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Histone Synthesis during the Early Stages of Germination in *Vicia faba* Embryonic Axes

Atsushi Kato [1], Keisuke Fukuei [2] and Shigeyuki Tanifuji [1]

[1] *Department of Botany, Faculty of Science, Hokkaido University, Sapporo 060, Japan*
[2] *Department of General Education, Kunitachi Music College, Tachikawa 190, Japan*

Histone synthesis during the early stages of germination of *Vicia faba* seeds was examined by labeling the detached embryonic axes with ^{3}H-lysine and ^{3}H-arginine and analyzing the extracted histones by polyacrylamide gel electrophoresis. All five plant histones were synthesized in the axes prior to the onset of very active incorporation of ^{3}H-thymidine into DNA and continued to be synthesized in all the germination stages examined. In vitro translation experiments, using a wheat germ cell-free system, indicated that the embryonic axes of dry *V. faba* seeds contained preformed mRNAs for various kinds of polypeptides, but were deficient in preformed mRNAs for histones. The histone synthesis in the axes was dependent on the transcription of new histone mRNAs. This study indicated that the synthesis of plant histones is more active on free polysomes than on membrane-bound polysomes.

Key words: Germination (seed) — In vitro translation — Plant histone synthesis — Preformed mRNA — *Vicia faba*.

Histone synthesis and DNA replication have been shown to occur concomitantly and to be closely interrelated in the somatic cells of animals (Robbins and Borun 1967, Spalding et al. 1966) and plants (Bloch et al. 1967). DNA-RNA hybridization and in vitro translation experiments have shown that histone mRNAs are associated with polysomes during only the S-period in synchronized HeLa cells (Detke et al. 1979, Gallwitz and Breindl 1972, Stein et al. 1975).

In contrast, a lack of simultaneous histone and DNA synthesis was observed in early-cleavage sea urchin embryos (Arceci and Gross 1977). Unfertilized sea urchin eggs contained large amounts of preformed mRNAs for histones, which were utilized during cleavage in the intervals outside of the S-period (Farquhar and McCarthy 1973, Skoultchi and Gross 1973). Adamson and Woodland (1974) showed that four histones other than H1 are synthesized in *Xenopus laevis* oocytes, in which no DNA replication takes place. Furthermore, the synthesis of one H1 component at the G1 stage has been reported in cultured animal cells, with BHK cells (Tarnowka et al. 1978).

Our earlier observations showed that the first DNA replication in the embryonic axes of germinating *V. faba* seeds took place in a partially synchronized fashion after a lag period and that syntheses of RNA, total proteins, histones and ribosomal proteins preceded the DNA synthetic phase (Jakob 1972, Jakob and Bovey 1969, Fukuei et al. 1977, 1978, Sakamaki et al. 1975). The present study investigated whether histone synthesis at the early stage of germination, which is not simultaneous with DNA replication, is dependent on preformed mRNAs in the embryonic axes of dry seeds, analogous to the case of sea urchin embryos, or whether it depends on the transcription of new histone mRNAs.

Abbreviations: mRNP, mRNA-containing ribonucleoprotein; TCA, trichloroacetic acid.

Materials and Methods

Plant materials—Embryonic axes of *Vicia faba* L. (cv. Wase-Soramame) were excised from seeds soaked for 18 hr in water at 27°C, and incubated aseptically for 6 or 18 hr in a liquid culture medium (Sakamaki et al. 1975). Some of the incubated axes were treated with ^{3}H-lysine or ^{3}H-arginine alone or mixed, for the final 2 or 5 hr of the incubation period. Embryonic axes were also excised manually from dry seeds. Wheat embryos (*Triticum aestivum* L.) were prepared by the procedure of Johnston and Stern (1957) using CCl_4-cyclohexane floatation.

Preparation of polysomal fractions and ribosomal fraction enriched with mRNP-particles—Free and membrane-bound polysomes and microsomes were prepared essentially according to Larkins and Davies (1975). Embryonic axes were ground in liquid nitrogen with a mortar and pestle and homogenized in buffer A [0.25 M sucrose, 200 mM Tris-HCl (pH 8.5), 60 mM KCl, 30 mM magnesium acetate, and 5 mM dithiothreitol]. After filtration through double layers of Miracloth, the filtrate was centrifuged at $2{,}000 \times g$ for 5 min, and then the supernatant was recentrifuged at $30{,}000 \times g$ for 20 min to obtain a postmicosomal fraction, designated as S-30. The sediment was resuspended in buffer A and again centrifuged at $30{,}000 \times g$ for 20 min and a microsomal fraction, P-30, was obtained.

Twenty eight milliliters of the S-30 fraction was placed on 10 ml of a 70% sucrose cushion made with buffer B [40 mM Tris-HCl (pH 8.5), 20 mM KCl, 10 mM magnesium acetate, and 5 mM dithiothreitol] and centrifuged at $123{,}000 \times g$ (average) for 4 hr in a RP-50-2 rotor of a a Hitachi ultracentrifuge. The sediment was a free polysomal fraction. To obtain a free ribosomal fraction enriched with mRNP-particles, 28 ml of the S-30 was placed on 10 ml of a 20% sucrose cushion and centrifuged at $160{,}000 \times g$ (average) for 4 hr. To prepare membrane-bound polysomes the P-30 fraction was suspended in buffer B containing 1% (v/v) Triton X-100, layered on 10 ml of 70% sucrose, and then centrifuged at $123{,}000 \times g$ (average) for 4 hr. The sediments of polysomes and microsomes were each suspended in a small volume of 20 mM HEPES-KOH (pH 7.6), 20 mM KCl, 5 mM magnesium acetate, and 5 mM dithiothreitol and used for in vitro translation experiments.

Extraction of polyA(+) RNA—Embryonic axes were ground in liquid nitrogen, homogenized with 100 mM Tris-HCl (pH 7.8), 100 mM NaCl and 1 mM EDTA containing 1% SDS and then centrifuged at $10{,}000 \times g$ for 10 min. To the supernatant, pronase E (Kaken Chem. Co., Tokyo) was added at 500 μg/ml and incubated at 37°C for 30 min. Next, an equal volume of chloroform-phenol (1 : 1, v/v) was added, stirred at room temperature for 30 min, and then the mixture was centrifuged at $11{,}000 \times g$ for 15 min. The aqueous phase was re-extracted with chloroform-phenol, and the RNA was precipitated by adding 2.5 volumes of cold ethanol. From this RNA preparation, polyA(+) RNA was obtained essentially by the method of Aviv and Leder (1972). The buffer used were modified according to Bantle et al. (1976) as follows: 10 mM Tris-HCl (pH 7.5), 500 mM NaCl and 1 mM EDTA for binding polyA(+) RNA to oligo-(dT) cellulose and 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA for its elution.

Cell-free protein synthesis—Wheat germ extracts were prepared according to Marcu and Dudock (1974). The concentration of the wheat S-30 fraction thus prepared was 75–90 A_{260} units/ml. A standard cell-free assay system (50 μl) contained 15 μl of wheat S-30 fraction, 20 mM HEPES-KOH (pH 7.6), 80 mM KCl, 3 mM magnesium acetate, 2 mM dithiothreitol, 1.5 mM ATP (neutralized with KOH), 40 μM GTP, 8 mM creatine phosphate, 40 μg/ml creatine phosphokinase, 25 μM each of 19 amino acids except for labeled amino acids, of which 1–10 μM were used (2–10 μCi/sample), and 15 μl of polysomes or polyA(+) RNA (Marcu and Dudock 1974, Roberts and Paterson 1973). Assays were carried out at 25°C for 60 min with polysomes and for 90 min with RNA, after which 5 μl aliquots of the reaction mixture were absorbed into Whatman 3 MM filter disks. Then the incorporation of radioactive amino acids into the hot-TCAinsoluble fraction was measured as described by Roberts and Paterson (1973).

Analysis of in vitro translation products—One set of duplicate assays was used to analyze whole polypeptides synthesized in the wheat cell-free system. Each sample was diluted with an equal volume of 2-fold concentrated electrophoresis sample buffer [to give a final concentration of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerine, and 0.001% bromophenol blue], heated at 95°C for 3 min, and then applied to a SDS-polyacrylamide slab gel (12.5% acrylamide, 0.33% bisacrylamide) (Laemmli 1970). The molecular weights of the polypeptides were estimated from the mobility of marker proteins: cytochrome *c*, myoglobin, trypsin inhibitor II, L- and H-chains of γ-globulin, and bovine serum albumin.

Each sample of the duplicate set was cooled in an ice-bath and mixed with an equal volume of 1 N H_2SO_4 and further diluted if required with 0.5 N H_2SO_4. The mixtures were shaken for 2 hr at about 4°C and then centrifuged at $15{,}000\times g$ for 15 min. The sediments were again extracted with 0.5 N H_2SO_4 for 1 hr at 4°C. Next, 9 volumes of cold (−20°C) acetone were added to the combined supernatants. After storage overnight at −20°C, the acid-extractable polypeptides were collected by centrifugation, successively washed with acidified acetone, acetone/ether (1 : 1, v/v) and ether as described previously (Fukuei et al. 1978), and then were also analysed by SDS-polyacrylamide gel electrophoresis.

The gels were prepared for fluorography according to the method of Bonner and Laskey (1974) and exposed to sheets of Fuji RX film.

Extraction of histones and ribosomal proteins—Histones and ribosomal proteins were extracted from the embryonic axes which had been treated with 40 μCi/ml ^{3}H-lysine for 5 hr after 33 hr of germination, as described previously (Fukuei et al. 1978).

Materials—Glassware used in this study was sterilized by dry heating at about 200°C and all buffers were autoclaved. L-[4,5-^{3}H]-leucine (55 Ci/mmol), L-[4,5-^{3}H]-lysine monohydrochloride (99 Ci/mmol), and L-[5(n)-^{3}H]-arginine monohydrochloride (19 Ci/mmol) were purchased from Amersham Japan Co.

Results

The level of incorporation of ^{3}H-thymidine into DNA was low in *V. faba* embryos after 24 hr of germination, but very high in 36-hr embryos (Fukuei et al. 1978). But an in vivo labeling experiment with a combination of ^{3}H-lysine and ^{3}H-arginine showed that the specific activities of the total histones extracted from the 24-hr embryos and 36-hr embryos, both of which had been labeled for 2 hr, were nearly the same, as shown in our previous paper (Fukuei et al. 1978). Fluorographs of the slab gels of the histones indicated that five polypeptides were synthesized, having mobilities identical with *V. faba* H1, H2A, H2B, H3 and H4 histones stained with Coomassie Brilliant Blue (Fig. 1). H1 histones separated into two bands under the conditions used for gel electrophoresis in this study, whether they were stained or radioactive. The fluorographs made after 24 and 36 hr germination showed no essential difference in the pattern of their histones labeled with ^{3}H-lysine and ^{3}H-arginine. However, less intense bands appeared in the region between H1 and H2A/H2B, particularly in the early stage embryos. It is not clear whether these acid-extractable labeled polypeptides correspond to non-histone chromatin proteins or contaminating ribosomal proteins.

Fluorographs of gels of whole translation products labeled with ^{3}H-leucine showed about 30 polypeptides synthesized by free and membrane-bound polysomes or microsomes, which were prepared from the 36-hr embryos (Fig. 2, lanes 2–4, Fig. 3, A and B). Their molecular weights ranged from 10,000 to 68,000 daltons. The same result was obtained with ^{3}H-lysine-labeled products, although their fluorographic patterns were different from those of ^{3}H-leucine-labeled ones, because of their dissimilar amino acid constitutions (Fig. 3).

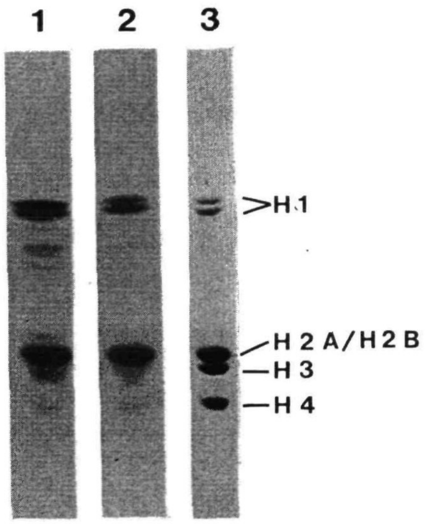

Fig. 1

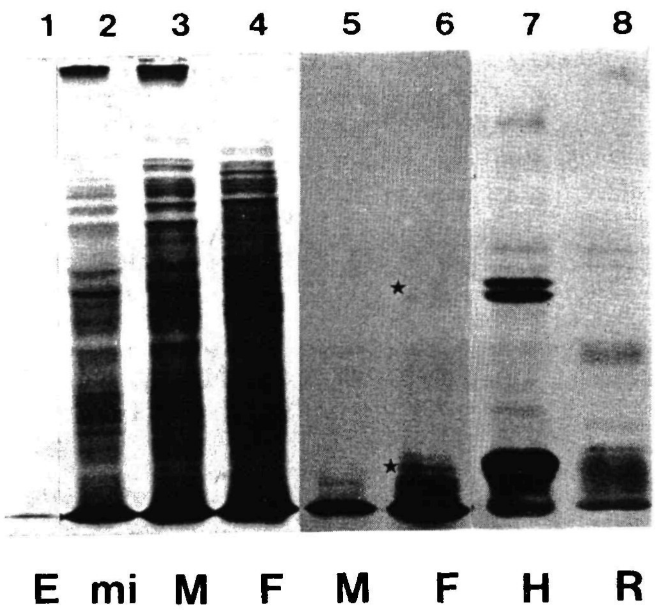

Fig. 2

Fig. 1 Gel electrophoresis of *Vicia faba* histones. Histones were extracted from *V. faba* embryonic axes treated with both ^{3}H-lysine and ^{3}H-arginine for 2 hr after 22 hr (lane 1) or 34 hr (lane 2) of germination and subjected to SDS-polyacrylamide gel electrophoresis, after which fluorographs were made. Authentic *V. faba* histones stained with Coomassie Brilliant Blue are shown at right (lane 3).

Fig. 2 Gel electrophoresis of ^{3}H-leucine-labeled polypeptides synthesized in the wheat cell-free system. Whole translation products (lanes 1–4) or H_2SO_4-extractable products (lanes 5 and 6) directed by 3.2 A_{260} units of either microsomes (mi, lane 2), membrane-bound polysomes (M, lanes 3 and 5), or free polysomes (F, lanes 4 and 6) were run on SDS-polyacrylamide gels and fluorographed. The products of endogenous templates in the cell-free system (E) are shown in the fluorograph at left (lane 1). Fluorographs of ^{3}H-lysine-labeled *V. faba* histones (H, lane 7) and ribosomal proteins (R, lane 8) are also shown. These polysomes and microsomes were prepared from embryos after 36 hr of germination. The asterisks indicate the polypeptides having mobilities identical to the lysine-rich histones H1 and H2A/H2B.

Generally the banding patterns of the products of membrane-bound polysomes were nearly identical to those of microsomes, but differed significantly from those of free polysomes. There were quantitative variations with several polypeptides between the products synthesized by free and membrane-bound polysomes, although clear qualitative differences could not be detected. ^{3}H-Leucine-labeled polypeptides of 38,000 and 51,000 daltons were synthesized in smaller amounts on free polysomes than on membrane-bound polysomes (Fig. 3, A and B). In contrast, several polypeptides between about 22,000 and 27,000 daltons as well as a 35,000 daltons component were synthesized in greater quantity by free polysomes (Fig. 3, A and B). In the case of ^{3}H-lysine labeling, some quantitative differences in the banding pattern were also observed (Fig. 3, C and D).

Although polysomes or microsomes with the same amount of absorbance at 260 nm were added to the cell-free systems and all their products were applied to the gel electrophoresis slabs, the incorporation of ^{3}H-leucine or ^{3}H-lysine into the whole translation products was always higher with free polysomes than with membrane-bound polysomes or microsomes.

Analysis of the acid-extractable polypeptides of the ^{3}H-leucine-labeled translation products

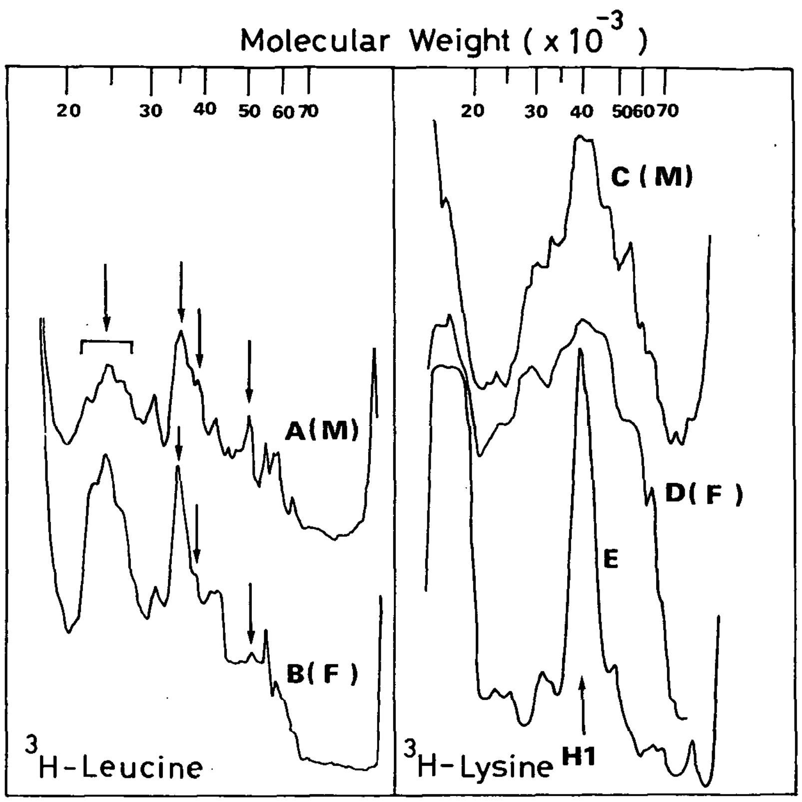

Fig. 3 Densitometric tracings of fluorographic films from slab gels. ^{3}H-Leucine-labeled (A and B) and ^{3}H-lysine-labeled (C and D) in vitro translation products and ^{3}H-lysine-labeled *V. faba* histones (E) were run on SDS-polyacrylamide gels and fluorographed. A and C are products of membrane-bound polysomes (M) and B and D are those of free polysomes (F). The polysomes were prepared after 36 hr of germination. A and B correspond to lanes 3 and 4 in Fig. 2. H1 indicates *Vicia* H1 histones.

by gel electrophoresis indicated that basic polypeptides with mobilities similar to those of *V. faba* histones and ribosomal proteins were synthesized mainly on free polysomes (Fig. 2, lane 6). ^{3}H-Lysine was incorporated to a much greater extent into the acid-extractable products than ^{3}H-leucine, and heavier labeling occurred in polypeptides corresponding in their mobilities to H1 and H2A/H2B lysine-rich histones (Fig. 4, lane 3). Similarly, ^{3}H-arginine was incorporated into the acid-extractable polypeptides, two of which had the same mobilities as arginine-rich H3 and H4 histones (Fig. 4, lane 6). The same amounts of free and membrane-bound polysomes were used in the in vitro translation experiments. Therefore, we concluded that *V. faba* histones, as well as some ribosomal proteins, were synthesized more actively on free polysomes.

Essentially the same results were obtained with free polysomes of the 24-hr embryos, in which histones were actively synthesized, as with those of the 36-hr embryos. However, when the free polysomal fraction prepared from dry seed embryos was added to the cell-free system, no appreciable ^{3}H-lysine and only a little ^{3}H-arginine was incorporated into acid-extractable polypeptides. This result suggests that histone mRNAs are not present in the dry embryos. In order to determine whether dry *V. faba* embryos contain preformed mRNA, total polyA(+) RNA was extracted from the embryonic axes at different stages, and their translation products were analyzed. Fig. 5 shows that the banding patterns of the in vitro translation products coded by the polyA(+)

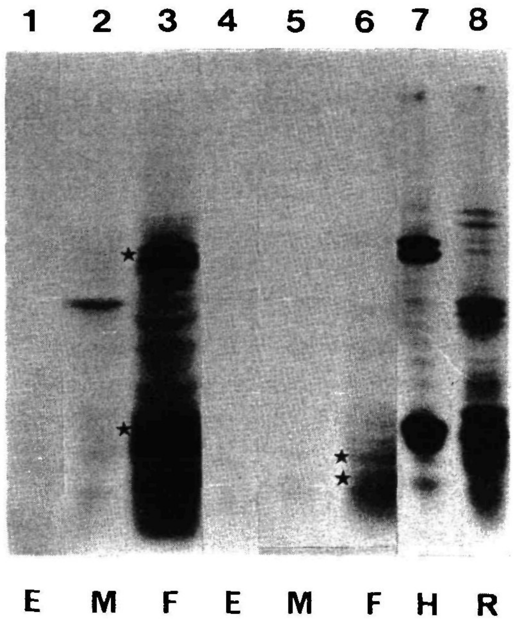

Fig. 4

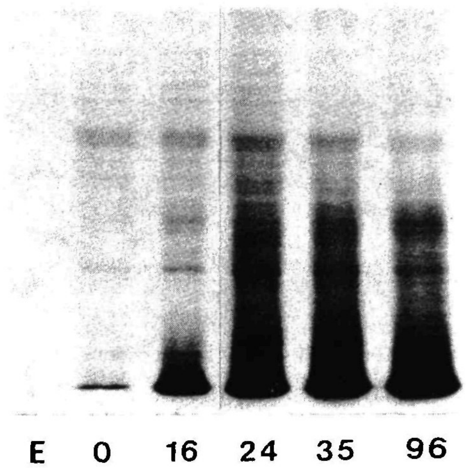

Fig. 5

Fig. 4 Gel electrophoresis of H_2SO_4-extractable translation products. Acid-soluble polypeptides were extracted from the whole translation products directed by membrane-bound polysomes (M), free polysomes (F) or endogenous templates (E) and analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 1–3 are ^3H-lysine labeled and lanes 4–6 are ^3H-arginine labeled. Lanes 7 and 8 are ^3H-lysine-labeled *V. faba* histones (H) and ribosomal proteins (R). The asterisks indicate the polypeptides having mobilities identical to the lysine-rich histones H1 and H2A/H2B (lane 3) or to the arginine-rich histones H3 and H4 (lane 4).

Fig. 5 Comparison of the composition of polyA(+) RNA at different stages of germination. Whole ^3H-leucine-labeled translation products coded by polyA(+) RNA obtained from *V. faba* embryonic axes at various times (0, 16, 24 and 35 hr) and from roots of 4-day-old seedlings (96 hr), as well as the endogenous templates (E), were run on SDS-polyacrylamide gels and fluorographed.

RNA resemble those of the translation products from polysomes. There was no change in the banding patterns during seed germination, except that a few new polypeptides could be detected in the roots of 4-day old seedlings (Fig. 5). The dry seed embryos were shown to contain polyA(+) RNA quite similar in its composition to that contained in the germinating embryos.

Next, mRNP-particles were isolated from dry embryos in order to examine their in vitro template activity. Dry seed embryos, whether of monocots or of dicots, contain monomer ribosomes but not polysomes. And most plant mRNP-particles containing preformed mRNA have smaller sedimentation values than 80*S* ribosomes (Peumans and Carlier 1977, Schultz et al. 1972, Weeks and Marcus 1971). Therefore, the ultracentrifugation procedure was modified to encourage the sedimentation of free mRNP-particles. When the mRNP-particle-enriched ribosomal fraction was subjected to sucrose density gradient centrifugation, the sedimentation profile of an absorbance of 260 nm did not show a marked accumulation of presumptive mRNP-particles in the region lighter than the 80*S* monomer ribosomes. However, ^3H-leucine incorporation into in vitro products was obviously increased by the addition of this fraction containing preformed mRNA from the dry seed embryos. At least 20 polypeptides could be detected, ranging in molecular weight from about 10,000 to about 65,000 daltons (Fig. 6, lane 2). Some of the polypeptides encoded by the preformed mRNP-particles could also be labeled with ^3H-methyl-methionine

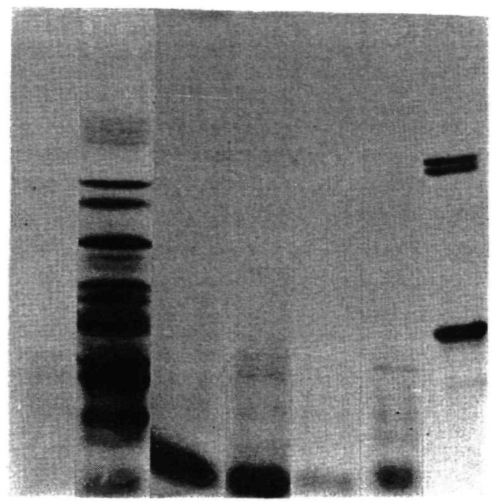

Fig. 6 Gel electrophoresis of translation products coded by mRNP particles from dry *V. faba* seeds. Whole translation products labeled with ^{3}H-leucine (lanes 1 and 2), ^{3}H-lysine (lanes 3 and 4) or ^{3}H-arginine (lanes 5 and 6), which were directed by mRNP-particle-enriched ribosomal preparations, were run on gels and fluorographed. The products of the endogenous templates (lanes 1, 3 and 5) were also analyzed. ^{3}H-Lysine-labeled histones (lane 7) are shown at right.

(data not shown), but not with ^{3}H-lysine and only slightly with ^{3}H-arginine. These results indicate that the embryonic axes of dry *V. faba* seeds contained preformed mRNAs for at least 20 polypeptides, but did not contain mRNAs for basic proteins in significant amounts. We concluded that histone synthesis in early germination is dependent on the histone mRNAs transcribed immediately after the imbibition of water. This conclusion was supported by the following observation. When the embryonic axes detached at the 16th hr of germination were incubated for 7 hr together with 400 μg/ml actinomycin D and then treated for 2 hr with ^{3}H-lysine, ^{3}H-arginine, and 400 μg/ml actinomycin D, the incorporation of the labeled amino acids into histones was inhibited by actinomycin D to a level of 28% of the control, confirming that histone synthesis was dependent on the newly transcribed RNA.

Discussion

Zauderer et al. (1973) reported that histone mRNAs in mouse myeloma cells were associated specifically with free polysomes and also with polysomes loosely bound with cellular membranes, but were not associated with tightly-bound polysomes. More efficient synthesis of histones by free polysomes was observed by Nabeshima et al. (1975) in regenerating rat liver. They also found that ribosomal structural proteins were synthesized more efficiently by free and loosely-bound polysomes. No such studies have been reported on plant histones. Our present study was able to show that *V. faba* histones and some ribosomal proteins were synthesized actively by free polysomes. When, however, fluorographs of the whole translation products were compared, no great qualitative difference could be detected between the products of the two types of polysomes, though some quantitative differences were observed. α-Amylase, a plant secretory protein, (Jones 1969), cellulase (Verma et al. 1975), zein, a storage protein, (Larkins et al. 1976), and oat globulin (Luthe and Peterson 1977) have all been found to be synthesized on membrane-bound polysomes. However less active synthesis by free polysomes was also observed in the cases of cellulase, zein and oat globulin.

There is much evidence for the presence of preformed mRNAs in dry seeds. mRNP-particles with the ability to stimulate amino acid incorporation in a cell-free system have been found in soybean seeds (Mori and Matsushita 1970), wheat and rye embryos (Peumans and Carlier 1977, Schultz et al. 1972, Weeks and Marcus 1971). Gordon and Payne (1976) demonstrated that polyA(+) RNA isolated from dry rye embryos, oil-seed rape and pea seeds coded polypeptides with molecular weights ranging from 10,000 to 70,000 daltons. They also reported the presence of RNA with template activity in dry *V. faba* seeds. Later, Caers et al. (1979) showed that the polyA(+) RNA present in dry wheat embryos had similar coding properties to those of embryos germinated for 6 or 8 hr, in which most of the RNA was shown to be newly synthesized. Our studies with *V. faba* embryos also demonstrated no qualitative changes in the polyA(+) RNA population during germination, except for the occurrence of a few new polypeptides in the roots of 4-day old seedlings. Dry *V. faba* embryos did contain preformed mRNAs in the form of mRNP-particles, which were mostly the mRNAs for polypeptides labeled with ^{3}H-leucine. When ^{3}H-lysine or ^{3}H-arginine was used, litlte or no labeling of translation products was obtained with the dry embryos, showing the lack of mRNAs for basic polypeptides in their preformed mRNA population. This situation is entirely different from that observed in sea urchin eggs, which contain large amounts of preformed histone mRNAs.

Since dry *V. faba* embryos do not contain histone mRNAs, histone synthesis in early germination (at 24 hr) would seem to require the previous transcription of histone mRNAs at a time during which it is known that no incorporation of ^{3}H-thymidine yet occurs. Translation of histones was shown to occur simultaneously with DNA replication, and transcription of histone mRNAs was shown to begin 2 hr earlier than the onset of DNA replication in synchronized HeLa cells (Borun et al. 1967). A functional relationship between histone synthesis and DNA replication also was suggested by the rapid and selective blocking of histone synthesis by inhibitors of DNA replication such as hydroxyurea (Butler and Mueller 1973) and cytosine arobinoside (Borun et al. 1967). Although they are coordinated, histone synthesis does not entirely depend on DNA replication. Russev et al. (1980) showed that the treatment of Ehrlich ascites tumor cells with hydroxyurea caused 95% inhibition of DNA replication and 60% inhibition of histone synthesis within 60 min, but thereafter histone synthesis was restored to a level of about 70% of the control during the following 4 hr without the recovery of DNA replication. Jackson and Chalkely (1981) reported that the treatment of cultured mammalian HTC cells with 5 mM hydroxyurea for a short period inhibited more than 90% of DNA replication, but only 50% of histone synthesis. Furthermore, as mentioned above, histone synthesis not coupled with DNA replication has been found in early cleavage stage sea urchin embryos and *Xenopus* oocytes. Germinating *V. faba* seed is suitable material for further study of the relationship between the transcription of histone mRNAs and DNA replication.

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