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Short Communication

Protein Synthesis Patterns[1]

RELEVANCE OF OLD AND NEW MESSENGER RNA IN GERMINATING MAIZE EMBRYOS

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ABSTRACT

The proteins synthesized during the first hours of seed imbibition were studied in axes and scutellum of maize embryos separately. Increase in fresh weight was followed in the embryonic axes through the germination period. Pulse labeling experiments with ^{14}C-amino acids were carried out at two stages of development: 0 to 6 and 18 to 24 hours in the presence and absence of α-amanitin. The proteins were analyzed by two-dimensional gel electrophoresis and fluorography. Results showed a major pattern of proteins common to both tissues, axes and scutellum ('house keeping' proteins), besides the specific proteins synthesized by each tissue. In the axes, the changes in proteins observed between the periods of 0 to 6 and 18 to 24 hours of development seem to be due both to newly synthesized mRNA as well as to delayed translation of stored mRNA species.

The existence of stored mRNA in embryos of different quiescent cereal seeds has been described (2, 16, 18). Many reports have been concerned with finding out the role of the stored mRNA of the seed embryos (for review, see 14). Some authors have postulated that these mRNA are required basically for rapid resumption of metabolic activity in the seeds during germination (2, 13); others support the idea that they have been specifically synthesized during the maturation period and stored as ribonucleoprotein particles to insure germination under unfavorable conditions (17), while still others suggest that stored mRNA is required for new RNA transcription, which in turn supports the actual germination process (9).

Two well-defined developmental steps are recognized during seed germination: it has been demonstrated that the early elongation period of the radicle is supported by translation of the stored mRNA, while the further sustained growth period seems to require newly formed mRNA (4, 7, 9). The specific mRNA species required by each process must be clarified since there is no complete agreement in regard to the similarity or discrepancy of the information encoded by the stored and newly synthesized mRNA (2, 3). Furthermore, this picture is more complicated considering the possibility of processing of preformed transcripts among the stored mRNA population; this process could be responsible for changes in the protein pattern observed at different stages of embryo development (11). The present paper is an attempt to analyze the type of protein synthesized and their origin during the early stages of maize germination. Proteins made at two stages of germination in two different tissues of the maize embryo are compared in the presence or absence of α-amanitin which interferes with the synthesis of mRNA (9).

MATERIALS AND METHODS

Biological Materials. Seeds of maize (*Zea mays* L.), hybrid H-30 provided by Dr. Joaquín Ortiz Cereceres from the Colegio de Postgraduados de Chapingo, México, were used in this work. Embryonary axes and scutellum were manually cut off from the seeds. Each experiment was carried out with 100 to 150 mg of tissue.

Incubation System. Tissue samples were imbibed between two filter papers in 0.2 ml of incubation medium (50 mM Tris-HCl buffer, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 2% sucrose, 0.5 mM of an equimolar mixture of the 20 amino acids and 10 μg/ml chloramphenicol (8), in sterile 25-ml Erlenmeyer flasks at 25°C. A mixture of ^{14}C-labeled amino acids (20–30 μCi New England Nuclear) was added to the samples per pulse. In some experiments, the tissues were pretreated with α-amanitin (10 μg/ml) (20 min under vacuum). Controls were also vacuum treated without α-amanitin.

After the incubation period, the tissues were washed with 50 ml of buffer containing: 50 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, and 5 mM β-mercaptoethanol, at 0°C. Samples were homogenized in 1.5 ml of the same buffer in a mortar and pestle, precooled at 0°C. In some experiments, 0.1 mM PMSF (phenylmethylsulfonyl fluoride) were added to the homogenizing buffer. The homogenate was centrifuged at 15,000*g* for 30 min. The supernatant was again centrifuged at 190,000*g* for 80 min. Soluble proteins from the supernatant were precipitated with 10% TCA, allowed to stand at 0°C for 3 h, and centrifuged at 3,000 rpm for further analysis.

Electrophoretic Procedure. The precipitated proteins were suspended in water, neutralized with 4 M KOH, and an equal volume of ampholite-buffer mixture (LKB) either pH 3.5 to 10 or 5 to 8 was added. Electrophoresis in two dimensions was carried out following O'Farrell's procedure (15), except that in the second dimension 0.2% SDS was added to the polyacrylamide gels. The stacking gel was 5% and gel gradient from 7 to 15% was used as the running gel. A Bio-Rad model 220 apparatus was used and 30,000 to 40,000 cpm were applied to each isoelectric focusing gel.

Slab gel electrophoresis in one dimension was carried out following the method of Laemmli (10), except that the running separator gel was a 7 to 15% acrylamide gradient in 50 mM Tris-HCl buffer 0.2% SDS, pH 8.8. The proteins were run at a constant current at 1.5 mamps per channel. An electrophoresis calibration kit from Pharmacia (range 14,000–94,000 D) was

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used.

Fluorography. The method of Bonner and Laskey (1) was followed, and the dried gels were exposed to a Kodak X-0 mat S-5 film at −70°C for 1 to 3 weeks.

Protein Determination. After 10% TCA precipitation, samples were neutralized with 4 M KOH and proteins were measured by the method of Lowry *et al.* (12). BSA (Sigma) was used as standard.

RESULTS

Two main periods of development were studied for synthesis of proteins: the 0- to 6-h period of imbibition, when the embryos just reinitiate metabolic activity after a long period of quiescence, and the 18- to 24-h period, when the axes initiate radicle protrusion (Fig. 1). The protein synthesized by the maize axes in those periods were pulse-labeled with ^{14}C-amino acid mixture, and analyzed by two-dimensional gel electrophoresis, using a 3.5 to 10 pH range. Quantitative and qualitative changes are apparent among those proteins in the correspondent fluorographing prints (Fig. 2). These patterns, which are highly reproducible, show that many proteins synthesized at the later period do not appear on the earlier one (dashed squares), although some, present in the 0- to 6-h period are not in the 18- to 24-h one (dashed circle). The same experiments were carried out in the presence of α-amanitin. Experimental conditions were selected in which uracil incorporation into polysomal poly A$^+$ RNA was dramatically inhibited (90%, unpublished data). Under these conditions, negligible inhibitory effect was noticed on the amount of ^{14}C-amino acid incorporated into proteins of the 0- to 6-h period, but significant inhibition was obtained for the 18- to 24-h period (Table I).

These data indicate that, for the first period studied, the proteins being synthesized belong to stored mRNA, while for the second one a significant fraction of the synthesized proteins are already translated from the new mRNA. The proteins formed on the 18- to 24-h period in the presence of α-amanitin were analyzed by two-dimensional gel electrophoresis; in these experiments, a 5 to 8 pH range was used in order to achieve a better resolution, since most of the proteins appeared located in the central region of the 3.5 to 10 pH gels. The fluorography pattern with α-amanitin was compared with the one obtained for this period in the absence of the antibiotic (Fig. 3). A striking result was obtained since, as it can be seen in this figure, the 18- to 24-h patterns with and without α-amanitin are very similar. Most of the proteins formed in the absence of α-amanitin are also seen in its presence, indicating that they are formed from stored mRNA species. Some spots, however, are absent (arrow) or dramatically depleted (dashed squares) in the α-amanitin pattern, suggesting they belong to the newly synthesized mRNA.

It is also interesting to note that, in the two-dimensional fluorography of the α-amanitin pattern (Fig. 3B), some spots are more noticeable than in the gel without α-amanitin and even some new spots are visible (dashed oval) (Fig. 3). This experiment was repeated using [^{35}S]methionine instead of ^{14}C-amino acid mixture, and the proteins were analyzed by one-dimensional gel electrophoresis. A new band can also be observed in the fluorography of proteins made in the presence of α-amanitin.

In order to find out which set of proteins are related to the 'house keeping' functions of the cells and which are actually involved in the sustained growth process of germination, ^{14}C-amino acid pulse-labeled experiments were carried in scutellum. This embryonary tissue is not expected to divide nor to develop into the new plantlet but rather to function as metabolic sustainer and transporter of metabolites for the embryonic axes. The capacity of scutellum to synthesize proteins during these periods is much lower than the one observed in the axes (from 3 to 5 times lower), (18). However, fluorographic analysis of the scutellum proteins synthesized during the 0- to 6-h and 18- to 24-h period can be clearly observed (Fig. 4). As expected, a large background of proteins common to axes and scutellum are seen on these fluorographic prints (Figs. 2 and 4). The 0- to 6-h patterns are, however, more similar among themselves than each one with their correspondent 18- to 24-h pattern. A common group of proteins for the two 18- to 24-h patterns can also be recognized in the fluorographies. However, these two patterns differ more between themselves than the corresponding 0- to 6-h patterns, suggesting a more specialized stage of differentiation for both tissues in the second period.

DISCUSSION

Early work in rye showed that the proteins synthesized from the stored and newly synthesized mRNAs, when translated *in*

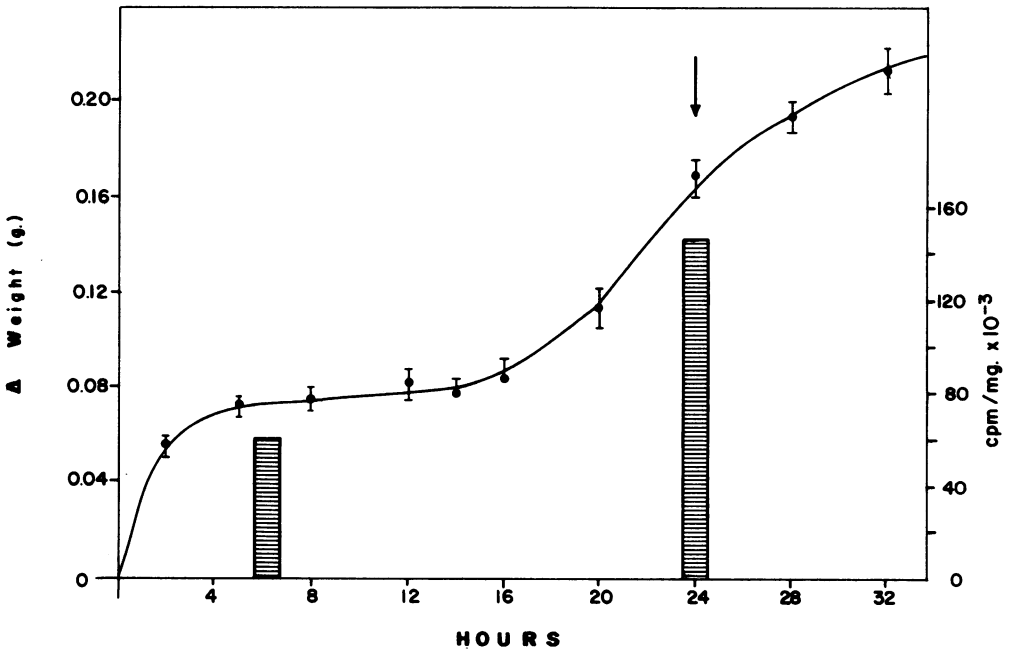

FIG. 1. Fresh weight increase of maize axes during germination. Each experimental value of the fresh weight represents average of at least three batches of 20 embryonary axes each. Vertical lines represent SD. Bars stand for ^{14}C-amino acid incorporation into TCA-precipitable material in a 6-h pulse-labeled experiment, after correction for total amino acid pool size. Arrow indicates average time for radicle protrusion.

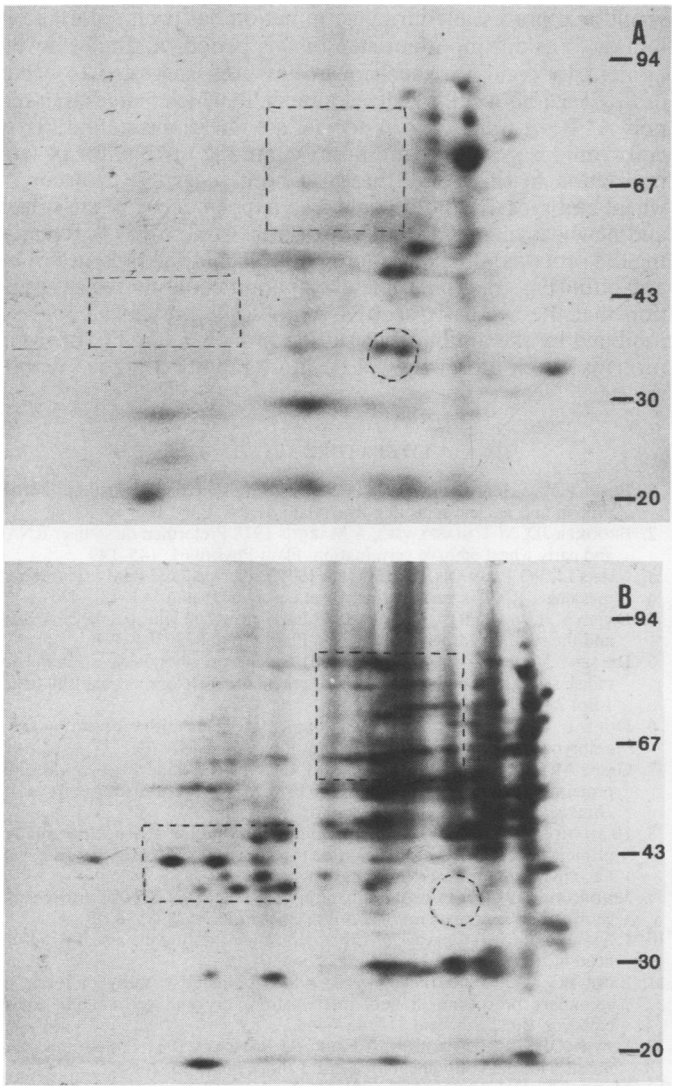

FIG. 2. Fluorographies of two-dimensional gel electroforetic analysis of axes proteins. A 6-h pulse-labeled ^{14}C-amino acids mixture (20 μCi) was given at 0 h (A) or 18 h (B) to batches of embryonary axes. Proteins were isolated as indicated in "Materials and Methods," and separated by gel electrophoresis in a 3.5 to 10 pH range, using 30,000 to 40,000 cpm in each isoelectric focusing gel. Numbers indicate the mol wt $\times 10^{-3}$ of reference polypeptides.

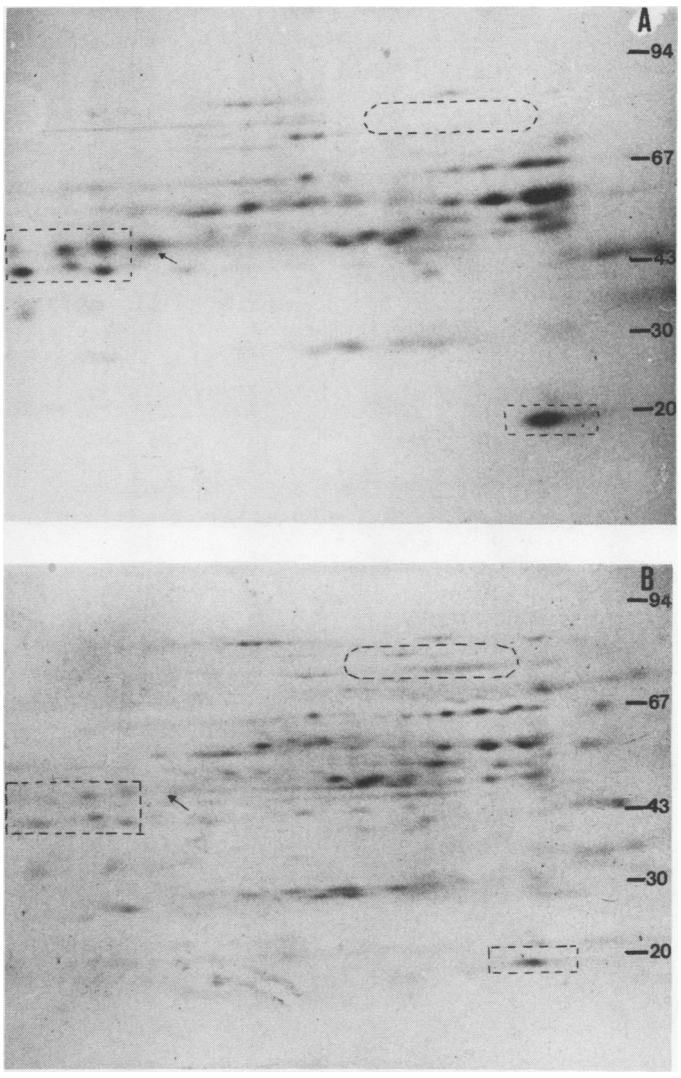

FIG. 3. Effect of α-amanitin in the proteins synthesized by embryonary axes during the 18- to 24-h period. Two separated groups of embryonary axes (100 mg each) were 6-h pulse-labeled with 20 μCi of ^{14}C-amino acids at 18 h. One of them was previously treated with α-amanitin (10 μg/ml at 0 time) (B). Proteins were separated by two-dimensional gel electrophoresis in a 5 to 8 pH range and detected by fluorography. Numbers indicate the mol wt $\times 10^{-3}$ of reference polypeptides.

Table I.

^{14}C-Amino Acid Pulse (6 h)	Amino Acid Incorporation into Proteins		Inhibition
	Control	+α-Amanitin	
	cpm/mg		%
0–6[a]	7,115	6,818	4
18–24[b]	36,650	21,255	42
18–24[a]	212,683	87,687	58

[a] α-Amanitin added at 0 h.
[b] α-Amanitin added at 18 h.

vitro, gave similar patterns (17). Furthermore, hybridization experiments with cDNA from early and late mRNAs of wheat embryos (2) showed no differences. Later, however, different patterns for proteins synthesized in early and late germination periods were found in wheat embryos (11), in castor bean (13), and in cotton (6). Our results also showed differences in the pattern of proteins synthesized during the early and late germination periods of maize axes as seen by the ^{14}C two-dimensional gels (Fig. 2). These differences are not due to artefacts or degradation during the sample processing since the same patterns are obtained in the presence of phenylmethylsulfonyl fluoride.

Although, both scutellum and axes constitute the seed embryo, each one has a different role during germination. The synthesized proteins in axes and scutellum, however, seem to be similar on the early stage of germination (0- to 6-h period) when both tissues are just reinitiating metabolic activity (Figs. 2A and 4A), suggesting that there is a common group of proteins at that time, regarded as the house keeping proteins (6), which should be present in the cells to fulfill the requirements for their basic metabolic functions. These proteins seem to be codified mainly by the stored mRNA (Table I).

A significant group of the proteins synthesized in the later period (18–24 h), when newly synthesized mRNA is being expressed, is not altered by the addition of α-amanitin to the embryos (Fig. 3), suggesting that they are translated from pre-

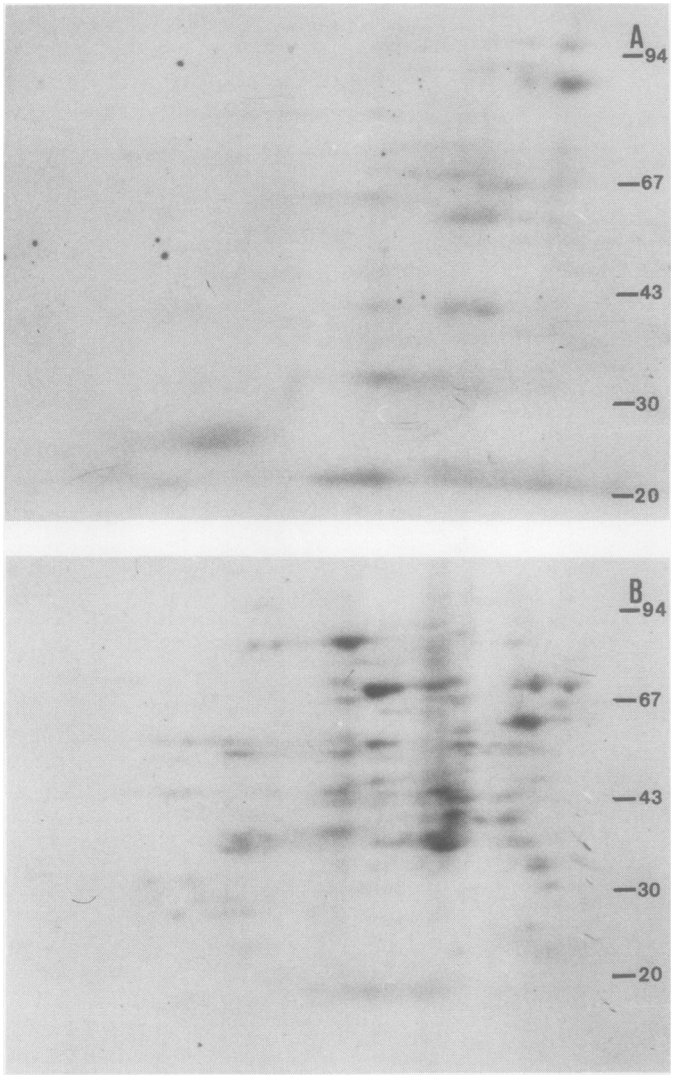

FIG. 4. Fluorographs of proteins synthesized by scutellum. Batches of 100 mg of scutellum were 6-h pulse-labeled with 20 μCi-amino acid mixture at either 0 h (A) or 18 h (B). Proteins were separated by two-dimensional gel electrophoresis in a 3.5 to 10 pH range and detected by fluorography. Number indicate the mol wt $\times 10^{-3}$ of reference polypeptides.

formed RNA transcripts of the stored mRNA population. However, since many of these proteins are not present in the 0- to 6-h pattern, the data might be interpreted as indicative of a selective mechanism present in the cells which modulates the translation of the stored transcripts; that is to say, some of the stored RNA species, though already present in the quiescent axes, are not translated until late in germination, probably due to posttranscriptional processing requirements. Evidence for active transcriptional processing during germination has been reported for radish (5) as an important step in this period. A similar developmentally regulated mechanism has been suggested to occur during sea urchin embryo development, in which maternal stored poly A^+ RNA transcripts in oocytes are not expressed until later embryonic stages (19). Composition among mRNA for *in vivo* translation in this period has also been suggested to occur in wheat embryos (2) and it could also happen between the stored and newly available (processed or synthesized) mRNA. Regarding the proteins which are more noticeable in the presence of α-amanitin (Fig. 3), a plausible explanation could be the assumption that the synthesis of mRNA for some proteases could be inhibited by the antibiotic, resulting in higher stability of some proteins which otherwise will be more rapidly degraded within the cell.

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