2,4-Dichlorophenoxyacetic Acid-enhanced Phosphorylation of Soybean Nuclear Proteins

MICHAEL G. MURRAY and JOE L. KEY
Department of Botany, University of Georgia, Athens, Georgia 30601

ABSTRACT

In vitro nuclear protein phosphorylation is enhanced in nuclei isolated from 2,4-dichlorophenoxyacetic acid (2,4-D)-treated mature soybean (Glycine max) hypocotyl relative to nuclei from untreated tissue. Increased nuclear protein phosphorylation correlates with increased levels of nuclear protein kinase activity. These changes generally parallel previously reported 2,4-D-enhanced RNA polymerase activity of these nuclei and the in vivo levels of RNA synthesis. Phosphate incorporation represents bona fide protein phosphorylation, with 87% of the label being identified as phosphoserine and 7% as phosphothreonine. Label from [γ-32P]adenosine 5'-triphosphate is incorporated primarily into various nonhistone fractions with the greatest accumulation in loosely associated fractions (either released during incubation with ATP or removed by 0.15 M NaCl). Although electrophoretic analysis on sodium dodecyl sulfate gels shows no differences in the protein profiles of the loosely associated or sodium dodecyl sulfate-soluble histone proteins, there are changes in the pattern of phosphorylation of other proteins, after 2,4-D treatment. Acid-soluble basic nuclear proteins are phosphorylated to a much lower extent than are the other nuclear protein fractions. While histone F, is subject to slight phosphorylation when nuclei are labeled in vitro, phosphorylation of the other histones is undetectable. One acid-soluble protein shows a substantial increase in quantity and in phosphorylation after 2,4-D treatment. This protein is similar in electrophoretic mobility to pea histone F, but its identity is unknown. Urea-acetic acid gels of the acid-soluble nuclear proteins show that auxin treatment results in increased quantities and in increased phosphorylation of various low mobility nonhistone basic proteins.

Evidence derived in numerous systems is compatible with the hypothesis that at least some chromosomal proteins participate in transcriptional regulation. Changes in the types and quantities and various modifications of these proteins (e.g. methylation, acetylation, phosphorylation, and thiolation) occur during passage through the cell cycle and during periods of increased template transcription (for reviews, see refs. 14 and 21). Histones were first shown to restrict template activity in 1962 (9). In view of their general lack of species and tissue specificity, histones are now thought to play primarily structural roles (21), thereby resulting in general template restriction. In contrast, nonhistone proteins by virtue of their heterogeneity and tissue and species specificity may participate more specifically in gene regulation (14, 21).

Since the initial observation that proteins isolated from lymphocyte nuclei were rich in phosphoserine (16), numerous correlations between increased chromosomal protein phosphorylation and gene activity have been observed (14). Transcription of rat liver chromatin by homologous RNA polymerase is stimulated by the addition of nonhistone phosphoprotein but not affected by dephosphorylated nonhistone protein (33). Dephosphorylation of Hela cell nonhistone protein prior to chromatin reconstitution results in a 50% reduction in the total number of sites available for initiation of transcription and an 80% reduction in the availability of histone genes for transcription (37). Keller et al. (12) have found a protein kinase activity associated with transcriptionally active chick oviduct chromatin, and increased protein kinase activity in nuclei has been shown to be correlated with activation of RNA synthesis in various rat tissues (4, 5).

Little information on nuclear protein phosphorylation in higher plants is available. van Loon et al. (41) have evaluated in vitro and in vivo chromatin protein phosphorylation in Lemma and in barley. Patterns of protein phosphorylation change during barley germination, although roots and shoots show similar profiles at any developmental stage. Chapman et al. (2) have shown that abscisic acid decreases nuclear protein kinase activity and causes altered phosphorylation profiles in Lemma. Kinetic stimulates in vitro protein phosphorylation in both cabbage and tobacco, but inhibits phosphorylation in vitro in nuclei and chloroplasts. Both tobacco and cabbage ribosomes contain protein kinase activities which are not affected by either cAMP, IAA, or gibberellic acid (27).

Application of the synthetic auxin, 2,4-D, to etiolated soybean seedlings causes very large increases in RNA synthesis in the mature region of the hypocotyl (13). Ribosomal RNA is preferentially synthesized in response to 2,4-D treatment (10, 13), and this increase is associated with a 5- to 8-fold increase in RNA polymerase I activity (8). While auxin does increase the synthesis of all species of RNA including short lived mRNA and heterodisperse nuclear RNA (13), RNA polymerase II activity is enhanced less than 30% if at all (8). While increased RNA polymerase I activity may account for the increase in RNA synthesis, changes in the level of RNA polymerase II alone do not seem sufficient to explain the enhanced synthesis of heterodisperse nuclear RNA and mRNA. Alteration of chromatin template availability is one mechanism by which 2,4-D could affect the synthesis of informational RNA without requiring increased RNA polymerase II activity. Such a model would be consistent with results indicating that chromatin isolated from IAA-treated lentil roots is more available for in vitro transcription by RNA polymerase II than is chromatin from untreated roots (38).

If chromatin template alterations are involved in 2,4-D-enhanced RNA synthesis in soybean, differences in chromosomal proteins and in their phosphorylation might be expected. Data presented here suggest that, concomitant with the 2,4-D en-
hancement of RNA synthesis, there are increases in the phosphorylation of several nuclear protein fractions in soybean, but no dramatic differences in the types of proteins associated with chromatin.

**MATERIALS AND METHODS**

**Plant Material.** Soybean seeds (Glycine max, var. Wayne), purchased from Noble Brothers Seed Co., Gibson City, Ill., were germinated in moist vermiculite after pretreatment for 5 min in 10% (v/v) Clorox. Seeds were dark-grown for 5 days, or 6 days when 72-hr 2,4-D-treated seedlings were required, in a growth chamber at 28 C. At specified times prior to harvest, "treated seedlings" were sprayed to runoff with 2.5 mm, 2,4-D (pH 6). Mature hypocotyl tissue from 0.5 cm below the cotyledon to 1 cm above the first secondary root was harvested and chilled to 4 C. Except where specified, all operations were carried out at 0 to 4 C and all buffers contained PMSF to minimize protease activity.

**Buffers.** Buffer A contained 50 mm Tris-HCl (pH 8), 10 mm MgCl₂, 250 mm sucrose, 0.5 mm PMSF, 10 mm MSH, and 1% (v/v) DMSO. Buffer B contained 10 mm Tris-HCl (pH 8), 1 mm MgCl₂, 0.5 mm PMSF, 10 mm MSH, and 1% (v/v) DMSO. Buffer C contained 10 mm Tris-HCl (pH 8), 0.5 mm PMSF, 10 mm MSH, and 1% (v/v) DMSO. Buffer D contained 25 mm MES-NaOH (pH 6), 20 mm KCl, 20 mm MgCl₂, 0.6 mm sucrose, 0.5 mm PMSF, 10 mm MSH, and 1% (v/v) DMSO. Buffer E contained 25 mm MES-NaOH (pH 6), 20 mm KCl, 20 mm MgCl₂, 30% (v/v) glycerol, 0.5 mm PMSF, 1% (v/v) DMSO, and 1.2 mm sucrose.

**Chromatin Isolation.** Chromatin was isolated by a modification of the method of Huang and Bonner (9) and described by Lin et al. (18). One hundred g of tissue were homogenized in 200 ml of buffer A at full speed for 1 min in a Willems polytron PT20-ST (Brinkmann Instruments, Inc.). After filtration through six layers of cheesecloth, the supernatant was centrifuged at 12,000g for 10 min. Chromatin was scraped from the underlying starch, suspended in 25 ml of buffer B containing 1% (v/v) Triton X-100 using a glass Teflon homogenizer, filtered through Miracloth (Calbiochem), and centrifuged at 12,000g for 10 min. The pellet was resuspended once more in buffer B plus 1% (v/v) Triton X-100, followed by two washes in buffer B. Chromatin was suspended in buffer C containing 1 mM sucrose and layered over a pad of 1.8 mM sucrose in buffer C. The gradient was stirred gently to disrupt the interface and centrifuged for 1 hr at 90,000g. The starch-free, gelatinous pellet was suspended in buffer C.

**Isolation of Nuclei.** Nuclei were isolated according to the method of Chen et al. (3). Fifty g of tissues were finely minced with razor blades and homogenized in 100 ml of buffer D using a Willems polytron Pr20-ST set at 4.5 for 1 min. Homogenates were filtered through six layers of cheesecloth and centrifuged for 30 min at 2,000g. Nucleolar pellets were scraped from the underlying starch and suspended by gentle vortexing in buffer D containing 0.25% (v/v) Triton X-100. The suspension was filtered through 25-μm Nitex mesh (Tet/Kressilk, Elmsford, N.Y.) onto a pad of buffer E. The gradient was stirred gently to disrupt the interface and spun at 12,000g for 30 min. The pellet, containing nuclei, some nucleoli, and some starch grains, was suspended in buffer D.

**DNA and Protein Determinations.** Samples of nuclei or chromatin were precipitated with cold 10% (w/v) trichloroacetic acid, and the precipitates were suspended in 0.1 N NaOH for Lowry protein determinations using BSA as a standard (19).

---

Additional aliquots were precipitated in cold 0.5 N HClO₄ for DNA determinations according to Burton (1) using CsCl-puriﬁed calf thymus DNA as a standard.

**Phosphorylation Assays.** Carrier-free [γ-32P]ATP was prepared enzymically (31). The reaction contained 100 mm Tris-HCl (pH 7.5), 10 mm MgCl₂, 10 mm DTT, 25 μM [γ-32P]ATP (specific radioactivity 50–100 cpm/pmol). Phosphorylation assays were initiated by the addition of 0.5 to 1 A₂₆₀ unit of chromatin in a final reaction volume of 0.4 ml. Nuclear phosphorylation assays contained, in addition, 300 mm sucrose, 10 mm KCl, and 20% (v/v) glycerol to maintain nuclear integrity during incubation; the assays were initiated by addition of 2 to 5 A₂₆₀ units of nuclei in a final reaction volume of 0.4 ml. For assays of solubilized protein kinase activity, 5-μl fractions containing protein kinase were added to 0.2 ml of the preceding assay mixture containing 500 μg/ml casein (Sigma) as substrate. Assays were incubated at 28 C and terminated with 4 ml of cold 10% (w/v) trichloroacetic acid containing 10 mm sodium pyrophosphate. Precipitates were collected on GF/A glass ﬁber discs (Whatman), washed with 20 ml of cold 5% (w/v) trichloroacetic acid, followed by 4 ml of 95% ethanol, dried, and radioactivity determined in toluene-based scintillator. All results are averages of triplicate samples and have been corrected for nonspecific trapping of 32P or [γ-32P]ATP on the ﬁlters.

Reaction mixtures were scaled up, and specific radioactivities increased (300–700 cpm/pmol), when large amounts of phosphorylated chromatin or nuclei were required for protein fractionation. Procedures for terminating such reactions varied and will be described subsequently.

**Nuclease Digestions.** Nuclei or chromatin were incubated with [γ-32P]ATP as described above for 30 min, diluted with 4 volumes of 25 mm Tris-HCl (pH 7.5), 0.5 mm PMSF, 1% (v/v) DMSO. After 5 min at 100 C, samples were divided, and pancreatic RNase A (EC 2.7.7.16; Sigma type IA) and RNase T₁ (EC 2.7.7.26; Sigma grade III) were added to ﬁnal concentrations of 50 μg/ml and 10 units/ml, respectively. Reactions were incubated at 37 C, and trichloroacetic acid and trichloroacetic acid and polyribonucleotide determinations were terminated with 25 μg/ml DNase I (EC 3.1.4.5; RNase-free, Worthington). After 12 hr of incubation at 24 C, aliquots were removed and the remaining mixture made to 1 N NaOH. Samples were heated to 100 C for 15 min and further aliquots removed. Water was added at each step to control samples, and counts have been corrected for volume changes. All aliquots were precipitated and processed for counting as in the phosphorylation assays.

**Identiﬁcation of Phosphorylated Amino Acids.** 32P-labeled chromatin was washed ﬁve times with cold 10% (w/v) trichloroacetic acid containing 10 mm sodium pyrophosphate. The acid-insoluble pellet was washed twice with chloroform-ethanol-ether (2:2:1), hydrolyzed in 0.5 N HClO₄ for 15 min at 90 C, and the residue washed once with cold 0.5 N HClO₄. Aliquots of the organic and HClO₄ washes were dried on filter paper and radioactivity was determined. The ﬁnal pellet was suspended in 10 ml 2 N HCl, and 4.25 mg each of O-phosphoserine and O-phosphothreonine (Sigma) were added. Samples were hydrolyzed in sealed glass ampoules for 12 or 24 hr at 100 C. Phosphoserine and phosphothreonine were determined by Dowex AG 50-8 (Bio-Rad) chromatography according to Schaeffer et al. (30). Aliquots of each fraction were neutralized with NaOH and counted in an aqueous scintillator. Total amino acid in each fraction was analyzed with Fluram (Roche Diagnostics) according to Udenfriend et al. (40). Counts recovered in O-phosphoserine and O-phosphothreonine were corrected for hydrolitic destruction of the internal standards.

**Nuclear Protein Fractionation.** After incubation with [γ-32P]ATP, nuclei were pelleted at 15,000g for 20 min. The protein in the reaction supernatant was precipitated with 10% trichloroacetic acid for 20 min at 0–4 C, heated to 90 C for 5 min, and then rapidly cooled. Precipitates were washed two times with ice, then dried in vacuo and counted in a liquid scintillator.
(w/v) trichloroacetic acid containing 10 mM sodium pyrophosphate. This fraction is referred to hereafter as supernatant protein. Nuclear pellets were washed once in 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM MSH, 0.5 mM PMSF, and 1% (v/v) DMSO for 30 min, followed by centrifugation at 15,000g for 20 min. These wash proteins were precipitated with cold 10% (w/v) trichloroacetic acid. To extract basic nuclear proteins, the pellet was suspended in 0.4 M H2SO4, 10 mM MSH, 0.5 mM PMSF, and 1% (v/v) DMSO, stirred at 4°C for 3 hr, and centrifuged at 15,000g for 15 min. Acid extraction was repeated overnight, and following centrifugation, supernatants were combined and precipitated with 4 volumes of 100% ethanol. SDS-soluble, nonhistone protein was extracted from the acid-dehistonized nuclear pellet with 1% (w/v) SDS, 10 mM Tris-HCl (pH 8.0), 10 mM MSH, 0.5 mM PMSF, and 1% (v/v) DMSO at 100°C.

Aliquots of the various protein fractions were precipitated in triplicate for counting on GF/A filters. Additional aliquots were removed for protein determinations. The remaining material was retained for acrylamide gel electrophoresis.

**Electrophoresis.** Electrophoresis of total, supernatant, salt wash, and nonhistonie protein was performed on gradient polyacrylamide SDS gels according to Schwartz and Roeder (32). Gel slabs (0.75 mm × 10 cm × 14 cm) were cast in a slab gel apparatus (Hoeffer Instruments Co., San Francisco). Separation gels contained from 8.75 to 15% (w/v) acrylamide (bis-acrylamide to acrylamide ratio, 0.8:30), 375 mM Tris-HCl (pH 8.8), 1% (w/v) SDS, and 0.1% (v/v) TEMED, and were polymerized with 2 mg/ml of ammonium persulfate. Protein samples were prepared by precipitation with cold 10% trichloroacetic acid (w/v) and then washing the precipitates once in 5% (w/v) trichloroacetic acid, once in ethanol-ether (2:1), and three times in acetone. The pellets were vacuum-dried, suspended in 75 to 100 µl of 1% (w/v) SDS, 10 mM DTT, 10 mM MSH, 2.5 mM Tris-glycine (pH 8.3), 0.1% (w/v) bromphenol blue, 0.5 mM PMSF, 1% (v/v) DMSO, and 10% (v/v) glycerol, and heated to 100°C for 5 min. Samples (about 20 µg of protein) were applied under the reservoir buffer (25 mM Tris-glycine [pH 8.3], 0.1% [w/v] SDS, 0.1% [v/v] MSH) and loaded at 3 mamp/slab for 1.5 to 2 hr until all tracking dye had entered the gel. Current was then increased to 30 mamp/slab and the run continued until tracking dye was near the bottom. Slabs were fixed in 50% (v/v) ethanol and 12% (w/v) trichloroacetic acid and then stained in 0.1% (w/v) Coomassie brilliant blue R (Sigma) in 10% (v/v) acetic acid, 40% ethanol for 1 hr. Slabs were diffusion-destained in 10% (v/v) acetic acid. Molecular weights were calculated from the mobilities of the following standards: γ-globulin, 160,000 daltons; BSA, 67,000 daltons; ovalbumin, 44,000 daltons; chymotrypsin, 23,000 daltons; myoglobin, 17,000 daltons; and Cyt c, 13,000 daltons.

Electrophoresis of acid-soluble basic protein was carried out in urea-acetic acid gels prepared according to the procedure of Poffenbarn and Chalkley (24) using the same slab gel apparatus. For polymerization, it was necessary to increase the ammonium persulfate concentration to 0.4% (w/v). Slabs were preelectrophoresed at 10 mamp/slab using basic fuchsin as a tracking dye. Ethanol-precipitated samples were washed once in ethanol-ether (2:1), three times with acetone, dried under vacuum, and suspended in freshly deionized 8 M urea, 0.9 N acetic acid, 10 mM MSH, 0.5 mM PMSF, and 1% (v/v) DMSO. Samples (about 4 µg of protein) were applied and electrophoresed at 5 mamp/slab until all of the tracking dye had entered the gel. Current was then increased to 30 mamp/slab until the tracking dye was near the bottom. Slabs were fixed, stained, and destained as described for SDS gels.

**Autoradiography.** Slabs were placed on Whatman 3MM paper in a large Büchner funnel, covered with one layer of Saran wrap, and dried under vacuum. A heat lamp was used over the funnel to speed drying. Dried gels were placed on Kodak "NoScreen" x-ray film and exposed for 1 to 5 days.

**RESULTS**

Incorporation of 32Pi by Chromatin. Initial studies on phosphorylation of soybean chromatin protein were conducted using chromatin isolated from tissue harvested after varying lengths of auxin treatment. In Figure 1, the kinetic activities of phosphate incorporation by chromatin isolated from untreated and 15-hr 2,4-D-treated soybean hypocotyls are compared. Both the initial rate of phosphate incorporation and the final level of incorporation are higher in chromatin isolated from treated tissue. If the incorporated phosphate is turning over, dilution of [γ-32P]ATP with unlabeled ATP after the reaction has proceeded for some time should result in a reduction of incorporated 32Pi. The results indicate that addition of 100-fold excess unlabeled ATP after the reaction has proceeded for 20 min does not reduce the level of incorporated label. Label incorporation continues when an equivalent dilution with H2O is made. These data show that the incorporated phosphate is not turning over in either treated or untreated chromatin preparations, and thus neither preparation contains appreciable levels of protein phosphatase. Calculation of the actual pmol of phosphate incorporated indicates constant incorporation whether ATP or H2O is added, and it is therefore apparent that ATP does not become rate-limiting before the end of the reaction. The observed reduction in the rate of incorporation with time of incubation must therefore reflect either substrate saturation or inactivation of protein kinase.

Chromatin phosphorylation requires the presence of ATP and Mg2+. Incorporation of 32Pi from [γ-32P]ATP can be competed quantitatively with unlabeled carrier ATP (Fig. 1, inset). Whether other nucleotide triphosphates will support chromatin phosphorylation is unknown, but purified protein kinase from soybean chromatin will not utilize either GTP, CTP, or UTP as

Fig. 1. In vitro chromatin phosphate turnover and competition of incorporation from [γ-32P]ATP by unlabeled ATP. Chromatin (0.1 A280 unit) isolated from 15-hr 2,4-D-treated (■) or untreated (△) tissue was incubated in the standard reaction mixture (0.4 ml) for the indicated times. After 20 min, either 100-fold excess unlabeled ATP or an equal volume (0.1 ml) of H2O was added. Values are the mean of three replications. In the inset, chromatin (0.1 A280 unit) was incubated in the standard reaction mixture (0.4 ml), modified to contain varying unlabeled ATP concentrations and constant [γ-32P]ATP concentrations. Values for three replications are shown. The line was fit by least squares regression.
a phosphate donor (22). Divalent cation requirements for phosphorilation by chromatin are shown in Figure 2. Maximal incorporation requires 10 mM Mg\(^{2+}\). Ten mM Mn\(^{2+}\) will support about 50% of the maximal activity seen with 10 mM Mg\(^{2+}\), but Ca\(^{2+}\) will not support phosphorilation at any concentration tested. Na\(^{+}\) alone will not support phosphorilation, and in the presence of 10 mM Mg\(^{2+}\) is slightly inhibitory at higher concentrations. Purified protein kinase isolated from soybean chromatin shows identical cation requirements and in addition is inhibited by K\(^{+}\) and NH\(_4\)\(^{+}\) (22). Neither cAMP at 10^{-5} or 10^{-8} M nor 2,4-D at 10^{-5} or 10^{-7} M has any significant effect on chromatin phosphorylation or on purified soybean chromatin protein kinase activity (22).

Identity of Labeled Products. To determine whether any of the label was incorporated into nucleic acids, samples of in vitro labeled chromatin and nuclei were subjected to sequential RNAse and DNase digestion; these results are shown in Table I. After 4 hr of RNAse digestion followed by 12 hr of DNase digestion, no significant amount of the label had been rendered acid-soluble. The absence of any incorporation into nucleic acids is expected, since phosphate in the \(\gamma\) position of ATP is incorporated only in the case of RNA chain initiation, and soybean chromatin does not initiate new RNA chains in vitro (Guilfoyle, personal communication). Following RNAse and DNase digestion, 95% of the incorporated phosphate is solubilized by incubation in 1 N NaOH for 10 min at 100 C (Table I). Because phosphoesters of serine and threonine are base-labile, chromatin was analyzed for the presence of phosphoserine and phosphothreonine. Samples of in vitro labeled chromatin were subjected to prolonged acid hydrolysis followed by Dowex AG 50-8 (Bio-Rad) chromatography to separate phosphorylated amino acids. During preparation prior to hydrolysis in 2 N HCl, about 7% of the label was removed by chloroform-ethanol-ether (2:2:1) and about 10% was solubilized after incubation in 0.5 N HClO\(_4\) at 90 C for 15 min (Table IIA). The \(^{32}\)P inorganic solvents may represent phospholipids which have been reported to be a component of chromatin (20). Extremely acid-labile N-phosphoryl-histidine and -lysin have been identified in Walker carcinoma histone (34). However, identical amounts of label were released from both cold trichloroacetic acid-precipitated and phosphate buffer-washed chromatin upon incubation in 10% (w/v) at 30 C (data not shown). N-phosphoryl derivatives are not stable in cold trichloroacetic acid, and thus the acid-labile phosphate in soybean chromatin is probably not in the form of N-phosphoryl-histidine and -lysin. The distribution of \(^{32}\)P in phosphoserine and phosphothreonine after acid hydrolysis and Dowex chromatography is summarized in Table IIB. After 24 hr of hydrolysis, 87% of the \(^{32}\)P is in phosphoserine and 7% in phosphothreonine. These data were corrected for destruction of each phosphoester during hydrolysis. The 6% of the label not recovered in either phosphoester may be due to partially hydrolyzed phosphopeptides; indeed, shorter hydrolys (14 hr) gave a larger proportion in this unidentified component. The proportions of phosphoserine and phosphothreonine in soybean chromatin are similar to those reported in a number of other plant systems (41). The proportion of phosphoserine to phosphothreonine was the same in chromatin from both auxin-treated and untreated tissue (data not shown).

Effect of Auxin Treatment on Nuclear Protein Phosphorylation. Nuclei were isolated from the mature region of the soybean hypocotyl after varying periods of auxin treatment and assayed for in vitro nuclear protein phosphorylation (Fig. 3a). Auxin treatment results in enhanced protein phosphorylation reaching a maximum of about 80% above control levels by 24 hr, and decreasing thereafter. The shape of the curve is identical whether phosphorylation is expressed in terms of phosphate incorporated per unit of protein or per unit of DNA (data not shown). The over-all protein to DNA mass ratio remains relatively constant at 15.6 ± 3.6 so in nuclei from any stage after auxin treatment. Hence, the increased total nuclear phosphorylation represents an increase in the specific activity of nuclear protein and not an increase in the quantity of protein in the nucleus.

Maximal RNA synthesis has been previously shown to occur between 24 and 48 hr after 2,4-D treatment (13). RNA content in this tissue as a function of time of 2,4-D treatment has been plotted on Figure 3a (taken from ref. 13 with permission from

---

Table I. Resistance to Nuclease Digestion of \(^{32}\)P-Labelled Chromatin Protein. (5 A\(_{250}\) unit each) were incubated in the standard reaction mixture (0.4 ml) modified to contain the indicated concentrations of MgCl\(_2\), MnCl\(_2\), CaCl\(_2\), or NaCl for 10 min. In one set, NaCl concentration varied while MgCl\(_2\) was held constant at 10 mM. The means of three replications are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nuclei</th>
<th>Chromatin (5 A(_{250}) unit each)</th>
<th>Nuclease 1,2,4 D</th>
<th>H(_2)O</th>
<th>Alkaline Incubation</th>
<th>Treatment</th>
<th>Nuclease 1,2,4 D</th>
<th>H(_2)O</th>
<th>Alkaline Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3868±145</td>
<td>100</td>
<td>10,205±201</td>
<td>100</td>
<td>4hr, RNase A, T(_2)</td>
<td>3720±96</td>
<td>96</td>
<td>9,558±90</td>
<td>94</td>
</tr>
</tbody>
</table>

---

Table II. Identity of Phosphoserine and Phosphothreonine in \(^{32}\)P-Labelled Chromatin Protein. (25 A\(_{250}\) unit) isolated from 24 hr to 24-D treated tissue was labelled in vitro with \(^{32}\)P-ATP and precipitated with cold 10% trichloroacetic acid. (A) precipitated chromatin was washed with chloroform:methanol:ether (2:2:1) and treated with 0.5 N HClO\(_4\), for 15 min at 90 C. Alliquots were removed at each step to determine precipitable counts remaining. (B) HClO\(_4\)-treated precipitated chromatin was suspended in 2 N HCl and hydrolysed for either 14 or 24 hr as described in Methods prior to Dowex-I chromatography. 31,040 cpm of 14 hr or 24,992 cpm of 24 hr hydrolysate were loaded onto 1 x 25 cm Dowex (Ag50-8, Stained) columns and eluted with 0.5 N HCl. Counts have been corrected for destruction of phosphoserine (74% and 82%) or 14 hr or 24 hr and phosphothreonine (19% or 50% after 14 or 24 hr) by the use of internal standards.

Table: 2. Treatment | cpm | \(^{32}\)P remaining | %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chomatrin</td>
<td>78,463</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Chlorofomethanol: ether (2:2:1 wash)</td>
<td>72,568</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>0.5 N HClO(_4), 15 min</td>
<td>90 C</td>
<td>64,225</td>
<td>82</td>
</tr>
</tbody>
</table>

Table: 3. A. Treatment | cpm | \(^{32}\)P remaining | %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chomatrin</td>
<td>78,463</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Chlorofomethanol: ether (2:2:1 wash)</td>
<td>72,568</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>0.5 N HClO(_4), 15 min</td>
<td>90 C</td>
<td>64,225</td>
<td>82</td>
</tr>
</tbody>
</table>

Table: 4. Fraction | cpm | %
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total recovered from Ag50-8</td>
<td>28,504</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>19,323</td>
</tr>
<tr>
<td>Phosphothreonine</td>
<td>970</td>
</tr>
<tr>
<td>Not attributable to either phosphate</td>
<td>8,216</td>
</tr>
</tbody>
</table>

Copyright © 1978 American Society of Plant Biologists. All rights reserved.
the authors) to demonstrate the correlation between maximal nuclear protein phosphorylation and the onset of increased RNA synthesis.

To examine the mechanism of the increased phosphate incorporation, nuclei prepared after varying periods of 2,4-D treatment were extracted with 250 mM ammonium sulfate and the extract assayed for protein kinase activity using casein as an artificial substrate (Fig. 3b). Protein kinase activity increases substantially after 12 hr of treatment and remains at high levels until 36 hr after treatment. This discrepancy between the period of maximum nuclear protein phosphorylation and maximum protein kinase activity has been observed in other systems and has been interpreted to indicate that other factors, such as the rate of phosphate turnover and substrate protein susceptibility to phosphorylation, are involved (4). Protein phosphatase activity in intact nuclei was not assayed. However, since chromatin isolated from auxin-treated tissue shows higher levels of in vitro phosphorylation (Fig. 1) as well as higher associated protein kinase activities (22), but no detectable protein phosphatase, it is probable that increased protein kinase activity is in part responsible for the response in intact nuclei. Regardless of the mechanism, it is clear that auxin treatment does lead to enhanced nuclear protein phosphorylation in vitro, and that maximal phosphorylation correlates with the onset of auxin-enhanced RNA synthesis (13).

**Protein Classes Phosphorylated.** Nuclei were isolated from mature soybean hypocotyl after 0 to 72 hr of 2,4-D treatment and incubated with \( [\gamma^{32}P]ATP \) to determine the extent of phosphorylation in various classes of nuclear protein. Table III describes protein recovery and \( ^{32}P \) recovery during extraction and fractionation of nuclear proteins. Since no significant differences were observed during the fractionation of nuclear proteins from either auxin-treated or untreated tissue, the results have been averaged. About 50% of the total protein containing 49% of the trichloroacetic acid-precipitable \( ^{32}P \) remains in the reaction supernatant and is thus loosely associated with the nucleus. Another 9% of the protein and 9% of the label are dissociated with 0.15 M NaCl. Acid-soluble histones and nonhistone basic proteins constitute some 9% of the total nuclear protein and 4% of the total label. Nonhistone chromosomal protein (about 20% of the nuclear protein and 18% of the \( ^{32}P \)) is removed from acid-dehisontized nuclei by 1% SDS.

To determine whether 2,4-D treatment causes a specific increase in the phosphorylation of any particular class of nuclear protein, specific activities of in vitro phosphorylated nuclear protein fractions were determined following isolation from mature tissue after 0 to 72 hr of 2,4-D treatment. Specific activities for each class of proteins were normalized (based on the specific activity of total nuclear protein from untreated nuclei) and averaged (Fig. 4). Although the standard deviations are large,
the increased specific activity of total nuclear protein up to 24 hr and the subsequent decrease by 72 hr are evident in the supernatant, salt wash, and SDS-soluble nonhistone protein fractions. Auxin treatment has lesser effect on phosphorylation of the acid-soluble basic nuclear proteins. Samples of each protein fraction were then subjected to acrylamide gel electrophoretic analysis.

**Electrophoretic Analysis of Nuclear Protein Phosphorylation.**

Protein profiles and corresponding autoradiographs of nuclear proteins after SDS-acrylamide gel electrophoresis are shown in Figure 5. The length of autoradiography varied for each fraction of nuclear protein. Hence, relative differences in exposure (and thus 32P incorporation) are comparable only among the different 2,4-D treatments within each set of gels. Protein profiles of total nuclear protein are virtually identical at all times after auxin treatment (Fig. 5a). There may be slight reductions in several high mol wt components (160,000 and 120,000) after 36 hr of treatment and an increase in a 20,000 dalton component between 24 and 72 hr. Autoradiographs show increased phosphorylation in 108,000, 48,000, 40,000, and 28,000 dalton components up to 36 hr, followed by decreases in all components by 72 hr. Histone bands are evident on the total nuclear protein gels, but there is little or no 32P in these regions.

The supernatant nuclear proteins show many similarities to total nuclear protein upon electrophoretic analysis (Fig. 5b). Auxin treatment has no effect on the supernatant nuclear protein profiles. Phosphorylation in this fraction appears primarily in proteins of mol wt lower than 67,000. Some increase in the phosphorylation of 48,000 and 30,000 dalton components is evident 24 hr after treatment. Salt wash nuclear proteins are identical at any time after auxin treatment except for a possible increase in a 44,000 dalton component after 24 hr (Fig. 5c). There is a striking increase in the phosphorylation of a 48,000 dalton protein by 24 hr, as well as increases in the labeling of several high mol wt components (160,000, 124,000, and 108,000).

No alterations in protein profiles after auxin treatment are evident in the SDS-soluble nonhistone protein fraction (Fig. 5d). Phosphorylation in this fraction occurs primarily in higher mol wt proteins (i.e. in those greater than 48,000). Auxin treatment results in an increase in 32P incorporation into 94,000, 77,000, and 48,000 dalton proteins. As with the other fractions, phosphorylation in all fractions decreases after 24 to 36 hr.

Basic proteins were analyzed on urea-acetic acid acrylamide gels (Fig. 5e). Because higher plant histones do not correspond precisely to animal histones, identification of histones F₁, F₄, F₅a, and F₂b is based on the work of Spiker et al. (36). Designation of F₁ was based on its constant ratio to the other histones and its preferential loss of Amido black stain when destined in ferric chloride solution (35). Some slight phosphorylation of histone F₁ is evident but only if gels are overloaded with respect to maximum histone resolution and autoradiographed for longer periods (3 weeks). In vitro phosphorylation of the other histones was undetectable.

Electrophoretic analysis shows the presence of low mobility nonhistone basic proteins in the acid-soluble fracture. These proteins increase in quantity and in the degree of phosphorylation after 2,4-D treatment. It is evident that one such protein band (designated as B on Fig. 5e) accounts for most of the incorporated phosphate in the acid-soluble fraction. While band B did show similar mobility to the most slowly migrating pea histone F₁ in urea-acetic acid polyacrylamide gels, its mobility in SDS gels indicated a mol wt of about 48,000. Furthermore, band B was not present in constant ratio to the histones and was not evident in histones extracted from soybean chromatin (data not shown). Thus, the present data are insufficient to establish with certainty whether band B is a member of the F₅a subgroup.

**DISCUSSION**

Early attempts at examining soybean nuclear protein phosphorylation involved in vivo labeling of hypocotyl sections with 32P, either in the presence or absence of 2,4-D. Label incorporation into proteins was minute relative to that incorporated into nucleic acids, making studies of protein phosphorylation impossible unless nucleic acid contamination in the protein fractions could be completely eliminated. In vitro labeling using γ-labeled ATP gives high phosphoprotein specific activity and reduces incorporation into nucleic acids because the label will only be incorporated into the 5' end of newly initiated RNA chains. However, soybean chromatin and nuclei appear not to initiate new RNA chains in vitro and indeed, no significant incorporation into nucleic acids could be detected. Protein phosphoesters account for at least 75% of the label incorporated by soybean chromatin according to the criterion of base lability. Further, 87% of the base-labile 32P is in phosphoserine and 7% in phosphothreonine. Thus, in vitro analysis using [32P]ATP does reflect bona fide protein phosphorylation. Nuclei isolated as described maintain sufficient structural and functional integrity so that they do contain both RNA polymerase I and II and carry out RNA chain elongation at high rates (3). However, uncertainty connected with the possible loss of nuclear components during isolation and the possibility that protein kinase activities are altered in vitro make extension of these results to the in vivo state difficult.

Nuclei and chromatin isolated from mature 2,4-D-treated hypocotyl show higher levels of in vitro phosphorylation than do similar preparations from untreated tissues. Maximal phosphorylation occurs 24 hr after auxin treatment and decreases thereafter. When crude nuclei extracts are assayed for protein kinase activity using casein as a substrate, enzyme specific activity generally reflects the levels of total nuclear protein phosphorylation. Because maximal nuclear protein kinase activity precedes and persists longer than protein phosphorylation, the increased protein kinase activity is not sufficient to explain the response. Changes in the rate of phosphate turnover, or in substrate susceptibility to phosphorylation may also be involved. Regardless of the mechanism, it is interesting that the onset of increased nuclear protein phosphorylation coincides with increased RNA polymerase I activity (8) and with increased rates of RNA synthesis in vivo (10, 13). An additional correlation was found between transcriptional activity and in vitro phosphorylation in nuclei isolated from the apical region of the hypocotyl. In this region, auxin treatment reduces both RNA synthesis (13) and protein phosphorylation (22). The data are significant in view of reports in other systems that protein phosphorylation is involved in the regulation of transcription (14, 37).

The distribution of 32P in nuclear protein fractions agrees with results in other plant systems (2) that in the majority of the label is incorporated into nonhistone protein. About 60% of the total incorporated label is either released into the supernatant or removed by 0.15 M NaCl and is thus loosely associated with the nucleus. No differences were observed in protein profiles for supernatant or salt-soluble nuclear proteins. There are increases in the total specific activity of these fractions resulting from increased phosphorylation of specific proteins in each fraction. In particular, auxin treatment causes increased phosphorylation of 40,000 and 48,000 dalton components up through 24 hr followed by a general decrease in all phosphorylation thereafter. These two proteins are particularly prominent in the salt fraction but are also evident in the supernatant fraction as well. Kleinsmith and Allfrey (15) observed that most of the phosphoprotein from in vitro labeled lymphocyte nuclei was Tris-soluble and concluded that these proteins were nuclear sap proteins. Recent studies show that particles consisting of heterogeneous nuclear RNA and protein may be extracted...
from rat liver nuclei with 0.1 M NaCl (25) and that these particles are released from nuclei after incubation in media containing ATP (29). Ribonuclear protein particles isolated from in vivo labeled rat brain nuclei contain a large amount of phosphoprotein (7). Any material released from soybean nuclei incubated with ATP would have been present in the supernatant fraction. In view of the observed auxin-enhanced RNA synthesis in soybean (13), it is possible that many of the proteins in the supernatant and in the salt-soluble nuclear protein fractions are involved in the packaging of RNA.

Compared to the other protein fractions, histone phosphorylation is low, if not nonexistent, in in vivo labeled soybean nuclei, and there is little detectable effect of auxin treatment. In vivo labeling in many systems has shown that histones F1, F2, and F3 are highly phosphorylated during cell proliferation (11). Histone modification generally occurs during or shortly after histone synthesis (6, 28) and most histone kinases have been shown to be localized in the cytoplasm (17). Only about 8% of the total cellular histone kinase activity could be detected in soybean nuclei. While some auxin-enhanced histone kinase activity was observed in intact nuclei, the level of activity was at least 10-fold lower than the nonhistone protein kinase activity (data not shown). Purified nonhistone protein kinase from soybean will phosphorylate histone about 10% as effectively as casein, and thus the histone kinase activity in nuclei is probably artifactual (22). Auxin does stimulate cell division in the soybean hypocotyl (13) and thus probably does cause increased histone phosphorylation in vivo, but such modification should not be detectable in in vitro labeled nuclei.

Autodiradiographs show that most of the phosphorylation in the acid-soluble basic protein fraction is in low mobility nonhistone basic proteins. The most highly phosphorylated protein (band B) shows a substantial increase in phosphorylation after auxin treatment. Although band B does show similar mobility to calf thymus histone F1, it is not thought to be histone because it is present in variable ratio to the identifiable histones. Furthermore, if band B is an F1 histone, the in vitro system is displaying a surprising degree of specificity, inasmuch as F2a and F3 would also be expected to be highly phosphorylated. It should be emphasized that while band B is the most highly phosphorylated basic nuclear protein, it represents a very small percentage of the total incorporated phosphate. Because acid extraction immediately followed salt extraction, it is possible that band B represents a low level of carryover from the salt extraction. Pederson (26) has suggested that many nuclear proteins found in chromatins or nuclei are artifacts from ribonuclear clear protein particles; additionally, extensive phosphorylation of preribosomal protein particles has been demonstrated in Novikov hepatoma cells (23). Phosphorylation of soybean ribosomal proteins has been demonstrated (39). Since auxin is known to cause increased ribosome synthesis in soybean (13), it is possible that many of these nonhistone basic proteins reflect auxin-enhanced ribosome synthesis.

The SDS-soluble nonhistone protein fraction most closely corresponds to the nonhistone nuclear acidic proteins believed to be responsible for specific template regulation in a number of other systems (14, 33, 37). Accordingly, this fraction was expected to show the most extensive changes in phosphorylation after 2,4-D treatment. While there are changes in the level of phosphorylation in several nonhistone proteins, the data show the most dramatic differences in phosphorylation occurring in the supernatant and salt wash fractions. This does not rule out the possibility that many of the tightly bound nonhistone proteins are involved in template regulation. One may postulate that many proteins involved in template regulation would have to be capable of interacting with a number of nuclear recognition sites and thus would not be tightly bound. If such were the case, many of these proteins may have been removed at earlier stages of fractionation. Extreme phosphoprotein heterogeneity and limitations of resolution in the gel systems used make conclusions on 2,4-D-specific regulatory phosphoproteins difficult.

The data indicate that the predominant effect of 2,4-D is an increase in the phosphorylation of protein fractions that are easily removed from the nuclei. It is clear that 2,4-D-enhanced phosphorylation takes place during a period when the cell is preparing to synthesize and export into the cytoplasm large amounts of mRNA and rRNA (10, 13). In view of evidence in other systems that RNA and mRNA are packaged into ribonuclear protein particles containing high amounts of phosphoprotein (7, 25, 29), the majority of the changes in phosphorylation in soybean nuclei may be a result of increased RNA synthesis rather than a cause. Examination of the function of ribonuclear protein phosphorylation in 2,4-D-enhanced RNA synthesis may be an interesting area for further investigation.

Acknowledgment—The authors wish to thank W. F. Thompson for his helpful comments on the manuscript.

LITERATURE CITED

13. KEY JL, CY LIN, EM GIFFORD, RD DENGLER 1966 Relation of 2,4-D-induced growth aberrations to changes in nuclear acid metabolism in soybean seedlings. Bot Gaz 127: 87-94
15. KLEINSMITH LJ, VG ALLREY 1969 Nuclear phosphoproteins. II. Metabolism of exogenous phosphoprotein by intact nuclei. Biochim Biophys Acta 175: 136-141

Fig. 5. Electrophoretic analysis of nuclear protein phosphorylation. Nuclear proteins were fractionated as described under "Methods" from in vitro labeled nuclei prepared from tissue treated with 2,4-D for the indicated times. Total nuclear protein, supernatant, salt wash, and SDS-soluble nonhistone protein were analyzed on 4 to 15% (w/v) acrylamide gradient SDS gels and autoradiographed as described under "Methods." Numbers shown in the gel are mol wt and mobilities of standards (γ-globulin, BSA, chromatins, myoglobin, and Cy5 c). Basic proteins were analyzed on 7 M urea gel with concave acid gels. Positive contact prints of the autoradiographs are shown and thus light areas represent 32Pi. (a) Total nuclear proteins, 20-hr autoradiography, soybean histone is shown on the right; (b) supernatant proteins, 36-hr autoradiography; (c) salt wash proteins, 36-hr autoradiography; (d) nonhistone proteins, 72-hr autoradiography, (e) basic proteins, 136-hr autoradiography, pea histone (gift from S. SPIKER), soybean histone (isolated from chromatin), and calf thymus histone (Sigma) are shown on the right.
22. PATEL NT, V HOLUBEC 1977 Dependence on the protein moiety of nuclear ribonucleoprotein particles on the extent of particle purification as studied by electrophoresis including a two-dimensional procedure. Biochim Biophys Acta 474: 524-535
26. SATO T, K ISHIKAWA, K OGATA 1977 Characterization of nascent ribonucleic protein particles released from isolated nuclei of regenerating rat liver. Biochim Biophys Acta 474: 536-548
32. SPEKER S, JL KEY 1976 Identification of histones Fm and F1 in stained gels. J Chromatogr 128: 244-248
36. TEPFER DA, DE FORS 1975 Phosphorylation of ribosomal protein in soybean. Phytochemistry 14: 1161-1165