CHAPTER I

LEVELS OF EUKARYOTIC INITIATION FACTOR-2 (eIF-2)

DURING EMBRYOGENESIS AND GERMINATION IN BARLEY EMBRYOS
INTRODUCTION

Embryogenesis is the first stage in the life history of a plant. During this stage, seed formation occurs which consists of development of an embryo and accumulation of a large food reserve. This is followed by desiccation of the tissue which in turn leads to a period of dormancy or quiescence, where neither growth nor development takes place.

This is a mechanism by which the plant tides over unfavourable seasons and conditions. However, in favourable conditions the seed germinates. Germination is a process by which the suspended growth and development is resumed in the seed leading to formation of a whole plant from the embryo. The growth and development of the embryo is supported by the food reserves of the seed.

Embryogenesis, in general, can be divided into 3 parts (Fig.8) (Review: Dure, 1975). The first part is the longest, which is about half the total number of days of embryogenesis. It is the growing phase of the seed consisting of rapid cell division consequently leading to growth of endosperm/cotyledons, embryo and testa. There is an increase in DNA, RNA and protein syntheses. During this stage, there is a preparation for synthesis of food reserves which occurs at the next stage. RNA and proteins that are required for synthesis of storage proteins and carbohydrates and for general cell metabolism
Figure 8. Events during embryogenesis of a seed.
are synthesized during this phase. There is a massive influx of sugars from other parts of the plant leading to maximum accumulation at the end of the phase.

The second stage is the synthetic stage. The sugars are converted to starch and a large amount of storage proteins are synthesized. RNA synthesis continues but those synthesized in this stage are qualitatively different from those synthesized in the first stage. Germination-specific mRNAs, which are synthesized at this stage, are not translated till the onset of germination. By the end of this phase, the endosperm/cotyledons and embryo attain the maximum size and weight and the seed is said to be fully mature.

The final phase of embryogenesis is the period of desiccation. The polysomes disintegrate and there is a decline in protein and RNA syntheses during this phase. Besides the carbohydrates and proteins stored in the endosperm/cotyledons, germination-specific mRNAs and essential components of translational machinery are also conserved. Presence of the stored mRNAs had been shown in many dry seeds (Review: Payne, 1976; Dure, 1977). As mentioned earlier, they are synthesized in the second stage of embryogenesis (Dure et al., 1981; Galau & Dure, 1981) and are not allowed to be translated until germination. The mechanism of conservation is not yet clear though there are various speculations. Dure's group reported that abscisic acid, a plant hormone, is responsible for the
inability of the mRNA to be utilized during embryogenesis (Ihle & Dure, 1972). It has been observed that cordycepin, an inhibitor of polyadenylation, prevents protein synthesis in the early hours of germination, indicating that the mRNA cannot be translated unless they are polyadenylated (Walbot et al., 1974, Harris & Dure, 1978). This led to the proposal that the latent mRNAs are unprocessed and therefore cannot be translated. At the onset of germination they are processed and made ready for protein synthesis. But this may not be true for all cases. Some seeds are insensitive to cordycepin treatment during early germination (Delseny et al., 1975; Spiegel and Marcus, 1975; Sopory et al., 1981). Moreover, poly (A) containing mRNAs have been isolated from various embryos and have also been translated \textit{in vitro}. (Payne, 1976). Recently, some mRNAs from cotton seeds have also been reported to be polyadenylated and translatable \textit{in vitro} (Dure et al., 1981). Stored mRNA are known to be present as mRNP particles (Peumans & Carlier, 1977). The proteins of the particles may prevent the mRNAs from being translated. Presence of translational inhibitors in seeds have been shown by some workers. Protein kinase that phosphorylates eIF-2\textalpha subunit has been reported in wheat germ and has been shown to be a potent inhibitor of \textit{in vitro} translation (Ranu, 1980a, Sierra et al., 1977). A cAMP-independent protein kinase isolated from barley embryo has been shown to inhibit protein synthesis very effectively in a cell-free system (Reddy et al., 1983). A protein inhibitor
of elongation of translation has also been reported in wheat germ (Roberts & Stewart, 1979).

Protein synthesis in early hours of germination is solely dependent on the conserved mRNA and is not dependent on new mRNA synthesis (Review: Payne, 1976; Dure, 1977). Moreover, treatment of RNA synthesis inhibitors, like α-amanitin and actinomycin D, does not affect early protein synthesis at all (Spiegel & Marcus, 1975; Dure & Waters, 1965; Bhat and Padayatty, 1974; Sopory et al., 1981; Weeks & Marcus, 1971). De novo synthesis of some germination-specific enzymes have also been demonstrated even when RNA synthesis is inhibited (Ihle & Dure, 1969, 1970; Delseny et al., 1975, 1976).

The translation of these mRNAs seem quite crucial for germination (Brooker et al., 1977). Inhibition of translation by cycloheximide in the first hour leads to very poor germination in barley seeds. RNA synthesis and RNA polymerase activity are also inhibited at a later stage of germination if the first hour of protein synthesis is curtailed (Puri-Avinashi, 1980).

Stored mRNAs are reported to be present in other developmental systems also. Unfertilized sea urchin eggs contain mRNAs which are unavailable for translation till fertilization (Alton & Lodish, 1977). There is a rapid increase in the rate of protein synthesis after fertilization, accompanied by shift of these mRNAs from free cytoplasmic mRNP particles into polysomes.
During the early stages of chick muscle development, myoblasts undergo extensive proliferation which is followed by myoblast fusion leading to myotube formation. Proliferating myoblasts contain a considerable amount of myosin mRNA that is stored as 70-90S RNP particles. They are translated only after the beginning of myoblast fusion (Heywood et al., 1975).

In case of Artemia salina, after fertilization, the egg undergoes cleavage resulting in a blastula which gives rise to gastrula. The gastrula may continue its development and eventually become an adult or it may encyst, desiccate and enter a dormant state (Macrae et al., 1979). The dormant embryo has a store of poly (A+) and poly (A-) mRNAs. The latter is far more than the former (Sierra et al., 1976). When the desiccated cyst is hydrated in proper environmental conditions, the dormant embryo resumes development. The development is accompanied by increase in polyadenylation and concomitant increase in protein synthesis. mRNA isolated from dormant embryos can be translated in vitro (Sierra et al., 1976; Grosfeld & Littaur, 1976; Nilsson & Hultin, 1975).

Dormant embryos of Artemia salina form inactive cell-free translation systems (Sierra et al., 1974). It is capable of elongation of polypeptide chain but cannot initiate new ones. This has been attributed to a deficiency of initiation factors in dormant embryos. eIF-2 activity has been shown by Ochoa's group to be very low in dormant embryos which increases about
20 folds in developing embryos (Filipowicz et al., 1975, 1976). Furthermore, the system can readily translate mRNA when supplemented with supernatant or ribosomal wash from developing system or full complement of highly purified reticulocyte initiation factors (Filipowickz et al., 1976). It has thus been concluded that the dormant embryos have the whole protein synthesizing machinery except for the initiation factors, activities of which increase after resumption of development.

In contrast to the above report, Wahba's group of researches have purified active eIF-2 from both dormant and developing embryos (Macrae et al., 1979). The factor, according to them, is present in equivalent amounts at all stages of development. Pure eIF-2 obtained through six steps of purification from ribosome wash of both stages have a molecular weight of 139,000 consisting of 3 subunits, 52,000, 45,000 and 42,000 daltons. Immunologically, eIF-2 from developing embryos cross reacts with antibody against eIF-2 from dormant embryos. Besides eIF-2, Co-eIF-2A and CoeIF-2B have been purified from both stages. Like eIF-2, level of activity of these two factors remains unchanged through development (Woodley et al., 1981). No explanation has been given by either of the groups for the controversy regarding the level of eIF-2 in dormant embryos.

It has been observed that GTP:GDP ratio increases drastically during development in Artemia salina. (Mehta et al.,
Moreover, it has been reported in heme-deficient reticulocyte lysate, the protein synthesizing ability of the system is restored by the addition of excess GTP (Konieczny and Safer, 1983). The high level of GTP is able to release GEF from eIF-2(P).GEF complex thus making it available for recycling of eIF-2 (Goss et al., 1984). Therefore, it has been proposed by Mehta et al., (1983) that increase of GTP during development may have a role in regulation of protein synthesis and development in Artemia salina. Varying levels of eIF-2 may not be the mechanism of regulation in this system after all.

Barley embryos, like Artemia salina dormant eggs, form an inefficient cell-free translation system which has been observed in our laboratory (unpublished data) and by others (Carlier & Peumans, 1976). It would be interesting to check the levels of eIF-2 activity at different stages of development in this system.
RESULTS & DISCUSSION

Protein synthesis during germination:

In barley embryos, protein synthesis during germination was studied by incorporation of radioactive amino acids into TCA insoluble fraction of the embryo extract. If the embryos are subjected to continuous labelling, there is a constant increase in incorporation of radio-activity into the proteins (Fig. 9). Rate of protein synthesis at different hours of germination was measured by 1 h pulse labelling at different time points. Figure 10 shows a steady increase in the rate of protein synthesis reaching maximum by about 24 h post-imbibition.

eIF-2 activity in embryos:

The assay of eIF-2 by ternary complex (eIF-2.GTP.Met-tRNA) formation was done according to Siekierka et al., (1982). The eIF-2 activity was found in the high-salt wash of ribosomal pellet (Table 1). However, if protease inhibitor (PMSF) was omitted from the buffers used for the extraction, the activity was found in the supernatant and was totally lost from the ribosomal pellet. Moreover, the total activity was drastically reduced when PMSF is not used. This could be a result of protease action which probably dislodges the initiation factor from the ribosomes.
FIGURE 9. Protein synthesis during germination. The embryos were germinated in a medium containing \( ^3\text{H} \) leucine and \( ^3\text{H} \) lysine 20 \( \mu \text{Ci/ml} \). At indicated times, the germination was stopped and radioactivity in the TCA insoluble fraction of the embryo extract was determined as described in Materials and Methods.
FIGURE 10. Rate of protein synthesis during germination. At indicated times, embryos were given 1 h pulse of $^{3}\text{H}$ lysine (60μCi/ml) Radioactivity in the TCA insoluble fraction was then determined as described in Materials and Methods.
TABLE 1

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<th>Specific Activity of eIF-2 when extraction was done</th>
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<tr>
<td></td>
<td>with PMSF</td>
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<tr>
<td>Post-ribosomal supernatant</td>
<td>0.425</td>
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eIF-2 activity in barley embryos extracted with or without PMSF. The eIF-2 was assayed by ternary complex formation using post-ribosomal supernatant and the high salt wash of the ribosomal pellet as described in 'Materials and Methods'.
Levels of eIF-2 activity during embryogenesis and germination:

The eIF-2 activity was very high in dry embryos which decreased to 40% by 6th h, then slowly decreased to 25% by 24th h of germination (Fig. 11). The eIF-2 activity during early stages of embryogenesis (25th day) was very low but increased rapidly after 30th day of fertilization. There was a 10-fold increase in activity from 25th day to 40th day of embryogenesis. The activity in 40 days-old embryos was as high as that of mature dry embryos (Fig. 12).

Weight of developing seeds during embryogenesis:

The average weight of whole seeds has been determined on different days after anthesis. There was a steady increase of net weight of the seeds till about 30th day. After which, it reduced drastically because of dehydration. As a result, 40 days old seed was completely dehydrated and weighed as much as the mature dry seed (Fig. 12).

The amount of eIF-2 in ungerminated dry embryos was very high as compared to the reported values from mammalian systems (Benne et al., 1979; Harbitz & Hauge, 1979). It compares very well with the reported values by Macrae et al., (1979) of developing and dormant embryos of *Artemia salina*. But it is very low as compared to that reported by Ochoa's group (Filipowicz et al., 1976) in developing embryos. In wheat germ also, the activity of eIF-2 is very high. It has been purified and
FIGURE 11. eIF-2 activity during germination.
FIGURE 12. eIF-2 activity during embryogenesis. Inset: Change in the weight of seeds (average of five) during embryogenesis.
characterized by two laboratories (Treadwell et al., 1974, 1978; Warthal et al., 1979). Its activity has been found in the post-ribosomal supernatant by both the groups. Neither of the groups used any protease inhibitors for eIF-2 extraction. Table 1 shows clearly that the absence of any protease inhibitor leads to dislodging of the eIF-2 from the ribosomes and there is also a lowering of total activity of eIF-2 in the system when PMSF is not used. This may explain the difference in location of the factor in wheat and barley embryos.

The high level of eIF-2 in dry mature embryos decreased to 25% by 24th h of germination (Fig. 11). The rate of protein synthesis, however, reached a maximum in 24th h of germination (Fig. 10). This suggests that the rate of protein synthesis in germinating embryos has little correlation with the amount of eIF-2. This result is similar to Artemia salina (Macrae et al., 1979) where the amount of eIF-2 remains unchanged during early development although there is a progressive increase in protein synthesis. But the results do not agree with Filipowicz et al., (1975) where a 20-fold increase in eIF-2 activity, with resumption of development and protein synthesis was reported. Presence of an inhibitor in the partially purified preparation of eIF-2 of dormant embryos (Filipowicz et al., 1975) which suppresses eIF-2 activity is ruled out because, when dormant embryo extract is supplemented with a mixture of supernatants of developed and dormant embryos, it shows as much translational
activity as when supplemented with that of developing embryo alone. But this still does not rule out the possibility of the existence of an inactivator of the inhibitor in the supernatant of developing embryo which could explain the difference in levels of eIF-2 at the two stages.

In 24th h of germination only 25% of the initial eIF-2 activity was present in barley embryo (Fig. 11). This amount of eIF-2 is obviously sufficient to sustain a good rate of protein synthesis. Even in rabbit reticulocytes which have the capacity to synthesize protein at a high rate, the specific activity of eIF-2 has been reported to be less than 0.04 in the ribosomal wash (Benne et al., 1979). This amount of eIF-2 activity is apparently sufficient to maintain a high rate of protein synthesis. This obviously indicates that the eIF-2 stored in the dry embryos is much in excess. This large store of eIF-2 is synthesised during embryogenesis, conserved through desiccation and used only during germination. It is probably stored in excess so that the developing system does not have to replenish the stock for several hours during early germination, the period when more crucial germination-specific proteins are being synthesized.

The developing seed reached a maximum weight by 30th day of embryogenesis after which dehydration started and was completed by the 40th day (Fig. 12). The synthetic phase of embryogenesis ends with the onset of dehydration on the 30th
day (Fig.8). Therefore, synthetic processes, including protein synthesis should be over by the 30th day. The large stock of eIF-2 would be synthesized during this synthetic phase but it is detected only after the onset of desiccation and reaches a high level by the time the embryo is completely dry. Presence of an inhibitor of eIF-2 activity before the desiccation phase could explain the phenomenon. The inhibitor may be inactivated by the process of dehydration. Another possibility is the existence of eIF-2 as an inactive precursor which gets activated at the onset of desiccation. The suppression of eIF-2 activity could be a mechanism by which the newly synthesized eIF-2 is stored during the metabolic phase of embryogenesis so that it will be conserved for use during germination.

As mentioned earlier, the barley extract makes a very poor in vitro translation system inspite of the presence of very high level of eIF-2 activity as in dormant eggs of Artemia salina (Sierra et al., 1974; Macrae et al., 1979). The presence of translation inhibitor(s) in the barley embryo cannot be ruled out to explain the phenomenon. A protein inhibitor, a cAMP-independent protein kinase, of translation has already been reported from barley embryos (Reddy, et al., 1983). Presence of an RNA inhibitor of protein synthesis has been discussed in the next chapter.