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Developmental Regulation of the Synthesis of Proteins Encoded by Stored mRNA in Radish Embryos[1]

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ABSTRACT

Major radish (*Raphanus sativus* L. cv National) proteins synthesized at the beginning of germination have been characterized by their migration in two-dimensional electrophoresis.

The use of 15-minute labelings shows that these proteins are encoded by stored mRNA. They undergo little or no posttranslational modification. Other proteins become detectable only after 1 hour of imbibition, and are probably encoded by newly synthesized mRNA. Comparison with proteins synthesized during embryogenesis, late germination, or with those present in dry embryos allows the classification of the proteins encoded by stored mRNA into two sets:

The first set is synthesized also during late embryogenesis and is present in dry embryos. Some of these polypeptides are no longer synthesized later in germination while the others continue to be synthesized. The corresponding stored mRNAs can be considered as remnants of mRNA actively translated during embryogenesis.

The second set is synthesized only during early germination. Their messengers appear during late embryogenesis although they are apparently not translated at this stage, but translation can be induced by a desiccation treatment. These polypeptides may play a particular role during early germination.

Dry seed embryos contain stored mRNA (8). However, neither the nature nor the function of proteins encoded by stored mRNA have been definitively established. Are they only remnants of mRNA specific to embryogenesis or do they play a particular role during early germination?

Several groups have suggested that most stored mRNA codes for storage proteins or other abundant seed proteins. Mori *et al.* (18) have shown that storage protein mRNAs are present in dry soybean seeds, while other authors (2, 16) have characterized a storage albumin in mung bean encoded by a stored messenger. Peumans *et al.* (21) have detected the mRNA of pea lectin in dry seeds. These results demonstrate that at least some stored mRNAs code for proteins already present in the seed and, consequently, do not play a specific role in early germination. However, these studies do not exclude the possibility that another fraction of stored mRNA codes for proteins necessary in early germination.

A more exhaustive characterization of mRNA expressed during embryogenesis and germination has been performed by Dure *et al.* (10) with cotton seeds. By analyzing mRNA/cDNA reassociation kinetics (13) or comparing two-dimensional electrophoresis patterns of polypeptides synthesized *in vivo* or *in vitro* (10), these authors have shown the presence of a specific set of mRNAs which accumulate during late embryogenesis and disappear during early germination. This work is presently the only one suggesting that some stored mRNAs code for proteins playing a specific role in early germination.

We have previously detected the presence of stored mRNA in dry radish embryos and analyzed their life time (6, 7). As a first step in the characterization of proteins encoded by stored mRNA in radish, we have analyzed by two-dimensional electrophoresis the polypeptides synthesized in embryos during the first 15 min of germination and in an *in vitro* translation system directed by stored mRNA. These polypeptides have been compared with those synthesized during late embryogenesis and late germination and with those present in dry embryos.

In this report, we show that the polypeptides synthesized during the first 15 min of germination are encoded by stored mRNA and can be classified into two sets.

MATERIALS AND METHODS

Plant Material. Radish seeds (cv National, rond rose à bout blanc) were obtained from Vilmorin. Intact embryo axes were harvested manually. They were germinated in distilled H_2O containing the labeling solution in some experiments. Seeds were also sown in a greenhouse and plants were grown in individual pots until flowering. In these conditions, it takes about 60 d after flowering to obtain mature dried seeds. Immature seeds were collected according to morphological features for staining or labeling.

For RNA extraction, dry embryos, embryo axes of 16-h-old seedlings, and immature seeds were frozen immediately in liquid nitrogen, freeze-dried, and stored at −70°C.

In a few experiments we artificially dehydrated immature seeds in jars covered with parafilm over activated silica gel at room temperature for 48 h.

***In Vivo* Protein Synthesis.** *In vivo* labelings were carried out in plastic cap (1 cm diameter) covered with parafilm, using [^{35}S] methionine (CEA, 575 Ci/mmol) in a dark incubator at 22°C. The labeling solution contained 3000 μCi/ml when dry embryos were labeled for 15 min or 30 min, 1500 μCi/ml when dry embryos, immature seeds, or artificially dehydrated immature seeds were labeled for 1 h, 300 μCi/ml when 2-, 4-, and 14-h postimbibition embryos were labeled for 2 h.

After the incubation period, the material was rinsed in distilled H_2O, decontaminated in 3% calcium hypochlorite, and rinsed in distilled H_2O. It was frozen and stored at −70°C until protein extraction.

Extraction of Proteins. Soluble proteins were extracted at 4°C from various developmental stages by homogenizing the material for 1 min in a 1-ml Eppendorf tube using a home-made, motor-driven (100 rpm) millstone, in 100 μl of a solution containing

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10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 100 μg/ml pancreatic DNase and 50 μg/ml pancreatic RNase. After standing on ice for 30 min, the mixture was centrifuged, the pellet was rehomogenized and reextracted with 50 μl of the same buffer for 15 min. The two supernatants were pooled and NP-40 added to 1%, ampholines (LKB 3.5–10) to 1%, β-mercaptoethanol to 1% and urea to saturation (9.5 M).

Extraction of RNA. RNA was extracted using autoclaved glassware throughout the extraction. Frozen material was ground to a powder with liquid nitrogen before adding the extraction buffer (0.1 M glycine-NaOH (pH 9.0), 0.15 M NaCl, 1% desoxycholate, 0.1% SDS, proteinase K to 60 μg/ml). An equal volume of phenol/chloroform (1:1) was added; the mixture was homogenized 2 min in a Waring Blendor and stirred 15 min at 4°C. The aqueous phase was recovered by centrifugation for 15 min at 8,000 rpm. Proteinase K was added to 20 μg/ml to this phase and the mixture stirred with an equal volume of phenol/chloroform for 15 min at 4°C. The aqueous phase was recovered by centrifugation. The phenol/chloroform extraction was repeated twice. The final aqueous phase was made 0.2 M NaCl and precipitated with two volumes of ethanol overnight at −20°C. The precipitate was collected by centrifugation, vacuum dried, dissolved in sterile water, and 4 M NaCl (pH 5.5) was added to a final concentration of 3 M to remove contaminating polysaccharides, DNA and low mol wt RNAs. The high mol wt RNAs were allowed to precipitate for 1 h at 0°C. The pellet was recovered by centrifugation, dissolved in sterile water, made 0.2 M NaCl, and precipitated by ethanol overnight at −20°C. The precipitate was collected as before, rinsed twice with 60% isopropanol, once with absolute ethanol, dried, dissolved in sterile water at 10 μg RNA/μl and used for *in vitro* protein synthesis.

***In Vitro* Protein Synthesis.** Cell free protein synthesis was carried out using the RNase-treated rabbit reticulocyte lysate obtained from Amersham. In a typical experiment 1 μl RNA (10 μg/μl) was added to 10 μl lysate containing 20 μCi of [^{35}S] methionine (CEA, 575 Ci/mmol) and incubated at 30°C for 90 min. At the end of the incubation period, a 1-μl aliquot was removed, the TCA precipitable radioactivity determined, and the remainder processed for gel electrophoresis. The samples were treated with 1 μl of RNase A+RNase T_1 (50 μg/ml each) for 15 min at 30°C and stored at −70°C. Before electrophoresis, NP-40 was added to 1%, ampholines (LKB 3.5–10) to 1%, β-mercaptoethanol to 1%, and urea to saturation (9.5 M).

Isoelectrofocusing. Proteins were separated by NEPHGE at pH 3.5 to 10 (20) or by IEF (19) at pH 5 to 7 in cylindrical gels (13.5 cm × 2 mm). NEPHGE gels contained 4% acrylamide, 9% urea, 2% NP-40, and 2% pH 3.5 to 10 LKB ampholines. Samples (20 μl) were loaded at the acid end of the gels, covered with 20 μl overlay solution (8 M urea, 1% pH 3.5–10 LKB ampholines, 2% NP-40). Electrodes consisted of 10 mM H_3PO_4 and 20 mM NaOH. The voltage was increased in steps (30 min at 100 v, 45 min at 200 v, 160 min at 300 v, and 60 min at 500 v) to 1,500 v h. IEF gels contained 4% acrylamide, 9 M urea, 2% NP-40, Pharmalyte ampholines (0.5% pH 3–10, 0.5% pH 4–6.5, 1% pH 5–8). Samples were loaded as described above, using 1 M glutamic acid and 30 mM ethanolamine as electrodes. The voltage was increased in steps (30 min at 100 v, 45 min at 200 v, 16 h at 300 v, and 1 h at 1,000 v) to 6,000 v h. Gels were subsequently equilibrated in 10 ml buffer 0 (10% glycerol, 5% β-mercaptoethanol, 2.5% SDS, 62.5 mM Tris-HCl [pH 6.8]) for 1 h and then frozen and stored at −70°C.

Denaturing Electrophoresis. After thawing, first-dimension cylindrical gels were dialyzed a further 1 h in 10 ml fresh buffer 0 containing 0.01% bromophenol blue. The second-dimension gels were prepared in a Bio-Rad apparatus (model 220) using 1.5-mm spacers. The separating gel was 12.5% acrylamide, 0.1% SDS, 0.37 M Tris-HCl (pH 8.8), and the stacking gel was 62.5 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 0.1% SDS, 10% glycerol, 1% Pharmacia IEF agarose (17). The stacking gel was poured at about 50°C and the first-dimension gel inserted directly on top. Migration was carried out at 20 mamp/gel until the bromophenol blue reached the lower buffer (about 4–5 h). The following mol wt markers were used: lysozyme (14,300), carbonic anhydrase (30,000), ovalbumin (46,000), BSA (69,000), phosphorylase b (92,500), and myosin (200,000) as the ^{14}C-methylated form (Amersham). The gels were subsequently treated for fluorography as described by Meyer and Chartier (17).

Amido Black and Silver Stainings. After electrophoresis, polyacrylamide gels were stained 1 h in amido black solution (0.5% amido black, 50% TCA, and 20% ethanol). Afterward, they were destained overnight in a solution containing 7% acetic acid, 20% ethanol, and 2% glycerol before photography.

Silver staining was performed as described by Wray *et al.* (23) with the following modification. The gel was soaked in 50% methanol overnight. Then, before silver staining as described by these authors, it was neutralized for 30 min with 100 ml of a solution containing 21 ml of 0.36% NaOH and 1.4 ml of 14.8 M NH_4OH.

If both staining and fluorography were necessary, the gel was first impregnated with PPO, then stained with amido black, destained, dried, and fluorographed.

RESULTS

Comparison of Proteins Synthesized *In Vivo* during the First 15 Minutes of Germination and in a Cell-Free System Directed by Stored mRNA. Proteins synthesized *in vivo* during the first 15 min of imbibition in radish embryos were separated by two-dimensional electrophoresis. The typical pattern is shown in Figure 1A. Subsequently, we focused our study on the 33 most radioactive polypeptides, which we call 'early germination' polypeptides. Figure 1B shows proteins translated *in vitro* by a cell-free system directed by mRNA extracted from unimbibed radish embryos. About 100 spots are present in these conditions.

Among the 33 most radioactive polypeptides synthesized *in vivo*, 23 comigrate with *in vitro* translation products (Fig. 1B, black arrows). Because migration is abnormal in the globin region of the *in vitro* pattern, we established the comigration of polypeptides 18, 22, 23, and 24 only by separating a mixture of *in vivo* and *in vitro* synthesized polypeptides in the same gel (results not shown). Nine polypeptides synthesized *in vivo* are undetectable by comigration in the *in vitro* pattern (Fig. 1A, white arrows). We can formulate several hypotheses to explain their absence.

(a) Cell-free protein synthesis systems are generally less efficient in the high mol wt region (mol wt > 50,000 D). This may explain why polypeptide 6 is less represented on *in vitro* gels than on the *in vivo* pattern, and is probably the reason for the absence of polypeptides 2, 3, 4, 5, 7, 9, and 10 on the *in vitro* pattern.

(b) Cell-free protein synthesis systems translate particular RNAs with greater or lesser efficiency. This is clearly illustrated by differences in the translation products obtained with a reticulocyte system and a wheat germ system directed by the same mRNAs (24).

(c) Proteins undergo *in vivo* co- and posttranslational modifications which modify their electrophoretic migration, as compared with *in vitro* products which are unmodified.

The absence of polypeptides 15 and 16 may be due to one or other of these reasons.

Because polypeptide 11 comigrates with a polypeptide encoded by an mRNA endogenous to the reticulocyte system, we cannot affirm that it is a direct translation product.

On the *in vitro* pattern we observe two polypeptides, a and a′, which do not comigrate with any spot on the *in vivo* gel but whose high radioactivity level indicates that they are products of many stored mRNAs. Nevertheless, none of the polypeptides

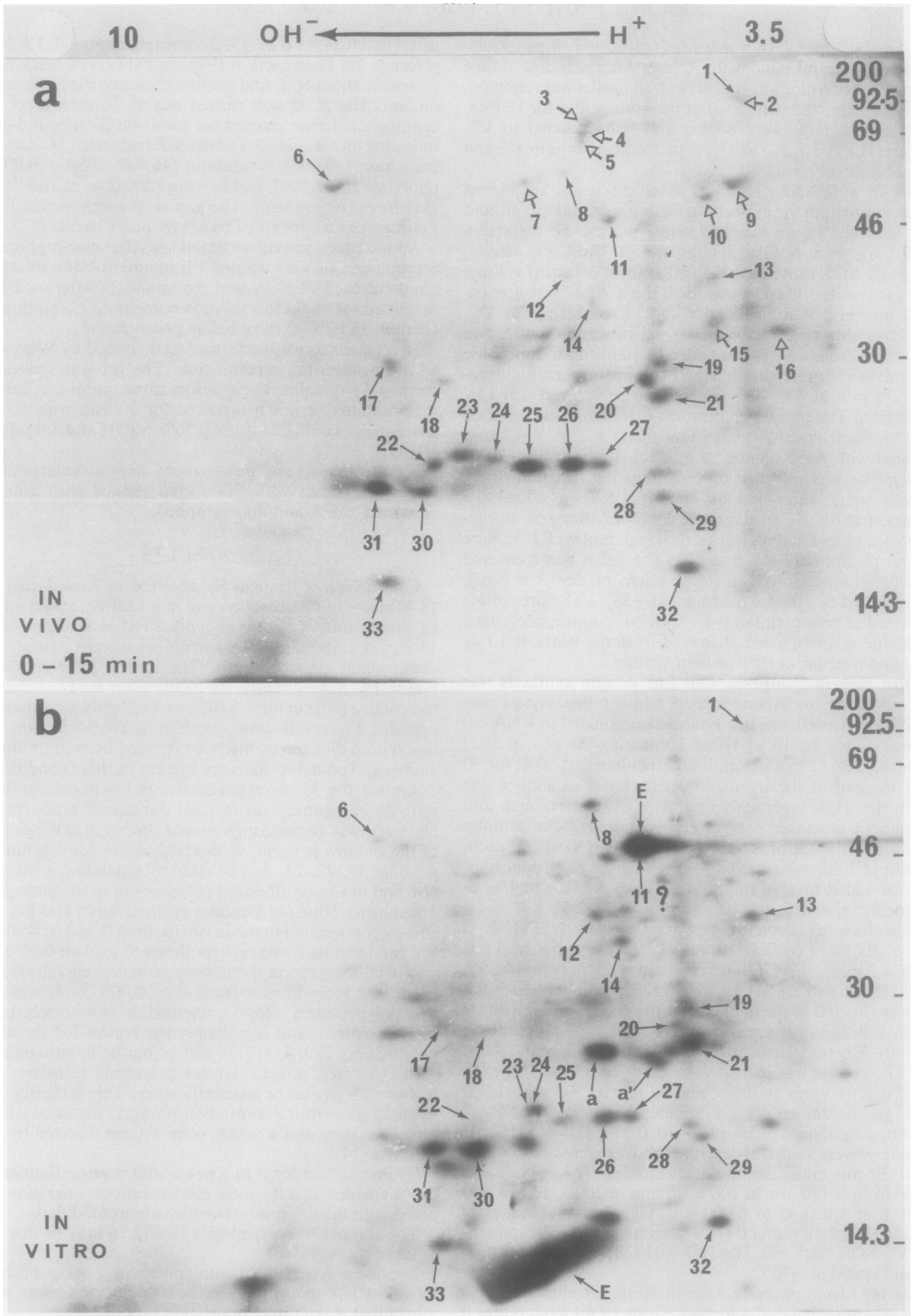

FIG. 1. Electrophoretic patterns of proteins synthesized *in vivo* during the first 15 min of germination (A) and in a cell-free system directed by stored mRNA (B). A, Arrows show the 33 most radioactive peptides synthesized *in vivo* and called early germination peptides. Black arrows indicate the peptides which comigrate with *in vitro* translation products and white arrows those which are absent on the *in vitro* pattern. B, E indicates the peptides which are coded by mRNA endogenous to the reticulocyte system. Peptides a and a′ are noted on *in vitro* pattern. The mol wt ($\times 10^{-3}$) of markers are indicated on the right of the patterns.

synthesized *in vivo*, and which apparently undergo posttranslational modification (white arrows), are radioactive enough to be the putative products of a or a'. In addition, these mRNAs are not polyadenylated, as has been shown in a previous study from our laboratory (15), in which poly-A^+ RNA was used to direct *in vitro* translation, as compared with this work, where we used total RNA.

These results clearly show that polypeptides are synthesized during the first 15 min of imbibition and that most of these polypeptides are direct translation products of the stored mRNA.

***In Vivo* Protein Synthesis at Later Stages of Germination.** To determine if polypeptides synthesized during the first 15 min of imbibition constitute a set of protein specific to early germination, we analyzed protein synthesis at later times, and followed the fate of these 33 polypeptides.

After 30 min germination low radioactive spots become detectable, due to the decrease in the background. Most probably, very short labeling (15 min) produces a high proportion of incomplete polypeptides which are responsible for this high background. The 33 early germination polypeptides continue to be synthesized without modification of their relative intensities (Fig. 2A). Nevertheless, after 1 h imbibition a slight modification in the relative radioactivity of the different polypeptides is obvious: for example, the radioactivity level of polypeptides 1 to 5, 7, 8, 12, and 14 increases. In addition, new polypeptides, which are not synthesized during the first 15 min of germination, become detectable but constitute only a limited proportion of the total protein synthesis (Fig. 2B).

After 4 h of germination (Fig. 2C), the 33 polypeptides are still synthesized but at this time polypeptides 6 and 21 show a greatly increased radioactivity level, while the level of radioactivity of polypeptides 20 and 22 to 27 drops drastically. At this time most of the radioactivity is present in polypeptides that are undetectable during early germination.

After 6 h of germination (Fig. 2D) only 9 of the 33 polypeptides translated from stored mRNA are detectable (white arrows). They continue to be synthesized after 16 h of germination.

In summary, these 33 polypeptides belong to two different sets: 24 are synthesized only at the beginning of germination while 9 continue to be synthesized later.

***In Vitro* Protein Synthesis during Late Germination.** To determine if the disappearance of 24 polypeptides among the 33 early germination polypeptides results from selective translation, post- or co-transcriptional modifications, or the disappearance of their mRNA, we translated *in vitro* mRNA extracted from embryo axes of 16-h-old seedlings.

The results presented in Figure 3A show that the corresponding mRNAs are no longer present. The 9 polypeptides synthesized after 6 h of germination are still synthesized after 16 h of imbibition (Fig. 3B). Among these, 7 are detectable on the *in vitro* gel. For the 2 others, polypeptide 15 possibly results from posttranslational modifications, as previously indicated, and consequently is not identifiable on *in vitro* gels. Polypeptide 29 shows only a very low radioactivity level on the *in vivo* gel and is, thus, probably synthesized at too low a level to be detected on the *in vitro* gel.

Polypeptides a and a' are still detectable on the *in vitro* pattern.

Apart from the seven mRNAs identical to stored mRNAs, it is evident that at 16 h of germination the majority of messenger activity is constituted by mRNAs absent in dry embryos. Moreover, the dissimilarity between the *in vivo* and *in vitro* patterns at 16 h of imbibition indicates that the bulk of these mRNAs code for polypeptides which are greatly modified *in vivo*.

Protein Patterns in Dry Embryos. To determine if certain of the 33 polypeptides synthesized during the first 15 min of imbibition are present in dry embryos, we have extracted embryo proteins, separated them by two-dimensional electrophoresis, and visualized them by amino black or silver staining.

Figure 4A shows the electrophoretic pattern of amido black-stained proteins. Twenty-three polypeptides among the 33 early germination polypeptides comigrate with proteins extracted from dry embryos.

For comparison, Figure 4C shows the pattern of early germination polypeptides of embryos labeled for 15 min. We have marked the 10 polypeptides which do not comigrate with amido black-stained polypeptides from dry embryos.

We attempted to detect these 10 polypeptides by silver staining (Fig. 4B), this method being considered to be more sensitive than amido black staining. In fact, the sensitivity of the silver method is very dependent on the proteins studied, certain proteins being less well stained with silver than with amido black. Nevertheless, some proteins which are minor after amido black staining are heavily stained by silver: for example, in the neutral region of the gel. In addition, some proteins which are undetectable after amido black staining become visible after silver staining; for example, certain high mol wt proteins and low mol wt basic proteins. However, none of the 10 polypeptides synthesized during early germination (Fig. 4C) and absent in amido black-stained gel (Fig. 4A) is detectable by silver staining (Fig. 4B). For these 10 polypeptides, two hypotheses can be formulated:

(a) The simplest hypothesis is that they are specifically synthesized during germination.

(b) The second hypothesis is that they are also synthesized during embryogenesis but are not detectable in dry embryos because of their low level (short life time or low level of synthesis), or because of posttranscriptional modifications which modify their electrophoretic migration.

In order to distinguish between these two hypotheses we analyzed protein synthesis during embryogenesis.

This analysis presents a methodological difficulty. The first part of this work was carried out using embryo axes. This structure does not exist in early embryogenesis and is difficult to separate in later phases. Thus, we have compared stored mRNA from embryo axes with that stored in complete seeds (embryo axes + cotyledons). Figure 7C (complete dry seed) is almost identical to Figure 1B (embryo axes). Thus, the analysis of protein synthesis during embryogenesis was carried out using complete immature seeds.

Characterization of Embryogenesis Stages. We chose three stages, noted stages 1, 2, and 3, in late embryogenesis. These immature seeds have the same size (3–4 mm diameter). From a morphological point of view, stage 1 seeds are translucent, neither embryo nor cotyledons being visible. In stage 2 seeds the white embryo and the two green cotyledons become visible. Stage 3 was chosen at the beginning of maturation drying. Stages 1 and 2 are characterizable by two-dimensional patterns after silver staining as shown in Figure 5. Stages 2 and 3 show protein patterns which are indistinguishable and present great similarity with the dry embryo pattern. Major storage proteins which have been previously characterized (14) are detectable from stage 2 onward. Using short labelings we attempted to find which of the 33 early germination proteins are synthesized during embryogenesis.

***In Vivo* Protein Synthesis during Embryogenesis.** Figure 6 shows the drastic modifications in protein synthesis that occur during embryogenesis. Among the 33 polypeptides synthesized in early germination, 20 are detectable in dry embryos by staining and are synthesized at stage 2 *in vivo* (Fig. 6B). The 3 remaining early germination polypeptides which are detectable in dry embryos (10, 28, and 32) were not detected. Among the 10 early germination polypeptides undetectable in dry embryos, 2 (7 and 8) are synthesized at stage 2.

In contrast polypeptides, 4, 5, and 22 to 27, are undetectable in all stages we have analyzed. We have verified that polypeptides

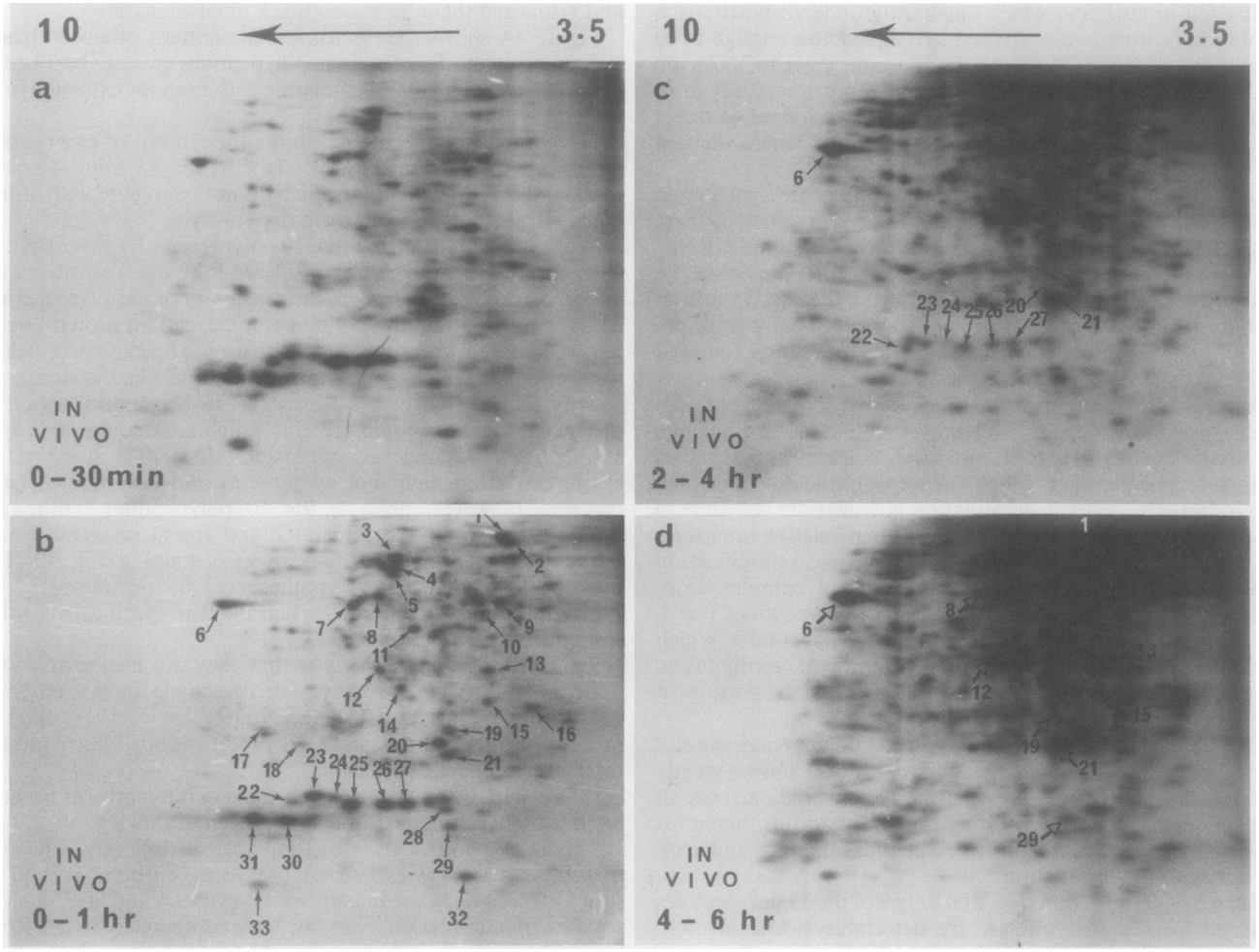

FIG. 2. Pattern modifications of proteins synthesized during germination. Embryos were labeled 0 to 30 min (A), 0 to 1 h (B), 2 to 4 h (C), and 4 to 6 h (D). Black arrows show on (B) the 33 early germination peptides and on (C) spots which increase or decrease in intensity. White arrows (D) indicate peptides which continue to be synthesized.

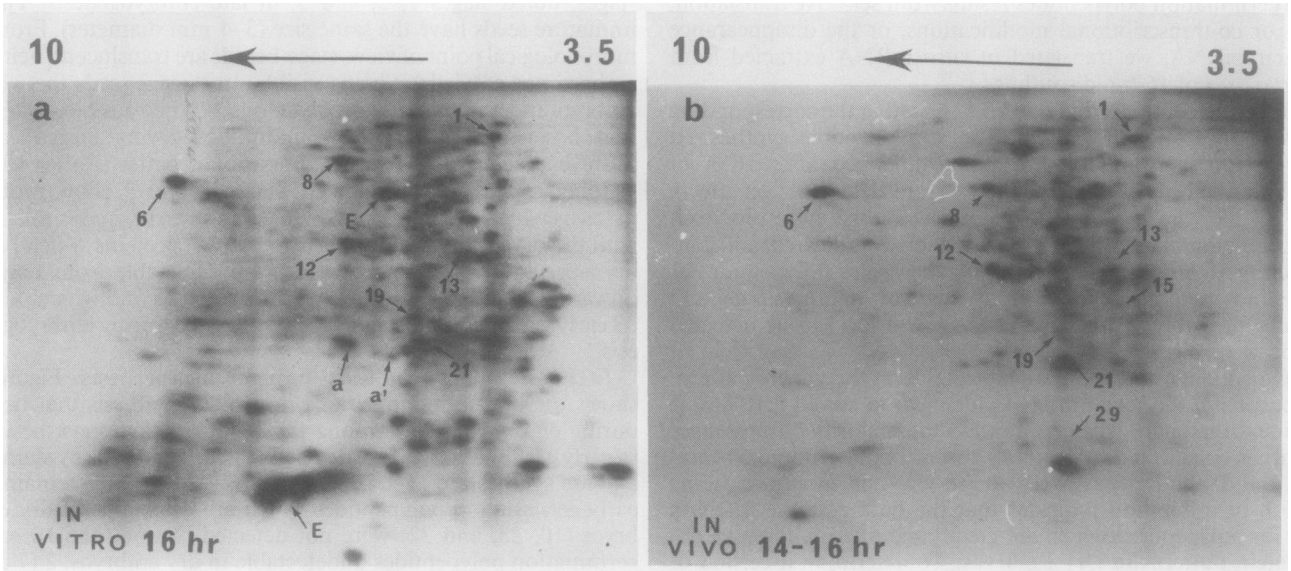

FIG. 3. *In vitro* (A) and *in vivo* (B) protein patterns during late germination. On pattern (B), black arrows show the early germination peptides which are still synthesized at this stage of germination, and on pattern (A), those which are detectable on the *in vitro* gel. E, peptides which are coded by mRNA endogenous to the reticulocyte system. Peptides a and a′ are noted on the *in vitro* pattern.

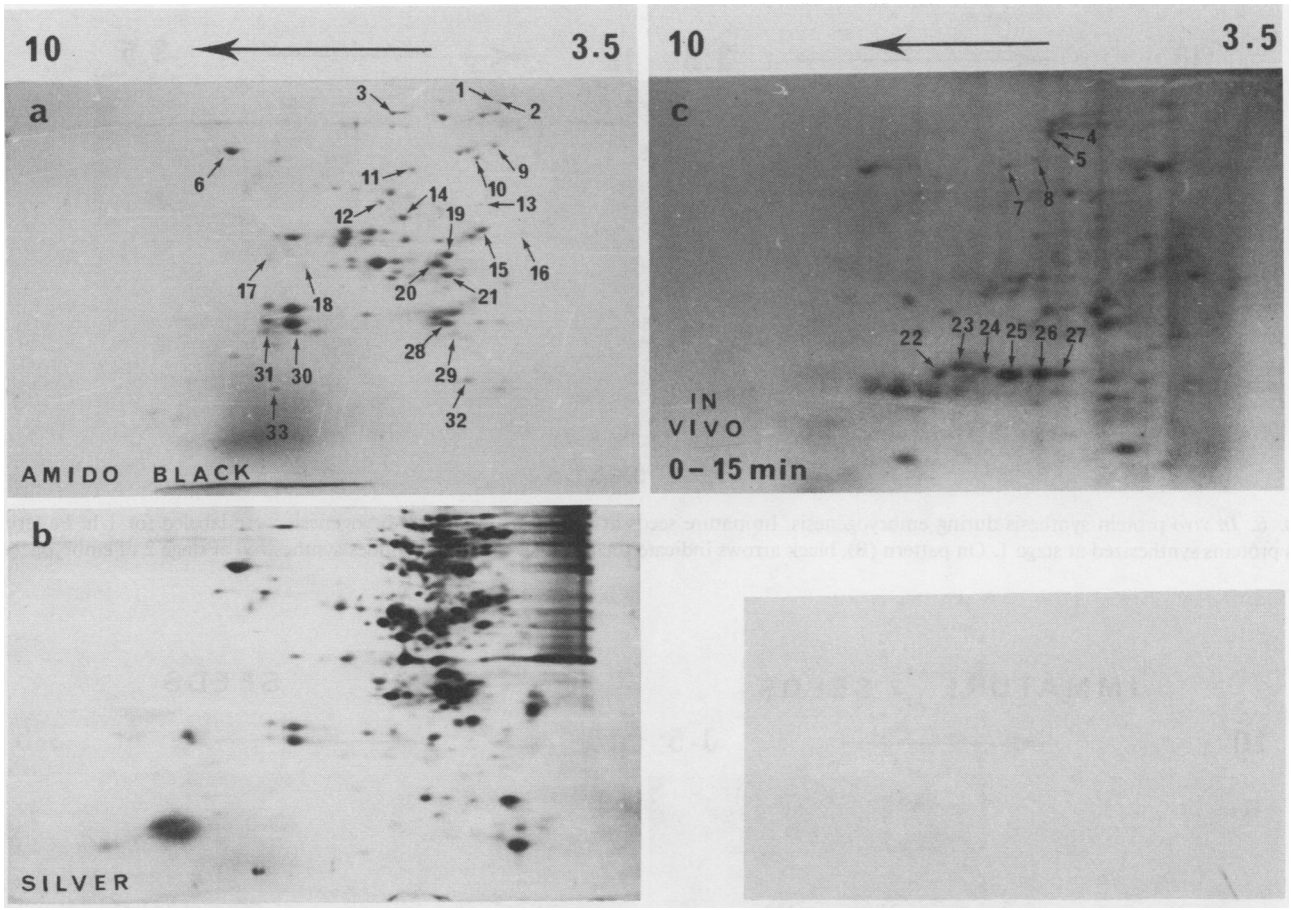

FIG. 4. Electrophoretic patterns of proteins in dry embryos after amido black (A) and silver (B) staining. On pattern (A), black arrows show the early germination peptides which comigrate with proteins extracted from dry embryos. Those which do not comigrate with amido black-stained proteins are marked on pattern (C), obtained with dry embryos labeled 15 min.

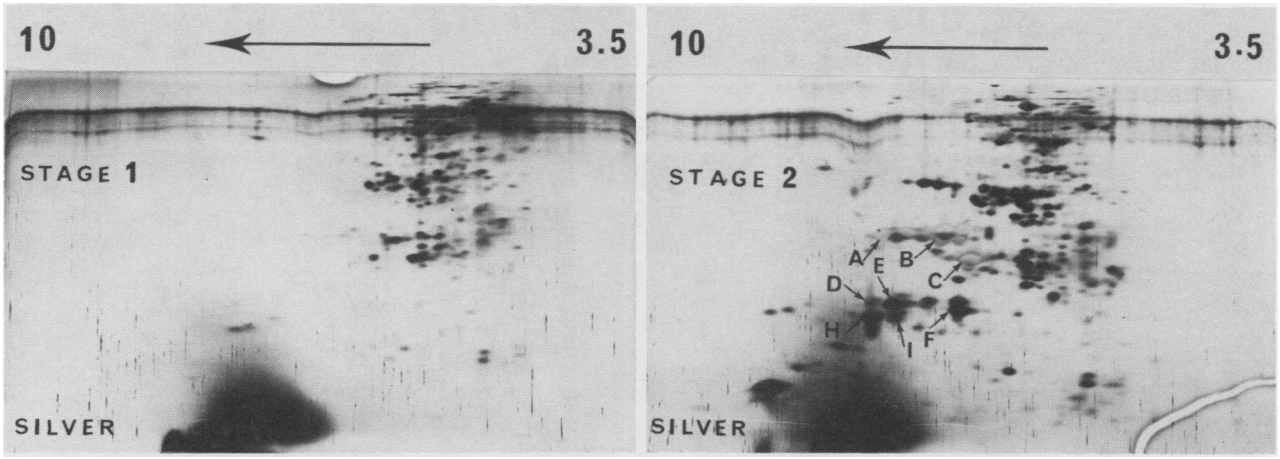

FIG. 5. Electrophoretic patterns of silver-stained proteins from immature seeds. Proteins from immature seeds at two stages of late embryogenesis were visualized by silver staining (left, stage 1; right, stage 2). Major storage proteins are indicated according to the nomenclature previously used (14).

in the 20,000 D region do not coincide with early germination polypeptides 22 to 27 (results not shown).

In summary, these 8 polypeptides appear to be specifically synthesized during early germination. We attempted to determine at which stage their mRNAs accumulate by translating mRNA *in vitro*.

Proteins Translated *In Vitro* from Immature Seed mRNA. RNAs from immature seeds at stage 2 were extracted and translated in a cell-free system (Fig. 7A). Storage protein precursors are probably situated in the 21,000 and 50,000 D regions, as has recently been proposed (3, 15). These major peptides are absent from the *in vitro* translation products of stored mRNA.

Among the 22 early germination polypeptides synthesized in stage 2 of embryogenesis, 18 are detectable on the *in vitro* pattern (Fig. 7A). For polypeptides 2, 3, 15, and 16, synthesized *in vivo* but unidentifiable on *in vitro* gels, several explanations can be

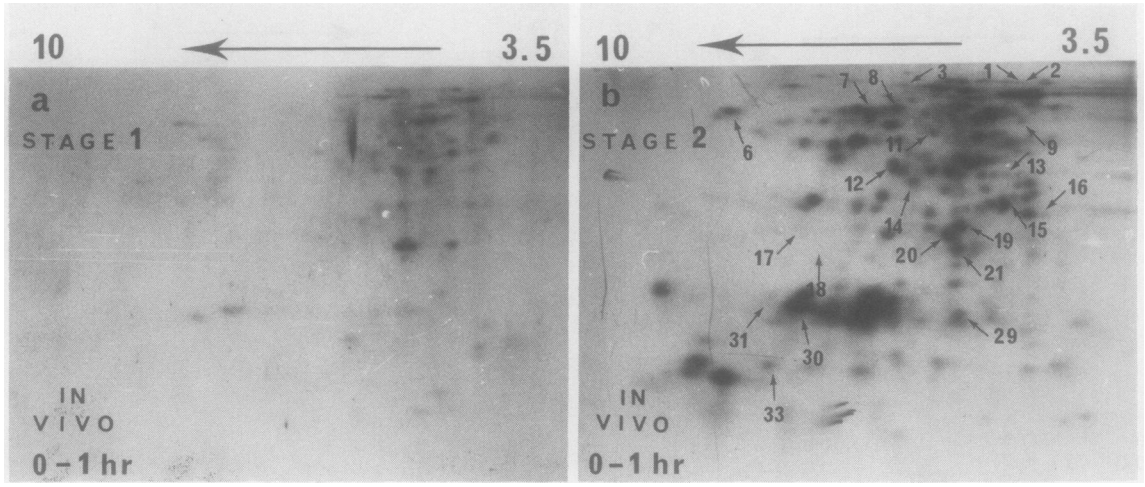

FIG. 6. *In vivo* protein synthesis during embryogenesis. Immature seeds at two stages of late embryogenesis were labeled for 1 h. Pattern (A) shows proteins synthesized at stage 1. On pattern (B), black arrows indicate the early germination peptides synthesized at stage 2 of embryogenesis.

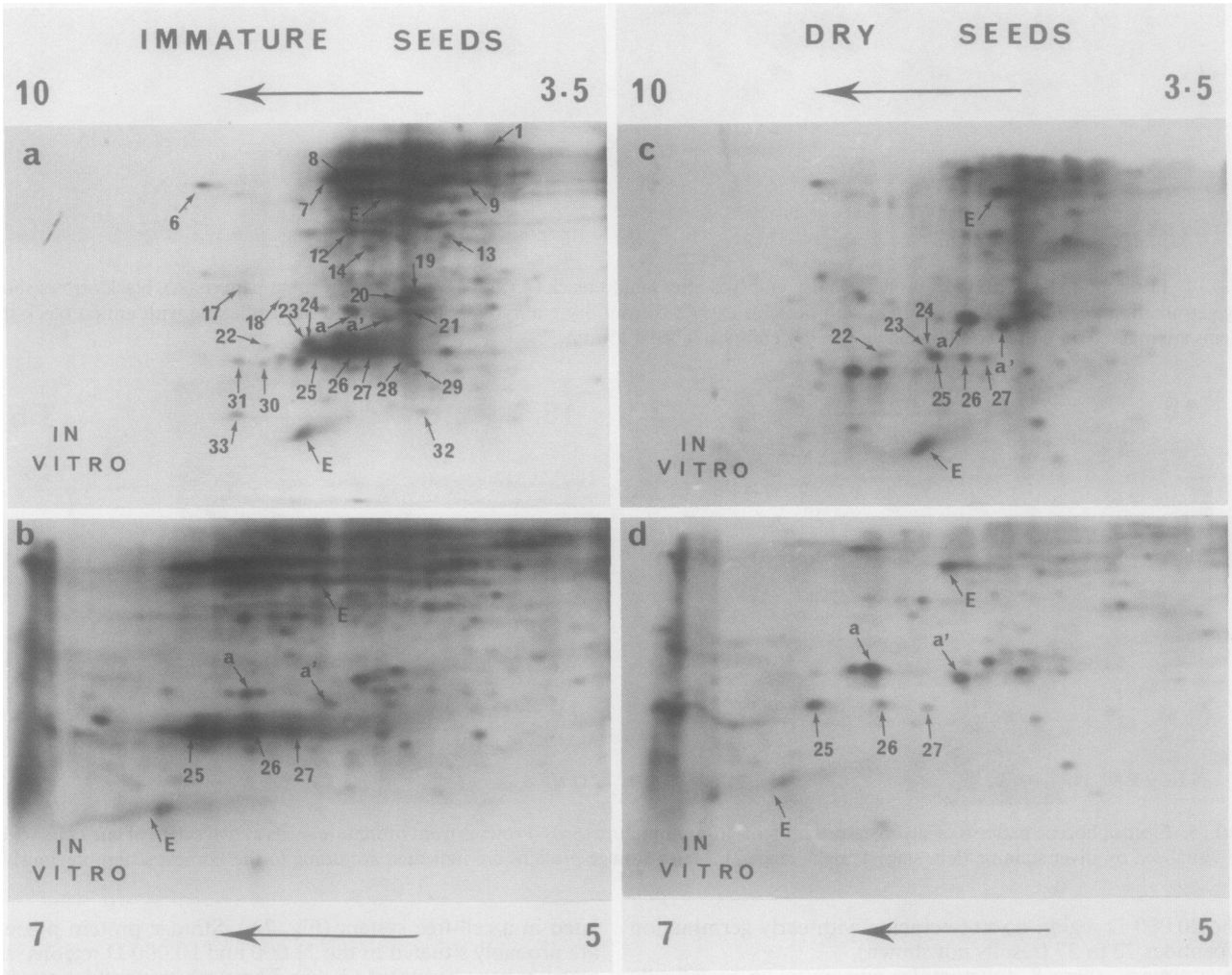

FIG. 7. Electrophoretic patterns of proteins translated *in vitro* from immature (A, B) and dry (C, D) seed mRNA. RNA from immature seeds at stage 2 and dry seeds were extracted and translated in the cell-free system. *In vitro* translation products were separated by NEPHGE (A, C) or IEF (B, D). On pattern (A), black arrows show the early germination peptides. On the other three patterns, black arrows indicate only those which are 'specific' to germination: 22 to 27 (C), 25 to 27 (B), and 25 to 27 (D). E, peptides which are coded by mRNA endogenous to the reticulocyte system. Peptides a and a' are noted on the four *in vitro* patterns.

formulated. For polypeptides 2 and 3 the background is too high to detect them. Polypeptide 15 is never detectable on *in vitro* gels, possibly because of posttranslational modifications. Polypeptide 16 presents only a very low radioactivity level on the *in vivo* gel and is undetectable on the *in vitro* pattern, probably due to a low level of synthesis. Polypeptides 28 and 32 are present on the *in vitro* gel although they are not detectably synthesized *in vivo*. Nevertheless, these polypeptides are present in dry embryos (Fig. 4A).

However, the most important information we can obtain from this gel (Fig. 7A) concerns the polypeptides specifically synthesized *in vivo* during early germination (4, 5, and 22–27). Due to the high radioactivity in the migration zone of polypeptides 23 to 27, we have used a pH 5 to 7 gradient in the first dimension in addition to our pH 3.5 to 10 gels to obtain a better resolution of this region (Fig. 7B). Polypeptides 22 to 27 are visible on these gels, indicating that the mRNAs of these 6 polypeptides are present. Polypeptides 4 and 5 migrate in a region of the gel where the background is very high and consequently we cannot determine whether their mRNAs are present at this stage of embryogenesis (Fig. 7A).

Polypeptides a and a′ are still detectable on the *in vitro* pattern from immature seeds.

Desiccation Treatment. As the mRNAs of polypeptides 22 to 27 are present in immature seeds (stage 2) but the corresponding polypeptides are not detectable after *in vivo* labeling, we tried to determine which factor is responsible for their appearance.

Maturation drying being the most notable change in seeds, we dehydrated immature seeds collected at stage 2 and subsequently labeled them for 1 h. In these conditions (Fig. 8), polypeptides 24, 26, and 27 are present on the electrophoretic pattern, whereas the presence of polypeptides 22, 23, and 25 is uncertain. In addition, polypeptides 10, 28, and 32, which are not detected *in vivo* at stage 2 of embryogenesis, are detectable on this protein pattern.

Origin and Fate of the Thirty-Three Most Radioactive Polypeptides Encoded by Stored mRNA. Table I presents a summary of the information we have obtained concerning the 33 major early germination polypeptides. (a) Twenty-five polypeptides are synthesized during late embryogenesis and are present in embryos. Of these, 16 are only synthesized at the beginning of imbibition. The other 9 (O) continue to be synthesized later in germination. (b) Eight polypeptides (□) are synthesized only during early germination. We have classed them into two groups. One group consists of polypeptides 4 and 5 (PM ≃ 70,000). Their mRNA is undetectable. In these conditions, their origin (direct translation or modification products) remains uncertain. The other group are polypeptides 22 to 27 (PM ≃ 20,000). They are

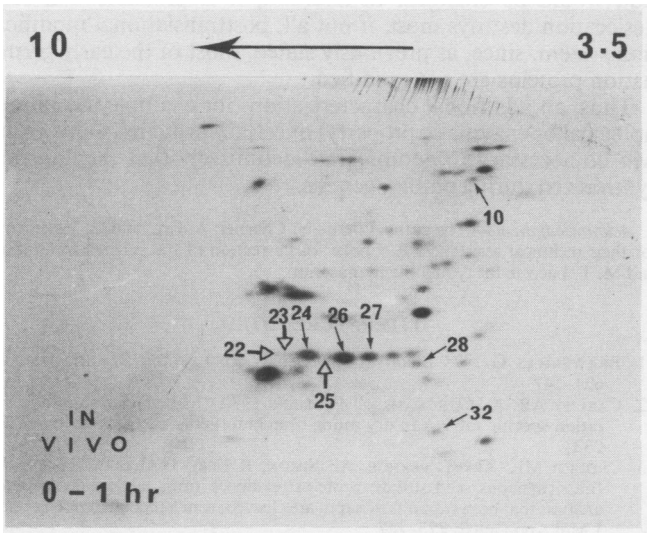

FIG. 8. Desiccation treatment. Immature seeds at stage 2 of embryogenesis were dehydrated and subsequently labeled for 1 h. Black arrows show early germination peptides which are not synthesized during late embryogenesis but induced by desiccation treatment. White arrows indicate the position of peptides whose presence is uncertain.

Table I. *Origin and Fate of the Thirty-Three Most Radioactive Peptides Coded by Stored mRNA*

The early germination peptides are noted 1 to 33 in the early germination column (vivo, 0–15 min). The late embryogenesis (left) and late germination (right) columns correspond to *in vivo* (vivo) and *in vitro* (vitro) peptides. For dry embryos, one column corresponds to the amido black-stained peptides (staining) and the other to the peptides coded by stored mRNA (vitro). The presence or absence of peptides is noted + or −; if the presence is uncertain, the peptide is marked ?; E indicates that the presence of one peptide is uncertain because it comigrates with a peptide which is coded by an mRNA endogenous to the reticulocyte system. The numbers (O) correspond to ubiquitous peptides; the numbers (□) to peptides 'specific' to germination. The others are synthesized during late embryogenesis but not during late germination.

Late Embryogenesis		Dry Embryos		Early Germination 0–15 min	Late Germination	
Vivo	Vitro	Staining	Vitro	Vivo	Vivo	Vitro
+	+	+	+	(1)	+	+
+	−	+	−	2	−	−
+	−	+	−	3	−	−
−	?	−	−	[4]	−	−
−	?	−	−	[5]	−	−
+	+	+	+	(6)	+	+
+	+	−	−	7	−	−
+	+	−	+	(8)	+	+
+	+	+	−	9	−	−
−	?	+	−	10	−	−
+	E	+	E	11	−	E
+	+	+	+	(12)	+	+
+	+	+	+	(13)	+	+
+	+	+	+	14	−	−
+	−	+	−	(15)	+	−
+	−	+	−	16	−	−
+	+	+	+	17	−	−
+	+	+	+	18	−	−
+	+	+	+	(19)	+	+
+	+	+	+	20	−	−
+	+	+	+	(21)	+	+
−	+	−	+	[22]	−	−
−	+	−	+	[23]	−	−
−	+	−	+	[24]	−	−
−	+	−	+	[25]	−	−
−	+	−	+	[26]	−	−
−	+	−	+	[27]	−	−
−	+	+	+	28	−	−
+	+	+	+	(29)	+	−
+	+	+	+	30	−	−
+	+	+	+	31	−	−
−	+	+	+	32	−	−
+	+	+	+	33	−	−

direct translation products. They are no longer synthesized during late germination because of the disappearance of their mRNAs. Their messengers are present during late embryogenesis but they are apparently not synthesized at this stage of embryogenesis.

DISCUSSION

We first attempted to determine the shortest pulse labeling compatible with the detection of early germination proteins. Radish embryos are apparently an excellent material for this purpose, since a distinct protein pattern can be obtained after only 15 min imbibition. This should be compared with the 40-min early germination labeling used for wheat embryos by Cuming and Lane (4) and Thompson and Lane (22) and the 6-h labeling used for cotton cotyledons by Dure *et al.* (10).

This very early and short labeling shows that the analyzed proteins are encoded by stored mRNA, since it has been demonstrated that eukaryotic mRNA synthesis (transcription, maturation, and transport) necessitates at least 15 to 20 min (1). This conclusion is reinforced by the superposition of the patterns of *in vivo* synthesized early germination polypeptides and *in vitro* translation products directed by stored mRNA.

In addition, the comparison of the patterns of *in vivo* and *in vitro* synthesized proteins shows that the early germination polypeptides undergo little or no posttranslational modification, when compared with the proteins synthesized during embryogenesis and those synthesized during late germination, where *in vivo* and *in vitro* patterns are very different. This may be a general characteristic of early germination polypeptides since a similar situation has been observed in wheat embryos by Thompson and Lane (22).

During the first period of imbibition, the protein pattern remains unchanged. Only later do new proteins become detectable and are most probably encoded by newly synthesized mRNA. These new proteins constitute the bulk of synthesis after 4 h of imbibition. Dommes and Van de Walle (9) have shown that newly synthesized mRNAs are associated with polysomes after 1 to 2 h of germination in maize embryos, and Cuming and Lane (4) have demonstrated considerable modification of protein synthesis between 1 and 5 h germination of wheat embryos. After 6 h, early germination proteins are barely detectable. This is in agreement with our previous study using cordycepin to block mRNA synthesis, where we determined a half-life of 100 min for stored mRNA during germination (6). Thus, the time course we describe here for radish is probably similar to that in other plants.

Among the abundant stored mRNAs, two, detected by their *in vitro* translation products (a and a′), have no corresponding *in vivo* product. Different hypotheses can be formulated.

(a) The corresponding *in vivo* products are synthesized and undergo posttranslational modification, changing their migration characteristics. Nevertheless, no highly radioactive spot, which could correspond to the modified form of a and a′, is present *in vivo* and absent *in vitro*.

(b) The relative translation rate of these particular messengers may be exceptionally high in the reticulocyte translation system. However, overexposure of *in vivo* gels does not show any radioactivity in the a and a′ regions.

(c) These messengers may not be translated at all *in vivo*. They have in fact at least two other characteristics which distinguish them from the other stored mRNAs. They are not degraded during late germination and they are not polyadenylated (15). Thus, it is possible that these sequences may not be true messengers, but degradation products of particularly abundant mRNAs present during embryogenesis.

The polypeptides expressed during early germination belong to two different sets.

The majority of the spots, representing almost half the incorporated radioactivity, correspond to proteins which are very abundant in the seed. It is unlikely that the additional synthesis of these polypeptides during early germination is of importance for the germination process. Thus, the corresponding mRNAs can be considered as relics of embryogenesis. The question of the presence or absence of storage protein mRNA in stored mRNA is controversial. On the one hand, Dure *et al.* (11) have shown that the mRNAs coding for the 70 and 67 kD storage protein precursors are undetectable, using *in vitro* synthesis, in stored mRNA from cotton seeds, and other workers (4, 22) have observed the same results in wheat. Our analysis of radish storage mRNA leads to the same conclusion. On the other hand, Mori *et al.* (18), using immuno-precipitation, have detected mRNA coding for storage proteins in dry soybeans. In fact, the discrepancy between these different reports may be due to the methodology. Electrophoretic analysis of polypeptides only permits the detection of major products, while immuno-precipitation allows the estimation of minute quantities of proteins. Similarly, Dure *et al.* (12) have recently shown by hybridization using cloned cDNA that trace amounts of storage protein mRNAs are present in dry cotton seeds. The general conclusion may be that mRNA coding for storage proteins do not belong to major stored mRNAs.

The second set is composed of proteins detectable only during early germination. These proteins are of particular interest because they may play a specific role during germination. Although they have been postulated since the discovery of stored mRNA in seeds, their existence has only been proven very recently by Dure *et al.* (10) in cotton using two-dimensional electrophoresis. The mRNAs coding for these proteins are synthesized during late embryogenesis, but it appears that translation of these messengers necessitates treatment with ABA (for cotton, [10]) or desiccation (for *Phaseolus vulgaris* [5]; and radish, this work). Nevertheless, these three reports, which establish the existence of this protein class, use the same methodology. The polypeptides specifically synthesized during early germination are characterized by their migration in a two-dimensional electrophoresis system. The absence of corresponding spots during embryogenesis does not definitively demonstrate that their mRNAs are not translated. Indeed, if polypeptides encoded by these mRNAs are synthesized and then posttranslationally modified, their migration would be changed. In addition, it is probable that seed desiccation destroys most, if not all, posttranslational modification system, since, as previously stated, most of the early germination proteins are not modified.

Thus, an additional characterization (for example, an immunological or enzymatic property) of certain proteins of this group will be necessary to demonstrate definitively that they are not synthesized during embryogenesis.

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