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Auxin-Induced Changes in the Population of Translatable Messenger RNA in Elongating Sections of Soybean Hypocotyl[1]

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LINDA L. ZURFLUH AND TOM J. GUILFOYLE
Department of Botany, University of Minnesota, St. Paul, Minnesota 55108

ABSTRACT

***In vitro* translation products of polyadenylated RNA from untreated and auxin-treated elongating sections of soybean (*Glycine max* var. Wayne) hypocotyl were analyzed by two-dimensional polyacrylamide gel electrophoresis. The levels of translatable messenger RNA for at least ten *in vitro* translation products are increased by auxin treatment. The induction by auxin occurs rapidly (within 15 minutes), and the amounts of the induced *in vitro* translation products increase with time of auxin treatment. Indoleacetic acid has the same effect on the population of translatable messenger RNA as 2,4-dichlorophenoxyacetic acid. The auxin-induced *in vitro* translation products disappear rapidly when Actinomycin D is present during the last two hours of a three-hour auxin treatment.**

Continued synthesis of RNA and protein is obligatory for auxin-induced cell extension in excised plant organs (*i.e.* hypocotyls, epicotyls, and coleoptiles) during steady-state growth (11 for review). In addition to this requirement for steady-state growth, protein synthesis is also required for the auxin-induced H^+ secretion which presumably elicits the rapid growth response (2, 7, 18). There is, however, little or no concrete information on the types of proteins required for auxin-induced cell extension and on the roles such proteins play in the process of cell extension. Even more basic questions remain unanswered; for example, does auxin simply maintain or enhance general protein synthesis in excised organs, or does auxin alter the types or enhance the synthesis of a limited number of polypeptides (*i.e.* "growth essential proteins")? If the synthesis of specific polypeptides required for cell extension is induced by auxin, we might expect these polypeptides to appear rapidly (*i.e.* within 10–15 min after auxin application [8]), to be continuously synthesized while auxin is present, and to disappear rapidly or discontinue synthesis when auxin is removed.

We have previously attempted to answer some of the questions referred to above. We analyzed the spectrum of polypeptides which accumulated in excised soybean hypocotyls incubated in the presence or absence of auxin (*e.g.* 2,4-D) for periods of labeling (*e.g.* [^{35}S]methionine) ranging from 1 to 3 h (22). The accumulation of a small number of specific polypeptides appeared to be induced by auxin when radioactively labeled hypocotyl extracts were analyzed by one- and two-dimensional polyacrylamide gel electrophoresis. Although some information about auxin-induced polypeptide synthesis can be gained from such experiments, *in vivo* radioactive labeling experiments allow one only to analyze the polypeptides that accumulate or disappear over the labeling period chosen and are limited by the amount of time required to achieve sufficient radiospecific activity of the polypeptides to allow analysis. Such experimental constraints make it infeasible to study rapid growth effects induced by auxin.

In contrast to *in vivo* radioactive labeling experiments, *in vitro* translation of purified messenger RNA allows one to assess the spectrum of polypeptides synthesized at discrete and very short times after auxin application. This approach is limited only by how faithfully the *in vitro* translation system mimics that which is occurring *in vivo* at any given time. We have thus taken such an approach to determine if auxin modifies the spectrum of polypeptides synthesized during auxin-induced cell extension of soybean hypocotyl. We have utilized both wheat germ and rabbit reticulocyte translation systems and have analyzed the *in vitro* translation products by two-dimensional polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Plant Material. Soybean seeds (*Glycine max* var. Wayne) were germinated in a 1:1 mixture of moistened vermiculite and Perlite in the dark at 30°C. After 72 h, the seedlings were harvested, and the 1.2-cm section from the elongating region of the hypocotyls (0.5–1.7 cm below the cotyledons) was excised. The sections were rinsed with deionized distilled H_2O and placed in incubation medium.

Growth of Hypocotyl Sections. Fifty sections in 10 ml incubation medium were incubated in 50-ml culture flasks at 30°C with continuous shaking. The incubation medium contained 2% (w/v) sucrose, 50 μg/ml chloramphenicol, and 10 mM K-phosphate (pH 6). After a 2-h preincubation (with one change of solution), the incubation solution was replaced with fresh incubation medium containing or lacking 5×10^{-5} M 2,4-D. At the end of the incubation period, the hypocotyl sections were rinsed and immediately frozen in liquid N_2. In some cases, the sections were first placed on ice and the 1-mm terminal segments were removed prior to freezing the sections in liquid N_2. Sections were stored at −70°C until utilized for RNA extractions.

Extraction of RNA and Isolation of Polyadenylated RNA. RNA was extracted from soybean hypocotyl sections according to the method of Lizardi and Engelberg (13) with the modifications described below. Silanized and baked glassware was used throughout the extraction. Frozen sections were ground to a fine powder in a mortar under liquid N_2. One g powdered tissue was mixed with 4 ml 2.4% (w/v) SDS, 50 mM Tris-HCl (pH 7.2), 0.1 M NaCl, 10 mM vanadyl sulfate ribonucleoside complex (4), and 50 μg/ml Proteinase K (E. M. Merck); and the mixture was incubated for 1 h at 37°C. The extract was filtered through Miracloth (Calbiochem), and the filtrate was centrifuged at $12{,}000g_{max}$ for 10 min at 4°C. The supernatant was filtered through Miracloth, and 0.7 volume of 3.5 M $NaClO_4$ (or 0.25 volume of 7.2 M $NaClO_4$) was added to the filtrate. The mixture was clarified by a brief incubation at 50°C, and nucleic acids were precipitated with ethanolic perchlorate reagent (21) and reprecipitated with isopropanol es-

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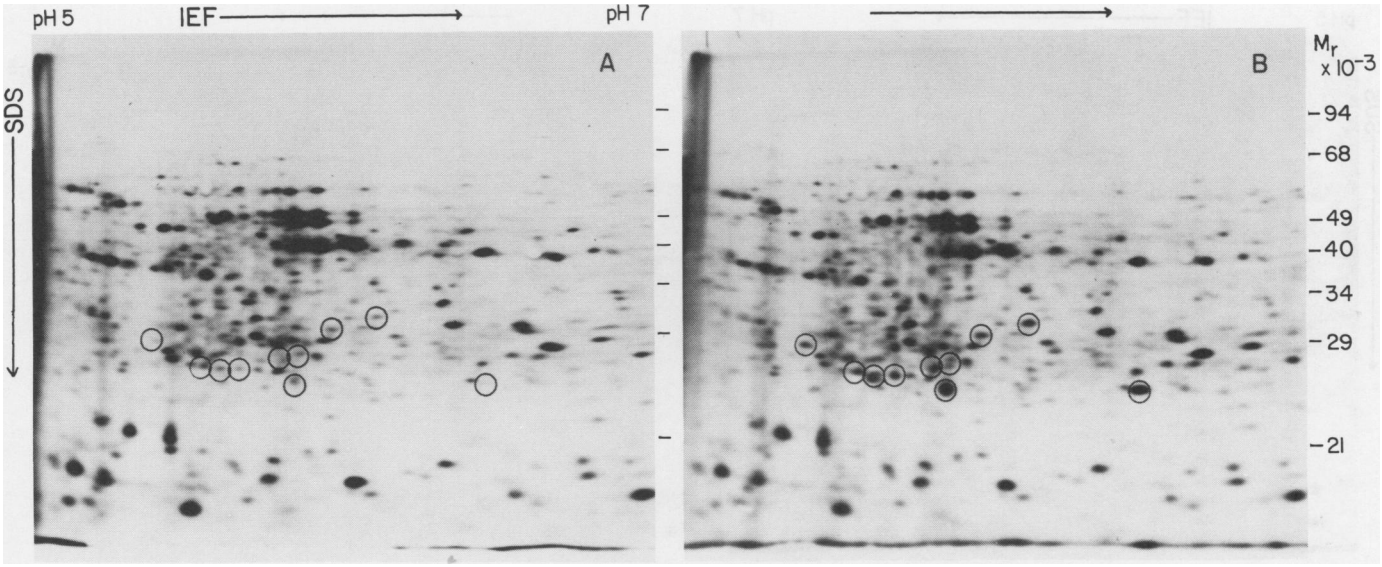

FIG. 1. Fluorographs of *in vitro* translation products obtained from polyadenylated RNA from elongating sections and resolved by two-dimensional polyacrylamide gel electrophoresis. Sections were incubated in the absence (A) or presence (B) of 2,4-D for 60 min. *In vitro* translation was performed in a wheat germ cell-free protein-synthesizing system. ^{35}S cpm loaded on first-dimension gel: (A) 990,000; (B) 1,150,000.

sentially as described (13). The nucleic acid pellet was dissolved in 0.15 M NaOAc[2] (pH 6), and high mol wt RNA (9, 16) was precipitated by adding 4 M NaOAc (pH 6) to a final concentration of 3 M essentially as described by Kern (9). The RNA pellet was dissolved in 0.15 M KOAc (pH 7), insoluble material was removed by centrifugation, and the RNA was precipitated with two volumes of ethanol at −20°C. The precipitated RNA was dissolved in water; and after the *A* at 260 nm was determined, the RNA was reprecipitated with ethanol.

Polyadenylated RNA was prepared by oligo(dT)-cellulose chromatography (Collaborative Research, Type T3) as described by Bantle *et al.* (1, Fig. 1). Absorbance measurements at 260 nm were made, and the polyadenylated RNA was reprecipitated in the presence of 0.15 M KOAc (pH 7) and two volumes of ethanol at −20°C. For use in translation assays, the precipitated polyadenylated RNA was dissolved in water.

Preparation of Wheat Germ Extract. Commercial wheat germ (General Mills, Vallejo, CA) was sieved to remove contaminating bran and endosperm fragments. The wheat germ extract was prepared according to the procedure of Marcu and Dudock (14) with the following modifications: (a) a 1:5 ratio of wheat germ to extraction buffer was used; (b) KOAc (pH 7) was substituted for KCl and 6 mM 2-mercaptoethanol was substituted for 1 mM DTT in the extraction and equilibration buffers; (c) the amount of extract applied to the column (22 × 1.5 cm Sephadex G-25, medium) was approximately 30% of the bed volume; (d) after the final extract was divided into small aliquots and immediately frozen in liquid N_2, it was stored at −70°C.

Wheat Germ Cell-Free Protein Synthesis. Immediately before use, the wheat germ extract was thawed and treated with 150 units/ml micrococcal nuclease (Worthington Biochemical Corp.) in the presence of 1 mM $CaCl_2$ at 20°C for 10 min; and the reaction was terminated by the addition of 2 mM EGTA (17). Translation assays were performed in a final volume of 25 μl. The mixture contained: 7.5 μl of the micrococcal nuclease-treated wheat germ extract; 20 mM Hepes (pH 7.6); 1 mM ATP; 0.1 mM GTP; 40 μg/ml creatine phosphokinase (Worthington Biochemical Corp.); 8 mM phosphocreatine; 100 mM KOAc (pH 7); 2.5 mM MgOAc; 2 mM DTT; 0.08 mM spermine; 10 μg/ml placental RNase inhibitor (19); 12.5 to 18.75 μCi [^{35}S]methionine (New England Nuclear or Amersham Corp., 900–1,200 Ci/mmol); 30 μM of each of the other 19 amino acids; and 30 μg/ml polyadenylated RNA. The mixture was incubated at 25°C for 90 min. Placental RNase inhibitor was isolated from human placenta as described by Blackburn (5).

Reticulocyte Lysate Cell-Free Protein Synthesis. Reticulocyte lysate was obtained from Green Hectares (Oregon, WI) and was stored at −70°C. Immediately before use, the lysate was thawed and made 2 mM DTT to stabilize the endogenous ribonuclease inhibitor (19). Messenger RNA-dependent lysate was prepared essentially as described by Pelham and Jackson (17) and was supplemented with 1.4 mM ATP, 0.07 mM GTP, and 0.34 mg/ml wheat germ tRNA. Translation assays were performed in a final volume of 25 μl and contained 16 μl of the messenger RNA dependent lysate, 2 μg/ml placental RNase inhibitor (19), 12.5 to 18.75 μCi [^{35}S]methionine, and 50 μg/ml polyadenylated RNA.

Preparation of *In Vitro* Translation Products for Electrophoresis. Cell-free protein synthesis was terminated by placing the mixture on ice. Unlabeled methionine was added to 1.2 mM, and an aliquot of the reaction mixture was taken to determine the amount of [^{35}S]methionine incorporation into protein. Five volumes of cold (−20°C) acetone (Mallinckrodt, SpectrAR) was added to the remainder, and the mixture was incubated overnight at −20°C. The acetone precipitate was pelleted by centrifugation (1 min, Beckman microfuge). The pellets were air-dried and were dissolved in 48 μl (or 72 μl, reticulocyte system) of Lysis Buffer (15) and stored at −70°C. Equal volumes of the solubilized pellets were loaded on first-dimension gels to facilitate consistent resolution of polypeptides from different samples.

Polyacrylamide Gel Electrophoresis. Two-dimensional polyacrylamide gel electrophoresis with isoelectric focusing in the first dimension and SDS in the second dimension was performed as described by O'Farrell (15) with the following modifications (communicated by D. C. Baulcombe and J. L. Key, University of Georgia, Athens, GA): (a) the polarity of the system was reversed (samples were loaded on the acidic end of the isoelectric focusing gel); (b) the upper (anode) buffer was 0.2% (v/v) H_2SO_4 and the lower (cathode) buffer was 0.5% (v/v) ethanolamine; and (c) isoelectric focusing was conducted for a total of 5,050 v-h (17 h at 250 v plus 1 h at 800 v). Second-dimension gels were 12% (w/v) polyacrylamide.

Fluorography. Slab gels were stained and destained, and processed for fluorography (6). The gels were dried onto Whatman

[2] Abbreviation: OAc, acetate.

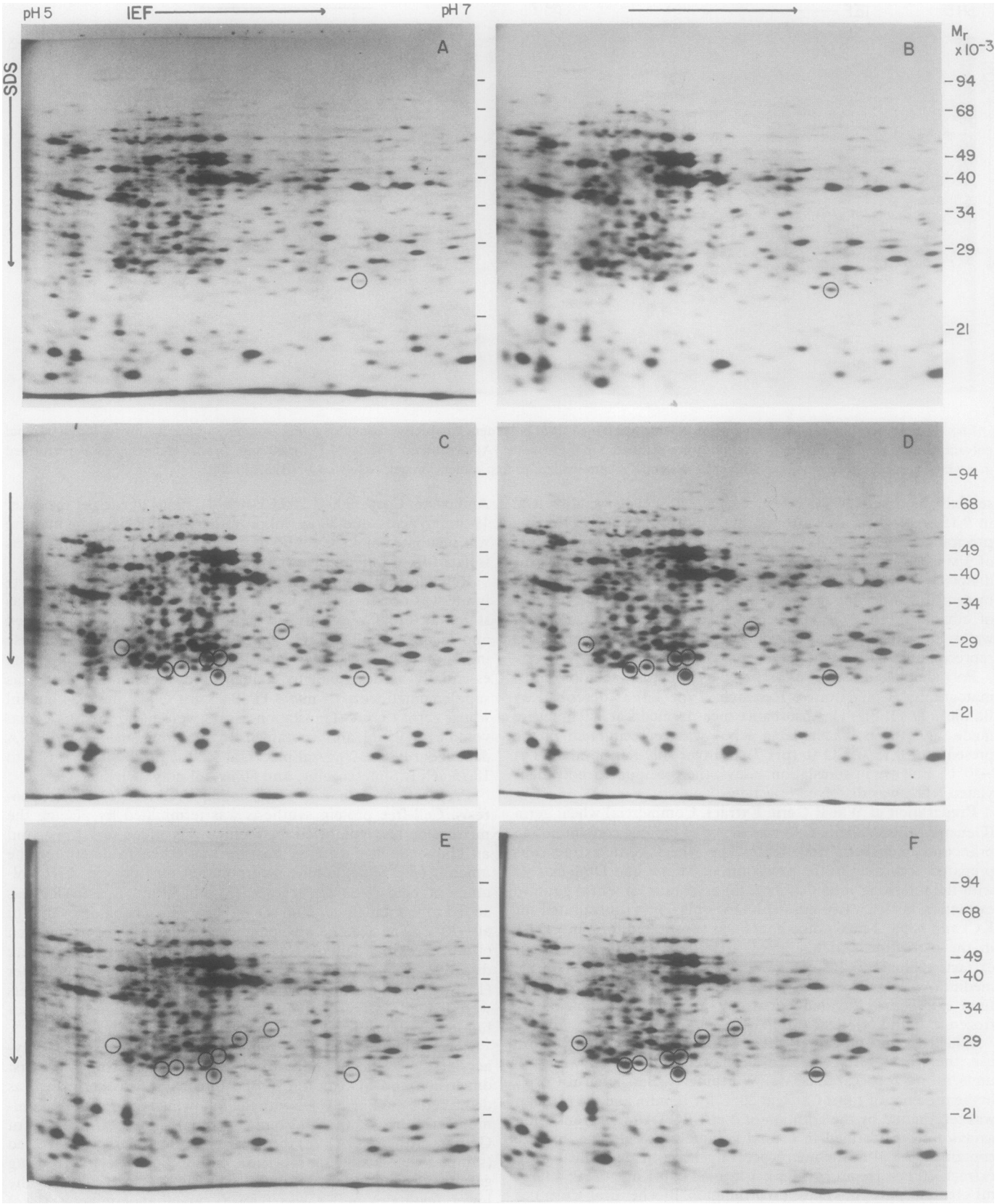

FIG. 2. Fluorographs of *in vitro* translation products obtained from polyadenylated RNA from elongating sections and resolved by two-dimensional polyacrylamide gel electrophoresis. Sections were incubated in the absence of 2,4-D for (A) 15 min, (C) 30 min, and (E) 45 min; or in the presence of 2,4-D for (B) 15 min, (D) 30 min, and (F) 45 min. *In vitro* translation was performed in a wheat germ cell-free protein-synthesizing system. ^{35}S cpm loaded on first-dimension gel: (A) 750,000; (B) 670,000; (C) 1,110,000; (D) 1,140,000; (E) 1,140,000; (F) 1,020,000.

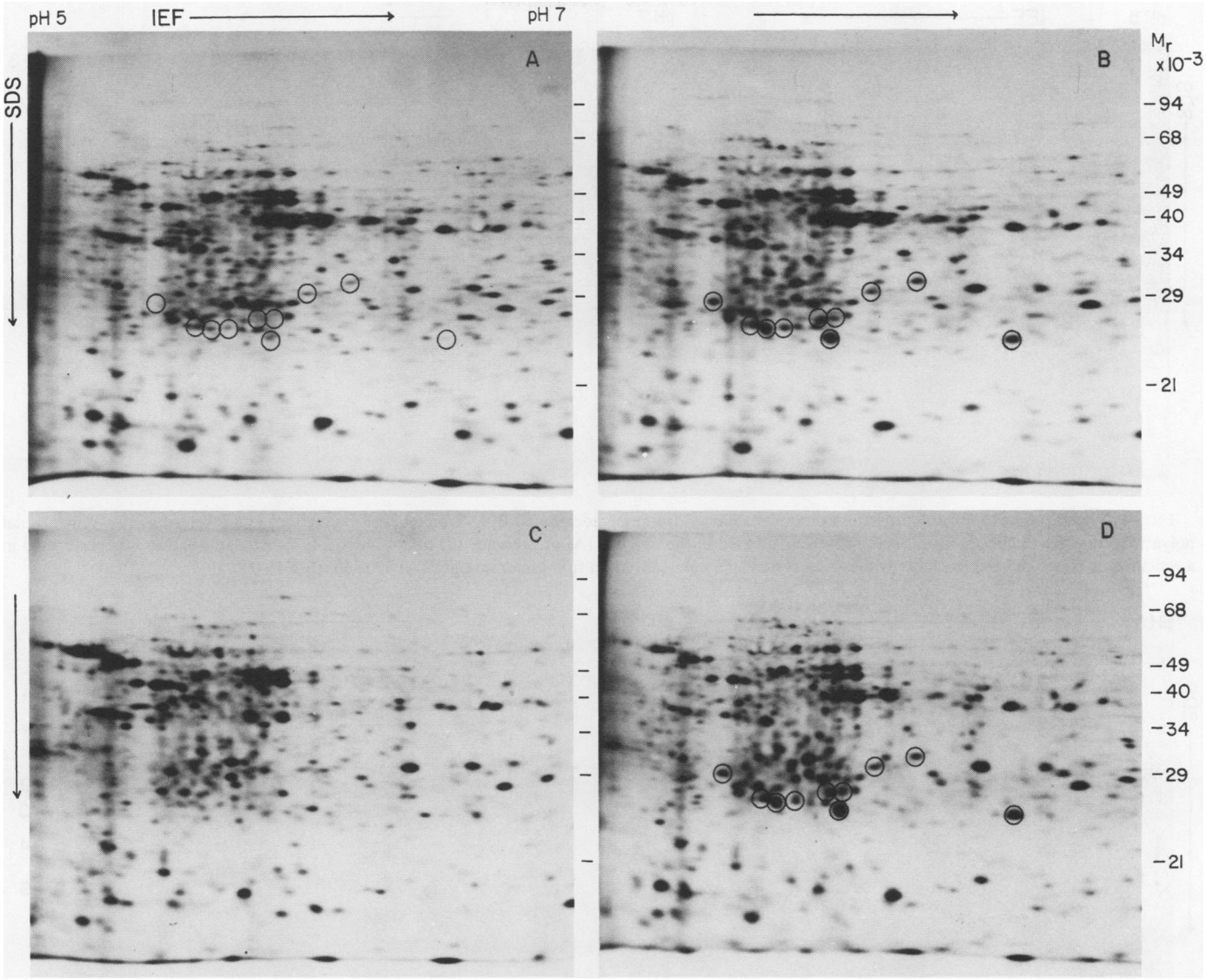

FIG. 3. Fluorographs of *in vitro* translation products obtained from polyadenylated RNA from elongating sections and resolved by two-dimensional polyacrylamide gel electrophoresis. Sections were incubated in the absence (A) or presence (B) of 2,4-D or in the presence of IAA (D) for 2 h. For (C), sections were not incubated. *In vitro* translation was performed in a wheat germ cell-free protein-synthesizing system. ^{35}S cpm loaded on first-dimension gel: (A) 1,250,000; (B) 1,140,000; (C) 1,450,000; (D) 1,110,000.

3MM paper under reduced pressure and exposed at −70°C to Kodak X-Omat R or AR x-ray film which had been prefogged to give a linear film response to radioactivity (12). The amount of hot TCA-precipitable radioactivity in each sample was used to calculate the exposure time of individual fluorographs. Fluorographs were exposed 1 day/2,000,000 cpm ^{35}S applied to the first-dimension gel. In this way, the product of radioactivity and exposure time was constant for all fluorographs.

RESULTS AND DISCUSSION

In vitro translation experiments were performed to examine the effects of auxin on the population of translatable messenger RNA in soybean hypocotyl sections. Preliminary results from the *in vitro* translation of total RNA isolated from elongating and basal sections which had been incubated in the presence or absence of 2,4-D for 1, 3, and 5 h indicated that auxin treatment caused an increase in the amount of several *in vitro* translation products (23). To examine better the early auxin effects on the population of translatable messenger RNA, we have preincubated sections for 2 h in a buffered solution to deplete the endogenous auxin and have translated polyadenylated RNA (rather than total RNA) in both wheat germ and reticulocyte lysate cell-free protein-synthesizing systems.

Comparison of the *in vitro* translation products of polyadenylated RNA isolated from elongating sections of soybean hypocotyl which were treated or untreated for 60 min (Fig. 1) reveals that 2,4-D treatment results in an increased amount of at least 10 *in vitro* translation products. The increased amount of at least one of these polypeptides is apparent in the *in vitro* translation products of polyadenylated RNA from elongating sections incubated in the presence of 2,4-D for as little as 15 min (Fig. 2, A and B), and increases in the amounts of all of these 2,4-D-specific *in vitro* translation products continue with time of treatment (Fig. 2, C and D [30-min treatment]; Fig. 2, E and F [45-min treatment]; Fig. 3, A and B [2-h treatment]). Most of the *in vitro* translation products (*e.g.* about 500 consistently observable radioactive polypeptide spots) remain apparently unchanged in amount during the period of 2,4-D treatment when compared with *in vitro* translation products of polyadenylated RNA from untreated sections. Thus, 2,4-D appears to induce the synthesis of only a small

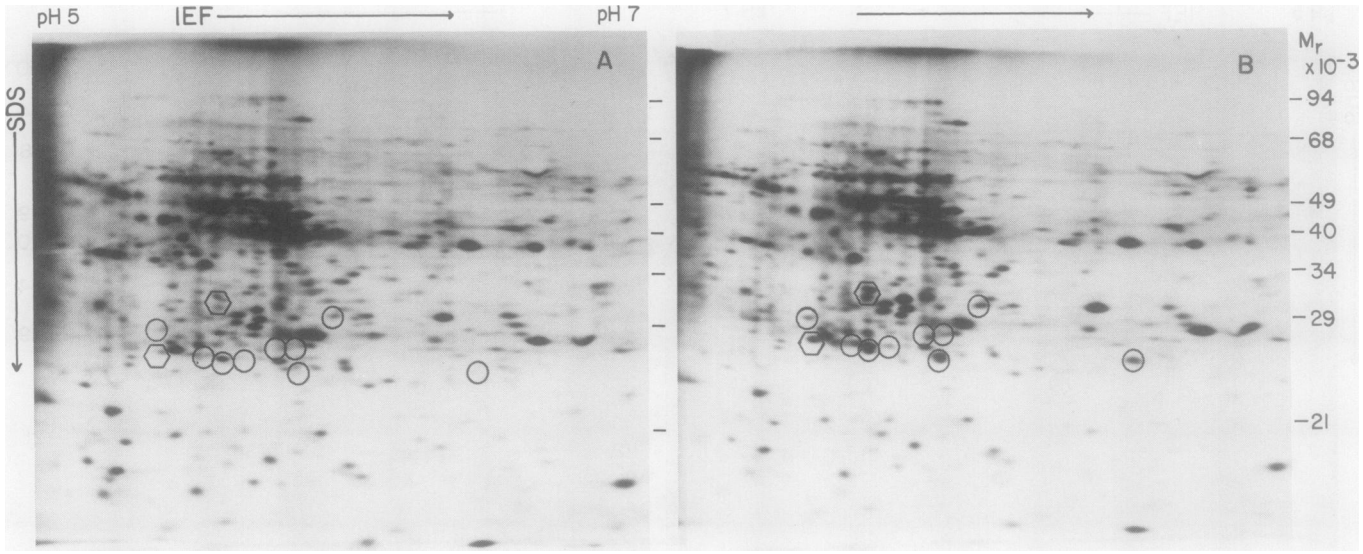

FIG. 4. Fluorographs of *in vitro* translation products obtained from polyadenylated RNA from elongating sections and resolved by two-dimensional polyacrylamide gel electrophoresis. Sections were incubated in the absence (A) or presence (B) of 2,4-D for 2 h. *In vitro* translation was performed in a reticulocyte lysate cell-free protein-synthesizing system. ^{35}S cpm loaded on first-dimension gel: (A) 800,000; (B) 910,000.

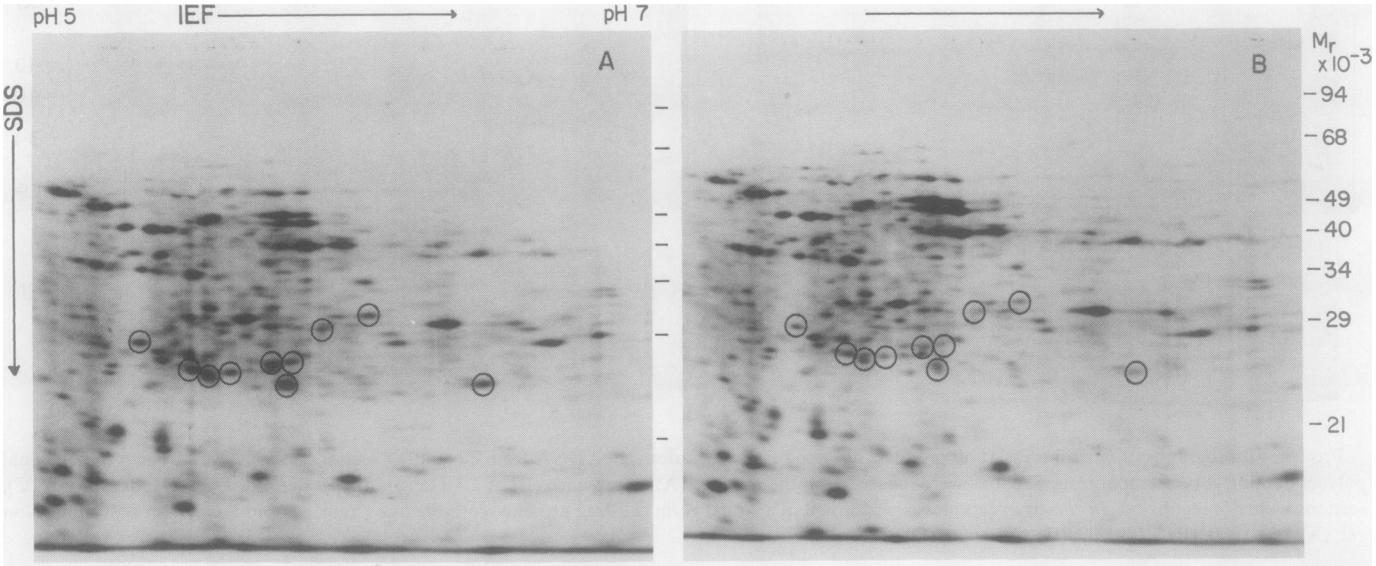

FIG. 5. Fluorographs of *in vitro* translation products obtained from polyadenylated RNA from elongating sections and resolved by two-dimensional polyacrylamide gel electrophoresis. Sections were incubated in the presence of 2,4-D for 3 h with (A) no additions or (B) 20 μg/ml Actinomycin D added after 1 h 2,4-D treatment. *In vitro* translation was performed in a wheat germ cell-free protein-synthesizing system. ^{35}S cpm loaded on first-dimension gel: (A) 1,400,000; (B) 1,180,000.

number (*e.g.* about 10 out of 500) of specific polypeptides in elongating soybean hypocotyl sections.

Much of the work by us and others which deals with auxin effects on the soybean hypocotyl has employed the synthetic auxin, 2,4-D, because it is more potent and more stable than IAA, the naturally-occurring auxin. The use of 2,4-D is, however, subject to criticism because it has not been established that 2,4-D induces a response that is identical to that induced by IAA. Therefore, we compared the *in vitro* translation products of polyadenylated RNA isolated from elongating hypocotyl sections which were incubated in the presence of either IAA (2×10^{-4} M) or 2,4-D (5×10^{-5} M). No differences in the *in vitro* translation products displayed on two-dimensional polyacrylamide gels were observed (Fig. 3, B and D). Thus, IAA induced the synthesis of a set of polypeptides identical with that induced by 2,4-D. This experiment provides a sensitive test which suggests that IAA and 2,4-D share the capacity to increase the levels of identical sets of translatable messenger RNA.

The use of excised hypocotyl sections to study auxin action is also subject to criticism because excision and incubation may modify the metabolism of the organ (20). This excision is required, however, in order to deplete the auxin supply in the organ so that auxin-induced cell extension can be studied. In order to assess the effects of excision and incubation on the population of translatable messenger RNA in the elongating region of soybean hypocotyl, we isolated polyadenylated RNA from elongating sections which were unincubated (excised from 3-day-old etiolated soybean seedlings and immediately frozen in liquid N_2). A comparison of the *in vitro* translation products of polyadenylated RNA from incubated and unincubated sections reveals that incubation results in

alterations in the population of translatable messenger RNA (Fig. 3C compared to Fig. 3, A, B, and D). This result suggests that some caution is required when attempting to correlate the effects of various agents (*e.g.* auxin) on organ sections with the role of that agent in the intact plant. Nevertheless, if appropriate controls are employed (*i.e.* identical incubation conditions for organ sections with auxin being the only variable), excised organs should provide insight into how the hormone functions in intact organs. Other studies (24) indicate that a specific set of *in vitro* translation products whose synthesis is induced by auxin is observed in both elongating and basal (mature) sections of soybean hypocotyl and in intact soybean hypocotyl treated with 2,4-D.

Because any cell-free protein-synthesizing system may be somewhat artifactual, we also assessed the effects of auxin on translatable messenger RNA by employing a second cell-free protein-synthesizing system, rabbit reticulocyte lysate. Although some of the messages exhibit differential translational efficiencies in wheat germ and reticulocyte lysate cell-free systems, most of the *in vitro* translation products of a particular polyadenylated RNA sample are present in both systems. Nine of the 10 *in vitro* translation products which are observed in increased amount as a result of a 1- to 2-h auxin treatment in the wheat germ system are likewise observed in increased amount in the reticulocyte lysate system (Fig. 4). There are at least three reticulocyte lysate *in vitro* translation products which are observed in increased amount as a result of auxin treatment that are not detected in gels of *in vitro* translation products of the wheat germ system. Thus, although the components of each system differ significantly, at least nine out of a total of 13 auxin-induced *in vitro* translation products are observed in both cell-free protein-synthesizing systems. This suggests that at least nine auxin-induced *in vitro* translation products represent authentic polypeptides and are not artifacts of a particular *in vitro* translation system (*i.e.* premature termination products).

Because auxin-specific *in vitro* translation products appear within 15 to 30 min after auxin application, we investigated whether their disappearance is also rapid. Incubation of elongating sections in the presence of 20 μg/ml Actinomycin D for the last 2 h of a 3-h 2,4-D treatment results in a decreased amount of the auxin-specific *in vitro* translation products when compared with products of polyadenylated RNA from sections incubated for 1 (Fig. 1) or 3 (Fig. 5) h in the presence of 2,4-D alone. Actinomycin D (10 μg/ml) has been shown to inhibit presumptive messenger RNA synthesis in elongating soybean hypocotyl sections (10). These results suggest that the messenger RNAs which encode the auxin-induced *in vitro* translation products have relatively short half-lives, possibly on the order of 1 to 1.5 h.

CONCLUSIONS

Our results show that auxin treatment alters the population of translatable messenger RNA in elongating sections of soybean hypocotyl. The levels of at least 10 translatable messenger RNAs are increased by auxin. The observed increases in the amounts of specific *in vitro* translation products as a result of auxin treatment may be elicited through the increased synthesis of a specific messenger RNA, through a more efficient processing of specific hnRNA to the mature messenger RNA, or through the decreased degradation of a specific messenger RNA. The increases may alternatively be effected by an increase in the *in vivo* translatability of a specific messenger RNA through some type of selective regulation which is also recognized *in vitro*. Specific cDNA clones of the auxin-regulated polyadenylated RNA sequences will be required to aid in distinguishing among these possibilities (3).

It is important to note that 2,4-D and IAA exert the same effect on the population of translatable messenger RNA. This result provides evidence that 2,4-D has a mode of action similar to IAA, at least in elongating soybean hypocotyl sections.

We have identified a group of polypeptides whose synthesis is regulated by auxin. We can determine how auxin regulates the synthesis of these polypeptides even though their cellular function is unknown. By constructing cDNA clones and utilizing recombinant DNA technology, we should eventually be able to determine whether auxin acts at the transcriptional level in regulating the synthesis of these proteins; we should be able to determine if these proteins and their respective messenger RNAs are regulated coordinately; and by DNA sequencing techniques with genomic DNA clones, we should be able to determine if auxin-regulated DNA sequences share homologous regulatory sequences.

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