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On the Role of Stored mRNA in Protein Synthesis in Embryonic Axes of Germinating *Vigna unguiculata* Seeds[1]

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ABSTRACT

Polyadenylated (poly A$^+$) RNAs were prepared from both dry and incubated embryonic axes of *Vigna unguiculata* seeds and were translated by a wheat germ translation system. Analysis with gel electrophoresis and fluorography showed that translation products of poly A$^+$ RNA from dry embryonic axes were nearly the same as those from 2-hour incubated axes but somewhat different from those of 4- to 24-hour incubated axes, and that translation products remained almost unchanged between the 4- and 24-hour stages of postimbibition. The results indicate the possibility that the stored mRNA (poly A$^+$ RNA from dry embryonic axes) directs the protein synthesis required for early stages of germination. This is supported by comparison of the *in vitro* translation products of poly A$^+$ RNAs with those of polysomal RNAs. Experiments with α-amanitin, a specific inhibitor of RNA polymerase II (J. Jendrisak 1980 J Biol Chem 255: 8529–8533), suggested that the synthesis of some of the stored mRNA species is resumed as early as 4 hours after the onset of imbibition.

When plant seeds germinate, a dramatic increase in metabolic activity occurs. Protein synthesis is rapidly activated after the onset of imbibition (2). Earlier studies have indicated that such protein synthesis in early germination stages may be programmed by 'stored' or 'preformed' mRNA that is transcribed during seed maturation and conserved in dry seeds prior to germination (19). To examine the role of stored mRNA in the protein synthesis of early germination stages, experiments involving translation of stored mRNA by *in vitro* systems have been undertaken by several research groups. Their results may be roughly divided into two categories. One ascribes a functional role to stored mRNA in seed germination and the other, a negative or very restricted role. The former is largely based on: (a) similarities between translation products or nucleotide sequences of mRNAs from dry and germinated seeds (3, 6); (b) insignificant effects of inhibitors of poly A$^+$ RNA[3] synthesis on the protein synthesis of early germination stages (11); and (c) efficient *in vitro* translation of poly A$^+$ RNA from dry seeds which produces polypeptides with a high molecular range (6, 10, 20). The alternative point of view is supported by evidence that stored mRNA-directed proteins consist mostly of low mol wt polypeptides or maturation-specific storage proteins and that these are replaced by new protein species shortly after imbibition starts (5, 7, 17, 21). The stored mRNA is considered to have played its role during seed maturation and occasionally termed 'residual' mRNA (9).

We have established the presence of stored mRNA in embryonic axes of dry *Vigna unguiculata* seeds by using inhibitors of transcription and translation (23). α-Amanitin added to embryonic axes from the onset of imbibition depressed poly A$^+$ RNA synthesis but had no significant effects on early protein synthesis. In the present work, we isolated stored mRNA from dry *V. unguiculata* embryonic axes, translated it in an *in vitro* system, and analyzed the translation products mainly by SDS-PAGE. Fluorographic patterns of stored mRNA-directed products were compared with those of polysomal RNA-directed products. Based on our results, the role of stored mRNA in the protein synthesis of early germination stages of *V. unguiculata* seeds is discussed.

MATERIALS AND METHODS

Plant Material and Incubation Conditions. Embryonic axes of *Vigna unguiculata* (L.) Walp seeds were allowed to imbibe water (or an inhibitor solution) and incubated on layers of wet filter paper at 27°C in the dark as described previously (23).

***In Vitro* Translation.** Poly A$^+$ RNA was prepared from embryonic axes according to the method of Aviv and Leder (1) with some modifications (23). Preparation of wheat germ extract (S-30) and cell free translations were carried out after the method of Marcu and Dudock (16) with some modifications. The complete reaction mixture for *in vitro* protein synthesis contained 15 μl of wheat germ extract (S-30), 20 mM Hepes-KOH (pH 8.0), 1 mM ATP, 200 μM GTP, 50 μM of all 18 amino acids (minus cysteine and either leucine or methionine), 100 μM DTT, 50 mM KCl, 8 mM phosphocreatine (neutralized with HCl), 50 mM creatine phosphokinase (dissolved in 50% glycerol), 0.1 mM cysteine (dissolved in 50 mM DTT), 0.5 mM spermidine, 5 μCi of L-[4,5-^{3}H]leucine (60 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom) or L-[^{35}S]methionine (1,435 Ci/mmol; Amersham) and 15 μl of a poly A$^+$ RNA preparation in a final volume of 50 μl. This mixture was incubated at 22°C. At indicated intervals, an aliquot (5 μl) was pipetted onto a filter paper disc (Toyo No. 4A). The filter paper was soaked in hot 5% TCA solution for 10 min, washed 3 times with cold 5% TCA and once with ethanol:ether (1:1), and dried under a far-red lamp. The radioactivity was determined in a Beckman liquid scintillation system CPM-200.

SDS-PAGE. Cell-free translation products of poly A$^+$ RNA or polysomal RNA were analyzed after the method of Laemmli (13). Proteins were stained with Coomassie brilliant blue. Fluorography was performed by the aid of EN^{3}HANCE according to the method of Laskey and Mills (14) using Fuji RX films.

Isolation of Polysomes. Polysomes were prepared according to the methods of Jackson and Larkins (12) with some modifications. Embryonic axes pulverized in liquid N_2 were homogenized

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[3] Abbreviation: poly A$^+$ RNA, polyadenylated RNA.

in a mortar and pestle with a polysome extraction buffer consisting of 0.2 M Tris-HCl (pH 8.5), 0.4 M KCl, 50 mM Mg-acetate, 25 mM EGTA, 0.25 M sucrose (RNase free), and 5 mM DTT. The homogenate was centrifuged at 13,000*g* for 15 min. Triton X-100 was added to the resulting supernatant to give a 1% concentration. The polysomes were pelleted from the supernatant through a 2.5-ml layer of 1.5 M sucrose in the polysome extraction buffer at 200,000*g* for 2 h. For analysis of polysome profiles, the polysome pellets were dissolved in the polysome extraction buffer and insoluble materials were removed by centrifugation at 13,000*g* for 10 min. Aliquots (0.2 ml) of the resulting supernatant were layered onto sucrose gradients, which had been formed by layering 0.75, 1.5, 1.5, and 0.75 ml of 125, 250, 375, and 500 mg/ml sucrose dissolved in a gradient buffer consisting of 40 mM Tris-HCl (pH 8.5), 20 mM KCl, and 10 mM Mg-acetate and allowing it to stand at 4°C overnight. The preparations were centrifuged at 40,000 rpm in a Hitachi RPS-50-2 rotor at 4°C for 90 min. The gradients were analyzed with an ISCO model D density gradient fractionator attached to a UA5 absorbance monitor. For measurement of polysomal RNA content, RNA was extracted from the polysomal fraction as described previously (23).

RESULTS

Partial Characterization of Poly A⁺ RNA-Directed Translation. The content of total RNA did not increase until the 12-h stage. The poly A⁺ RNA content increased after the onset of imbibition and was 460% of the original value by the 12-h stage (Table I).

Table I. *Change in RNA Content during Incubation of Vigna unguiculata Axes*

Imbibition Time	Total RNA	Poly A⁺ RNA	Polysomal RNA[a]
h		*μg/embryonic axis*	
0	58.1	0.19 (0.32)[b]	1.50 (2.6)
4	66.0	0.35 (0.53)	8.77 (13.3)
12	55.1	0.87 (1.58)	21.2 (38.5)
24	83.6	0.65 (0.78)	——[c]

[a] Polysome fraction was isolated after the method of Jackson and Larkins (12), and RNA was extracted from the fraction as described previously (23). [b] The value in parentheses denotes per cent of total RNA. [c] ——, Not determined.

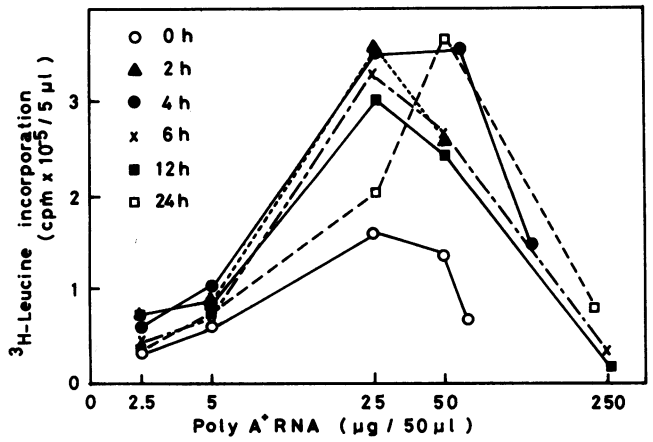

FIG. 1. Translation activity of poly A⁺ RNAs from dry and incubated *V. unguiculata* embryonic axes in a wheat germ system. Poly A⁺ RNAs were prepared from axes 0 (dry), 2, 4, 6, 12, and 24 h after the onset of imbibition.

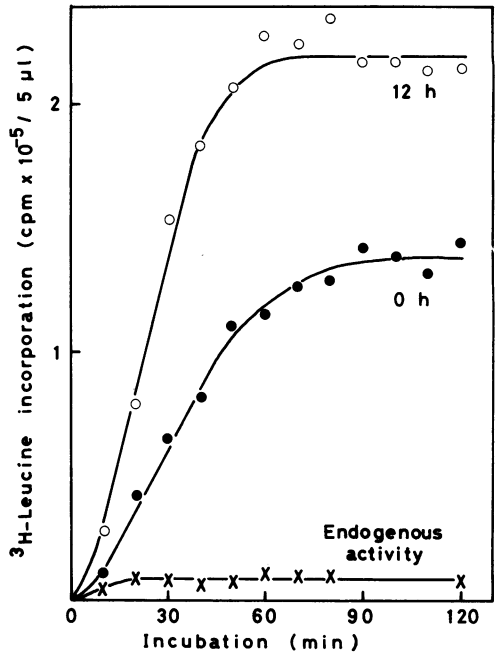

FIG. 2. Time course of protein synthesis in a wheat germ system by poly A⁺ RNAs prepared from 0 (dry) and 12-h incubated embryonic axes. Endogenous activity: poly A⁺ RNA not added.

The translation activities of poly A⁺ RNAs isolated from both dry and incubated embryonic axes were dependent on poly A⁺ RNA concentrations (Fig. 1): each poly A⁺ RNA showed a narrow range of optimum concentration (25–50 μg/50 μl of the reaction mixture). Higher concentrations of poly A⁺ RNA (250 μg/50 μl) hardly stimulated amino acid incorporation. Poly A⁺ RNAs from dry axes and 2-, 6-, and 12-h incubated axes had nearly the same optimum concentration of 25 μg/50 μl; poly A⁺ RNAs from 4- and 24-h incubated axes were optimal at 50 μg/50 μl. Poly A⁺ RNA from dry embryonic axes clearly stimulated amino acid incorporation, but its translation activity at the optimum poly A⁺ RNA concentration was obviously lower than that of incubated axes.

Figure 2 shows the time course of *in vitro* protein synthesis under the optimum conditions. Poly A⁺ RNA from 12-h incubated embryonic axes stimulated amino acid incorporation by 20-fold over the endogenous activity. The rate of translation was linear with respect to the reaction time until 60 min and then reached a plateau. The poly A⁺ RNA isolated from dry embryonic axes also stimulated protein synthesis by about 15-fold, indicating the presence of stored mRNA in embryonic axes of dry *V. unguiculata* seeds.

SDS-PAGE Analysis of Poly A⁺ RNA Translation Products. Translation products directed by poly A⁺ RNA from dry and incubated embryonic axes were analyzed by SDS-PAGE and visualized by fluorography (Fig. 3). The results demonstrate again that stored mRNA occurs in dry embryonic axes. Since the mol wt of the products ranged up to 1×10^5 D, the stored mRNA is unlikely to represent the degraded residue of mRNAs which had functioned during seed formation. Translation products of poly A⁺ RNAs from dry and 2-h incubated axes gave similar fluorographic patterns. However, translation products of poly A⁺ RNA from 4-h incubated axes differed from those of dry axes. The major polypeptide bands directed by the stored mRNA (indicated by arrows in Fig. 3) were not observed among the products directed by poly A⁺ RNA from 4-h incubated axes. Translation

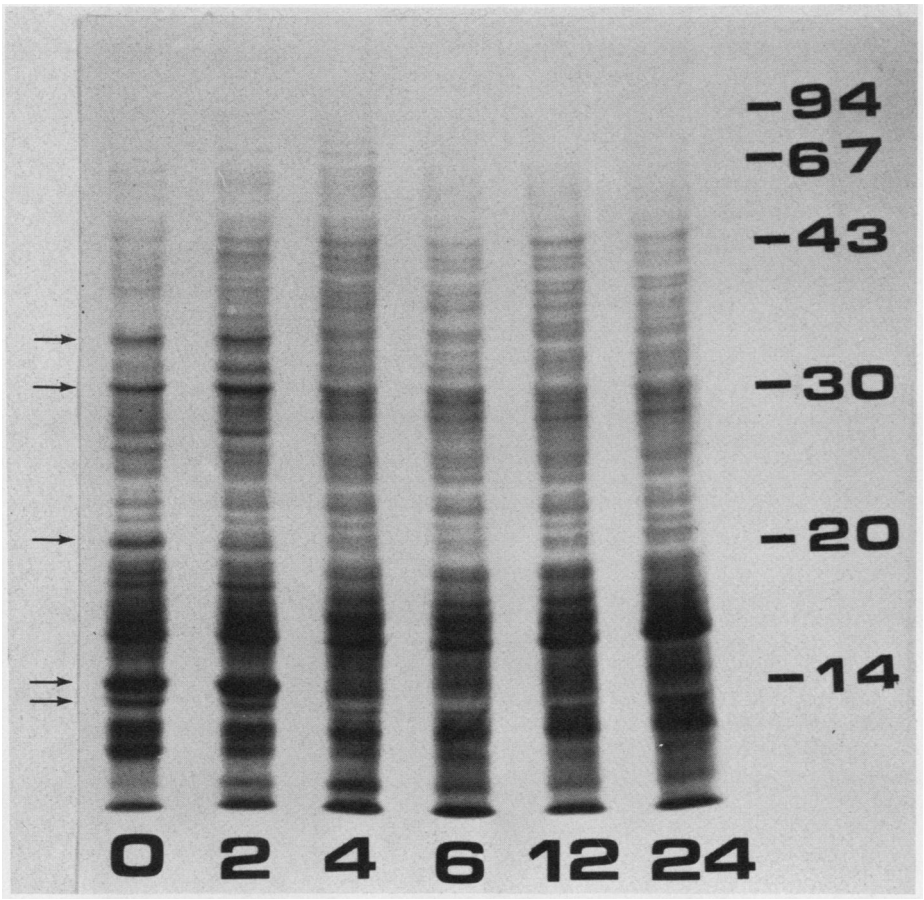

FIG. 3. Fluorograms of SDS-PAGE of translation products directed by poly A$^+$ RNAs from axes 0 (dry), 2, 4, 6, 12, and 24 h after the onset of imbibition. Arrows indicate the products specific for stored mRNA. [^{3}H]Leucine was used as the radioactive precursor.

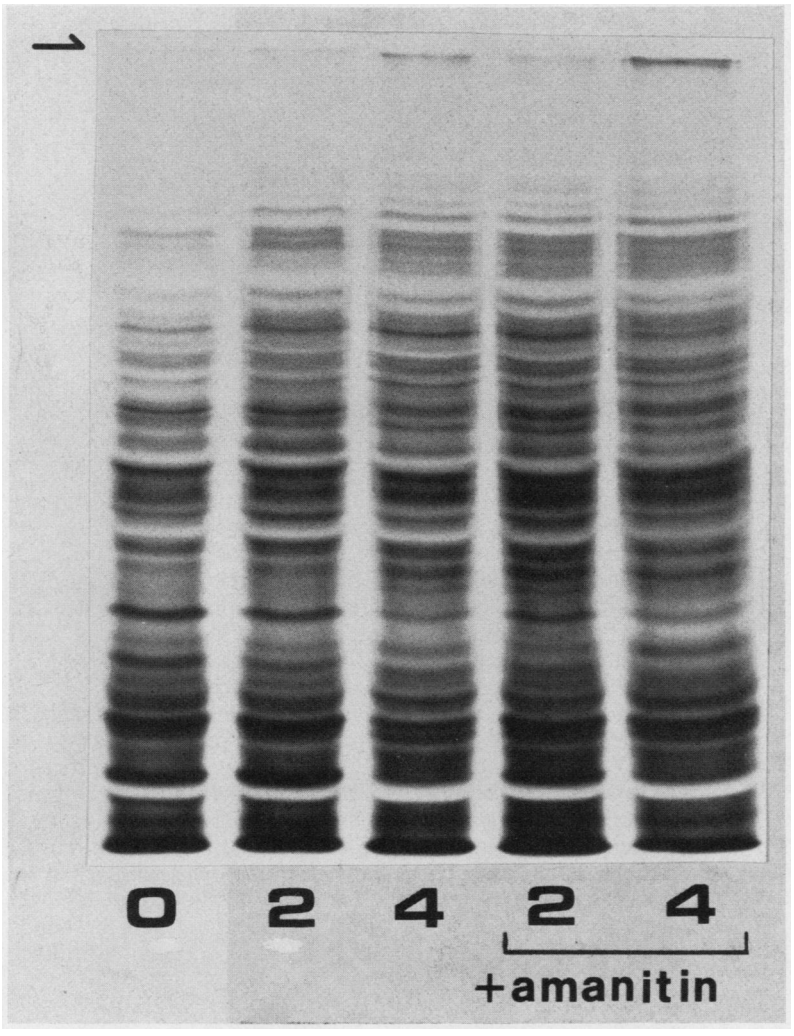

FIG. 4. Effect of α-amanitin on translation products of poly A$^+$ RNAs. The translation products were analyzed by SDS-PAGE and visualized by fluorography. Poly A$^+$ RNAs were prepared from embryonic axes incubated for 0 (dry), 2, and 4 h in the presence and absence of α-amanitin (5 μg/ml). [^{3}H]Leucine was used as the radioactive precursor. Electrophoretic conditions were the same as for Figure 3. The arrow indicates the front.

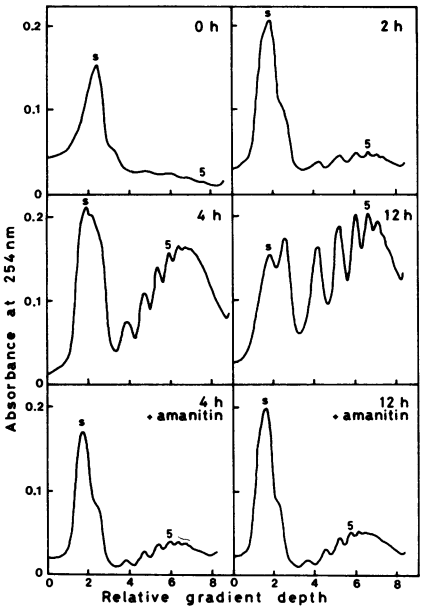

FIG. 5. Sucrose density gradient profiles of the ribosomal pellets from embryonic axes 0 (dry), 2, 4, and 12 h after the onset of imbibition and those from axes incubated for 4 and 12 h in the presence of α-amanitin (5 μg/ml). *s* and *5* denote the respective positions of monomers and polymers of size class 5.

products remained almost unchanged between the 4- and 24-h stages. It should be noted that no new polypeptide bands were detected among translation products of poly A$^+$ RNAs from 4- to 24-h incubated axes.

Next, embryonic axes were incubated for 2 and 4 h in the presence and absence of α-amanitin (5 μg/ml); then translation products of poly A$^+$ RNAs from these axes were compared. Virtually no differences between the translation products of poly A$^+$ RNA from nontreated and α-amanitin-treated embryonic axes were observed (Fig. 4), indicating that mRNAs coding for new protein species were insignificantly synthesized at least until 4 h after the onset of imbibition.

Changes in Polysome Profiles and Translation of Polysomal RNA. The RNA content of the polysomal fraction from dry embryonic axes was very low, but it increased by 14-fold between the 4- and 12-h stages. At the 12-h stage, more than one-third of the total RNA was polysomal (Table I). Dry axes contained few polysomes, but polysomes were gradually formed as incubation proceeded (Fig. 5). After 4 h, polysome formation became obvious, and, by the 12-h stage, the relative amount of polysomes was larger than that of monosomes of degraded subunits. In embryonic axes incubated for 4 or 12 h in the presence of α-amanitin (5 μg/ml), polysome formation was strongly depressed to the level of the 2-h stage. This suggests that, even at stages as early as 4 h, polysomes are partly supported by newly synthesized mRNA. Figure 6 shows a comparison of *in vitro* translation products of polysomal RNA with those of the corresponding poly A$^+$ RNA. The polysomal RNA from dry embryonic axes directed *in vitro* protein synthesis, but its activity was lower than that of incubated axes (about 55%). Their products corresponded to those of the poly A$^+$ RNAs from dry axes. The major bands of polysomal RNA-translation products of dry axes rapidly disappeared 4 h after the onset of imbibition, and this coincides with the results obtained with poly A$^+$ RNA. Newly synthesized protein bands were not detected in products of the polysomal RNA from axes of the 4-h stage.

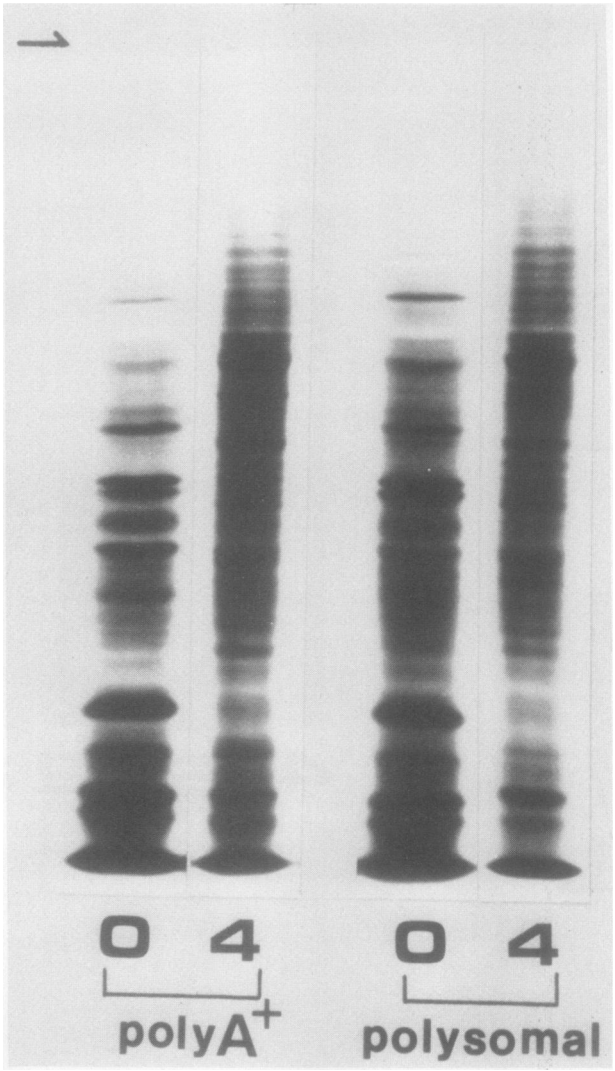

FIG. 6. Fluorograms of SDS-PAGE of translation products directed by poly A$^+$ RNAs and polysomal RNAs, both of which were prepared from embryonic axes 0 (dry) and 4 h after the onset of imbibition. [^{35}S] Methionine was used as the radioactive precursor. Electrophoretic conditions were the same as for Figure 3. The arrow indicates the front.

DISCUSSION

The poly A$^+$ RNA isolated from dry embryonic axes had a high level of translation activity when examined by a wheat germ translation system (Figs. 1 and 2). This confirms the occurrence of stored mRNA in *V. unguiculata* embryonic axes. However, the translation activity of poly A$^+$ RNA from dry axes was only half the activity of poly A$^+$ RNAs from the 2- to 12-h stages of postimbibition when compared at optimum RNA concentrations in the reaction mixture (Fig. 2), suggesting that some qualitative changes in mRNA occur upon imbibition.

SDS-PAGE analysis of *in vitro* translation products of stored mRNA (poly A$^+$ RNA from dry axes) showed the presence of some polypeptide bands that are detected only in dry axes (Fig. 3). These bands disappeared rapidly as the imbibition proceded (4 to 12 h after the onset of imbibition) and could be attributed to maturation-specific proteins. The mRNAs directing these proteins may be analogous to those found in *Vigna aureus* (5) and soybean (18). In particular, one major polypeptide band has a mol wt (1.4×10^4 D, Fig. 3) similar to that of storage albumin from *V. aureus* embryonic axes (15). Except for these few bands, the fluorographic pattern of poly A$^+$ RNA-translation products

from dry axes is very similar to the patterns from the 4- to 24-h incubated axes (Fig. 3). These results suggest that the stored mRNA positively directs the protein synthesis required for early stages of germination. This is further substantiated by comparison of the *in vitro* translation products of poly A^+ RNAs with those of corresponding polysomal RNAs (Fig. 6). Moreover, the addition of α-amanitin to axes clearly inhibited the synthesis of poly A^+ RNA (23) as well as the polysome formation (Fig. 5) at the 4-h stage of postimbibition, but this same treatment hardly affected the fluorographic pattern of the *in vitro* translation products (Fig. 4).

The stored mRNA found in radish embryonic axes (7), wheat embryos (4, 24), maize embryos (8), and castor bean seeds (17, 21) disappears promptly after the onset of imbibition; instead, newly formed mRNA plays the functional role in the protein synthesis of early germination stages. The present results with *Vigna unguiculata* do not agree with these reported observations.

Evidence obtained in the present studies together with our previous results (23) support the following view of stored mRNA in *V. unguiculata* embryonic axes: stored mRNA of the dry embryonic axes is responsible for protein synthesis during the first 24 h after the onset of imbibition, and in addition, the synthesis of some of these mRNA species is resumed as early as 4 h after the onset of imbibition. This supports the idea that imbibition (rehydration) and germination are part of the continuous developmental process from embryogenesis to plant growth. Therefore, there seem to be no marked metabolic changes between the late stage of seed maturation and the early stage of germination. However, this view of stored mRNA may not apply to storage organs of seeds, such as cotyledons, in which a marked alteration of metabolism occurs between seed maturation and germination.

Recently, Sánchez de Jiménez and Aguilar (22) reported that, in the axes of germinating maize embryos, the changes in proteins observed between the periods of 0 to 6 h and 18 to 24 h post imbibition seem to be due to newly synthesized mRNA as well as to delayed translation of stored mRNA species. These observations agree with those reported in this paper.

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