Plant Physiol. (1979) 64, 630–634
0032-0889/79/64/0630/05/$00.50/0

Developmental Changes in the Activity of Messenger RNA Isolated from Germinating Castor Bean Endosperm[1]

Received for publication January 22, 1979 and in revised form May 5, 1979

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ABSTRACT

The capacity of polyadenylated RNA from developing castor bean endosperm to program protein synthesis in a wheat germ cell-free translational system has been examined. Although the use of micrococcal nuclease-treated wheat germ extracts demonstrated a low but significant content of translatable mRNA in dry seeds, a large scale increase in total translational capacity was observed during germination. The cellular content of translatable mRNA peaked on the 4th day of germination and subsequently declined. It is concluded that protein synthesis in castor bean endosperm cells during germination is directed by newly transcribed mRNA.

The germination of plant seeds initiates a series of biochemical events, one of the earliest of which is a rapid increase in protein biosynthetic capacity (12, 13). Although it is clear that a variety of mRNAs are translated during the early stages of germination, the time at which these messengers are transcribed is less well understood. Considerable data have accumulated which suggest that dry seeds already contain preformed mRNA resulting from transcription during seed formation (for review, see 15). Most of the studies which support this concept have been carried out during the early stages of hydration and germination. It is not clear to what extent such long lived mRNA is responsible for directing protein synthesis during germination or at what developmental stage its role is taken over by newly transcribed mRNA.

Recently we have shown that the polyadenylated RNA fraction isolated from the endosperm tissue of 3-day-old castor bean seedlings actively directs protein synthesis in a mRNA-dependent cell-free translational system derived from rabbit reticulocytes (6). At this developmental stage, endosperm cells are actively synthesizing enzymes responsible for gluconeogenesis from stored triglyceride (1, 2, 4). Gluconeogenic enzymes (specifically, the glyoxysomal matrix proteins) were shown to comprise a significant proportion (15–20%) of the total translational products. In the present study we have assessed the rate of synthesis of endosperm mRNA during early seedling growth by extracting and translating poly(A^+) RNA[2] at various developmental stages. Although poly(A^+) RNA was readily detected in dry seeds, the results indicate that protein synthesis during germination is largely, perhaps completely, dependent on newly transcribed mRNA.

MATERIALS AND METHODS

Preparation of Poly(A^+) RNA. Endosperm tissue was excised from 35 seeds at various developmental stages which had been carefully selected for uniformity. Endosperm halves were frozen and ground to a powder in liquid N_2 and then added to 3 volumes of 150 mM NaCl, 50 mM Tris-HCl (pH 9.0), 5 mM EDTA, and 5% (w/v) SDS and briefly homogenized in a Waring Blendor. Deproteinization by phenol-chloroform (1:1) extraction, precipitation of RNA with ethanol, removal of contaminating polysaccharide by washing with 3 M Na-acetate (pH 5.5), and isolation of poly(A^+) RNA by oligo(dT)-cellulose chromatography were performed as described previously (6). Poly(A^+) RNA was precipitated at −20 C with 2.5 volumes of ethanol, redissolved in sterile distilled H_2O at a concentration of 12 A_{260} units (600 μg RNA)/ml and stored at −80 C.

Preparation of Wheat Germ Extracts. A wheat germ S30 fraction was prepared as described by Roberts and Patterson (17) but without preincubation. The wheat germ S30 extracts routinely had concentrations greater than 100 A_{260} units/ml, and were stored as small aliquots in liquid N_2. When appropriate, endogenous mRNA present in the wheat germ S30 extracts was destroyed by preincubation with Ca^{2+}-dependent micrococcal nuclease as described by Pelham and Jackson (16) for the rabbit reticulocyte lysate system. Immediately after thawing, wheat germ S30 extracts were incubated for 10 min at room temperature after adding $CaCl_2$ (1 mM final concentration) and micrococcal nuclease at a final concentration of 10 μg/ml. Nuclease activity was then blocked by the addition of ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid to a final concentration of 2 mM.

Cell-free Protein Synthesis. The translational system contained, in a final volume of 50 μl, 20 mM 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid (adjusted to pH 7.5 with KOH), 2 mM DTT, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 10 μg creatine phosphokinase, 0.25 mM spermidine, 2.25 mM Mg-acetate, 100 mM KCl, 30 μM of 19 unlabeled amino acids (methionine being excluded), 10 to 20 μCi [^{35}S]methionine (specific radioactivity up to 1,000 Ci/mmol), and 20 μl wheat germ S30 extract. Protein synthesis was initiated by adding poly(A^+) RNA (0.06 A_{260} units unless otherwise stated). Incubation was at 27 C and at intervals 5-μl samples were removed. Hot trichloroacetic acid-insoluble radioactivity was determined by liquid scintillation counting in Bray's scintillant (7) after processing the samples according to the procedure of Mans and Novelli (11).

Polyacrylamide Gel Electrophoresis. After incubation for 60 min, 25 μl of the cell-free translational mixture was mixed with an equal volume of 10% (w/w) trichloroacetic acid and kept on ice for 30 min. The precipitate was collected by centrifugation and dissolved by incubation (20 min at 37 C followed by 2 min at 100 C) in sample buffer containing 10% (w/v) SDS, 10% (w/w) sucrose, 17% (v/v) β-mercaptoethanol, 2 mM EDTA, 50 mM Tris-HCl (pH 8.3), and bromophenol blue as tracker dye. The reduced proteins were alkylated by incubation for 60 min at 30 C with 25 mM 2-iodoacetamide before loading the sample into a slot on a 10 to 15% polyacrylamide gradient slab gel containing 0.1% SDS and topped with a 5% acrylamide stacking gel. Gels were prepared as described by Laemmli (10). After electrophoresis, the gels were

[1] This work was supported by the Science Research Council through Grant GR/A 3703.1.

[2] Abbreviations: poly(A^+) and poly(A^-) RNA: RNA with and without attached polyadenylic acid sequences.

stained and destained, and fluorograms were prepared as described by Bonner and Laskey (3).

Immunoprecipitation. Antibodies against the total matrix proteins separated from isolated glyoxysomes were raised in rabbits as described previously (5).

Six μg of carrier glyoxysomal matrix protein was added to 50 μl of cell-free translational mixture which was adjusted to 0.1 M Tris-HCl (pH 8.5), 0.8 M NaCl, 1% (v/v) Triton X-100, and 100 units/ml of Trasylol. Ten μl of the rabbit antiglyoxysomal matrix protein IgG fraction (17.5 mg protein/ml) or preimmune IgG fraction (25 mg protein/ml) was added to the mixture. After incubation at 37 C for 1 h, 60 μl of the IgG fraction of goat antiserum to rabbit IgG (11.5 mg protein/ml) was added and the mixture was incubated further at 4 C for 24 h. Immunoprecipitates were collected by centrifugation, washed three times in 10 mM Tris-HCl (pH 8.5), 0.5 M NaCl, 1% (v/v) Triton X-100 and twice in 10 mM Tris-HCl (pH 8.5), dissolved in 10 mM Tris-HCl (pH 8.5), containing 2% SDS and counted.

Isolation of Polysomes. Endosperm tissue was frozen and ground to a powder in liquid N_2. The tissue was briefly homogenized in 5 volumes of 150 mM Tris-acetate (pH 8.5), 200 mM RNAase-free sucrose, 50 mM KCl, 20 mM Mg-acetate, 0.4% (v/v) Nonidet P-40 which contained 30 mg/ml sodium heparin. The homogenate was filtered through sterilized cheesecloth and centrifuged for 10 min at 23,000*g* and 2 C. The supernatant was layered over 2 ml of 1 to 5 M sucrose solution containing 50 mM Tris-acetate (pH 8.5), 20 mM KCl, and 10 mM Mg acetate in a 6.5-ml centrifuge tube. Ribosomes were pelleted by centrifuging for 3.5 h at 100,000*g* and 2 C in the 40.3 rotor on a Beckman L2-65B ultracentrifuge. Pellets were resuspended in 50 mM Tris-acetate (pH 7.6), 30 mM KCl, 10 mM Mg-acetate, and 5 mM β-mercaptoethanol. Aliquots containing 100 to 120 μg of RNA were layered onto 17.5-ml sucrose gradients increasing linearly in concentration from 10 to 30% (w/v) sucrose. Sucrose solutions were prepared in the polysome resuspension buffer. Gradients were centrifuged for 1.75 h at 96,000*g* (average) and 2 C in a SW 27 rotor. The A_{260} profile across the centrifuged gradients was obtained using an ISCO model 185 gradient fractionator linked to a UV monitor.

RNA Determination. RNA content was determined spectrophotometrically assuming that 1 A_{260} unit corresponds to 50 μg of RNA. This figure was determined using a calibration curve prepared for purified yeast RNA. One A_{260} unit defines that amount of RNA which has an A of 1.0 at 260 nm in a 1-cm light path cuvette.

Materials. [^{35}S]Methionine was obtained from the Radiochemical Centre, Amersham, U. K., Nonidet P-40 from Shell Chemical Co., Sittingbourne, U. K., oligo(dT)-cellulose from Collaborative Research, Waltham, Mass., micrococcal nuclease from Boehringer Mannheim, Germany, and goat antibodies to rabbit IgG from Miles Laboratories, Elkhart, Ind. Sodium heparin (166 units/mg) was a generous gift from Leo Pharmaceutical Products, Ballerup, Denmark, and wheat germ, obtained from General Mills, Minneapolis, Minnesota, was kindly provided by Dr. C. J. Leaver, University of Edinburgh, U. K.

RESULTS AND DISCUSSION

Characterization of Translational System. The poly(A$^+$) RNA isolated from the endosperm of germinating castor bean seedlings was translated when added to wheat germ S30 extracts in the presence of appropriate cofactors. That fraction of the total cellular RNA which did not bind to oligo(dT)-cellulose during isolation, designated poly(A$^-$) RNA, was not translated when added to the translational system in equivalent or increased concentrations. Accordingly we regard the poly(A$^+$) RNA fraction as a preparation which contains the bulk of the active mRNA isolated from the tissue. In the translational system, protein synthesis was dependent on the presence of Mg^{2+}, K^+, and an energy-generating system. Synthesis was severely inhibited by the addition of cycloheximide, but was relatively insensitive to the presence of *D-threo*-chloramphenicol. Synthesis was also completely inhibited when mRNA was added to the translational system in the presence of either ribonuclease or the initiation inhibitor, aurintricarboxylic acid. Optimum concentrations of added Mg^{2+} and K^+ were determined to be 2.25 and 100 mM, respectively. The observed rate of protein synthesis was linearly proportional to the amount of added poly(A$^+$) RNA up to an optimal concentration of 60 μg/ml, but higher concentrations resulted in a marked decrease in translation (Fig. 1).

Developmental Changes in Cellular mRNA Activity. An analysis of the total cellular RNA isolated from the endosperm tissue excised from 35 seeds at various developmental stages is shown in Figure 2a. Although we appreciate that phenol-detergent extraction of nucleic acid is probably not quantitative and that the efficiency of extraction may vary somewhat during seedling development, it is probable that a net accumulation of total cellular RNA occurs during the first 4 days of germination. The poly(A$^+$) RNA was isolated from the total RNA by affinity chromatography on oligo(dT)-cellulose. Although a significant poly(A$^+$) RNA content was found at all developmental stages examined, it also increased rapidly during germination to reach a peak on the 4th day (Fig. 2b). Cellular poly(A$^+$) RNA content, therefore, increased in phase with the synthesis of gluconeogenic enzymes and the organelles which house them (2). Poly(A$^+$) RNA was readily detected in dry seeds and cellular content appeared to decline during imbibition.

We assume that the isolated poly(A$^+$) RNA fractions contained most of the cellular mRNA present at each developmental stage and that the activity of these fractions in cell-free translational assays gave an indication of the amount of translatable mRNA present in the tissue at the time of isolation. The results obtained from translating poly(A$^+$) RNA isolated during development, at a concentration of 60 μg/ml, are shown in Figure 3. A striking increase in messenger activity was observed over the first 4 days of germination which subsequently declined. These data are complicated by the fact that the concentration of active mRNA within the constant concentration of total poly(A$^+$) RNA added to each translational assay cannot be accurately assessed at every stage. It is possible that the concentration of translatable mRNA may have been limiting at certain stages, particularly at the onset of germination. However, varying the amount of poly(A$^+$) RNA added to the translational assay at each developmental stage did not significantly affect the results shown in Figure 3. Mixing experiments

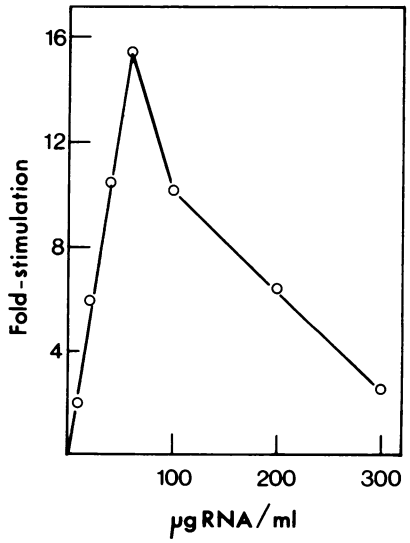

FIG. 1. Effect of varying the concentration of poly(A$^+$) RNA from 3-day-old endosperm on the rate of polypeptide synthesis in wheat germ S30 extracts. Incubation was for 30 min at 27 C.

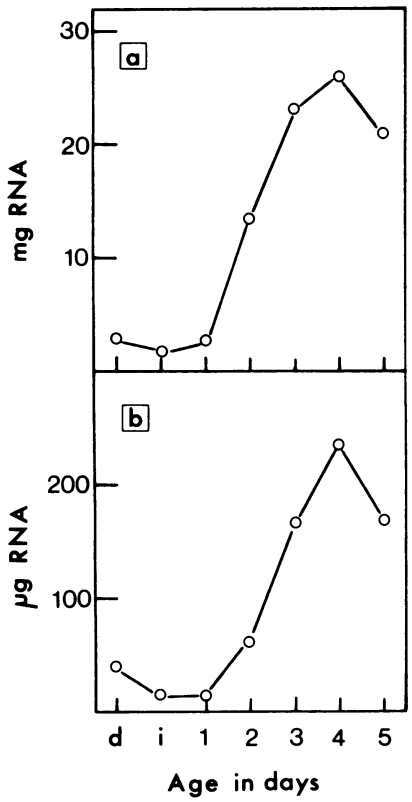

FIG. 2. Effect of seedling age on the amount of (a) total RNA and (b) poly(A$^+$) RNA extracted from 70 endosperm halves. d and i signify dry and imbibed seeds, respectively.

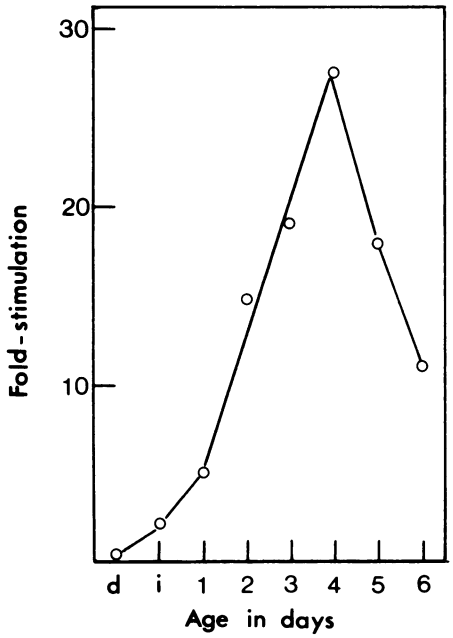

FIG. 3. Stimulation of polypeptide synthesis in wheat germ S30 extracts by the addition of 60 µg/ml of poly(A$^+$) RNA isolated from endosperm tissue at different developmental stages. d and i signify dry and imbibed seeds, respectively.

in which poly(A$^+$) RNA fractions from more than one stage were translated in the same assay established that inhibitory factors were not present in the poly(A$^+$) RNA preparations from the early and late stages examined. Because the developmental stages showing greatest mRNA activity at fixed poly(A$^+$) RNA concentration in the translational assays (Fig. 3) correspond with stages showing maximum cellular poly(A$^+$) RNA content (Fig. 2b), we conclude that there is a striking increase in the total translational capacity of the endosperm during the early stages of seedling growth. We further conclude that protein synthesis in germinating castor bean endosperm is largely directed by newly transcribed mRNA. Since the observed developmental increase in total cellular RNA (Fig. 2a) is due primarily to increases in 18S and 25S ribosomal RNA species (18), it also appears that this protein synthesis occurs mainly on newly assembled ribosomes.

The translational products formed at each stage, under the direction of 60 µg/ml (polyA$^+$) RNA, were separated by polyacrylamide gel electrophoresis in the presence of SDS and visualized by fluorography. Numerous discrete polypeptides of widely different mol wt were formed (Fig. 4). Antiserum raised against glyoxysomal matrix proteins was added to the total translational products obtained at each stage and immunoreactive polypeptides were precipitated. As shown in Table I, glyoxysomal proteins

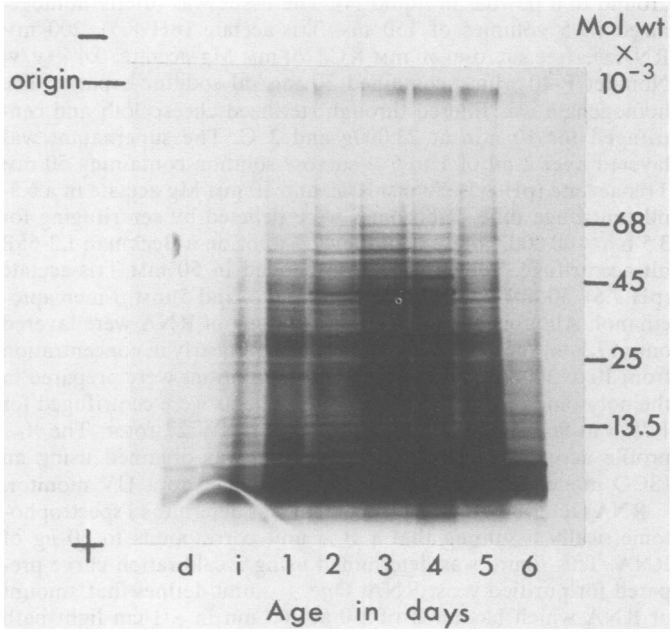

FIG. 4. Characterization of products obtained from the translation of poly(A$^+$) RNA (60 µg/ml) isolated from endosperm tissue at different developmental stages. Analysis was by SDS-polyacrylamide gel electrophoresis and fluorography. d and i signify dry and imbibed seeds, respectively. Mol wt standards used were: BSA, 68,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; ribonuclease A, 13,500.

Table I. *Immunoprecipitation of Glyoxysomal Proteins from Total Translational Products during Development*

Tissue Age[1]	Total Products	Immunoprecipitated Products[2]	% of Total in Immunoprecipitates
days	*cpm × 10^{-3}*		
Seed, dry	95	1.16	1.7
Seed, imbibed	148	9.96	6.7
1	400	31.2	7.8
2	539	48.5	9.0
3	760	107.4	14.0
4	876	132.8	15.1
5	624	106.2	17.0
6	268	24.0	8.9

[1] Poly(A$^+$) RNA was translated at a concentration of 60 µg/ml at each stage.

[2] Corrected for nonspecific precipitation obtained with preimmune serum which was 0.5 to 1% of the total products at each stage.

accounted for a significant proportion of the *in vitro* products at all stages, particularly between days 3 and 5 when maximum rates of cellular glyoxysome biosynthesis are known to occur (2, 9).

The data shown in Figure 3 were obtained by subtracting the counts incorporated by the endogenous wheat germ mRNA from the total counts incorporated in the presence of added castor bean endosperm mRNA. Because of this endogenous activity, it was not possible to demonstrate an increase in the rate of protein synthesis resulting from the addition of poly(A^+) RNA isolated from dry seeds. In order to facilitate the translation of any active mRNA present in dry seed poly(A^+) RNA we destroyed endogenous mRNA present in the wheat germ S30 extracts by nuclease treatment. The procedure employed was that recently described by Pelham and Jackson (16) for the preparation of mRNA-dependent rabbit reticulocyte lysates. The results of the nuclease treatment are shown in Figure 5. Typical activities of wheat germ S30 extracts and the stimulation due to the addition of poly(A^+) RNA isolated from 4-day-old endosperm tissue are shown. Pretreatment of these extracts with Ca^{2+}-dependent micrococcal nuclease completely eliminated endogenous mRNA activity. The addition of day-4 (polyA$^+$) RNA showed that these treated extracts still retained the capacity to translate exogenous mRNA. A comparison of the rates of translation of added endosperm mRNA in untreated and nuclease-treated wheat germ S30 extracts established that the latter translated exogenous mRNA at 66% of the efficiency observed for untreated extracts. This figure is in close agreement with the efficiency observed by Pelham and Jackson (16) when exogenous 9S globin mRNA was added to nuclease-treated rabbit reticulocyte lysates. When dry seed poly(A^+) RNA was added to translational systems prepared using nuclease-treated, mRNA-dependent wheat germ extracts, a low but significant incorporation of radioactivity into protein was observed (Fig. 6). We therefore conclude that dry castor bean seeds, in keeping with recent results obtained with other seeds (for review, see 15) contain a small amount of translatable mRNA.

Polysome profiles obtained at different developmental stages were also consistent with the main conclusions obtained in the present study. We experienced considerable difficulty in obtaining undegraded polysomes from the endosperm tissue at all developmental stages, presumably due to the high levels of ribonuclease activity present in this tissue. From a variety of ribonuclease inhibitors employed, only heparin (14) consistently gave reasonable profiles, and even in the presence of this compound we are uncertain to what extent polysome degradation may still have occurred. In the case of dry seeds, only monosomes appeared to be present. Imbibed seeds showed evidence of polysome assembly to a limited extent, while polysomes were clearly observed in the case of endosperm tissue excised from 3-day-old seedlings.

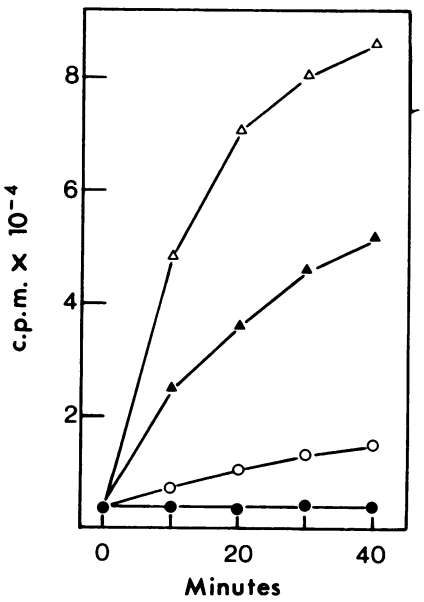

FIG. 5. Effect of micrococcal nuclease treatment on polypeptide synthesis in wheat germ S30 extracts. Endogenous mRNA activity (○) and the activity due to the addition of 60 μg/ml of poly(A^+) RNA from 4-day-old endosperm (△) in untreated wheat germ extracts, and endogenous activity (●) and the activity due to the addition of 60 μg/ml of poly(A^+) RNA from 4-day-old endosperm (▲) in nuclease-treated extracts.

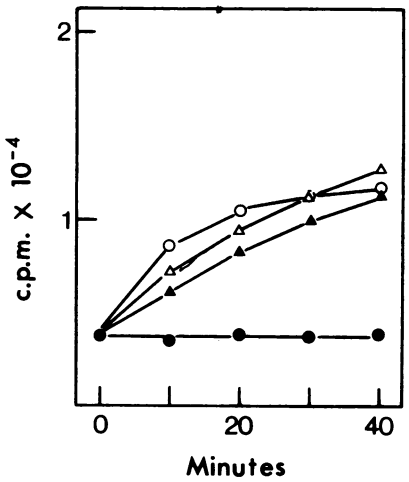

FIG. 6. Time course for polypeptide synthesis in nuclease-treated wheat germ S30 extracts supplemented with 40 μg/ml (○), 80 μg/ml (△), and 120 μg/ml (▲) of poly(A^+) RNA from the endosperm of dry seeds, and the endogenous activity in the absence of added RNA (●).

CONCLUSIONS

Many studies into the biochemistry of seed germination have provided evidence for the presence of long lived mRNA species stored in the dry seeds (8, 15, 19). At the present time there is little information regarding the nature and role of the proteins which are coded for by preformed mRNA. In particular, it is not clear whether preformed mRNA codes for proteins uniquely involved in the germination process, or whether such proteins are coded for by newly formed mRNA which is itself transcribed during germination.

In the present study we have shown that although preformed mRNA can be detected in dry castor bean seeds, a large scale increase in the cellular content of active endosperm mRNA occurs during the early stages of germination. The endosperm tissue has a limited lifetime in germinating seedlings and rapidly senesces once gluconeogenesis from fat is replaced by cotyledonary photosynthesis as the principal mode of seedling nutrition. Germinating castor bean endosperm cells achieve a large scale *de novo* synthesis of gluconeogenic enzymes and organelles in the absence of net protein synthesis or cell division (2). We conclude that the synthesis of these proteins uniquely formed at this developmental stage is directed by mRNA newly transcribed from derepressed genes.

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