Regulation and \textit{in Vitro} Translation of Messenger Ribonucleic Acid for Cellulase from Auxin-treated Pea Epicotyls* \\

(Received for publication, June 11, 1974)

Desh Pal S. Verma, Gordon A. MacLachlan, Henry Byrne,† and Dawn Ewing§

From the Department of Biology, McGill University, Montreal, Quebec, Canada, H3C 3G1

SUMMARY

Polysomal RNA was isolated from pea epicotyls treated with (2,4-dichlorophenoxy)acetic acid, and fractionated on oligo(dT)-cellulose to yield poly(A)-containing RNA. This RNA fraction was translated in a wheat embryo cell-free system and found to have more than 90% of the messenger activity in total polysomal RNA. Immunoprecipitation of the translation product by monospecific antibodies to pea cellulases (β-1,4-glucan 4-glucanohydrolase, EC 3.2.1.4) indicated that cellulase was synthesized in this system. The immunoprecipitate co-migrated with the buffer-soluble cellulase component in sodium dodecyl sulfate-gel electrophoresis. Buffer-insoluble cellulase was not detected in the \textit{in vitro} translation products. Fractionation of mRNA from membrane-bound and free polysomes and their subsequent translation indicated preferential synthesis of buffer-soluble cellulase on membrane-bound polysomes. With the above techniques for assaying buffer-soluble cellulase mRNA, a 10-fold increase in the level of this messenger per tissue segment was observed within 48 hours following (2,4-dichlorophenoxy)acetic acid treatment. There was no evidence for pre-existing untranslatable message for cellulase in control tissues. Since there was no delay in the appearance of mRNA for cellulase, compared to a 24-hour lag in the increase of cellulase activity, it is suggested that translational as well as transcriptional controls are exerted on the biosynthesis of cellulase \textit{in vivo}. Analysis of the rates of peptide chain initiation and elongation, both \textit{in vivo} and \textit{in vitro}, indicated that peptide chain elongation may be rate-limiting during the lag phase of cellulase development.

Treatment of pea epicotyls with auxin-type growth regulators such as the hormone indoleacetic acid, or the analogue (2,4-dichlorophenoxy)acetic acid, results in a specific increase in cellulase activity (1–5). This enzyme activity has now been resolved into two separate components (6). One is buffer-soluble and the other buffer-insoluble, and both appear in about equal amounts in response to auxin treatment. It was shown (4, 7) that a crude microsomal fraction isolated from indoleacetic acid-treated tissue supported \textit{in vitro} protein synthesis with a concomitant increase in cellulase activity. Tentatively it was concluded that this fraction contained polyribosomes which could synthesize cellulase \textit{in vitro}. However, these experiments did not provide direct evidence for cellulase synthesis or \textit{de novo} synthesis of its mRNA in response to hormone treatment. We describe here the isolation and \textit{in vitro} translation of mRNA for cellulase from (2,4-dichlorophenoxy)acetic acid-treated peas.

Several messenger RNAs have now been isolated from animal tissues on the basis of the presence of poly(A), and translated \textit{in vitro} in homologous or heterologous systems (8–13). Similarly, there is evidence that plant mRNA contains poly(A) (14, 15) and we have recently isolated a message for leghemoglobin from nitrogen-fixing soybean root nodules and translated it in the wheat embryo cell-free system (16). The synthesis and detection of a specific enzyme is more difficult, since the product is likely to represent a relatively small percentage of the total protein (17, 18). This necessitates the use of extremely sensitive techniques. In cases of inducible enzymes, pretreatment of tissue with inducer may result in increased availability of translatable messenger for the enzyme-protein (10, 18, 19).

In this study, monospecific antibodies were prepared against pure cellulase (6) and used to precipitate products synthesized in a heterologous wheat embryo cell-free protein-synthesizing system directed by poly(A)-containing RNA from (2,4-dichlorophenoxy)acetic acid-treated peas. The immunoprecipitate was characterized by co-electrophoresis with authentic cellulase on sodium dodecyl sulfate gels. With this assay technique, cellulase mRNA was found to appear earlier than detectable enzyme activity in the tissue, suggesting that both translational and transcriptional controls are exerted on cellulase biosynthesis following auxin treatment.

\textbf{EXPERIMENTAL PROCEDURE}

\textit{Materials}—[\textit{H}]poly(U), K+ salt (specific activity 47 Ci per mole of phosphorus), was from Schraczmann; [\textit{U-14C}]leucine (specific activity 300 mCi per mmol), \textit{l-[\textit{4,5,3-3H}]leucine} (specific activity 30 Ci per mmol), and [\textit{2-3H}]glycine (specific activity 9.4 Ci per mmol) were from New England Nuclear. Cretinine phosphate kinase (Li salt) was from Boehringer-Mannheim Biochemicals. Oligo(dT)-cellulose was from Collaborative Research Inc.,

* This study was supported by a grant from the National Research Council of Canada (to G.A.M.) and scholarships from the National Research Council (H.B.) and the McConnell Foundation (D.E.).

† Present address, Biology Department, Carleton University, Ottawa, Canada.

§ Present address, Biology Department, McMaster University, Hamilton, Ontario, Canada.

---

This is an Open Access article under the CC BY license.
Waltham, Mass., and tobegy mosaic virus RNA was a gift from Dr. A. Marcus, Institute for Cancer Research, Philadelphia.

**Growth and Treatment of Plants—** *Pisum sativum, L.* var. Alaska seeds were grown in darkness for 7 to 8 days and intact seedlings were treated with (2,4-dichlorophenoxy)acetic acid as described in the preceding paper (6). Subapical segments (10 mm) were excised and used as source of celluus and polysomes.

**Isolation of Total Cellular mRNA**—Twenty subapical segments from (2,4-dichlorophenoxy)acetic acid-treated epicotyls were homogenized in 6 ml of extraction medium: 150 mM Tris-acetate, pH 8.5, 200 mM sucrose, 50 mM KCl, 20 mM magnesium acetate, 1 mM mercaptoethanol, and 0.4% Nonidet P-40 (Shell Chemical Co.). The slurry was centrifuged for 10 min at 20,000 × g and the supernatant was layered on a 3 ml cushion of 24% sucrose, 50 mM Tris-acetate, pH 8.5, 20 mM KCl, and 10 mM magnesium acetate. After centrifuging for 90 min at 105,000 × g, the polysomal pellet was resuspended with a Dounce homogenizer in 0.4 M NaCl, 10 mM Tris-acetate, pH 7.6, and RNA was precipitated with 2.5 volumes of 100% ethanol at 20°C. The pellets were collected on Whatman 3MM filters, washed three times with phosphate-buffered saline and either counted directly following hot trichloroacetic acid precipitation or dissolved in sodium dodecyl sulfate buffer (25) in preparation for electrophoresis.

**Isolation of Poly(A)-containing RNA**—From Polysomes—The polysomal fraction was collected on a 3 ml cushion of 40% sucrose containing 150 mM Tris-acetate, pH 9.0, 0.1 mM NaCl, 2 mM NaEDTA, and was then made 1% with sodium dodecyl sulfate at room temperature. RNA was extracted with chloroform-phenol (12) and fractionated on oligo(dT)-cellulose as described previously (16). Samples were applied to column in 0.4 M NaCl, 10 mM Tris-acetate, pH 7.6, and 50% sodium dodecyl sulfate and washed with the same solution minus sodium dodecyl sulfate to remove all nonadenosine RNA. The fractions which eluted subsequently with 10 mM Tris-acetate, pH 7.6, contained most of the poly(A) RNA. Fractions were pooled, made 2% with potassium acetate, pH 5.5, and precipitated with 2.5 volumes of ethanol at 20°C. The RNA was collected by centrifugation, dried in air and dissolved in water.

**Polysome Preparation**—Polysomes were isolated with high ionic strength buffer at high pH (20, 21). Twenty tissue segments were frozen in liquid nitrogen immediately after excision, ground with a mortar, and homogenized in 6 ml of extraction medium: 150 mM Tris-acetate, pH 8.5, 200 mM sucrose, 50 mM KCl, 20 mM magnesium acetate, 1 mM mercaptoethanol, and 0.4% Nonidet P-40 (Shell Chemical Co.). The slurry was centrifuged for 10 min at 20,000 × g and the supernatant was layered on a 3 ml cushion of 24% sucrose, 50 mM Tris-acetate, pH 8.5, 20 mM KCl, and 10 mM magnesium acetate. After centrifuging for 90 min at 105,000 × g, the polysomal pellet was resuspended with a Dounce homogenizer in 0.4 M NaCl, 10 mM Tris-acetate, pH 7.6, and RNA was precipitated with 2.5 volumes of 100% ethanol at 20°C. The pellets were collected on Whatman 3MM filters, washed three times with phosphate-buffered saline and either counted directly following hot trichloroacetic acid precipitation or dissolved in sodium dodecyl sulfate buffer (25) in preparation for electrophoresis.

**Translation of Pea mRNA in Wheat Embryo Cell-free System—** Poly(A)-containing RNA prepared from pea polysomes was translated in a standard amino acid incorporation system from wheat (24) which contained in a final volume of 0.4 ml: 100 to 200 µg of ribosomes, 20 µg of TRNA, 0.08 ml of postribosomal supernatant, 45 mM KCl, 3.5 mM magnesium acetate, 1 mM ATP, 25 µM GTP, 8 mM creatine phosphate, 16 µg of creatine phosphate kinase, 25 mM Tris-acetate, pH 8.0, 2 mM dithiothreitol, and 0.1 µCi of [3H]leucine. Tobacco mosaic virus RNA was used as control messenger. All incubations were carried out for 60 min at 30°C. Reactions were stopped by adding 0.2 ml of 15% trichloroacetic acid containing 1 ml unlabeled leucine and 5.0 ml of 5% trichloroacetic acid. Radioactivity in hot trichloroacetic acid precipitable material was measured as described above.

**Immuno precipitation Reaction**—Antisera to purified BS and BI cells and labeled peptides were prepared as described previously (6). Precipitation of celluus by antibodes was performed as follows. Polyclonal antibody from (2,4-dichlorophenoxy)acetic acid-treated peas was prepared in the wheat embryo cell-free system, and after incubation unlabeled amino acid plus 10 µg each of purified BS and BI celluus were added. The naseent peptides and ribosomes were removed by centrifuging for 1 hour at 105,000 × g. The precipitate was collected by centrifuging at 25,000 × g for 20 min, dissolved in a small volume of Tris-acetate buffer at 10 volts per cm for 120 min at 25°C. Antiserum was applied to a column of BS or BI cellulase and either counted directly following hot trichloroacetic acid precipitation or dissolved in sodium dodecyl sulfate buffer (25) in preparation for electrophoresis.

**Immuno electrophoresis**—The monospecificity of antisierum to BS cellulus was tested by imm unc eletrophoresis. Slides were coated with 1% agarose (Sigma) in sodium barbital buffer, pH 8.6, 0.5 ionic strength. Pure and crude BS celluus were applied in separate wells and electrophoresis was performed in the above buffer at 10 volts per cm for 24 hours at 2°C. The slides were washed, dried, and stained with acid fuchsin.

**Sodium Dodecyl Sulfate Gel Electrophoresis—** Purified BS and BI celluus were labeled in vitro with [3H]dimethylsulfate and subjected to electrophoresis on 7.5% sodium dodecyl sulfate gels as described previously (6). The immunoprecipitated product from in vitro protein synthesis was washed three times with phosphate-buffered saline, solubilized in sodium dodecyl sulfate buffer (25), and run on duplicate gels. Gels were cut into 2-mm slices and radioactivity was measured by solubilizing the gels in H2O2 and counting in PCS (Amersham-Searle).

**Relative Rate of Peptide Chain Elongation in Vivo**—Epicotyls from control and (2,4-dichlorophenoxy)acetic acid-treated peas were supplied with 20 µl of 0.5 µCi of [3H]leucine and 1.5 µCi of [3H]leucine, respectively, for 40 min through the base of the inter node 3 cm below the apex. Subapical segments were excised, mixed together, and polysomes were isolated as described above. More than 95% of radioactivity in hot trichloroacetic acid-precipitated protein was associated with polysomes and the postribosomal supernatant. Labeled material from the supernatant was considered to represent newly generated complete protein chains (26). Polyomone-bound label was regarded as mainly nascent protein, since puromycin or EDTA treatment released more than 95% of the radioactivity, which was then precipitable by hot trichloroacetic acid. Ribosomal proteins did not become significant in the soluble fraction. Protein synthesis was washed three times with phosphate-buffered saline and either counted directly following hot trichloroacetic acid precipitation or dissolved in sodium dodecyl sulfate buffer (25) in preparation for electrophoresis.

**Peptide Chain Elongation in Vitro**—A heterologous system from wheat and pea was used to study rates of peptide chain initiation and elongation. Pea polysomes were isolated as described above and resuspended in a medium containing 20% glycerol, 10 mM Tris-
acetate, pH 7.5, 20 mM KCl, 1 mM magnesium acetate, and 4 mM mercaptoethanol. Aliquots of polysomes (100 µg of RNA) were incubated in the wheat embryo cell-free system (minus ribosomes) as described above. Under these conditions, polysomes primarily complete nascent chains (elongation) and there is little (10 to 20%) reinitiation as tested by use of the inhibitor aurintricarboxylic acid (24, 27).

Before testing for the presence of soluble elongation factor activity, pea supernatant was partially purified to remove any inhibitors (28) as follows. Fifty subapical segments were frozen in liquid nitrogen, ground in a mortar, and homogenized in 6 ml of 50 mM Tris-acetate, pH 7.5, 1 mM dithiothreitol, 400 mM sucrose, and 1 mM magnesium acetate. The slurry was further homogenized with a Polytron for 2 sec at a setting of 4. The brei was centrifuged as described above to pellet ribosomes and the postribosomal supernatant was fractionated with a saturated solution of ammonium sulfate adjusted to pH 7.4 with NH₄OH (29). The precipitate between 40% and 65% saturation was dissolved in 0.4 ml of 10 mM Tris-acetate, pH 7.6, 50 mM KCl, and 4 mM mercaptoethanol, and desalted on a column (3 × 0.9 cm) of Sephadex G-25 (fine) which had been equilibrated with the same buffer. This preparation had no inhibitory effect on the complete wheat incorporation system, and it was able to replace the wheat supernatant insofar as it supported peptide chain elongation in that system.

RESULTS

Generation of Cellulase after (2,4-Dichlorophenoxy)acetic Acid Treatment—Total cellulase activity increased several fold after (2,4-dichlorophenoxy)acetic acid treatment of intact pea epicotyls (Fig. 1). This increase was comparable to that observed following treatment of the decapitated epicotyl with the hormone indoleacetic acid (1-5). The activity was distributed almost equally between BS and BI cellulase (cf. 6) throughout the course of development. Both enzyme activities showed a distinct lag of about 24 hours, after which they increased at a linear rate. The parallel kinetics, and identical responses to inhibitors of protein and nucleic acid synthesis (data not shown), imply a common mode of biosynthesis of the two enzymes.

Crude microsomes isolated from hormone-treated peas have been shown (7) to support in vitro protein synthesis and generate cellulase activity during incubation. This suggested the presence of polysomes containing mRNA for cellulase in the microsomal fraction. Density gradient profiles of ribosomal preparations from control and (2,4-dichlorophenoxy)acetic acid-treated pea epicotyls (Fig. 2) showed that a high percentage (80-90%) of ribosomes were present as polysomes. This was due to a marked increase in the net amount of ribosomes in auxin-treated tissue and not due to conversion of monosomes to polysomes. In order to investigate whether a specific mRNA for cellulase is formed in response to auxin treatment, we have attempted to isolate and translate the messenger RNA in vitro in a heterologous cell-free system from wheat embryos.

Isolation of Pea mRNA—When total polysomal RNA was isolated from control and (2,4-dichlorophenoxy)acetic acid-treated pea epicotyls as described under “Experimental Procedure,” and fractionated on an oligo(dT)-cellulose column, the bulk of the RNA passed through the column (Fig. 3). Subsequent washing with lower salt concentrations resulted in elution of a small peak of RNA. The presence of poly(A) in these fractions was tested by hybridization of the unlabeled RNA to [3H]poly(U). As shown in Fig. 3, most of the poly(A)-containing RNA eluted with buffer at low ionic strength. This was the effective fraction in supporting in vitro protein synthesis in the wheat embryo cell-free system (Table I).

Translation of Pea mRNA—Characteristics of the wheat embryo cell-free system used for in vitro protein synthesis are given in Table I. It was completely dependent on addition of exogenous mRNA, and the process of initiation (30), since the inhibitor of initiation, aurintricarboxylic acid, prevented translation (see also Refs. 24, 27). Peptide chain initiation and

Fig. 1. Increase in cellulase activity following (2,4-dichlorophenoxy)acetic acid treatment of intact pea epicotyls. Seven-day-old dark-grown peas were treated with (2,4-dichlorophenoxy)acetic acid as described under “Experimental Procedure” and cellulase was isolated from 20 subapical segments (30 mm). The total cellulase activity was resolved into buffer-soluble (BS) and buffer-insoluble (BI) components (6). Cellulase levels in control (untreated) tissue did not change.

Fig. 2. Polysome profiles from apical 10-mm segments of (A) control and (B) (2,4-dichlorophenoxy)acetic acid (2,4-D)-treated (24 hours) intact pea epicotyls. Polysomes were isolated from 20 segments immediately after excision as described under “Experimental Procedure.” (2,4-Dichlorophenoxy)acetic acid treatment resulted in 90% more ribosomes per segment within 24 hours and size distribution of polysomes remained unchanged (80 to 90% polysomes).
Fig. 3. Oligo(dT)-cellulose chromatography of polysomal RNA. Polysomes were prepared from control or (2,4-dichlorophenoxy)acetic acid-treated tissue (48 hours) and RNA was extracted from polysomes as described under “Experimental Procedure.” RNA was dissolved in 0.4 M NaCl, 10 mM Tris, pH 7.6, and 0.5% sodium dodecyl sulfate, and applied at A to an oligo(dT)-cellulose column which was equilibrated in the same buffer system. At B, the column was washed with this buffer system minus sodium dodecyl sulfate until no further RNA was eluted. The small amount of RNA which remained bound to the column was eluted starting at C with low ionic strength buffer, (0.01 M Tris, pH 7.6). The presence of poly(A) in RNA fraction was detected by hybridization to [3H]poly(U). The fractions which eluted from the column starting at C had most of the poly(A)-containing RNA. It was precipitated with ethanol and used for translation studies. Controls with mRNA or rRNA did not bind any poly(U).

Characterization of Translation Product—The presence of cell-free-mRNA available in the translation products directed by pea mRNA was tested with antisera prepared against purified BS and BI cellulases as described under “Experimental Procedure.” Following translation, reaction mixtures were centrifuged to remove ribosomes and nascent protein, and antisera were added to the supernatant. As shown in Table II, there was very little immunoprecipitation in the product formed in vitro in a system directed by control pea mRNA. However, in similar preparations the use of mRNA from (2,4-dichlorophenoxy)acetic acid-treated peas, 63% of the total radioactivity was precipitated. This immunoprecipitate was analyzed by sodium dodecyl sulfate-gel electrophoresis along with pure BS and BI cellulases which had been labeled with [3H]dimethylsulfate in vitro (6). As shown in Fig. 4, the bulk of the immunoprecipitated radioactivity co-migrated with BS cellulase but no radioactivity coincided with 13s cellulase. Since the antiserum to BS cellulase was considered to be the BS cellulase component.

Fig. 3. Oligo(dT)-cellulose chromatography of polysomal RNA. Polysomes were prepared from control or (2,4-dichlorophenoxy)acetic acid-treated tissue (48 hours) and RNA was extracted from polysomes as described under “Experimental Procedure.” RNA was dissolved in 0.4 M NaCl, 10 mM Tris, pH 7.6, and 0.5% sodium dodecyl sulfate, and applied at A to an oligo(dT)-cellulose column which was equilibrated in the same buffer system. At B, the column was washed with this buffer system minus sodium dodecyl sulfate until no further RNA was eluted. The small amount of RNA which remained bound to the column was eluted starting at C with low ionic strength buffer, (0.01 M Tris, pH 7.6). The presence of poly(A) in RNA fraction was detected by hybridization to [3H]poly(U). The fractions which eluted from the column starting at C had most of the poly(A)-containing RNA. It was precipitated with ethanol and used for translation studies. Controls with mRNA or rRNA did not bind any poly(U).

Characterization of Translation Product—The presence of cellulase-like material in the translation products directed by pea mRNA was tested with antisera prepared against purified BS and BI cellulases as described under “Experimental Procedure.” Following translation, reaction mixtures were centrifuged to remove ribosomes and nascent protein, and antisera were added to the supernatant. As shown in Table II, there was very little immunoprecipitation in the product formed in vitro in a system directed by control pea mRNA. However, in similar preparations the use of mRNA from (2,4-dichlorophenoxy)acetic acid-treated peas, 63% of the total radioactivity was precipitated. This immunoprecipitate was analyzed by sodium dodecyl sulfate-gel electrophoresis along with pure BS and BI cellulases which had been labeled with [3H]dimethylsulfate in vitro (6). As shown in Fig. 4, the bulk of the immunoprecipitated radioactivity co-migrated with BS cellulase but no radioactivity coincided with 13s cellulase. Since the antiserum to BS cellulase was monospecific, i.e. only one precipitation band was observed, it was concluded that the immunoprecipitated material was BS cellulase.

Table I

| Reaction components | Incorporation of [3H]leucine for 60 min at 30°C (cpm) |
|---------------------|--------------------------------------------------|
| Complete System     |                                                  |
| Tobacco mosaic virus RNA as messenger (5 μg)      | 25,400                                           |
| Poly(A)-containing pea RNA (6.4 μg)               | 23,300                                           |
| Poly(A)-minus pea RNA (60 μg)                     | 1,550                                            |
| Deletions           |                                                  |
| Messenger RNA       | 1,200                                            |
| Wheat ribosomes     | 252                                              |
| Supernatant factors | 140                                              |
| Energy-generating system | 53                                      |
| Additions           |                                                  |
| 19 Amino acid mixture | 21,100                                       |
| Pea ribosomal wash  | 21,000                                           |
| Arabinosecetic acid (50 μL)                        | 250                                              |

Table II

Identification of cellulase by immunoprecipitation of product synthesized in cell-free systems directed by exogenous mRNA

Pea RNA was isolated from control and (2,4-dichlorophenoxy)acetic acid-treated tissue as described in Fig. 3 and poly(A)-containing RNA was translated in standard wheat embryo cell-free systems as in Table I (with 200 μL as the total volume per reaction mixture). Immunoprecipitation reactions were performed with anti-BS and anti-BI cellulase sera as described in Fig. 4. Aliquots treated with control rabbit serum precipitated 50 to 150 cpm.

| Source of messenger RNA | Total incorporation | Anti-cellulase precipitate |
|-------------------------|---------------------|---------------------------|
| Tobacco mosaic virus RNA (5 μg) | 13,500 | 52 | 0.4 |
| mRNA from untreated pea (2.3 μg) | 10,900 | 96 | 0.8 |
| mRNA from (2,4-dichlorophenoxy)acetic acid-treated pea (18 μg) | 19,000 | 1200 | 6.3 |
were allowed to react with antisera prepared against US cellu-
rate from (2,4-dichlorophenoxy)acetic acid-treated pea epicot-

Further studies were carried out on the biosynthesis and regula-
tion of BS cellulase only.

Although both cellulases are generated in vitro in near equal
amounts after (2,4-dichlorophenoxy)acetic acid treatment (Fig.
1), the buffer-insoluble component was not detected in the pro-
tein synthesized in vitro. Since the technique used in these ex-
periments would detect only soluble proteins, it is possible that
BI cellulase peptides were synthesized but removed along with
nascent peptides and ribosomes because they were insoluble.
Alternatively, the newly synthesized molecule may have required
further modification before antisera would recognize it, or its
nascent peptides and ribosomes because they were insoluble.

While both cellulases are generated in vivo in near equal
cellulase levels following (2,4-dichlorophenoxy)acetic acid
treatment, translated in the wheat embryo cell-free system, and the product was immuno-
lase as above. Table III shows that total incorporation sup-
ported by the membrane-bound mRNA was comparable to that
by mRNA from free polysomes, but there was a much higher
percentage of radioactivity (4-fold) precipitated by anti-BS
cellulase serum in the product made by the former. These
results indicate that BS cellulase is preferentially synthesized
on membrane-bound polysomes.

\[ \text{Table III} \]

| Source of mRNA | Total incorporation per 4 \( \mu \)g of mRNA | Anti-BS cellulase precipitation |
|---------------|---------------------------------------------|--------------------------------|
| Free polysomes | 4170                                        | 178                           | 4.2 |
| Membrane-bound polysomes | 4270 | 654 | 15.3 |

---

**Fig. 5. Immunoelectrophoresis of BS cellulase.** Purified BS

![Image of BS cellulase immunoelectrophoresis](image)

**FIG. 5.** Immunoelectrophoresis of BS cellulase. Purified BS

cellulase \((P)\) and crude buffer-soluble extract \((C)\) were subjected
to electrophoresis on an agarose slide as described under "Experi-
mental Procedure," and antiserum to BS cellulase was applied as
a strip between the two samples (boundary indicated). Reaction
was allowed to proceed at 4° for 24 hours and slides were dried and
stained (6).

**TABLE III**

**Synthesis of buffer-soluble cellulase in vitro with poly(A)-containing
RNA from free and membrane-bound polysomes**

Polysomes from 20 segments of (2,4-dichlorophenoxy)acetic
acid-treated pea epicotyls (48 hours) were separated into free and
membrane-bound components as follows. Tissue was homogen-
edized in 0.05 M Tris, pH 8.2, 0.5 m KCl, and 0.005 m magnesium
acetate, and centrifugated at 23,000 \( \times g \) to yield a supernatant
containing free polysomes. The pellet was resuspended in 0.15 m
Tris acetate, pH 7.5, 0.05 M KCl, 10 mm magnesium acetate, and
0.5% Nonidet P-40 detergent (Shell Chemical Co.), and cen-
trifugated at 23,000 \( \times g \) to yield a supernatant containing

demand-bound polysomes. Both polysome preparations were
pelleted through 3 ml of sucrose cushion as described under "Ex-
perimental Procedure," and total polysomal RNA was fraction-
ated on oligo(dT)-cellulose as in Fig. 3. The yield of poly(A)-
containing RNA was 22.2 and 12.0 \( \mu \)g/20 segments from free and

---

**Fig. 4. Sodium dodecyl sulfate gel electrophoresis of the prod-
uct from in vitro translation of cellulase mRNA which was preci-
cipitated by cellulase antisera.** Poly(A)-containing RNA pre-
pared as in Fig. 3 from polysomes of (2,4-dichlorophenoxy)acetic
acid-treated (48 hours) pea epicotyls was translated as in Table I
with 4.2 \( \mu \)g of mRNA per incubation mixture (four samples, 400 \( \mu \)l
each). Both \([\text{H}]\)leucine and \([\text{H}]\)glycine were employed as source
of label since glycine represented a large fraction (12%) of total
amino acids in soluble cellulase (6). After incubation, carrier BS
and BI purified polysomes were added, and the ribosomes were
removed by centrifugation. The supernatant was dialyzed against
phosphate buffer-saline and concentrated by lyophilization to 0.8
ml. Aliquots (0.2 ml) were allowed to react with antisera against
BS and BI cellulases. The immunoprecipitates were pooled and
washed three times with phosphate buffer-saline and dissolved in
sodium dodecyl sulfate buffer (25). They were applied to 7.5% acrylamide gels in sodium dodecyl sulfate (6 \( \times 0.6 \) cm) and sub-
jected to electrophoresis in sodium dodecyl sulfate buffer (6). Purified BS and BI cellulases were methylated with \([\text{H}]\)dimethyl
sulfate and subjected to electrophoresis as markers on duplicate
gels. Gels were fractionated and radioactivity was determined
as described under "Experimental Procedure."
This RNA is also regulated at the level of translation in vivo. The per-
centage of pre-existing nonfunctional mRNA for cellulase in zero
time control tissue. The fact that a several-fold increase in
precipitable counts with the total translation product, assuming equal
efficiency for translation of all messages.
precipitated with anti-BS cellulase serum. The levels of mRNA
for BS cellulase in relation to BS cellulase activity are shown in
Fig. 6. Following (2,4-dichlorophenoxy)acetic acid treatment,
there is a linear increase in amount of translatable mRNA for
BS cellulase during the first 48 hours, whereas cellulase activity
develops linearly only after a lag of about 24 hours. The
poly(A)-containing fraction was isolated using oligo(dT)-cellulose
chromatography and translated in the standard wheat embryo
cell-free system. The product synthesized in vitro was allowed to
react with antiserum to BS cellulase as in Fig. 4 in order to assay
the level of mRNA for this enzyme. The percentage of mRNA
for cellulase was calculated by comparing anti-BS-cellulase pre-
cipitable counts with the total translation product, assuming equal
initiation rates for translation of all messages.

Dichlorophenoxy)acetic Acid Treatment—Preliminary data on the rate of peptide chain elongation during (2,4-dichlorophenoxy)acetic acid treatment of pea epicotyls indicated that the total amount of polysomes increased about 50% within 24 hours (Fig. 2), whereas the absolute rate of protein synthesis per segment did not increase proportionately during this period. In order to investigate modulation of protein synthesis at the translational level, the relative rates of peptide chain initiation and elongation were determined in control and (2,4-dichlorophenoxy)acetic acid-treated tissue.

There is little likelihood that initiation of protein synthesis is rate limiting in the intact epicotyl since the percentage of total ribosomes present as polysomes remained very high with or without (2,4-dichlorophenoxy)acetic acid-treatment (Fig. 2). No more than 90% ribosomes could be recruited into polysomes in this tissue even when the rate of peptide chain elongation was deliberately retarded (by treatment with cycloheximide) in an effort to increase the number of ribosomes per messenger. If the rate of initiation was limiting, then treatment with cycloheximide should cause a marked change in polysome size distribution (cf. 26, 32). In the intact pea tissue, cycloheximide (used at a level which partially inhibits protein synthesis) did not alter the polysome profile, indicating that peptide chain initiation was not rate limiting.

The relative rate of peptide chain elongation in vivo was measured by a double labeling ratio method as described by Palmiter (26). Differentially labeled tissue segments from control and (2,4-dichlorophenoxy)acetic acid-treated peas were combined and the amounts of labeled protein in polysomes (nascent) and soluble fractions (released) were compared (see “Experimental Procedure”). This method assumes that differences in labeling in the two tissues are not accompanied by major changes in mean molecular weight of the proteins synthesized. The fact that polysome profiles from control and (2,4-dichlorophenoxy)acetic acid-treated tissues showed similar size distributions of polysomes (Fig. 2, A and B), imply that the mRNA populations were comparable with respect to length. Moreover, the mean molecular weight of proteins synthesized by control and (2,4-dichlorophenoxy)acetic acid-treated tissues was very close as measured by sodium dodecyl sulfate gel electrophoresis. The results (Table IV) show that (2,4-dichlorophenoxy)acetic acid-treatment causes a transient suppression of about 30% in the relative rate at which polysomes complete their peptide chains in vivo.

In order to measure translational capacity of pea polysomes in vitro, assays were conducted using wheat supernatant in which concentrations of initiation and elongation factors were non-limiting (21). Under these conditions, polysomes primarily complete nascent chains and there is little reinitiation (24). As shown in Table V the rate of translation of pea polysomes was

| Time after (2,4-dichlorophenoxy)acetic acid treatment | Relative rate of peptide chain elongation (compared to zero time control) | Reduction in elongation rate |
|------------------------------------------------------|---------------------------------------------------------------|-----------------------------|
| 0 hr                                                  | 1.01                                                         | %                           |
| 12 hr                                                 | 0.75                                                         | 25                          |
| 24 hr                                                 | 0.68                                                         | 32                          |
| 48 hr                                                 | 0.93                                                         | 7                           |

24-Hour control (untreated) 0.98 2

TABLE IV

Effect of (2,4-dichlorophenoxy)acetic acid treatment on the relative rate of peptide chain elongation in vivo

D. P. S. Verma, unpublished data.
Evidence for the synthesis of a specific cellulase protein in this heterologous system was obtained by identification of the translation product with antiserum prepared against purified cellulase. Since the total cellulase (RI + 1%) represented only a small fraction of total protein, several criteria were used to ensure homogeneity of the purified enzymes before preparation of antiserum. The fraction was free of any inhibitor of protein synthesis as found in crude pea supernatant (28).

| Time after (2,4-dichlorophenoxy)acetic acid treatment | Wheat supernatant plus polysomes from (2,4-dichlorophenoxy)acetic acid-treated peas | Supernatant from (2,4-dichlorophenoxy)acetic acid-treated peas plus control pea polysomes |
|------------------------------------------------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| hr                                                   | cpm/100 pg polysomes                                                            |                                                                                   |
| 0                                                    | 15,900                                                                           | 3,470                                                                             |
| 12                                                   | 14,400                                                                           | 2,900                                                                             |
| 24                                                   | 15,500                                                                           | 1,800                                                                             |
| 48                                                   | 14,200                                                                           | 4,210                                                                             |

Lack of detection of B1 cellulase in the in vitro translation products does not necessarily indicate that mRNA for this enzyme was not present or not translated in the system. Newly formed B1 cellulase may have been insoluble in the reaction medium (which contained only approximately 0.05 M total salts) and thus removed from the bulk of the soluble translation products along with ribosomes and nascent peptides. Alternatively, the newly synthesized B1 peptides may have been different from the native molecule and not recognized by B1 antiserum. Concurrent increase of mRNA for B1 and BS cellulases probably occurs since BS and B1 cellulase activities develop with parallel kinetics (Fig. 1) and there is no precursor-product relationship between them (6). Translation of the mRNA for B1 cellulase, however, may depend on more complex cellular organization, e.g. a membrane site into which newly completed buffer-insoluble peptides are lodged. Efforts to locate this messenger and translate it are in progress.

With respect to the intracellular site for cellulase biosynthesis, the mRNA isolated from membrane-bound polysomes contained most of the BS cellulase message (Table III). Like other secreted proteins (35), cellulase may be preferentially synthesized on membranes and transported to the cell wall through Golgi or endoplasmic reticulum vesicles (36). Preferential synthesis of another secreted protein (rat growth hormone) in vitro has also been demonstrated (37) with mRNA from membrane-bound polysomes.

**Induction of Cellulase mRNA**—Cellulase activity has been shown to be specifically generated in pea epicotyl tissue as a result of auxin treatment (1–5). Evidence that this increase in cellulase is due to the appearance of specific mRNA was provided by assaying level of mRNA in a cell-free system for translation. In order to rule out the possibility that a non-functional reserve of mRNA (not in polysomes) pre-existed in untreated tissue, mRNA was prepared from total cellular RNA, and poly(A)-containing RNA was tested for ability to support translation in vitro synthesis of cellulase. Fig. 6 demonstrates that at zero time there was relatively little mRNA for cellulase in untreated tissue, and that the messenger level increased almost linearly for at least 48 hours following (2,4-dichlorophenoxy)acetic acid treatment. Unlike some systems, e.g. ovalbumin induction (19) where the development of ovalbumin mRNA and the protein are coincident, a specific increase in cellulase mRNA occurs before the appearance of cellulase activity. Effects of inhibitors of RNA and protein synthesis in vivo (1–3) showed that both RNA and protein synthesis are essential for development of cellulase activity. Thus present data is consistent with the contention that cellulase mRNA is synthesized de novo as a result of (2,4-dichlorophenoxy)acetic acid treatment.

**Regulation of Cellulase Synthesis following Auxin Treatment**—One possible explanation for an increase in mRNA for cellulase before the increase in enzyme activity following (2,4-dichlorophenoxy)acetic acid-treatment (Fig. 6) is inefficiency in the translation mechanism during the early period (0 to 24 hours). The initiation steps in translation did not appear to be rate limiting during that time since the polysome levels were very high (80 to 90%) with or without auxin treatments (Fig. 2). However, analysis of the rate of peptide chain elongation both in vivo (Table IV) and in vitro (Table V) indicated that a transient reduction of 30 to 40% occurred during the lag period of cellulase development. Elongation rates returned to normal (zero time) levels when cellulase activity began to rise linearly.
(24 to 48 hours). Reasons for this temporary reduction in rate of translation are not fully understood. The available evidence (Table V) suggests that polysomes themselves are equally active in control and (2, 4-dichlorophenoxy)acetic-acid-treated tissue when supplemented with exogenous soluble factors from wheat supernatant, but similar factors from pea appear to become rate limiting. Hormonal effects on translation have also been observed in other plant tissues, e.g. in cotton embryos, where the message for carboxypeptidase is available but not translated in the presence of the hormone abscisic acid (38, 39).

Since the appearance of cellulase in auxin-treated peas was detected by assaying for activity only and not for enzyme protein, the interpretation of translational controls in this system should be treated as tentative. Other mechanisms may be regulating cellulase biosynthesis in this system. For example, differential turnover rates of mRNA or enzyme, or both, could operate during development in such a way that net mRNA levels increase before enzyme. Hormones have been shown to affect polysome levels (40, 41) and changes in bound ribonuclease activity (42) during growth in the epicotyl. The availability of ribosomes to attach to mRNA could also be a limiting factor at both transcriptional and translational levels. Increases as a result of auxin treatment, and the process appears to be regulated at both transcriptional and translational levels. Finally, little is known about compartmentation of functional messenger in eucaryotic tissue.

The present study shows that a specific mRNA for cellulase increases as a result of auxin treatment, and the process appears to be regulated at both transcriptional and translational levels.

Acknowledgments—We wish to thank Dr. A. Marcus, Institute for Cancer Research, Philadelphia, for supplying wheat embryos and tobacco mosaic virus-RNA during initial stages of this work, and Dr. D. Fromson and Mr. N. Christou for helpful discussion.

REFERENCES

1. FAN, D. F., AND MACLACHLAN, G. A. (1966) Can. J. Bot. 44, 1025–1034
2. FAN, D. F., AND MACLACHLAN, G. A. (1967) Plant Physiol. 42, 1114–1122
3. FAN, D. F. (1967) Ph.D. thesis, McGill University
4. MACLACHLAN, G. A., DAVIES, E., AND FAN, D. F. (1968) in Biochemistry and Physiology of Plant Growth Substances, (Wightman, F. and Settfield, G., eds) pp. 443–453, Rungo Press, Ottawa
5. DAVIES, E., AND MACLACHLAN, G. A. (1968) Arch. Biochem. Biophys. 120, 595–600
6. BYRN, H., CHRISTOU, N. V., VERMA, D. P. S., AND MACLACHLAN, G. A. (1975) J. Biol. Chem. 260, 1012–1018
7. DAVIES, E., AND MACLACHLAN, G. A. (1969) Arch. Biochem. Biophys. 129, 381–387
8. LOCKARD, R. E., AND LARDER, J. B. (1969) Biochem. Biophys. Res. Commun. 37, 204–212
9. PEMBERTON, R. E., HOUSEN, D., LODISH, H. F., AND BAGLI- ONI, C. (1972) Nature New Biol. 236, 99–102
10. PALMITER, R. D., AND SMITH, L. T. (1973) Mol. Biol. Rep. 1, 129–134
11. O'MALLEY, B. W., RODENFIELD, G. C., COMSTOCK, J. P., AND MEANS, A. R. (1972) Nature New Biol. 240, 45–48
12. AVIV, H., AND LEDER, P. (1972) Proc. Nat. Acad. Sci. U. S. A. 69, 1408–1412
13. MORRISON, T. G., AND LODISH, H. F. (1973) Proc. Nat Acad. Sci. U. S. A. 70, 315–319
14. HOOGEN, T. J. V., MERCE, J. F. B., AND GOODWIN, P. B. (1973) Nature New Biol. 240, 68–80
15. MANAHAN, C. O., APP, A. A., AND STILL, C. C. (1973) Biochem. Biophys. Res. Commun. 53, 588–595
16. VERMA, D. P. S., NASH, D. T., AND SHULMAN, H. M. (1974) Nature 251, 74–77
17. SHAFRITZ, D. A., DRETSDALE, J. W., AND ISSELBACHER, K. J. (1972) J. Biol. Chem. 248, 3229–3227
18. SCHUTZ, G., BEATO, M., AND FEIGELSON, P. (1973) Proc. Nat. Acad. Sci. U. S. A. 70, 1918–1921
19. PALMITER, R. D. (1973) J. Biol. Chem. 248, 8260–8270
20. VERMA, D. P. S., AND MARCUS, A. (1974) Plant Physiol. 53, 233–237
21. DAVIES, E., LARKINS, B. A., AND KNIGHT, R. H. (1972) Plant Physiol. 50, 581–584
22. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951) J. Biol. Chem. 193, 265–275
23. VERMA, D. P. S., AND MARCUS, A. (1973) Develop. Biol. 30, 104–114
24. WICKS, J. D., AND MARCUS, A. (1971) Biochim. Biophys. Acta 232, 671–684
25. PALMITER, R. D., OKA, T., AND SCHIMKE, R. T. (1971) J. Biol. Chem. 246, 724–737
26. PALMITER, R. D. (1979) J. Biol. Chem. 247, 6770–6780
27. MARCUS, A., BEWLEY, J. D., AND WEEKS, D. P. (1970) Science 167, 1735–1736
28. COLTAS, A., AND PARTHIER, B. (1971) Biochem. Physiol. Pflanzen 162, 60–74
29. LIN, C. Y., AND KEY, J. L. (1973) Phytochemistry 12, 43–53
30. WICKS, J. D., VERMA, D. P. S., SEAL, S. N., AND MARCUS, A. (1972) Nature 236, 167–168
31. SEAL, S. N., BEWLEY, J. D., AND MARCUS, A. (1972) J. Biol. Chem. 247, 2592–2597
32. STANWICK, C. P. (1966) Biochem. Biophys. Res. Commun. 24, 1222–1226
33. EFRON, D., AND MARCUS, A. (1973) Fed. Eur. Biochem. Soc. Lett. 33, 23–27
34. LUNDQVIST, R. E., LAZAR, J. M., KLEIN, W. H., AND CLARK, J. M., JR. (1972) Biochemistry 11, 2014–2019
35. TATA, J. R. (1973) in Karolinia Symposia on Research Methods in Reproductive Endocrinology (Diefenbeuk, E., ed) pp. 192–224, Karoliniska Institute, Stockholm
36. MOLLENHAUER, H. H., AND MORRISON, T. G. (1973) Ann. Rev. Plant Physiol. 24, 27–49
37. BANKROFT, F. C., GUANO-JER, W. V., AND ZUBAY, G. (1973) Proc. Nat. Acad. Sci. U. S. A. 70, 3646–3649
38. IHN, J. N., AND DURF, L. S., III (1970) Biochem. Biophys. Res. Commun. 36, 955–1001
39. IHN, J. N., AND DURF, L. S., III (1972) J. Biol. Chem. 247, 5045–5055
40. TREWAVAS, A. (1968) Phytochemistry 7, 673–681
41. DAVIES, E., AND LARKINS, B. A. (1973) Plant Physiol. 52, 339–345
42. BIRMINGHAM, B. C., AND MACLACHLAN, G. A. (1971) Plant Physiol. 50, 371–374