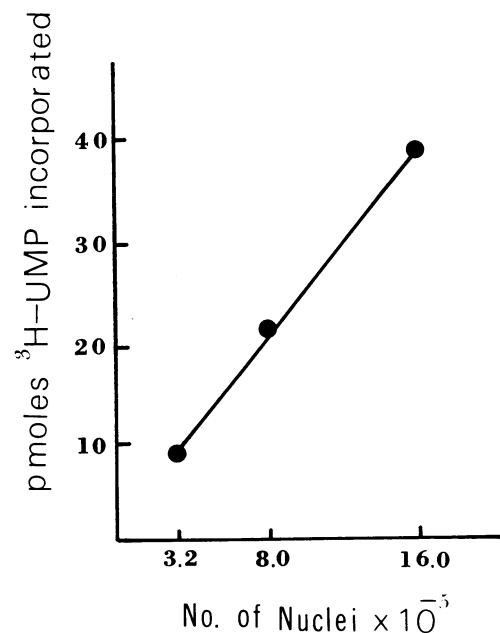

FIG. 3. Relationship between the RNA synthesis and the concentration of nuclei from untreated tissues. The indicated numbers of nuclei were incubated for 20 min in the assay mixtures containing 50 mM (NH$_4$)$_2$SO$_4$.

DISCUSSION

We have previously reported (20) that soybean chromatin isolated by conventional methodology results in a select fraction of nucleoprotein rich in RNA polymerase I whereas most of the RNA polymerase II is not pelleted even at high centrifugal forces. The isolation of plant nuclei is required to study hormonal regulation of nuclear-bound RNA polymerases other than the nucleolar RNA polymerase (RNA polymerase I). Nuclei isolated from soybeans which were sprayed with 2,4-D show a specific enhancement of RNA polymerases I while neither RNA polymerase II nor DNA polymerase are significantly affected. This enhanced RNA polymerase I activity coincides with a dramatic increase in RNA and protein content and nucleolar swelling in nuclei isolated from auxin-treated soybean. Enhanced RNA polymerase I activity following estradiol administration in animals is similarly associated with a large increase in RNA and protein content in the nucleus (40) and nucleolar enlargement (19).

Plant nuclei possess RNA polymerase I and II which have similar properties to RNA polymerases in isolated animal nuclei (14). RNA polymerase I is optimally active at low ionic strength (50 mM $(NH_4)_2SO_4$) and is insensitive to α-amanitin. RNA polymerase II is optimally active at high ionic strength (200 mM $(NH_4)_2SO_4$) and is completely sensitive to low levels of α-amanitin. RNA polymerase III which has been reported to synthesize tRNA and 5S RNA in animal nuclei (41) has not been detected in this study. The relative activities of RNA polymerase I and II in isolated soybean nuclei are maintained following solubilization and chromatography of the enzymes on DEAE-cellulose.

Total RNA polymerase activity in isolated soybean nuclei is significantly greater (based on either DNA or protein content) than activity in isolated soybean chromatin (7). The greater RNA polymerase activity in isolated nuclei results primarily from the recovery of RNA polymerase II in nuclear
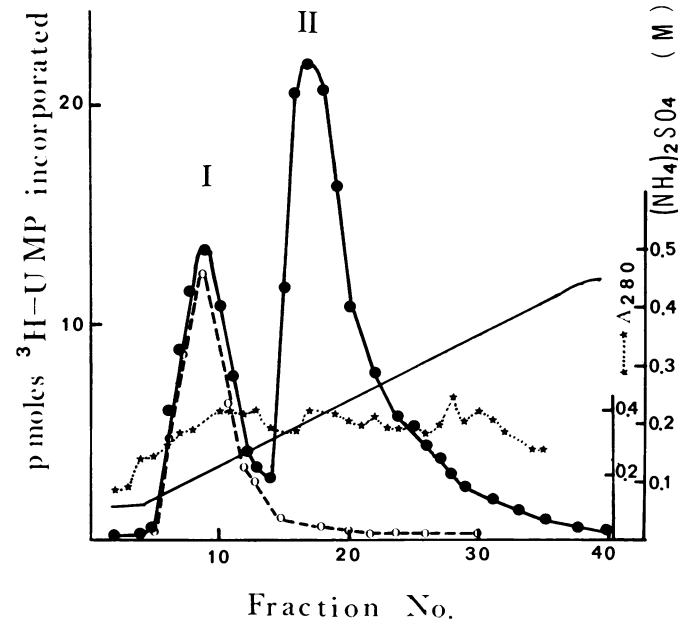

Fraction No.

FIG. 4. DEAE-cellulose profile of RNA polymerase solubilized from untreated soybean nuclei. Protein solubilized from untreated soybean nuclei was loaded onto DEAE-cellulose and fractionated with a linear gradient of $(NH_4)_2SO_4$ (50–500 mM). 0.05 ml from each 1-ml fraction was assayed for 20 min. ●, No α-amanitin; ○, 4 μg/ml α-amanitin; ★, A_{280}/ml.

Table III. ^{3}H-TTP Incorporation by Purified Nuclei

The reaction mixtures were incubated for 30 min at 28 C under the standard assay conditions. The number of nuclei used per assay was 5×10^5. DNase (20 μg) was added at the end of 30-min incubation period and the incubation was continued for 30 min. In the untreated experiment there was an incorporation of 2130 cpm, and 2340 cpm in the auxin-treated.

Reaction Medium	Incorporation	
	Untreated nuclei	Auxin-treated nuclei
	%	
Complete	100	100
−dATP	53	55
−dCTP	44	45
−dGTP	43	41
−dATP, dCTP, dGTP	31	31
−dATP, dCTP, dGTP; +ATP, CTP, GTP	37	32
+DNase	51	

preparations and failure to recover this enzyme activity in chromatin preparations.

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