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Early auxin-regulated polyadenylylated mRNA sequences in pea stem tissue

(indoleacetic acid/cell elongation/cell-free translation/gene expression/hormone action)

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ABSTRACT **Polyadenylylated mRNA from etiolated pea stem segments treated with or without 20 μM indoleacetic acid (IAA) for various periods of time was assayed by translating it in a wheat germ extract containing [^{35}S]methionine and separating the translation products by two-dimensional gel electrophoresis. Within 2 hr IAA causes at least five mRNA sequences to increase in translational activity, relative to initial levels and to simultaneous controls; three of these rise significantly within 20 min after exposure of tissue to IAA but are apparently not elevated at 10 min, whereas the others begin to increase at successive times later than 30 min, and still others begin to change only later than 2 hr. These observations indicate an early, highly selective IAA regulation of mRNA amounts or activities, becoming progressively more extensive with time. The earliest detected enhancement seems close to the primary action of IAA but appears not to be rapid enough to be responsible for auxin induction of cell enlargement.**

For more than a decade, it has seemed likely that action of the plant growth hormone auxin, or indoleacetic acid (IAA), involves stimulated or modified gene expression, at least in the long term (1–3). After auxin treatments of several hours, a number of effects on (*i*) the amounts of or (*ii*) radioisotope incorporation into RNAs and proteins have been reported (3–10). However, short-term auxin effects on gene expression (up to 1 hr of exposure to the hormone) have not been detected (11), even though auxin fully stimulates growth or elongation within 10–15 min (12, 13). This paper reports the occurrence of early IAA effects on mRNAs in pea stem segments (a classical system for studying auxin action on growth), observed by using an *in vitro* mRNA translation system and two-dimensional electrophoretic separation of translation products.

MATERIALS AND METHODS

Plant Material. Pea seeds (*Pisum sativum* cv. Alaska) were planted in vermiculite and germinated for 6.5 days at 25°C in the dark, except for brief exposures to dim red light during watering. A segment, 8 mm long, beginning 3 mm below the top of the apical hook was cut from the third internode of each seedling. During experimental manipulation, segments were handled under dim red light. All glassware was autoclaved before use. Each sample of 50 segments (*ca.* 1 g fresh weight) was kept at 25°C in darkness in 5 ml of 15 mM sucrose containing chloramphenicol (50 μg/ml) for 1.5 hr and then for 0.5 hr in 5 ml of incubation buffer (1 mM citrate/1 mM Pipes/15 mM sucrose/1 mM KCl/50 μg of chloramphenicol per ml, pH 6.0) to deplete segments of endogenous auxin. They were then kept in incubation buffer with or without 20 μM IAA for indicated periods of time. After incubation all segments were frozen in liquid nitrogen and stored at −70°C.

Purification of Poly(A)$^+$mRNA. Total nucleic acids were extracted from the frozen segments (14), and poly(A)$^+$mRNA was isolated by affinity chromatography on oligo(dT)-cellulose (15), except that the final elution buffer was at 50°C. The preparations had an A_{260}/A_{280} ratio of 2.0–2.3. Concentration of RNA was measured by absorbance at 260 nm, assuming 20 A_{260} units/mg of RNA.

Cell-Free Protein Synthesis. Wheat germ lysate was prepared (16), and the S23 extract was preincubated with Ca^{2+}-dependent micrococcal nuclease (17) to remove endogenous mRNA. *In vitro* translation (18) was conducted for 60 min at 29°C in 25 μl of 20 mM Hepes (pH 7.5) containing 1.2 mM ATP, 80 μM GTP, 9.6 mM creatine phosphate, 1.5 μg of creatinine phosphokinase, 80 μM spermine, 2 μg of wheat germ tRNA, 2 mM $Mg(OAc)_2$, 150 mM KCl, 20 μM of 19 unlabeled amino acids (except methionine), 50–70 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine (specific activity, >1000 Ci/mmol), 2.5 μg of poly(A)$^+$mRNA, 10 μl of nuclease-treated wheat germ extract, and 50 ng of purified placental RNase inhibitor (19). In preliminary experiments, the indicated concentrations of K^+, Mg^{2+}, poly(A)$^+$mRNA, and RNase inhibitor were determined to give maximum [^{35}S]methionine incorporation in the system. Pea epicotyl poly(A)$^+$mRNA increased the rate of [^{35}S]methionine incorporation into polypeptides by the wheat germ extract 80- to 100-fold above that obtained without mRNA addition.

Gel Electrophoresis. Translation products were separated in two dimensions (20) by nonequilibrium pH gradient electrophoresis in the first dimension and a 12–20% (wt/vol) acrylamide gradient with $NaDodSO_4$-containing buffers (21) in the second dimension. Normally 10 μl of translation mixture containing 5–6 × 10^6 cpm of incorporated ^{35}S, mixed with 10 μl of "IEF sample buffer" (20) and 10 mg of urea, was loaded onto the first-dimension gel. After electrophoresis, dried gels were autoradiographed by exposing to Kodak X-Omat XR-2 film for about 4 days. Circular sections were cut from dried gels at the location of individual polypeptide autoradiographic spots. Each section was digested (22), and its radioactivity was determined by liquid scintillation with about 90% efficiency.

RESULTS

Fig. 1 shows portions of autoradiograms of two-dimensional separations of [^{35}S]methionine-labeled polypeptides obtained by cell-free translation of pea internode poly(A)$^+$mRNA in the wheat germ system. The poly(A)$^+$mRNA specifies approximately 250 distinguishable polypeptides with M_rs ranging from 12,000–100,000 (only part of the complete range of M_r and pH are illustrated in Fig. 1). All the labeled polypeptides in Fig. 1 are specified by pea mRNA: in the absence of added mRNA only one feebly labeled M_r 15,000 polypeptide was detected.

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Abbreviation: IAA, indoleacetic acid.

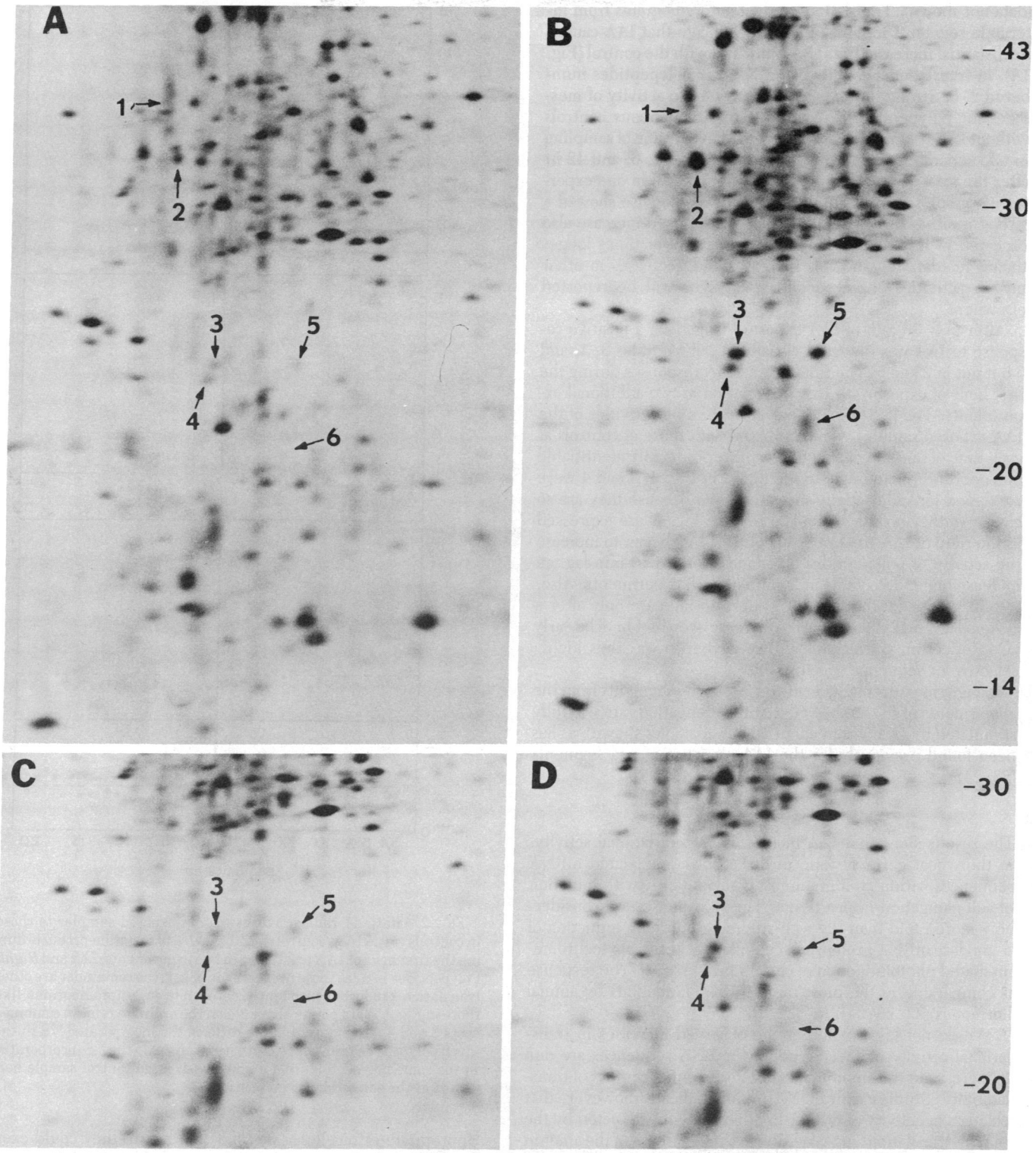

FIG. 1. Portions of autoradiograms of electrophoretically separated [^{35}S]methionine-labeled *in vitro* translation products specified by poly(A)$^+$RNA from pea stem segments. First (horizontal) direction, nonequilibrium pH gradient; second (vertical) direction, NaDodSO$_4$/acrylamide gradient. (*A* and *B*) Translation products with a M_r range of 14,000–50,000 and a pH range from 4.0 (left) to 8.0 (right). (*C* and *D*) Translation products with M_rs of 18,000–30,000 from comparable electropherograms. Poly(A)$^+$mRNA was from segments kept 2 hr without auxin after cutting (*C*) and from segments treated as in *C* but with either an additional 30-min incubation with 20 μM IAA (*D*) or an additional 2-hr incubation without (*A*) and with (*B*) 20 μM IAA. M_rs of reference proteins are shown on the right $\times 10^{-3}$: α-lactalbumin, 14; soybean trypsin inhibitor, 20; carbonic anhydrase, 30; ovalbumin, 43.

Comparison of translation products of mRNA from internodes as initially harvested and after the incubation procedure showed that very few qualitative changes in mRNA occur even after 12 hr of incubation (data not shown).

Auxin-Regulated mRNA Sequences. The total translational activity of poly(A)$^+$mRNA from IAA-treated and untreated tissues (per μg of RNA added to the *in vitro* translation system) was found to be essentially the same at all times of sampling

(data not shown). Translation product autoradiograms from internode segments incubated for 2 hr indicate that IAA caused a substantial increase (Fig. 1*B*), compared with the control (Fig. 1*A*), in translational activity of mRNAs for polypeptides numbered 2–6. Strong positive IAA effects on the activity of messages for polypeptides 2–6 relative to simultaneous controls without IAA treatment were observed at every time of sampling in two separate experiments, each sampled at 2, 6, and 12 hr after the start of IAA treatment (data not shown; in one experiment samples were also taken at 4 hr and likewise showed a strong positive IAA effect). A large IAA-induced increase also occurred in mRNA activity for polypeptide 1 at times longer than 2 hr (data not shown). Increases and decreases in other mRNA activities observed later than 2 hr will be reported elsewhere.

After only 30 min of IAA exposure (Fig. 1*D*), a positive response to IAA was detected visually in polypeptides 3, 4, and 5 but not in 2 and 6. The timing of mRNA responses during the first hour of exposure to IAA was studied in two additional experiments. The [^{35}S]methionine-incorporating activities of the IAA-exposed samples were compared with those of controls at each time of sampling for 21 different, reproducibly identifiable polypeptides (including 1–6 identified in Fig. 1; 3 and 4 were combined for radioactivity measurements because they are so close together on the gels). The results (Fig. 2) are expressed as the ratio of IAA treatment to control. IAA began to increase the activity of polypeptides 3, 4, and 5 after a 10-min lag, an increase being detectable at 20 min in both experiments. Also, in both, polypeptide doublet 6 began to increase only after a 30-min lag, and 2 did not start to increase even by 1 hr. The early auxin effect on translatable mRNA activities was thus highly selective or sequence specific (Fig. 2).

Length measurements made on internode segments from the experiments of Fig. 2 showed that the elongation rate was fully stimulated by IAA within about 10 min (data not shown), as has been found previously for the IAA response of pea segments (23, 24).

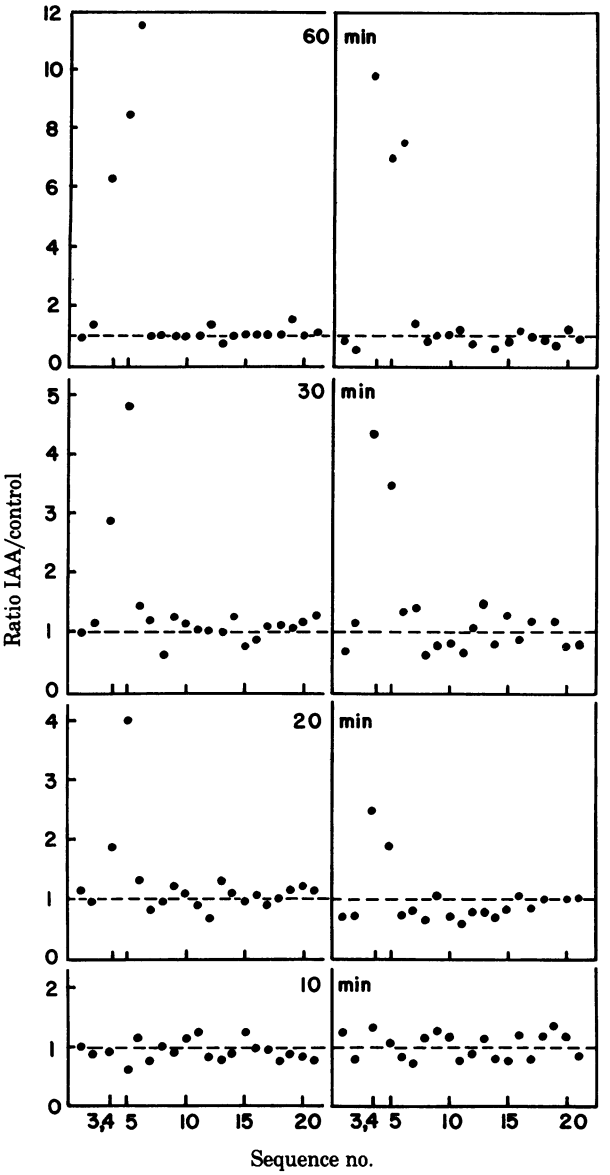

FIG. 2. Ratio of radioactivities in IAA-exposed samples to those in controls (ratio IAA/control) for 21 *in vitro* translation products during the first hour of IAA treatment in two experiments, *Left* and *Right*. Polypeptides 1–6 are those numbered in Fig. 1; the remainder are other translation products consistently detectable in autoradiograms like those in Fig. 1. Data points above 3,4 on the ordinate refer to combined spots 3 and 4. Each datum is the ratio of the incorporated ^{35}S in the given polypeptide from the IAA-treated sample, to the ^{35}S incorporated in the same polypeptide from a non-IAA-treated control sample harvested at the same time of incubation.

DISCUSSION

The results show that IAA increases the translational activity, in the wheat germ system, of three polyadenylylated mRNA sequences within 20 min, almost concomitantly with initiation of cell elongation in pea tissue. These responses are considerably more rapid than the well-studied gibberellin induction of α-amylase mRNA in aleurone cells (25, 26) or the phytochrome-mediated photoinduction of certain mRNAs (27). The response is comparable to the most rapid mRNA inductions by animal hormones (22, 28–31).

We do not know whether the observed increases in translational activity of specific poly(A)$^+$mRNA sequences are due to increase in the amount of mRNA or to enhancement of translatability. Studies with cDNA probes indicate, however, that changes in activity of mRNA in other systems, detected by the *in vitro* translation assay, are due to a net increase in the amount of mRNA (32–34). If an increase in mRNA levels is occurring here, it could be due to activation of transcription, activation of posttranscriptional processing, or selective stabilization of mRNA.

Rapid though they are, the earliest auxin-induced increases in mRNA activity presently detected in pea stem tissue do not occur prior to the onset of the elongation response (23, 24). Therefore, it seems impossible that the observed mRNA changes could lead to auxin stimulation of cell enlargement or of the H^+ pumping that apparently mediates it (35), especially because these messages would first have to be translated and their translation products possibly processed or moved to an appropriate intracellular site of action before they could exert an effect on H^+ pumping. Moreover, the auxin-stimulated mRNAs continue to increase for at least 1 hr (Fig. 2), whereas the growth rate reaches a maximum within 20 min. It is, of course, possible that minor mRNAs undetected by present methods respond even more rapidly to auxin and induce H^+ pumping and, thus, cell enlargement in the manner just indicated, but on the basis of the present findings we cannot advocate this.

We do not yet know the function of the polypeptides whose mRNAs show an early response to IAA. Previously reported IAA effects on enzyme activities such as cellulase (5), the synthase involved in ethylene production (36, 37), or the aspartyl

conjugating system for IAA (38), occur only over a time scale of several hours and, if based on mRNA changes, more plausibly involve some of the later-responding mRNA sequences, such as those for polypeptides 1 and 2. The early-responding sequences increase prior to the "second phase" of the auxin cell-enlargement response (13), which Vanderhoef (39) considers involves auxin-regulated gene expression. The early mRNA changes may well participate at least in this second phase.

Present results indicate a progressive increase, with time of hormone exposure, in the number of mRNA sequences responding to auxin. This suggests a temporal program of gene expression induced by auxin or else a "cascade" response in which some early-induced mRNA's code for regulatory polypeptides that affect the transcription or translatability of other messages, as has been suggested for development of certain animal hormone responses. Such a mechanism might explain why auxin-sensitive tissues show an early cell-enlargement response, followed hours later by a much more general stimulation of macromolecule biosynthesis and, in suitably competent tissues, eventually by cell division and other morphogenetic responses.

Although the earliest mRNA changes induced by auxin may not cause the initiation of cell enlargement, the speed of these increases seems to place them relatively close to primary auxin action. It will be of interest to know whether these increases are induced by auxin-stimulated H^+ pumping or represent a more direct action of the hormone.

Zurfluh and Guilfoyle (40) have recently obtained similar mRNA responses to IAA and 2,4-dichlorophenoxyacetic acid in soybean hypocotyl segments. These are the fastest mRNA responses yet reported for any plant growth regulator.

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