Auxin-Induced Deoxyribonucleic Acid Dependent Ribonucleic Acid Polymerase Activities in Mature Soybean Hypocotyl†

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ABSTRACT: When 3-day-old etiolated soybean seedlings are treated with the synthetic auxin, 2,4-dichlorophenoxyacetic acid, cells of the mature hypocotyl swell and proliferate abnormally. By 48 h after auxin application ribonucleic acid (RNA) polymerase I and II levels increase by about 10–20- and 6-fold, respectively, on a fresh weight tissue basis and about 3–6- and 2-fold, respectively, on a tissue deoxyribonucleic acid (DNA) basis. [^{35}S]Methionine incorporation into RNA polymerase subunits suggests that this increase in levels of RNA polymerases results from de novo synthesis of the enzymes. No alteration in subunit structure or patterns of incorporation of [^{35}S]methionine into RNA polymerase subunits is detected following auxin treatment. No differences in the phosphorylation patterns of RNA polymerase subunits are detected after hormone treatment. These results indicate that although the levels of RNA polymerases I and II may regulate, in part, the rates of transcription during physiological or developmental transitions, alteration or modification of RNA polymerase subunit structure does not appear to be involved in transcriptional regulation in the auxin-induced soybean hypocotyl.

Application of the auxin–herbicide, 2,4-D,[1] to 3-day-old etiolated soybean seedlings induces radial enlargement and proliferation of mature hypocotyl cells. The abnormal swelling and cellular proliferation in the mature hypocotyl is preceded by a large accumulation of RNA in the tissue (Key et al., 1966; Key, 1969). Although the accumulation of ribosomal RNA is most dramatic, both messenger RNA and transfer RNA also increase. The increase in RNA is paralleled by enhanced chromatin-bound or nuclear-associated RNA synthetic activity (O'Brien et al., 1968; Guilfoyle et al., 1975) which is primarily due to an increase in RNA polymerase I activity (Guilfoyle & Hanson, 1973; Guilfoyle et al., 1975). On the basis of an equivalent amount of nuclear DNA or protein, there is a 5–10-fold increase in chromatin-bound RNA polymerase I activity but little or no increase in RNA polymerase II activity associated with isolated nuclei (Guilfoyle et al., 1975; Lin et al., 1976). On a specific activity basis, soluble RNA polymerase II also remains at a fairly constant level following auxin treatment (Guilfoyle et al., 1975). Subunit structural analysis and DNA–RNA hybridization studies substantiate that enhanced chromatin-directed RNA synthetic activity observed following 2,4-D application is due to increased rates of precursor ribosomal RNA synthesis catalyzed by RNA polymerase I (Guilfoyle et al., 1976; Gurley et al., 1976).

In this study, I have analyzed the levels and subunit structures of soybean RNA polymerases I and II as well as the patterns of incorporation of [^{35}S]methinione and [^{32}P]-orthophosphate into RNA polymerase subunits during various times after auxin treatment. Results indicate that there is a 10–20-fold and a 6-fold increase in RNA polymerases I and II, respectively, within 48 h after auxin application. No alteration in the subunit structures or the patterns of incorporation of [^{35}S]methionine or [^{32}P]orthophosphate into RNA polymerase subunits was detected following 2,4-D treatment.

Experimental Procedures

Materials. Soybean seed (*Glycine max* var. Wayne) was germinated in moist vermiculite–perlite (1:1 v/v) in the dark at 30 °C for the times indicated. Treated seedlings were sprayed to runoff with a 2.5×10^{-3} M solution (pH 6.0) of 2,4-D at 72 h after planting. The mature hypocotyls (Lin et al., 1976) from untreated and 2,4-D treated seedlings were harvested at the times indicated and placed on ice prior to tissue disruption.

All materials used in the construction of polyacrylamide gels were purchased from Bio-Rad Laboratories. Enzyme-grade ammonium sulfate was obtained from Schwarz/Mann. Tris–base, glycine, dithiothreitol, 2-mercaptoethanol, nucleoside triphosphates, α-amanitin, calf thymus DNA (type IV), and phenylmethanesulfonyl fluoride were purchased from Sigma Chemical Co. Ethylene glycol and glycerol were Baker Analyzed Reagents. DEAE-cellulose (DE-52) and phosphocellulose (P-11) were from Whatman. Polymin P [poly(ethylenimine)] was obtained from Eastman. [5-^{3}H]UTP (18 Ci/mmol), [^{35}S]methionine (600 Ci/mmol), and [^{32}P]orthophosphate (carrier free) were from New England Nuclear. Kodak X-Omat R X-ray film was purchased from Eastman Kodak.

Calf thymus DNA (type IV from Sigma) was further purified as described by Guilfoyle & Jendrisak (1978). Heparin–Sepharose was prepared from sodium heparin (Grand Island Biological Co.) and Sepharose 4B (Pharmacia) as described by Bickle et al. (1977) except that 1 M glycine was substituted for triethylamine to block any remaining reactive groups after the gel was incubated with heparin.

Buffers. Homogenizing buffer was 50 mM Tris-HCl (pH 7.9 at 4 °C), 1 mM $MgCl_2$, 15 mM 2-mercaptoethanol, and 0.1 mM ethylenediaminetetraacetic acid. Chromatography buffer was 50 mM Tris-HCl (pH 7.9 at 4 °C), 1 mM ethylenediaminetetraacetic acid, 15 mM 2-mercaptoethanol, and 25% ethylene glycol. Storage buffer was 50 mM Tris-HCl (pH 7.9 at 4 °C), 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 50% glycerol. Antibody buffer was 25 mM sodium phosphate (pH 7.2) and 1% glycerol.

RNA Polymerase I and II Purification. Mature soybean hypocotyls were homogenized with a Polytron PT20ST in 2

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[1] Abbreviations used: 2,4-D, 2,4-dichlorophenoxyacetic acid; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxyethyl)-1,3-propanediol.

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volumes of homogenizing buffer at maximum speed for 2 min. The homogenate was filtered through eight layers of cheesecloth, and this was followed by filtration through one layer of Miracloth (Calbiochem). After filtration, Triton X-100 was added with rapid stirring to a final concentration of 0.5%, and the solution was centrifuged at 10000 rpm (Beckman JA-10 rotor) for 30 min. The chromatin pellet was utilized for the purification of RNA polymerase I (Guilfoyle et al., 1976), and the supernatant was utilized for the purification of RNA polymerase II (Guilfoyle & Jendrisak, 1978), since >90% of RNA polymerases I and II are associated with the chromatin and supernatant, respectively (Guilfoyle et al., 1975; Guilfoyle & Key, 1977).

Chromatin-bound RNA polymerase I was solubilized and precipitated with ammonium sulfate as described previously (Guilfoyle et al., 1976). The ammonium sulfate precipitate was suspended in chromatography buffer, adjusted to 0.25 M ammonium sulfate, and applied to a 10 × 0.75 cm column of heparin–Sepharose equilibrated with chromatography buffer containing 0.25 M ammonium sulfate. The column was washed with an identical buffer until no protein was detectable in the eluate and then developed with a 25 mL plus 25 mL gradient (0.25–1.0 M ammonium sulfate in chromatography buffer). One-milliliter fractions were collected and 25-μL aliquots were assayed for RNA polymerase activity. RNA polymerase I activity eluted as a broad peak between 0.35 and 0.55 M ammonium sulfate. Fractions containing RNA polymerase activity were pooled, dialyzed to 0.05 M ammonium sulfate in chromatography buffer, and applied to a 10 × 0.75 cm column of DEAE-cellulose equilibrated with chromatography buffer containing 0.05 M ammonium sulfate. After the column was washed extensively with an identical buffer, RNA polymerase I activity was eluted with a 25 mL plus 25 mL gradient (0.05–0.50 M ammonium sulfate in chromatography buffer). One-milliliter fractions were collected and 25-μL aliquots were assayed for RNA polymerase activity. RNA polymerase I activity eluted at ~0.125 M ammonium sulfate. Fractions containing activity were combined, dialyzed to 0.10 M ammonium sulfate in chromatography buffer, and applied to a 5 × 0.75 cm column of phosphocellulose equilibrated with chromatography buffer containing 0.10 M ammonium sulfate. The column was washed extensively with 0.125 M ammonium sulfate in chromatography buffer, and RNA polymerase I was eluted with 0.30 M ammonium sulfate in chromatography buffer. Half-milliliter fractions were collected and 25-μL aliquots were assayed for RNA polymerase activity. Active fractions were individually dialyzed against storage buffer and frozen at −70 °C or further purified by glycerol gradient centrifugation (Spindler et al., 1978).

RNA polymerase II was purified from the supernatant fraction of the hypocotyl homogenate by the method of Jendrisak & Burgess (1975) as modified by Guilfoyle & Jendrisak (1978). Purified enzymes were concentrated by dialysis against storage buffer and stored at −70 °C.

All purification procedures were conducted at 4 °C. RNA polymerase activities were assayed throughout purification as described by Guilfoyle et al. (1975). Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

In Vivo Labeling of RNA Polymerases with [^{35}S]-Methionine and [^{32}P]Orthophosphate. Three- to four-day-old etiolated soybean seedlings were severed 6 cm below the apical hook (just above the root–shoot transition zone), and rootless seedlings were placed upright in 20-mL glass scintillation vials with the severed ends of the hypocotyls submerged in 5 mL of solution. Untreated seedlings were incubated in distilled water containing 50 μg/mL chloramphenicol. Treated seedlings were incubated in a solution of 50 μg/mL chloramphenicol and 10^{-4} M 2,4-D. The vials containing 15 rootless seedlings were incubated at 30 °C in the dark after being placed in 2-L beakers which were covered with perforated saran wrap. Under these incubation conditions, the rootless seedlings exhibited similar growth characteristics, nucleic acid and protein accumulation, and chromatin-bound RNA polymerase activities as intact seedlings (T. J. Guilfoyle, unpublished experiments; Guilfoyle & Key, 1977). Radioactive label was added at the times indicated in the figure legends. After incubation, the radioactively labeled seedlings were severed 1 cm below the apical hook, and the mature hypocotyls were combined either with 50–100 g of 5-day-old soybean hypocotyls which had been treated for 48 h with 2,4-D or with 5 g of ungerminated embryonic axes from soybean seed. The labeled untreated and 2,4-D-treated hypocotyls along with carrier tissue were then homogenized and purified through the DEAE-cellulose step of purification for either RNA polymerase I or II.

Immunoprecipitation of RNA Polymerases I and II. Antibodies to soybean RNA polymerases I and II were raised in New Zealand white rabbits, and the IgG fractions were purified from the sera as described by Linn et al. (1973). The IgG fraction (5 mg/mL) was dialyzed against antibody buffer containing 100 mM NaCl and stored at −70 °C. Radioactively labeled RNA polymerases I and II were purified through DEAE-cellulose purification steps and were immunoprecipitated with excess antibody to soybean RNA polymerase I or II by incubation at 4 °C for 24 h in a buffer containing 50 mM Tris-HCl (pH 7.2 at 4 °C), 1 mM 2-mercaptoethanol, 25% ethylene glycol, 1 mM ethylenediaminetetraacetic acid, 250 mM ammonium sulfate, 1% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride. The immunoprecipitates were pelleted in a Beckman microfuge, and the pellets were washed twice in antibody buffer containing 250 mM ammonium sulfate, 1% Triton X-100, and 1 mM phenylmethanesulfonyl fluoride. The washed immunoprecipitates were suspended in 50 μL of sodium dodecyl sulfate sample buffer (Laemmli, 1970) and boiled for 3 min. The samples were then analyzed by polyacrylamide gel electrophoresis in the presence of dodecyl sulfate (Laemmli, 1970).

Polyacrylamide Gel Electrophoresis. One-dimensional polyacrylamide gel electrophoresis was performed in the presence of dodecyl sulfate on 0.75 mm thick slab gels by the method of Laemmli (1970). Polyacrylamide gels were stained with Coomassie brilliant blue R and destained as described by Burgess & Jendrisak (1975). Destained gels containing radioactive RNA polymerase were photographed and dried onto Whatman filter paper under vacuum.

Fluorography and Autoradiography. Fluorography was performed according to the method of Bonner & Laskey (1974). Gels dried on Whatman filter paper were fluorographed or autoradiographed at −70 °C using Kodak X-Omat R X-ray film.

Results

Levels of RNA Polymerases I and II in Untreated and Auxin-Treated Soybean Hypocotyls. After a 6–12-hour lag following 2,4-D application, there is a relatively linear increase in DNA content in mature soybean hypocotyls up to 48 h after treatment (Figure 1). By 48 h after auxin application, DNA content is ~3-fold greater in treated hypocotyls compared to untreated hypocotyls. Earlier studies indicated that an ~4-fold increase in chromatin-bound RNA polymerase I activity

Table I: Summary of the Purification of RNA Polymerase I from 1-kg Amounts of Soybean Hypocotyls Treated with Auxin for 0, 12, 24, and 48 h

fraction	protein (mg) after auxin treatment for				specific activity (units/mg)[a] after auxin treatment for				yield (%) after auxin treatment for			
	0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
solubilized[b] chromatin	15.3	31	49	125	0.7	0.95	1.3	1.7	100	100	100	100
heparin-Sepharose	1.0	3.0	5.2	12.2	8.0	8.2	8.7	12.0	75	84	71	67
DEAE-cellulose	0.3	0.7	1.5	4.6	23.5	24.1	25.0	29.0	66	57	59	62
phosphocellulose	0.03	0.07	0.12	0.4	195	200	215	220	55	48	40	41
glycerol gradient centrifugation	0.01			0.25	325			330	30			38

[a] Specific activity is defined as 1 nmol of UMP incorporated into RNA 30 min^{-1} mg of $protein^{-1}$. [b] Purification procedures are described under Experimental Procedures.

Table II: Summary of the Purification of RNA Polymerase II from 1-kg Amounts of Soybean Hypocotyls Treated with Auxin for 0, 12, 24, and 48 h

fraction[b]	protein (mg) after auxin treatment for				specific activity (units/mg)[a] after auxin treatment for				yield (%) after auxin treatment for			
	0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h	0 h	12 h	24 h	48 h
crude extract	5236	7110	12950	30500	0.060	0.067	0.080	0.078				
$(NH_4)_2SO_4$ precipitation	119	158	259	628	3.9	4.5	5.3	5.0	100	100	100	100
DEAE-cellulose	1.4	2.0	3.2	7.8	210	225	256	250	63	63	60	62
phosphocellulose	0.31	0.45	0.78	1.8	510	517	515	520	34	33	29	30

[a] Specific activity is defined in Table I. [b] Purification procedures are similar to those described by Guilfoyle & Jendrisak (1978).

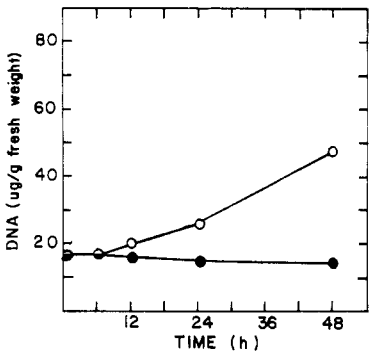

FIGURE 1: Increased DNA content in mature soybean hypocotyl following auxin application. DNA concentrations in mature hypocotyl were determined at the times indicated by the method of Burton (1968). (●) Untreated hypocotyls; (○) auxin-treated hypocotyls.

can be observed by 6 h after treatment with auxin, a period prior to DNA accumulation in the tissue (Guilfoyle et al., 1975). However, chromatin-bound RNA polymerase activities do not necessarily reflect the levels of enzyme present in a tissue or organ since the chromatin template may modulate RNA polymerase activities (Lin et al., 1976; Guilfoyle & Key, 1977). To gain some insight into how auxin enhances RNA synthesis in the mature soybean hypocotyl, I have monitored the levels of RNA polymerases I and II isolated from equivalent amounts (based on tissue fresh weight) of untreated and 2,4-D-treated tissue.

Tables I and II summarize the overall purification of RNA polymerases I and II to homogeneity from untreated and auxin-treated soybean hypocotyls. With the purification procedures summarized in Tables I and II, recoveries are 30–40% for RNA polymerase I and 30–35% for RNA polymerase II for both untreated and 2,4-D-treated hypocotyls. There are no apparent differences in recoveries or stabilities of either RNA polymerase I or RNA polymerase II from untreated and treated tissues. The intrinsic specific activities of purified RNA polymerases I and II are unaltered by auxin treatment. During purification of the RNA polymerases to homogeneity, I have found no differences in the concentrations of ammonium sulfate required to elute either RNA polymerase I or II from DEAE-cellulose or phosphocellulose for enzymes prepared from untreated and 2,4-D-treated hypocotyls. In addition, no alterations in divalent cation optima, ionic strength optima, or template requirements of the RNA polymerases are observed at any stage of purification of the enzymes following auxin treatment (data not shown).

By 12 h after auxin treatment, purification data (Tables I and II) indicate that there is a >100% increase in the amount of RNA polymerase I and a 50% increase in the amount of RNA polymerase II. At 48 h after auxin treatment, RNA polymerase I has increased by 10–20-fold and RNA polymerase II has increased by ~6-fold. Since the DNA content in the tissue has increased by ~3-fold by 48 h after auxin application (Figure 1), much of the increase in the amount of RNA polymerases can be accounted for by an increase in cell number (assuming that increased DNA content corresponds to cell division). However, if the amounts of RNA polymerases are monitored on the basis of equivalent amounts of tissue DNA, there is still about a 3–6-fold increase in RNA polymerase I and a 2-fold increase in RNA polymerase II observed 48 h after 2,4-D treatment.

Subunit Structures of RNA Polymerases Purified from Untreated and Auxin-Treated Soybean Hypocotyls. The polypeptides associated with purified RNA polymerases I and II from untreated and auxin-treated soybean hypocotyls are shown in Figures 2 and 3. RNA polymerase I purified from untreated hypocotyls and from hypocotyls treated for 48 h with 2,4-D possess identical polypeptides with apparent molecular weights of 195 000, 120 000, 45 000, 27 000, 22 000, 19 000, and 17 000 (Figure 2). These putative subunit molecular weights are only approximate since they are based on analyses

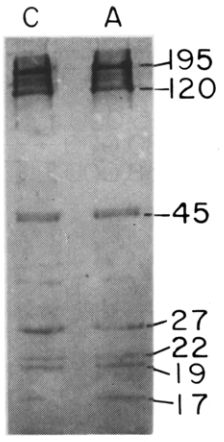

FIGURE 2: Polypeptides associated with RNA polymerase I from untreated and auxin-treated soybean hypocotyl. Polypeptides were resolved by electrophoresis on exponential gradient (10–16%) polyacrylamide gels in the presence of dodecyl sulfate. Samples are from the glycerol gradient step (Table I) of purification. Approximate molecular mass in kilodaltons of polypeptides are indicated adjacent to the figure. (C) is enzyme purified from control or untreated hypocotyl; (A) is enzyme purified from hypocotyl treated for 48 h with auxin.

with Tris–glycine buffered dodecyl sulfate–polyacrylamide gels with soybean RNA polymerase II and *Escherichia coli* RNA polymerase subunits as molecular weight standards (Guilfoyle & Jendrisak, 1978). Due to the relatively small amounts of RNA polymerase I present in soybean hypocotyl tissue, it was not feasible to determine the subunit structure of the enzyme at intermediate stages after auxin application.

Because of the large amounts of RNA polymerase II present in untreated and auxin-treated hypocotyl, it was possible to analyze the subunit structure of this enzyme at various stages after auxin application. RNA polymerase II enzymes purified from soybean hypocotyl at 0, 12, 24, and 48 h after auxin application have identical subunit structures (Figure 3). Each enzyme possesses subunits of about 180 000, 138 000, 42 000, 27 000, 22 000, 19 000, 17 600, 17 000, 16 200, and 16 100. Subunit structures have also been analyzed by two-dimensional polyacrylamide gel electrophoresis as described by Jendrisak & Burgess (1977), and no differences in subunit charge or molecular weight have been detected for RNA polymerase II at any stage after auxin treatment (data not shown). A 14 000 polypeptide (Guilfoyle & Jendrisak, 1978) is also associated with each class II enzyme but is not visible in Figure 3. Figure 3 also shows that RNA polymerase II enzymes purified from both untreated and auxin-treated hypocotyl are largely class IIB enzymes (e.g., the enzymes have a largest subunit of 180 000 in contrast to class IIA enzymes which have a largest subunit of ~215 000). Whether the 180 000 subunit exists in vivo is questionable, however, since at least a portion of the class IIB enzyme has been shown to arise artificially from the class IIA enzyme during purification procedures (Guilfoyle & Malcolm, 1980; Greenleaf et al., 1976; Dezelee et al., 1976).

In Vivo Labeling of RNA Polymerases with [^{35}S]-*Methionine.* To determine whether the increased amounts of RNA polymerases found in auxin-treated hypocotyl result from increased rates of de novo synthesis and to determine whether RNA polymerase subunits are synthesized in relatively stoichiometric amounts in auxin-treated and untreated hypocotyl, I analyzed the labeling patterns of RNA polymerase I and II subunits from hypocotyls incubated as rootless seedlings in the presence of [^{35}S]methionine. After in vivo labeling with [^{35}S]methionine, RNA polymerases I and II were purified through the DEAE-cellulose steps (see Experimental Procedures and Tables I and II) and immunoprecipitated with antibody raised against either RNA polymerase I or RNA polymerase II from soybean. Figures 4 and 5 show that auxin treatment results in a several-fold increase of [^{35}S]methionine incorporation into RNA polymerase I and II subunits. For determination of the relative level of [^{35}S]methionine incorporation into RNA polymerases I and II, the Coomassie blue stained gels and corresponding fluorograms were first scanned by densitometry. The areas under the 195 000 and 120 000 peaks of RNA polymerase I and the 180 000 and 138 000 peaks of RNA polymerase II for both stained gels and fluorograms were determined by cutting out the peaks and weighing them (Burgess & Jendrisak, 1975). The ratios of the areas under the fluorogram to the stained peaks for the analogous polypeptides in RNA polymerases from untreated and auxin-treated hypocotyl were then calculated. The ratios indicate that there is about an 8–10-fold increase in [^{35}S]-methionine incorporation into the 195 000 and 120 000 subunits of RNA polymerase I and a 2–3-fold increase in [^{35}S]-methionine incorporation into the 180 000 and 138 000 subunits of RNA polymerase II during the first 24 h after auxin treatment. This probably represents a minimal estimate of the relative increase in de novo synthesis of RNA polymerases induced by auxin since the pool size of amino acids increases following auxin application (T. J. Guilfoyle, unpublished experiments).

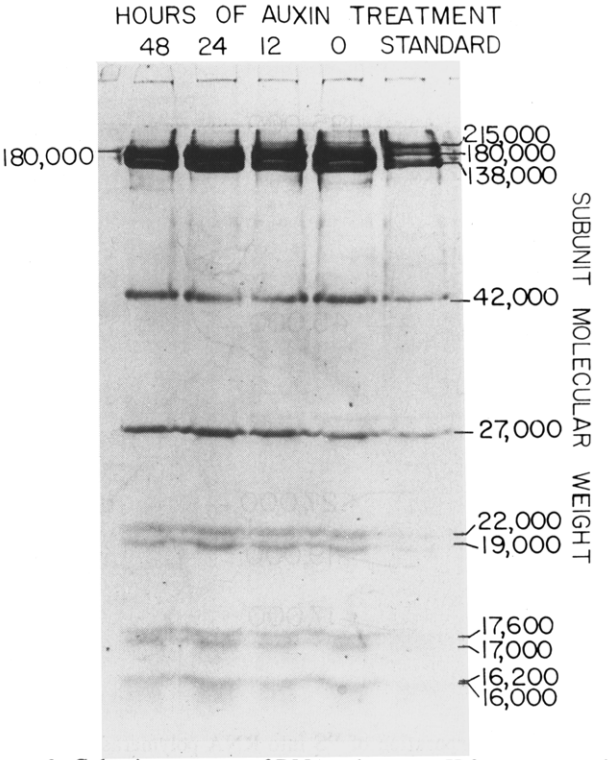

FIGURE 3: Subunit structures of RNA polymerase II from untreated and auxin-treated soybean hypocotyl. RNA polymerase II enzymes were purified according to the scheme summarized in Table II, and 3-μg amounts of each enzyme were subjected to electrophoresis in the presence of dodecyl sulfate. Polyacrylamide gels are 15% acrylamide. Numbers above the lanes indicate the time after auxin treatment. Numbers adjacent to the figure indicate approximate subunit molecular weights. The standard is RNA polymerase II purified from soybean axes germinated for 15 h (Guilfoyle & Malcolm, 1980); this enzyme contains equal quantities of 215 000 and 180 000 subunits (IIA and IIB).

In Figure 4, the polypeptides between 195 000 and 120 000 which are observed on both fluorograms and stained gels apparently arise as breakdown products of the 195 000 subunit. Peptide mapping using the method of Cleveland et al. (1977)

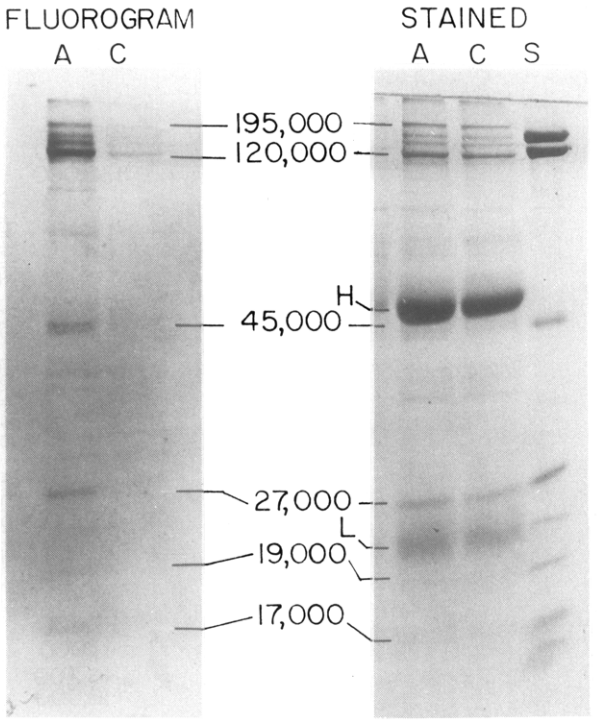

FIGURE 4: Incorporation of ^{35}S into RNA polymerase I subunits. Soybean rootless seedlings were labeled for 24 h in the presence of [^{35}S]methionine (0.5 mC_i/3 mL). The labeled mature hypocotyls (10 g) were combined with 100 g of unlabeled 48-h auxin-treated hypocotyls, and RNA polymerase I was purified through the DEAE-cellulose step of purification as summarized in Table I. Approximately 10 units of C (untreated control) and A (auxin-treated) were immunoprecipitated with antibodies raised against soybean RNA polymerase I. The immunoprecipitates were solubilized in dodecyl sulfate and subjected to electrophoresis on 10–16% exponential gradient polyacrylamide gels in the presence of dodecyl sulfate. ^{35}S immunoprecipitated from 10 units of C and A was 50 and 520 cpm, respectively, as determined by liquid scintillation counting. Approximate molecular weights of putative soybean RNA polymerase I subunits are indicated adjacent to the figures. H and L are the heavy and light chains of the IgG fraction. Fluorographs and stained polyacrylamide gels of the immunoprecipitates are displayed in the figure. (S) is soybean RNA polymerase II used as a molecular weight standard.

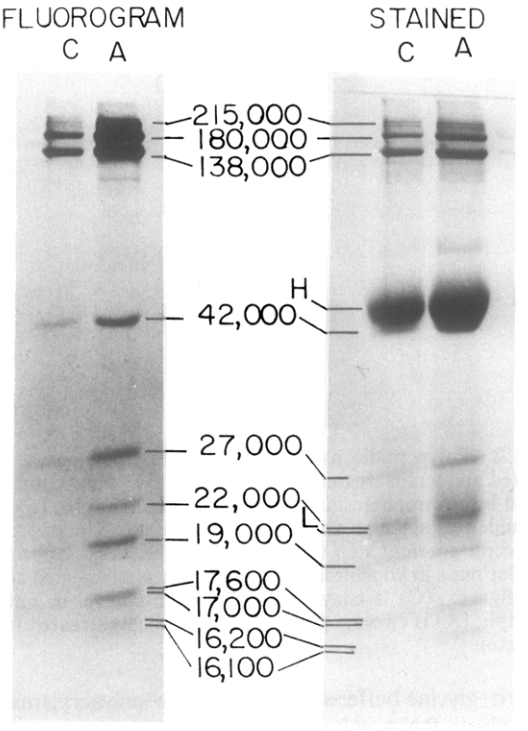

FIGURE 5: Incorporation of ^{35}S into RNA polymerase II subunits. See Figure 4 for details. Approximately 50 units of C (untreated control) and A (auxin-treated) was immunoprecipitated with antibodies raised against soybean RNA polymerase II. ^{35}S immunoprecipitated from 50 units of C and A was 275 and 1770 cpm, respectively. Fluorographs and stained polyacrylamide gels of immunoprecipitates are displayed in the figure.

with *Staphylococcus aureus* V8 protease indicates that the polypeptides between 195 000 and 120 000 subunits of RNA polymerase I possess similar peptide fragmentation patterns compared to the 195 000 subunit (data not shown). These polypeptides are probably generated artificially during purification of the class I enzyme. RNA polymerase I from untreated and auxin-treated hypocotyl displays similar degradation products of the 195 000 subunit.

Figures 4 and 5 also indicate that auxin application does not result in any preferential incorporation of label into any subunit compared to enzyme from untreated hypocotyl. In both untreated and auxin-treated tissue, [^{35}S]methionine incorporation into any RNA polymerase subunit is nearly proportional to the molecular weight of that subunit.

In Vivo Labeling of RNA Polymerases with [^{32}P]Orthophosphate. Since phosphorylation of RNA polymerases has been suggested as a mechanism for regulating eukaryotic RNA polymerase activities (Jungmann & Kranias, 1977), I analyzed the phosphorylation patterns of RNA polymerases from untreated and auxin-treated hypocotyl. RNA polymerases were labeled with ^{32}P by incubating rootless seedlings with [^{32}P]-orthophosphate in the presence or absence of auxin, and RNA polymerases were immunoprecipitated after purification through the DEAE-cellulose step (see Experimental Procedures). Although phosphorylation of RNA polymerase I subunits was not detected by employing this labeling procedure (data not shown), the 215 000 and 42 000 subunits of RNA polymerase II were labeled with ^{32}P in both untreated and auxin-treated hypocotyl (Figure 6). It is possible, however, that other subunits could be phosphorylated but not be detected by the exposure period used here. In contrast to the subunit composition observed with RNA polymerase II purified to homogeneity, immunoprecipitation of partially purified RNA polymerase II results in recovery of some 215 000 subunit or class IIA enzyme (Guilfoyle & Malcolm, 1980). No ^{32}P is associated with the 180 000 subunit of RNA polymerase II which is derived from the 215 000 subunit (Guilfoyle & Jendrisak, 1978), suggesting that the phosphorylated site is cleaved from the subunit during proteolysis. Although immunoprecipitation of ^{32}P-labeled RNA polymerase II was carried out in the presence of unlabeled extracts from ungerminated embryonic axes as indicated in Figure 6 (this results in the large amount of 215 000 subunit observed on stained gels since embryonic axes contain almost exclusively class IIA enzyme), immunoprecipitation of the labeled enzyme in the presence of unlabeled hypocotyl extracts (which yield almost exclusively class IIB enzyme) resulted in an identical pattern of subunit phosphorylation (data not shown).

Discussion

From the results presented here, it has been demonstrated that auxin treatment of mature soybean hypocotyl induces the proliferation of hypocotyl tissue and the de novo synthesis of RNA polymerases I and II. A 50% increase in RNA polymerase II and a 100% increase in RNA polymerase I are detected by 12 h after auxin application, a period which precedes significant increases in DNA synthesis and cell division. By 48 h after application of auxin the amount of RNA polymerase I has increased by about 10–20-fold and the amount of RNA

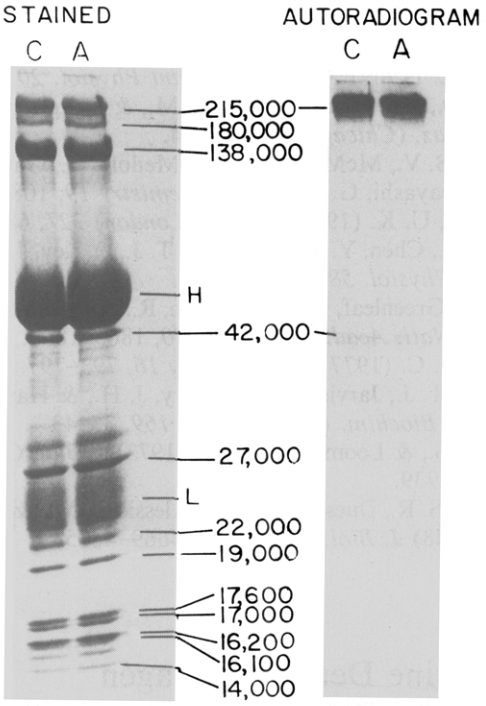

FIGURE 6: Incorporation or ^{32}P into RNA polymerase II subunits. Details on labeling are given under Experimental Procedures and in Figure 4 except that [^{32}P]orthophosphate was used in place of [^{35}S]methionine. Labeling was for 24 h with 5 mC_i/3 mL. C is untreated control and A is 24-h auxin-treated hypocotyls. Five grams of unlabeled, ungerminated soybean embryonic axes (embryonic axes contain almost exclusively RNA polymerase IIA with a 215000 dalton subunit) was mixed with 5 g of labeled tissue prior to purification of RNA polymerase II. Approximately 50 units of RNA polymerase II was immunoprecipitated from the DEAE-cellulose step (Table II) of purification with antibodies raised against soybean RNA polymerase II. Fifty units of both C and A contained ~150 cpm of ^{32}P as determined by liquid scintillation counting.

polymerase II has increased by ~6-fold; at this time DNA content has increased ~3-fold on the basis of equivalent fresh weight of mature hypocotyl. Incorporation of [^{35}S]methionine into RNA polymerase subunits suggests that the increase in amounts of enzyme after auxin treatment is due to de novo synthesis of the RNA polymerases, and auxin does not result in preferential synthesis of any subunit nor does the growth substance induce the synthesis of any novel polypeptide associated with the immunoprecipitates of RNA polymerases. No differences were detected in the phosphorylation patterns of RNA polymerases in untreated and auxin-treated tissues. Thus, it appears that the dramatic increases in RNA synthesis (Key, 1969) and chromatin-bound or nuclear-associated RNA polymerase activities (Guilfoyle et al., 1975) following auxin treatment of mature soybean hypocotyls may be correlated, at least in part, with increased amounts of RNA polymerases in the tissue, but no apparent modification of RNA polymerase subunit structures occurs during the activation of RNA synthesis and RNA polymerase activities.

We had reported earlier (Guilfoyle et al., 1975) that when auxin induces cellular proliferation of the mature, quiescent soybean hypocotyl, there is a 5–10-fold increase in chromatin-bound or template-engaged RNA polymerase I activity but little or no increase in template-engaged RNA polymerase II activity based on equivalent DNA or protein contents or equivalent number of nuclei (Lin et al., 1976). The results on the amounts of RNA polymerases in soybean hypocotyl presented here are based on equivalent tissue fresh weights and indicate that there is a 10–20-fold and 6-fold increase in class I and II enzymes, respectively, by 48 h after auxin application. Since the DNA content of the tissue was also analyzed at several stages following 2,4-D treatment, it can be determined that there is a 3–6-fold increase in the amount of RNA polymerase I and an ~2-fold increase in the amount of RNA polymerase II at 48 h post auxin treatment based on equivalent amounts of tissue DNA. When results from chromatin-bound or template-engaged RNA polymerase experiments (Guilfoyle et al., 1975; Lin et al., 1976; Guilfoyle & Key, 1977) are analyzed along with the data presented here, it is apparent that increases in the amounts of RNA polymerases alone do not explain the levels of RNA polymerase I and II activities expressed in isolated chromatin or nuclei. Thus, it appears that the chromatin template plays a major role in regulating the rate of transcription during auxin-induced cellular proliferation.

In this paper, I have analyzed RNA polymerase I and II enzymes for both quantitative and qualitative changes during the auxin-induced growth transition where relatively quiescent hypocotyl tissue undergoes abnormal proliferation. Quantitative changes in specific classes of RNA polymerases have been reported in many eukaryotic tissues which respond to growth-promoting hormones or drugs (Fuhrman & Gill, 1976; Jaehning et al., 1975; Hardin et al., 1976; Mauck, 1977); however, this is the first detailed analysis of possible qualitative changes in RNA polymerases during hormone-induced growth or proliferation. Prior to this report, studies on possible qualitative changes in RNA polymerases have been limited to developmental transitions in lower eukaryotes such as cellular differentiation in *Dictyostelium discoideum* (Pong & Loomis, 1973), *Acanthamoeba castellanii* (Detke & Paule, 1978a,b), and *Histoplasma capsulatum* (Kumar et al., 1980). The results presented here on soybean RNA polymerases are in general agreement with studies on *Dictyostelium* (Pong & Loomis, 1973) and *Acanthamoeba* (Detke & Paule, 1978a,b) which indicate that transitions in development and growth do not result in qualitative alteration of RNA polymerases.

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A Major Intermolecular Cross-Linking Site in Bovine Dentine Collagen Involving the $\alpha 2$ Chain and Stabilizing the 4*D* Overlap†

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ABSTRACT: Approximately 20% of the radioactivity incorporated into the dentine collagen of unerupted bovine molars after reduction with tritiated sodium borohydride was recovered in a cyanogen bromide peptide fraction of M_r 61 000 following chromatography on agarose A5m. After rechromatography on agarose A1.5m, this fraction was resolved into ten components by gel isoelectric focusing. Of these components, nine (the most acidic) were tritiated and contained the reduced cross-links dihydroxylysinonorleucine and hydroxylysinonorleucine. The amino acid compositions were consistent with the identification of each of these components as α2CB3,5 linked to one or two small peptides. By limited Edman degradation, with and without prior digestion with pyroglutamate aminopeptidase (EC 3.4.11.8), these small peptides were identified as α1CB0,1 and α2CB1, occurring in a ratio of approximately 2:1. Specific cleavage with cathepsin D revealed that all the cross-link was associated with the C-terminal one-third of the α2 chain, thus fixing the displacement of the participating molecules at 4*D*. The content of the known reducible cross-links present in these peptides, calculated from the specific activity of the reductant, was sufficient to account for only 10–20% of the cross-linking actually found, suggesting that stabilization is mainly through nonreducible cross-links of as yet undetermined structure. By quantitative analysis of homoserine content and semiquantitative amino-terminal analyses, it was determined that virtually all of the α2 chain of bovine dentine collagen is cross-linked in this manner. One cross-link per molecule in this location could make a major contribution to the mechanical stability of the insoluble collagen fibrils in this tissue.

In the form in which it fulfills a structural and mechanical role in the organism, collagen consists of fibers composed of many individual collagen monomers aligned in a parallel, polarized fashion. The presence of covalent intermolecular cross-links between these monomers is believed to be responsible for the insolubility of fibrillar collagen in nondenaturing solvents and for the physical properties (for example, tensile strength) which are essential for function in vivo. All vertebrate collagens so far studied contain cross-links of the Schiff base type formed by reaction of lysine or hydroxylysine with aldehydes resulting from oxidative deamination of the ϵ-amino group of lysine or hydroxylysine (Tanzer, 1976). This reaction is catalyzed by the enzyme lysyl oxidase (Pinnell & Martin, 1968; Siegel & Martin, 1970; Siegel et al., 1970). The cross-link formed from hydroxylysine and δ-hydroxy-α-aminoadipic δ-semialdehyde (usually referred to as hydroxyallysine) predominates in bone and dentine (Mechanic et al., 1971; Davis & Bailey, 1971). This may rearrange to the corresponding keto amine, hydroxylysino-5-ketonorleucine (Robins et al., 1973; Mechanic et al., 1974; Robins & Bailey, 1975). This species is isolated after reduction with sodium borohydride as dihydroxylysinonorleucine (DHLNL). Hydroxylysinonorleucine (HLNL) is also found but at lower levels. Both these reducible cross-links are generally considered to be intermediates which are subsequently converted to stable nonreducible forms (Robins et al., 1973) by processes which are, as yet, incompletely understood.

Information on both the number and the location, as well as on the chemistry, of the cross-links is essential for a complete understanding of their role in collagen structure and metabolism. Two cross-linking sites are well established for type

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