Cellular and Molecular Biology ™ **53**, N°3, 64-73
DOI 10.1170/T798

ISSN 1165-158X
2007 Cell. Mol. Biol.™

IN VITRO RECONSTITUTION OF LEGUMIN (11S) mRNA AND BINDING PROTEINS AS RELATED TO POST-TRANSCRIPTIONAL REGULATION OF PROTEIN SYNTHESIS IN DEVELOPING ALFALFA EMBRYOS

S. K. PRAMANIK[1]✉, A. L. WILLIAMS[1] AND J. D. BEWLEY[2]

[1]Department of Biology, Morgan State University, 1700 E. Cold Spring Lane, Baltimore, MD 21251
[2]Department of Botany, University of Guelph, Guelph, Ontario, N1G 2W1, CANADA
✉ Saroj K. Pramanik, Department of Biology, Morgan State University, 1700 E. Cold Spring Lane, Baltimore, MD – 21251, Tel. No.: (443) 885 - 3709 Fax No.: (443) 885- 8285 Email: spramani@jewel.morgan.edu

Received July 30th, 2006; Accepted December 30th, 2006; Published May 15th, 2007

Abstract– There is undetectable transcription of 11S storage protein (medicagin) mRNA by nuclei isolated from pre-cotyledonary-stage somatic embryos of alfalfa (*Medicago sativa* L). However, this message exists at steady-state levels in the embryos at this stage of development without concomitant synthesis of the storage protein. At the pre-cotyledonary stage, therefore, the transcriptional rate for 11S mRNA is low; what message is transcribed is sequestered in the form of mRNP complexes and is not recruited into polysomes *in vivo* (33). Both transcription (*in vivo* and in isolated nuclei) and translation of the 11S mRNA are evident at the onset of cotyledon development in somatic and zygotic embryos, reaching a maximum during expansion of the cotyledons and then declining as the embryos mature. Pre-cotyledonary-stage somatic embryos which do not utilize the 11S-mRNA in polysomes lack certain mRNA-binding proteins (32, 36 and 38 kD) which are present at later stages of development. These mRNA-binding proteins may be responsible for the initiation of large polysome formation since they were exclusively present in the translational extracts of cotyledonary somatic and zygotic embryos in which there was no repression of storage protein synthesis. In contrast, the pre-cotyledonary somatic embryos contained a different set of 11S-mRNA-binding proteins (28, 50, 55, and 62 kD) whose presence in the cotyledonary stage embryos was very rare or non-existent; these could be responsible for preventing translation.

Key words: Embryogenesis, Translational control, messenger ribonucleoproteins, alfalfa (*Medicago sativa* L.), legumin (11S)

INTRODUCTION

We previously observed that storage protein messages, including legumin (11S) mRNAs, are undertranslated during the pre-cotyledonary stages of alfalfa (*Medicago sativa* L.) somatic embryo development; later these mRNAs are actively recruited into polysomes, at the cotyledonary stage (33), with a concomitant synthesis of more message. We observed that there are several proteins (15 - 150 kD) which are associated with the cytoplasmic untranslated mRNAs in the non-polysomal fraction. Among them, the 30, 43, 55, and 65 kD proteins are unique to the non-polysomal fraction of pre-cotyledonary stage somatic embryos of alfalfa (6, 31), and these may be involved in the suppression of the storage protein synthesis at this stage of development. The presence of protein kinase activity in the non-polysomal fraction may play a role in regulating the recruitment of messages into the protein synthesizing (32).

The selective recruitment of mRNA into the polysomes of eukaryotic systems is a means of post-transcriptional regulation. In erythroblasts, for example, approximately 200 different mRNAs are actively translated whereas about 1200 others remain silent, among which 99% are poly(A)-binding protein mRNAs (17, 20, 25, 26). These untranslated mRNAs normally remain in the cytoplasm during and after cell differentiation and they are in association with a special set of regulatory proteins. This associated complex of mRNA and protein in the cytoplasm is the free-mRNP (messenger ribonucleoprotein) fraction, or the non-polysomal (also called repressed) fraction. Both the mRNP and deproteinized mRNAs can stimulate cell-free translation and, hence, the mRNAs within the complex are

potentially active (13, 36). This raises a series of questions: What is the physiological significance of stable mRNPs? How does a specific mRNA remain in the cytoplasm even after differentiation without being recruited into polysomes? What is the special intrinsic property of an mRNA that determines whether it will be translated only under a defined physiological condition? There are few answers to these questions. Global mRNA-associated proteins have been identified in several eukaryotes, mainly in animal tissues (3) and a few in plants (1, 6, 9, 11, 38) using the technique of mRNA-protein cross-linking *in situ*.

The reconstitution of mRNPs *in vitro* using UV-irradiation and a defined sequence of an mRNA has allowed for the identification of some special regulatory mRNA binding proteins; e.g. Greenberg and Carrol, 1985 (16) have characterized the globin-mRNA-binding proteins in a cell-free translation system. The most extensively studied regulatory protein of this type is the ferritin repressor protein which binds to the 5′- untranslated region of ferritin mRNA and regulates its translation (8, 29). There are several other mRNAs whose expression is also regulated at the translational level due to interactions with proteins, e.g. *rev* protein in the human immunodeficiency virus (10, 14), polio virus (22, 27), transferrin receptor mRNA (19, 34), the poly (A)-binding proteins (5, 35, 40), and the translational repression due to activation of nuclear kappa B by phosphorylated translation initiation factor 2 (eIF-2) (12). Global mRNA translational repression is also mediated by endoplasmic stress (21).

In this report, we have reconstituted mRNAs *in vitro* using legumin messenger RNA and cell-free translation extracts from developing embryos of alfalfa. We show that there is a specific association between legumin RNA and proteins, which is related to the type and stage of embryo development. This relationship could play a role in the post-transcriptional regulation of storage protein synthesis.

MATERIALS AND METHODS

Embryo Culture & Labelling of Proteins

Zygotic Embryos: Alfalfa (*Medicago sativa* L.)(cv.Excalibur) plants were grown in a growth cabinet at 25°C, 16/8 h light and dark cycle respectively. Flowers were manually pollinated for embryo formation and they were collected at different stages of development (39) for experiments.

Somatic Embryos: Calli were induced on embryogenic petioles in the presence of the hormones 2,4-D (4.6 µM) and kinetin (1.0 µM) on solid B5H medium (7) at 25°C during a 16/8 h light (7.5×10^{-5} mol m^{-2} sec^{-1}) / dark cycle. After 2 to 4 weeks, when pigmentation had occurred, the calli were transferred to suspension culture (B5G medium containing 4.6µM 2, 4-D, and 0.5µM NAA). When the suspension culture became thick (in about 7 - 10 d) it was sieved through 500 and 200 µm meshes, and cells collected on the 200 µm mesh were spread onto solidified hormone-free Boi2Y (15) medium for embryo development. Embryos at different stages of development were collected according to the procedure described by Atanassov and Brown, 1984 (2).

Isolation of Nuclei

The procedure described by Luthe and Quatrano, 1980 (24) was followed for the isolation of nuclei. Embryos were ground with a mortar and pestle in liquid N. The powder was further pulverized in a Duall ground-glass homogenizer with 5 ml Honda buffer (20 mM HEPES-KOH pH 7.9, 0.44 M sucrose, 2.5 % Ficoll, 5.0 % Dextran T-40, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5 % Triton X-100, 2 mM spermine) per gram of embryos. The slurry was filtered through three layers of 74 µm mesh polyester sieve cloth, and then the filtrate was centrifuged at 4000xg for 10 min at 2°C. The pellet was considered as crude nuclei since it could be contaminated with other organelles. Pure nuclei were obtained by resuspending the crude nuclei in 4.5 ml Honda buffer without spermine and then centrifuging (4000xg for 30 min at 2°C) on a Percoll (Pharmacia) step gradient which had 4.5 ml each of 40, 60, and 80 % Percoll over 2 M sucrose in 20 mM HEPES buffer pH 7.9 and 10 mM $MgCl_2$. The pellet was considered to be of pure nuclei. It was then washed three times with a buffer (50 mM TRIS-HCl pH 8.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 25% glycerol). Finally, nuclei were resuspended in the washing buffer and stored as aliquots at -80°C.

Nuclei Transcription and RNA Isolation

Nuclei transcription and RNA isolation were carried out as described in Pramanik *et al.*, 1989 (30). Essentially, stored nuclei (20 µl) were resuspended in 5 vol (100 µl) transcription cocktail (20 mM HEPES-KOH pH 7.9, 2 mM ATP, 0.5 mM each GTP and CTP, 50 µM UTP, 50 µCi [α-^{32}P]-UTP, 1 mM dithiothreitol (DTT), 2.5 mM Mg acetate, 60 mM KCl, 2 mM creatine phosphate, 0.5 µg/ml creatine kinase and 100 U human placental inhibitor) and the reaction was at 25°C for 1 h. Newly transcribed mRNA was purified from the nuclei by adding 80 µg tRNA and then digested with RNase-free DNaseI (6 µg) for 1 h at 25°C, followed by proteinase K digestion at 37°C for 1 h in the presence of 0.1% SDS and, finally, phenol:chloroform extraction and ethanol precipitation. Incorporation of radioactivity and the quantity of RNA synthesized were measured by cold-TCA-count precipitation.

DNA-Excess RNA Hybridization

The incorporation of ^{32}P-UTP into specific mRNAs was determined by DNA-excess (250 ng insert) RNA hybridization. The specific cDNA insert was denatured by boiling in 1xTE (10 mM TRIS-HCl pH 7.5, and 1 mM EDTA) for 10 min and rapid chilling on ice to avoid reannealing. It was then brought to hybridization buffer conditions (80% formamide, 0.4 M NaCl, 1 mM EDTA, 40 mM PIPES pH 6.4) and radiolabelled nuclear transcripts synthesized for different times from various stage embryos were added as a probe. The mixture was then allowed to

hybridize at 42°C overnight, in a final reaction volume of 50 µl. At the end of the hybridization, 300 µl chilled S1 nuclease buffer (0.25 M NaCl, 3 mM $ZnSO_4$, 30 mM Na acetate pH 4.5) was added. Non-hybridized ^{32}P-labelled RNA and single-stranded cDNA were digested with $2x10^3$ U/ml S1 nuclease at 37°C for 60 min. This mixture was either separated on urea:acrylamide gel electrophoresis or filtered directly onto Zeta-probe membrane (BioRad), previously soaked with 5xSSC (1xSSC = 0.15 M NaCl and 0.015 M Na citrate), by using the dot blot apparatus "Hybridot" (BRL Inc.). Each well was rinsed with another 0.5 ml 5xSSC and vacuum dried. Finally, the membrane was immersed into several volumes of 5xSSC to wash off the entire unincorporated and S1-digested radiolabelled transcript by checking the counts of the washings. It was baked for 2 h at 80°C, washed again with 0.1% SDS and 0.1xSSC for 2 x 20 min at 60°C and then exposed to X-ray film (Kodak XRP) for autoradiography.

Preparation of Cell-free extracts for in vitro translation

Various stages of embryos were used for the preparation of cell-free translational extracts for *in vitro* reconstitution of polysomes. The methodology described by Pramanik and Bag, 1989 (30) was used. In short, embryos were washed 3 times with chilled buffer containing 0.15 M NaCl, 25 mM HEPES-KOH (pH 7.5) and 10 mM DTT and then resuspended in 3 vol hypotonic buffer containing 10 mM HEPES-KOH (pH 7.5), 1 mM DTT, 0.5 mM spermine, 15 mM KCl, 1.5 mM Mg acetate and homogenized in a Duall-ground glass homogenizer. The homogenate was centrifuged at 30,000xg for 20 min and the supernatant was passed through a column (15 x 1 cm) containing coarse Sephadex G-25 equilibrated with 25 mM HEPES-KOH (pH 7.5), 120 mM KCl, 1.5 mM Mg acetate, and 1 mM DTT. The column was eluted with the same buffer and collected as 200 µl fractions. Those showing the highest A_{260} (i.e., polysomal and initiation complexes) were pooled together for *in vitro* translation.

Endogenous mRNA-free translational extracts were prepared by treatment with micrococcal-nuclease. A 100-µl cell-free extract was incubated with 25 U micrococcal nuclease (Pharmacia) in the presence of 0.3 mM $CaCl_2$, 2 mM Mg acetate, 2 mM DTT, 100 mM K acetate, 0.2 mM GTP, 1 mM ATP, 15 mM creatine phosphate, 8 µg creatine kinase, 10 µM each 19 amino acids (except methionine) in a final volume of 200 µl. After 10 min incubation at 20°C, nuclease activity was stopped by adding 0.6 mM EGTA. The final preparation was stored at -80°C as aliquots.

In Vitro Transcription

The legumin cDNA (11S) insert was purified from the pea legumin clone pCD43 and then placed under the control of a dual promoter (T7 and SP6) in plasmid pSPT18 (Pharmacia). This newly constructed plasmid was used for *in vitro* transcription reactions as per the procedure described by the supplier. A typical 20 µl reaction mixture contained 4 µl 5 x transcription buffer (200 mM TRIS-HCl pH 7.9, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl), 0.5 mM DTT, 20 U RNasin, 1000 µM each of ATP, GTP, CTP, 0.5 µM UTP, 50 µCi [α-^{32}P]-UTP (sp act 3000 µCi/mM) and 20 U of T7 RNA polymerase. The reaction was incubated at 37°C for 1 h. The newly transcribed mRNA was purified from the template plasmid DNA by incubating with RNase-free DNase I in the presence of 60 µg tRNA at 37°C for 30 min. The mixture was incubated further at 37°C for 30 min with proteinase K and 0.1% SDS. Finally, it was extracted with a phenol:chloroform mixture and chloroform alone. The aqueous layer was used for isopropanol precipitation at -20°C overnight. The transcribed RNA was recovered by repeated centrifugation followed by 70% ethanol washing. It was then dissolved in water and the incorporated radioactivity was measured as cold TCA-precipitated counts in the presence of 10 mM phosphate buffer, pH 7.2.

Ultra Violet-Cross Linking Between mRNA and Proteins in Reconstituted Polysomes

A flow diagram illustrating the various steps of the technique is given in Fig 1. Cell-free translation extracts (100 µl containing 50 µg protein) prepared from various developmental stages of alfalfa embryos were incubated with ^{32}P-labelled ($1.5\text{-}2.5x10^5$) legumin mRNA (0.5 - 1.0 ng) at 30°C for 15 min in a 96-well tissue-culture plate. This quick incubation period allowed the formation of initiation complexes and polysomes. The reaction was stopped by placing on ice and ribosome movement arrested by adding 50 µg/ml cycloheximide. Samples were then irradiated with UV light at 2°C for 10 min in a closed chamber with an incident dose of 4000µW/cm^2 (37) using a mineralight lamp model r52-G (Ultraviolet Products Inc, San Gabriel, USA). UV-irradiated samples were further incubated at 37°C for 30 min with a nuclease cocktail (micrococcal nuclease, RNase A and T1) to remove the non-crosslinked region of mRNA. It was brought to Laemmli's, 1970 (23) buffer conditions and then cross-linked mRNP-proteins were separated by SDS-PAGE (12% gel). After electrophoresis, the gel was incubated for 30 - 60 min at 85°C with 10% TCA and 10 mM phosphoric acid to remove the unincorporated or small fragments of ^{32}P-mRNA. Cross-linked proteins trapped with ^{32}P-labelled mRNA were made visible by autoradiography of the gels using Kodak XAR or XRP film.

Sucrose Density Gradient Analysis of Reconstituted Polysomes

The sedimentation profile of ^{32}P-labelled legumin mRNA in the reconstituted polysomes made from cell-free-translation extracts of developing alfalfa embryos (see previous section) was measured after sucrose density gradient separation. In this assay, ^{32}P-labelled reconstituted polysomes were layered over a 10 - 40% sucrose density gradient in 50 mM TRIS-HCl (pH 7.5), 100 mM NaCl, and 1 mM $MgCl_2$ which was then centrifuged in an SW 41 rotor (Beckman) at 100,000xg for 3.5 h at 2°C. At the end of centrifugation, each gradient was collected as 0.5 ml fractions. The cold TCA-precipitable ^{32}P-labelled legumin mRNA was counted in each fraction. Fractions 1 - 6, 7 - 10, and 11 - 20 were considered as mRNP, monosomes and small polysomes, and large polysomes, respectively.

RESULTS AND DISCUSSION

RNA Synthesis in Isolated Nuclei of Developing Embryos

Previously we observed that storage protein mRNAs are present during the entire period of development in somatic embryos of alfalfa, and in the cotyledonary stages of zygotic embryogenesis (33). The steady-state message content increases only during expansion of the cotyledons when messages are recruited by the polysomes for protein synthesis. However, these

mRNAs are not recruited into the polysomes during the pre-cotyledonary (mixture of globular and heart) stages of somatic embryogenesis but are sequestered in the non-polysomal fraction in the form of mRNP-complexes. The messages can be translated *in vitro* upon deproteinization, either in a wheat germ extract or a rabbit reticulocyte system (33). The cytoplasmic non-polysomal mRNAs from pre-cotyledonary stage somatic embryos are associated with a special set of proteins which are absent from the mRNAs of polysomal origin (31). These proteins may control the selective recognization and modulation of the expression of specific mRNAs. Our previous observations do not explain whether the non-translated storage protein mRNAs present in the pre-cotyledonary somatic embryos are in transit from the nucleus to the polysomes, are excess mRNAs in equilibrium with the polysomes, or are mRNAs masked by regulatory proteins which inhibit their recruitment into polysomes. We first tested these possibilities using the kinetics of nuclear transcription followed by DNA-excess dot-blot hybridization.

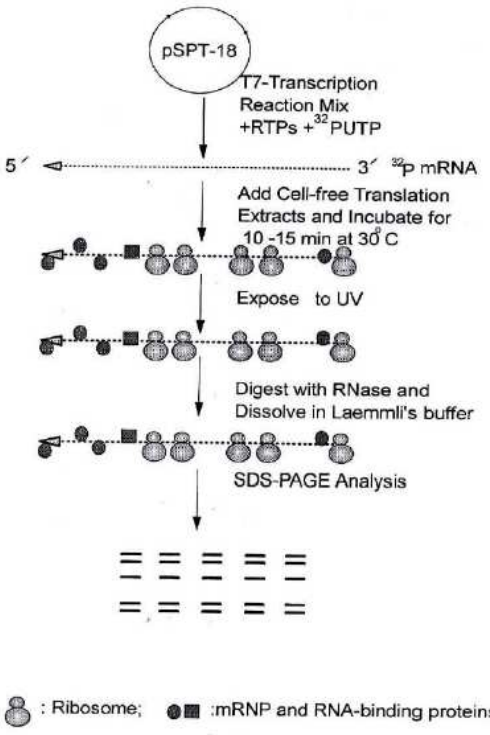

Figure 1. A summary of the technique of *in vitro* reconstitution of legumin mRNA and proteins in the cell-free translation extracts.

The rates of nuclear transcription at different stages of alfalfa embryo development are shown in Fig 2. When an equal number ($1x10^6$) of nuclei extracted from embryos at various stages of development was incubated in an *in vitro* transcription system, a lag period of incorporation of 15 min was noticed. Immediately after that, there was a more or less linear incorporation of acid-precipitable ^{32}P-UTP for a further 30 min, followed by a steady-state rate except in the nuclei preparations from somatic d 3 and 5 embryos which showed a more linear transcription rate for almost 120 min. The nuclei from fully-expanded cotyledons of somatic (d 10) or zygotic (stage VI - VII) embryos showed a slower rate of transcription than those of pre-cotyledonary stage, d 3 and 5, somatics. At the late cotyledonary stages of somatic embryogenesis (d 14 and 16) there was a lower rate of transcription. A very poor rate of transcription was also noted for very early (d 0) somatic embryos (globular stage). The transcription rate of leaf nuclei was almost the same as that of d 14 - 16 somatic embryos (Fig. 2).

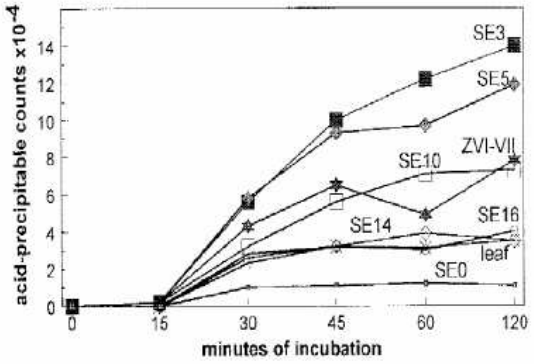

Figure 2. Kinetics of transcription in isolated nuclei from developing somatic and zygotic embryos and from leaf tissue.
Nuclei ($1x10^6$) suspended in 100 µl transcription cocktail were used for the transcription reaction. At different time intervals 5 µl were withdrawn from the reaction mixture, spotted on Whatman 541 filter paper, and then cold-TCA-precipitable radioactivity was measured.

The rate of transcription seemed to vary with the composition of the medium to which the somatic embryos were transferred and the developmental age of the embryo. An accelerated rate of transcription occurred from d 3 - 5 (Fig. 2) after the embryos were transferred from suspension culture medium (B5G) to a hormone-free Boi2Y medium, which supported the formation and development of the embryos. The Boi2Y medium promoted cellular differentiation and the formation of viable embryos, thereby, enhancing transcription. Yet, *in vivo*, the 11S mRNAs, although present, were not translated at this stage of development (33).

The poorer rat of transcription at the later stages of development, when the cotyledons were fully expanded, was probably due to the aging and maturity of the embryos, when most of the transcriptional machineries became inactive.

Analysis of Legumin (11S)-Specific mRNA during Transcription in Isolated Nuclei by DNA Excess RNA Hybridization

Newly synthesized ^{32}P-labelled transcripts were withdrawn from the *in vitro* transcription system at different times from the start of incubation and total ^{32}P-labelled RNA was purified. This was then used as a probe for hybridization against a pea legumin (11S) cDNA insert (pCD 43 cDNA). A parallel experiment was also conducted in which hybridization of *in vitro* transcripts was against either soybean actin (pSAc 43) or ribosomal RNA (pGMr1) gene inserts. After hybridization, dot blots were scanned with a gel scanner (Hoeffer Scientific Instruments) and absorption plotted as a bar diagram, using relative units of absorption (Fig. 3).

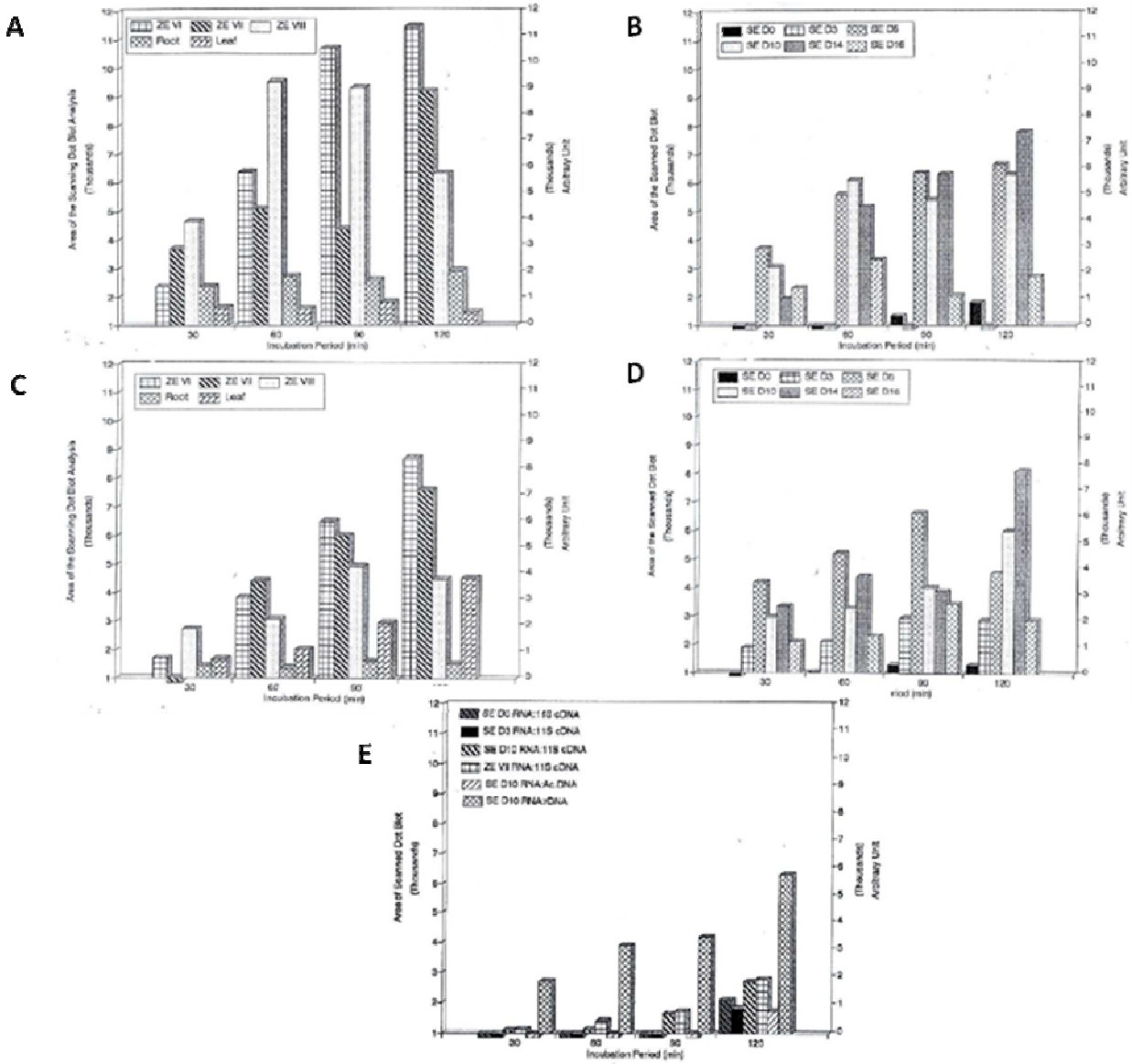

Figure 3. Expression of legumin (11S, medicagin) storage protein, actin and ribosomal RNA in developing somatic and zygotic embryos, roots and leaves.
Detection of individual mRNAs was measured by DNA-excess RNA hybridization as described in Materials & Methods. Kinetics of the hybridization plotted in the form of bar diagram using incubation period, min (x-axis) against the scanned area of the incorporated radioactivity in the dot blot, in arbitrary unit (y-axis). D: day; Z(E): zygotic (embryos); SE: somatic embryos (0, 3, 10 etc., number of d of development); Stg: stage of zygotic embryos development; Ac: actin; rDNA: ribosomal cDNA.

The legumin (Fig. 3 A & B) and the control actin Fig.3 (C & D) mRNA were transcribed by isolated nuclei from both somatic and zygotic embryos during their development. However, the legumin mRNA was barely detectable in nuclei from either the leaf or the root using low-stringency washing, and not at all in these from pre-cotyledonary-stage somatic embryos (d 0 - 3) nuclei. Actin mRNA was detectable in nuclei from the leaf and root, more predominantly in the former, and also in both embryo types. The slight appearance of legumin mRNAs in nuclear transcripts from either the leaf or root could be due to non-specific binding of the probe which was not eliminated by low-stringency washing.

Using northern blot analysis and high-stringency washing, no steady-state legumin mRNA was detected in either the leaf or root tissue, but remained present on blots from zygotic and somatic embryos. On the other hand, actin mRNA was detected in both leaf and root tissue even after high-stringency washing (Fig 4).

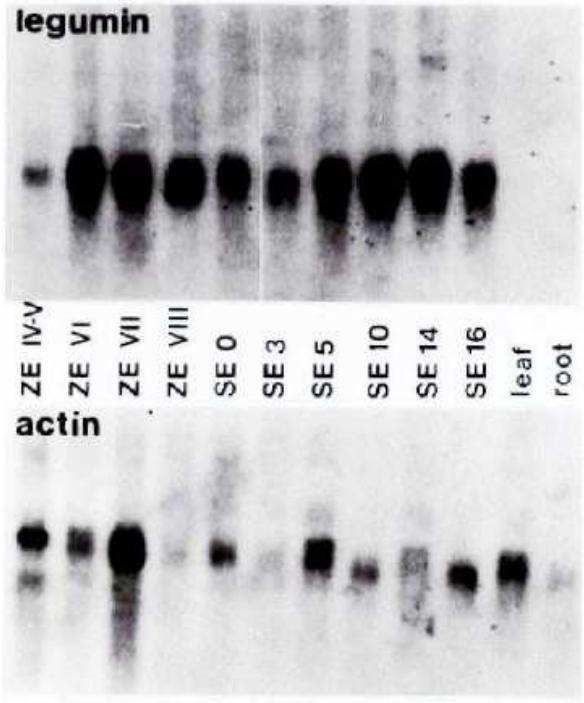

Figure 4. Steady-state mRNA analysis of developing embryos using northern blot technique.
A: Probed with ^{32}P-labelled legumin cDNA (pCD 43) clone. B: Probed with ^{32}P-labelled soybean actin genomic DNA (pSAc 43) clone. Lanes: Zygotic Embryos (ZE) - stage IV / V, ZE - stage VI, ZE - stage VII, ZE - stage VIII, Somatic Embryos (SE) - 0 d, SE - 3 d, SE - 5 d, SE - 10 d, SE - 14 d, SE - 16 d, leaf, root.

The synthesis by isolated nuclei from zygotic and somatic embryos of legumin and actin mRNAs was largely inhibited in the presence of 0.5 µg/ml α-amanitin (Fig. 3E), a fungal cyclic peptide which inhibits RNA polymerase II activity at low concentrations, without interfering the RNA polymerase III (18). Thus, transcription of ribosomal RNA genes occurred in the presence of α-amanitin during the incubation of isolated nuclei using a preparation from 10-d somatic embryos. α-Amanitin considerably diminished synthesis of the legumin and actin mRNAs; this is an indication of the fidelity of the *in vitro* transcription system.

Transcription of legumin mRNA during somatic embryogenesis occurred immediately after the onset of cotyledon formation, which was from d 5 onwards (Fig. 3). At this stage, the legumin mRNAs were recruited into polysomes and synthesized the corresponding storage protein. This process continued as long as the cotyledons underwent expansion (to d 14) and then declined as the embryos reached maturity (d 16) (33).

By comparing legumin transcription between developing somatic and zygotic embryos, there is a strong indication that zygotic embryos always support more transcription than the somatics. Stage VI zygotic embryos showed the maximum of legumin transcription, followed by a decline at later stages. On the other hand, in somatic embryos the legumin mRNAs started to be transcribed on d 5 and onwards, reaching the highest transcription at d 14, before starting to decline (Fig 3). The poorer legumin transcription in somatic embryos could be due to the composition of the medium in which they were allowed to differentiate. This is presumably less stimulatory for the completion of embryogenesis than the nutritional supply that zygotic embryos received from their parent plant.

In Vitro Reconstitution of Proteins with Legumin mRNA in mRNPs and Polysomes

It was of interest to determine why the legumin mRNA that was transcribed during the pre-cotyledonary stages of somatic embryos development was not translated *in vivo*. This could be due to an intrinsic property of the message, to defective translation machinery, or a combination of these. To ascertain which of these possibilities was most likely; an experiment involving *in vitro* reconstitution of mRNPs was carried out. Legumin message labelled with ^{32}P was transcribed by placing a pea legumin cDNA (pCD43) insert under the control of T7 promoter, to make a sense strand, in the plasmid vector

pSPT18 (Pharmacia). These mRNAs were then incubated separately with cell-free translational extracts prepared from pre-cotyledonary- and cotyledonary-stage somatic embryos and cotyledonary-stage zygotic embryos (details in Materials and Methods). After translation was underway (i.e., immediately after the formation of polysomes, which took 10 - 15 min), the reaction was stopped by adding 50 µg/ml cycloheximide to arrest polysome movement, and intimately-associated polysomal and non-polysomal and proteins were cross-linked by UV-irradiation.

The legumin mRNA became UV-cross-linked to several proteins in the cell-free translational extracts prepared from pre-cotyledonary- and cotyledonary stage-somatic and zygotic embryos (Fig 5). The greatest difference in the types of proteins which cross-linked was between the pre-cotyledonary-stage and cotyledonary-stage somatic embryos with proteins of 32, 36 and 38 kD being very predominant in the latter, and those of 21 - 23, 28, 50, 55, and 62 kD being enriched in, or exclusive to, the former. The proteins in the translational extracts of cotyledonary-stage zygotic embryos which became cross-linked to the legumin mRNA included the most predominant ones in the cotyledonary-stage somatic embryos (36 - 38 kD), but not those most evident in the pre-cotyledonary-stage embryos or they were present only faintly. Some of the faint bands on the autoradiograph of the cotyledonary-stage zygotic embryos were also present on that of the cotyledonary-stage somatics.

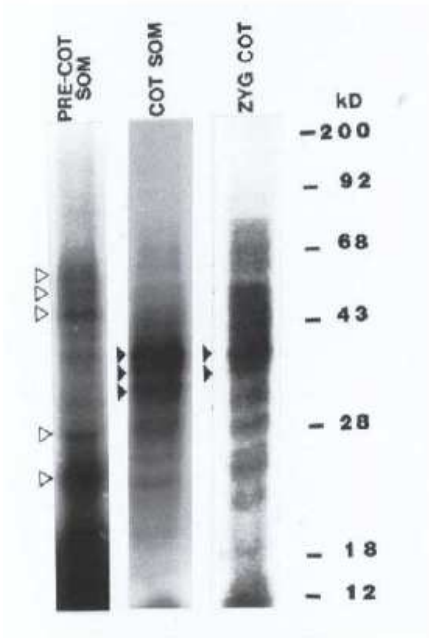

Figure 5. The association of proteins and legumin mRNA into an mRNP-complex using cell-free translational extracts of somatic and zygotic embryos of alfalfa. ^{32}P-labelled legumin mRNA was incubated with cell-free translational extracts from developing zygotic and somatic embryos and exposed to UV to make covalent linkages between the mRNA and intimately-associated proteins. The resultant complex was digested with a nuclease cocktail, and the proteins associated with ^{32}P-mRNA were resolved by SDS-PAGE.

The UV-cross-linked proteins from pre-cotyledonary-stage somatic, cotyledonary-stage somatic and zygotic embryos are shown. Proteins from the extracts of cotyledonary-stage embryos which cross-link with the mRNA are indicated by the dark arrows and those from the pre-cotyledonary stage by the open arrows. Mol. mass markers in kD shown on the right of C.

We determined if there is a relationship between the ability of legumin mRNA to be translated, and the activity of the cell-free translated extracts from the zygotic embryos, and the somatic embryos at the two stages of development. Legumin mRNA was incubated with cell-free-translational extracts in the presence of ^{35}S-methionine for various times, and the incorporation of label was measured in hot TCA precipitates. The efficiency of legumin mRNA utilization was least in pre-cotyledonary-stage somatic embryos (SE - 0) compared to cotyledonary-stage somatic (SE - 10) and zygotic (ZE - VII) embryos (Table 1).

Table 1. Efficiency of legumin mRNA utilization by various stages of cell-free-translation extracts using ^{35}S-methionine and measuring the hot TCA-precipitable counts at different times of incubation.

Incubation period (min)	SE 0	SE 10 (cpm/µl)	ZE VII
30	909	2523	2706
60	756	2202	3411
90	821	2511	3250
120	661	2199	4184

100 ng of legumin mRNA was used for in vitro translation in a reaction mixture of 50 µl volume containing equal amounts of A_{280} cell-free-extracts. 1 µl was removed from the reaction mixture at different times, spotted onto Whatman 541 filter paper and boiled for 20 min in 15% TCA. After boiling, filter papers were washed with several volumes of 15 % TCA until the washings had a normal background count. Finally, the filters were washed with ethanol and acetone, and counted in toluene-omnifluor. SE 0 and 10: Somatic Embryos day 0 and 10; ZE VII: Zygotic Embryos Stage VII).

While it must remain a speculation at this time, it is possible that defective translation of the legumin message during the pre-cotyledonary stages of somatic embryogenesis is due to a deficiency in the translationally-active legumin-message-binding protein complex. That is, the 36 and 38 kD proteins are required (and perhaps 32 kD), but are present only in the later cotyledonary-stage embryos, when legumin is synthesized. The presence of other mRNA-binding proteins (21 - 23, 28, 50, 55 and 62 kD) might result in the masking of the legumin message, thus, forming free mRNP complexes and thereby preventing initiation of translation at the pre-cotyledonary stage of somatic embryo development. This 62 kD protein may be our previously reported (31) autophosphorylated 65 kD protein kinase C which might show a lower molecular mass on this gel could be due to normal small variations in the migrations of proteins and markers during gel electrophoresis.

As development proceeds, the masking proteins are either turned over and are no longer present, or they cannot compete with newly synthesized translationally-active legumin mRNA-binding proteins (32, 36 and 38 kD) which positively regulate translation. Such associations between proteins and storage protein mRNAs during embryogenesis are a novel finding regardless of their cellular function.

Sucrose Density Gradient Analysis of In-Vitro-Reconstituted Legumin Polysomes

As we noted in the reconstituted polysomal experiments, that there is a variation in the legumin mRNA binding proteins between pre-cotyledonary- and cotyledonary-stage embryos. However, we do not know whether these differences in the binding of proteins to mRNA allow for the formation of larger polysomes or prevent the initiation of polysome formation. To check this, the distribution of ^{32}P-legumin mRNA in the reconstituted polysomes made from cell-free-translational extracts was measured by separation on a 10 - 40% sucrose density gradient. The distribution of acid-precipitable radioactivity (^{32}P-labelled legumin mRNA) in the reconstituted polysomes showed three major peaks: free mRNP (fractions 1 - 6, region I), monosomes and small polysomes (fractions 7 - 10, region II), and a large polysomes (fractions 11 - 20, region III) (Fig. 6).

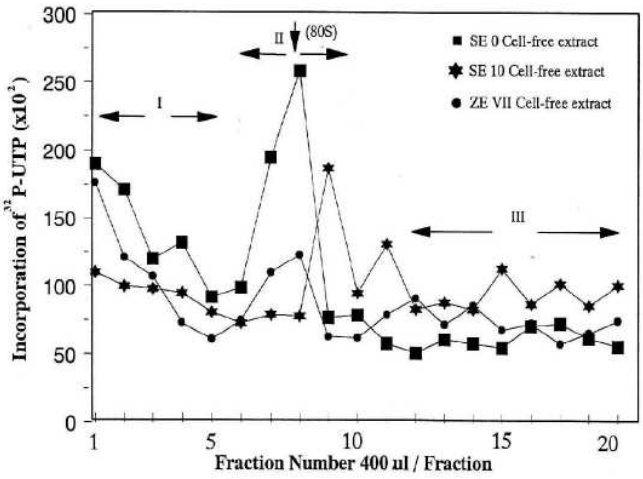

Figure 6. Sedimentation profile of reconstituted legumin-mRNA-containing polysomes in sucrose density gradients. ^{32}P-labelled legumin mRNA was incubated for a brief period (10 - 15 min) with cell-free translation extract prepared from various stages of developing alfalfa embryos to allow the formation of the initiation and polysome complexes and then analyzed on a 10 - 40% sucrose gradient. Centrifugation was for 3.5 h at 2°C at 100,000xg in a Beckman SW 41 rotor. The sample was fractionated (0.5 ml / fraction) using an Autodensi-flow IIC gradient maker (Buchler). Each fraction was plotted against the amount of TCA- precipitable ^{32}P-labelled legumin mRNA. Fractions 1 - 6, mRNP-complexes (region I); 7 - 10 monosomes and small polysomes (region II); 11 - 20 large polysomes (region III).

The translational extracts from both the cotyledonary-stage somatic and zygotic embryos allowed the formation of larger polysomes containing legumin mRNA (region III) *in vitro*, whereas the translational extract from the pre-cotyledonary somatic embryos prevented this. Rather it enhanced the formation of monosomes and small polysomes (region II) and mRNPs (I) compared to the cotyledonary translational extracts.

These findings are consistent with the suggestion that there is discrimination in the recruitment of mRNAs into polysomes at different stages of embryo development, with the legumin message being excluded during the pre-cotyledonary stage of somatic embryogenesis. Translational extracts prepared from this stage presumably lack some of the legumin mRNA-binding proteins which may be involved in translation in cotyledonary-stage embryos. Moreover, the pre-cotyledonary extracts may contain some regulatory proteins which bind to the legumin mRNA and negatively regulate its translation. Such regulatory proteins were not present in the reconstituted legumin mRNP-

complexes made from cotyledonary translation extracts of somatic or zygotic embryos (Fig. 5). We have also reported that cell-free-translational extracts prepared from pre-cotyledonary somatic embryos contain about 100-fold more protein kinase activity as compared with those from cotyledonary zygotic embryos (31). This kinase activity could covalently modify some of the mRNA-binding proteins and, thereby, prevent their initiation. Discrimination in mRNA utilization in polysomal complexes is a common phenomenon in animal systems, but parallel studies on plants are very rare, and none have addressed the association between potentially regulatory proteins and newly synthesized mRNA. In sea urchin egg and ascites cells (28) there is a marked degree of discrimination in the utilization of specific mRNAs by polysomes. These messages exist in the cytoplasm as free mRNPs or "masked" messages. Here we show that a similar situation can occur during embryogenesis, particularly during the early stages of somatic embryogenesis and we have started to identify the discriminatory factors in the polysomal fraction which may be responsible for the selective recruitment of legumin mRNA for translation.

Acknowledgements-This work was supported by grant A2210 from the Natural Science Engineering Research Council (NSERC) of Canada to JDB. We also express our gratitude to Dr. Rod Casey, John Innes Centre, Norwich, UK, for providing the clone pCD43 of pea legumin.

REFERENCES

1. Ajtkjozhin, M.A., Polimbetova, N.S. and Akhanov, A.U., Nuclear ribonucleoprotein particles of higher plants. *FEBS Lett.* 1975, 54: 212-216.

2. Atanassov, A. and Brown, D.C.W., Plant regeneration from suspension culture and mesophyll protoplasts of *Medicago sativa* L.. *Plant Cell Tissue Organ Culture*, 1984, 3: 149-162.

3. Bag, J., mRNA and mRNP. In: Translation in Eukaryotes, Trachsel H. (ed) CRC Press, Boca Raton, Florida, 1991, pp 71 -96.

4. Bag, J., Cytoplasmic mRNA-protein complexes of chicken muscle cells and their role in protein synthesis. *Eur. J. Biochem.* 1984, 141: 247-254.

5. Berger, L.C., Bag, J. and Sells, B.H. Identification of proteins associating with poly (A)-binding-protein mRNA. *Eur. J. Biochem.*, 1992, 204: 733-743.

6. Bewley, J.D., Pramanik, S.K. and Krochko, J.E., Regulation of storage protein synthesis during development of somatic and zygotic embryos of alfalfa (*Medicago sativa*): Transcriptional and post-transcriptional control. In*: Proceedings of the 4th International Conference on Seed Science and Technology*, ASFIS, Paris, France, 1993, 1: 29-38.

7. Bingham, E.T., Hurley, L.V., Kaatz, D.M. and Sanders, J., Breeding alfalfa which regenerates from callus tissue culture. *Crop Sci.*, 1975, 15: 719-721.

8. Brown, P.H., Daniels-McQueen, S., Walden, W., Patino, M.M., Gaffield, L., Bielser, D. and Thach, R.E., Requirements for the translational repression of ferritin transcripts in wheatgerm extracts by a 90 kD protein from rabbit liver. *J. Biol. Chem.*, 1989, 264: 13383-13386.

9. Cerny, R.E., Qi, Y., Aydt, C.M., Huang, S., Listello, J.J., Fabbri, B.J., Conner, T.W., Crossland, L. and Huang, J., RNA-binding protein-mediated translational repression of transgene expression in plants. *Plant Mol. Biol*., 2003, 52:357-69.

10. Cochrane, A.W., Chen, C.H. and Rosen, C.A.,. Specific interaction of the human immunodefficiency virus *Rev* protein with a structured region in the *Env* mRNA. *Proc. Natl. Acad. Sci. USA* 1990, 87, 1198-1202.

11. David-Assael, O., Saul, H., Saul, V., Mizrachy-Dagri, T., Berezin, I., Brook, E., andShaul, O., Expression of AtMHX, an Arabidopsis vacuolar metal transporter, is repressed by the 5' untranslated region of its gene. *J Exp Bot*. 2005, 56: 1039-47.

12. Deng, J., Lu, P.D., Zhang, Y., Scheuner, D., Kaufman, R.J., Sonenberg, N., Harding, H.P. and Ron, D., Translational repression mediates activation of nuclear factor kappa B by phosphorylated translation initiation factor 2. *Mol Cell Biol*. 2004, 24:10161-8.

13. Ernst, V. and Arnstein, H., Synthesis of α and β globin directed by messenger ribonucleoprotein from rabbit reticulocytes. *Biochimica Biophysica Acta* 1975, 378, 251-259.

14. Gale Jr., M., Tan, S.L. and Katze, M.G., Translational control of viral gene expression in eukaryotes. Microbiology and Molecular Biology Reviews, 2000, 64: 239-280.

15. Gamborg, O., Miller, R. and Ojima K., Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.*, 1968, 50: 151-158.

16. Greenberg, J.R. and Carroll III, E., Reconstitution of functional mRNA-protein complexes in a rabbit reticulocyte cell-free translation system. *Mol. Cell. Biol.* 1985, 5: 342-351.

17. Imaizumi-Scherrer, M.T., Maundrell, K., Civelli, O.C. and Scherrer, K., Transcriptional and post-transcriptional regulation of duck erythroblasts. *Dev.. Biol.* 1982, 93: 126-138.

18. Jacob, S.T., Sajdel, E.M. and Munro, H.N., Specific action of α-amanitin on mammalian RNA polymerase protein. *Nature* 1970, 225: 60.

19. Koeller, D.M., Casey, J.L., Hentze, M.W., Gerhardt, E.M., Chan, L.L-N, Klausner, R.D. and Harford, J.B.,. A cytosolic protein binds to structural elements within the iron regulatory region of the transferrin receptor mRNA. *Proc. Natl. Acad. Sci. USA* 1989, 86: 3574-3578.

20. Kubota, S., Mukudai, Y., Moritani, N.H., Nakao, K., Kawata, K. and Takigawa M., Translational repression by the *cis*-acting element of structure-anchored repression (CAESAR) of human ctgf/ccn2 mRNA, *FEBS Lett.*, 2005, 579: 3751-3758

21. Kawai, T., Fan, J., Mazan-Mamczarz, K. and Gorospe, M., Global mRNA stabilization preferentially linked to translational repression during the endoplasmic reticulum stress response. *Mol Cell Biol.* 2004, 24:6773-87.

22. Kauder, S.E. and Racaniello, G., Poliovirus tropism and attenuation are determined after internal ribosome entry. *J. Clin. Invest.* 2004, 113: 1743 – 1753.

23. Laemmli, U.K., Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970, 227: 680-685.

24. Luthe, D.S. and Quatrano RS. Transcription in isolated wheat nuclei. I. Isolation of nuclei and elimination of

endogenous ribonuclease activity. *Plant Physiol.* 1980, 65:305-308.

25. Maundrell, K., Imaizumi-Scherrer, M.T., Maxell, S., Civelli, O. and Scherrer, K., Messenger RNA for the 73000-dalton Poly (A)-binding protein occurs as translationally repressed mRNP in duck reticulocyte. *J. Biol. Chem.* 1983, 258: 1387-1390.

26. McCarthy, J.E.G. and Kollmus, H., Cytoplasmic mRNA-protein interactions in eukaryotic gene expression. *Trends Biochem. Sci.* 1995, 20: 191-197.

27. Merrovitch, K., Pelletier, J. and Sonenberg, N., 1989. A cellular protein that binds to the 5'-noncoding region of polyovirus RNA: implications for internal translation initiation. *Genes Dev.* 1989, 3: 1026-1034.

28. Moon, R.T., Danilchik, M.V. and Hille, M.B. 1982. An assessment of the masked message hypothesis: sea urchin egg messenger ribonucleoprotein complexes are efficient templates for in vitro protein synthesis. *Dev. Biol.* 1982, 93: 389-403.

29. Muckenthaler, M., Gray, N.K. and Hentze, M.W., IRP-1 Binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the Cap-binding Complex eIF 4F. *Mol. Cell* 1998, 2: 283 – 388.

30. Pramanik, S.K. and Bag, J., Expression of muscle-specific proteins is necessary to regulate translation of the mRNA for a 40-kDa housekeeping polypeptide in rat L6 Cells. *Eur. J. Biochem.* 1989, 182: 687-698.

31. Pramanik, S.K. and Bewley JD. 1996. Post-transcriptional regulation of protein synthesis during alfalfa embryogenesis. Proteins associated with the cytoplasmic polysomal and non-polysomal mRNAs (messenger ribonucleoprotein complex). *J. Exp. Bot.* 1996, 47:1871 - 1879.

32. Pramanik, S.K., Williams, A.L., and Bewley, J.D., Endogenous Protein Kinase –C Activity and Phosphorylated Proteins in Messenger Ribonucleoprotein Complexes of Developing Embryos of Alfalfa. *Cell. Mol. Biol*. 2004, 50: 773 - 781.

33. Pramanik, S.K., Krochko, J.E. and Bewley, J.D., Distribution of cytosolic mRNAs between polysomal and ribonucleoprotein complex fractions in alfalfa embryos. Stage specific translational repression of storage protein synthesis during early somatic embryo development. *Plant Physiol*. 1992, 99: 1590-1596.

34. Schalinske, K.L., Blemings, K.P., Steffen, D.W., Chen, O.S. and Eisenstein, R.S., Iron regulatory protein 1 is not required for the modulation of ferritin and transferring receptor expression by iron in a murine pro-B lymphocyte cell line. *Proc.Natl.Acad.Sci.USA*, 1997, 94, 10681 – 10686.

35. Seli, E., Lalioti, M.D., Flaherty, S.M., Sakkas, D., Terzi, N., Steitz, J.A., An embryonic poy (A) binding protein 9ePAB) is expressed in mouse oocytes and early preimplantation embryos. *Proc.Natl.Acad.Sci.USA*, 2005, 102: 367 – 372.

36. Stolovich, M., Lerer, T., Bolkier, Y., Cohen, H. and Meyuhas, O.,. Lithium can relieve translational repression of TOP mRNAs elicited by various blocks along the cell cycle in a glycogen synthase kinase-3- and S6-kinase-independent manner. *J. Biol. Chem*. 2005, 280:5336-42

37. Wagenmakers, A.J.M., Reinder, R.J., and Van Venrooij, W.J.,. 1980. Cross-linking of mRNA to proteins by irradiation of intact cells with ultraviolet light. *Euro. J. Biochem.* 1980, 112: 323-330.

38. Wiesem, A., Elzinga, N., Wobbes, B. and Smeekens, S., Sucrose-induced translational repression of plant bZIP-type transcription factors. *Biochem. Soc. Trans.* 2005, 33:272-5.

39. Xu, N., Coulter, K.M., Krochko, J.E. and Bewley, J.D., .Morphological stages and storage protein accumulation in developing alfalfa (*Medicago sativa* L) seeds. *Seed Sci. Res.* 1991, 1, 119-125.

40. Yohn, C.B., Cohen, A., Danon, A., Mayfield, S.P., A poly (A) binding protein functions in the chloroplast as a message-specific translation factor. *Proc. Natl .Acad. Sci* (USA) 1998, 95: 2238-2243