Expression of maize eukaryotic initiation factor (eIF) iso4E is regulated at the translational level

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Mechanisms for regulation of gene expression at the translational level have been reported at specific developmental stages in eukaryotes. Control of eukaryotic initiation factor (eIF) 4E availability by insulin/growth factors constitutes a main point of translational regulation. The aim of the present research was to understand the regulatory mechanism(s) behind the differential expression of two main 4E factors present in maize embryonic axes during germination. *De novo* synthesis of eIFiso4E initiates earlier and is faster than that of eIF4E in maize axes. Insulin addition to maize axes stimulated *de novo* synthesis of the eIFiso4E protein, but not that of eIF4E. Specific recruitment of the eIFiso4E transcript into polysomes was observed in these axes after insulin stimulation. Inhibitors of the insulin signal-transduction pathway, wortmannin and rapamycin, reversed the

insulin effect. *In vitro* translation of maize poly(A)⁺ RNAs by S6 ribosomal protein (rp)-phosphorylated ribosomes demonstrated a strong increase in eIFiso4E synthesis, as compared with its translation by S6 rp-non-phosphorylated ribosomes. Other mRNAs from the poly(A)⁺ RNA set, including the eIF4E mRNA, did not show differential translation with regard to the S6-phosphorylated status of the ribosomes. The overall results indicate that eIFiso4E, but not eIF4E, cell content is regulated by *de novo* synthesis in maize axes during germination, most probably by specific mRNA recruitment into polysomes via a signal-transduction pathway involving S6 rp phosphorylation.

Key words: polysome, S6 ribosomal protein phosphorylation, 5'-tract of oligopyrimidines, transcript recruitment.

INTRODUCTION

One of the most important targets of protein-synthesis regulation in eukaryotes is the eukaryotic initiation factor (eIF) 4E, known as cap-binding protein [1]. Plants have shown to contain two main isoforms of this protein, named eIF4E and eIFiso4E [2]. Differential expression patterns at the protein level have been found for these factors at specific developmental stages [3]. The eIFiso4E protein is present at higher levels with respect to eIF4E in wheat-germ extract [4] and maize embryonic axes [5], both representing quiescent tissues. In *Arabidopsis thaliana*, it was demonstrated that the corresponding mRNA isoforms display differential tissue distribution: eIFiso4E is expressed only in floral organs and developing tissues, whereas eIF4E is in most mature plant tissues [6].

In several eukaryotes, other than plants, the level of free eIF4E is regulated by 4E-binding proteins (4E-BPs), which form inactive complexes with this factor. Phosphorylation of the binding proteins releases the active 4E factor [7]. In mammals, the presence of at least three different isoforms of 4E-BP has been reported [8,9]. Furthermore, in these organisms the enhancement or inhibition of eIF4E activity via 4E-BP interaction is regulated by hormonal stimuli, particularly insulin [10,11].

In plants, the mechanism regulating eIF4E-isoform activity is especially intriguing, since no 4E-BPs seem to be present in these organisms. It has been proposed that regulation of the relative abundance of the two eIF4E isoforms would account for differential patterns of protein synthesis associated with specific developmental stages or environmental conditions [3,6]. However, at present there is no information on the regulatory mechanism of eIF4E and eIFiso4E activity [12].

During early maize germination, significant *de novo* synthesis of eIFiso4E, but not of the eIF4E protein, has been demonstrated, suggesting differential mechanisms regulating the expression of these isoforms at this developmental stage [5]. Previous work in our laboratory has shown that a mechanism of translational control regulates ribosomal protein synthesis in maize axes. This pathway, which involves the enhancement of S6 ribosomal protein (rp) phosphorylation on the 40 S ribosomal subunit, was demonstrated to be insulin-stimulated [13] in a similar manner to that reported for other eukaryotes [10]. Indeed, in animal cells, insulin and several growth factors/mitogens are known to stimulate this signal-transduction pathway that also induces preferential translation of the so-called 5'TOP mRNAs, with a specific tract of oligopyrimidines (TOP) in their 5'-untranslated region. Transcripts encoding proteins of the translational apparatus, such as eukaryotic elongation factor 1 α and rp mRNAs, have been found among the 5'TOP mRNAs [14–16].

Taking into account the apparent absence of 4E-BPs and the differential expression patterns of the two 4E isoforms in plants, it was considered relevant to test whether maize eIFiso4E and/or eIF4E cell content is regulated at the translational level, by the signal-transduction mechanism mentioned above.

EXPERIMENTAL PROCEDURES

Biological system

Maize (*Zea mays* L.) seeds of a Mexican land race variety (Chalqueño) were used for all experiments. The seeds were germinated in a Griffin incubator for 3, 6, 9 or 21 h by water imbibition on moisturized cotton, in the dark at 25 °C. The

Abbreviations used: eIF, eukaryotic initiation factor; CHX, cycloheximide; 4E-BP, 4E-binding protein; RRL, rabbit reticulocyte lysate; rp, ribosomal protein; TOP, tract of oligopyrimidines.

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embryonic axes were excised manually and incubated for 3 h in sterile MS [17] liquid medium ($1.5 \text{ ml} \cdot \text{g}^{-1}$ of axes $^{-1}$) for each treatment [13].

De novo protein-synthesis system

De novo synthesis of eIF4E and eIFiso4E proteins was analysed by addition of [^{35}S]methionine ($11.1 \times 10^6 \text{ Bq} \cdot \text{ml}^{-1}$) to the axes in the incubation medium (MS), supplemented or not with insulin ($200 \mu\text{M}$) for the last 2 h of incubation. To inhibit *de novo* protein synthesis, $70 \mu\text{M}$ cycloheximide (CHX) was added to the medium during the same period, a dose sufficient to block 95% of protein synthesis in maize axes [18]. For some experiments, the excised axes were preincubated for 1 h with $0.01 \mu\text{M}$ of either wortmannin or rapamycin prior to insulin addition. At the end of the incubation period, axes were washed and the eIF4F complexes (composed of eIF4E and eIF4G proteins) were purified by $^7\text{mGTP}$ -Sephacryl affinity chromatography. To this end, total cytoplasmic proteins contained in the post-ribosomal supernatant of 100 embryonic axes were incubated with $150 \mu\text{l}$ of $^7\text{mGTP}$ -Sephacryl (Sigma, St. Louis, MO, U.S.A.) and the retained factors eluted with $100 \mu\text{M}$ $^7\text{mGTP}$, as described previously [5]. Under the experimental conditions used, neither eIFiso4E nor eIF4E protein was detected by Western blotting in the supernatant after the $^7\text{mGTP}$ -Sephacryl treatment. The patterns of *de novo*-synthesized proteins produced under labelling conditions were analysed by fluorography of the dried SDS gels.

Identification of proteins by SDS/PAGE and Western blotting

The $^7\text{mGTP}$ -bound proteins specifically eluted from the affinity column were concentrated in Amicon Centricon 10 concentrators (Millipore, Bedford, MA, U.S.A.) and resolved by SDS/PAGE (13% gels). The proteins were blotted on to a PVDF membrane (Millipore), which was blocked with 5% dried skimmed milk for 1 h, followed by overnight incubation at 4°C with mouse polyclonal antiserum against wheat eIF4E or eIFiso4E, kindly donated by Dr K. S. Browning (University of Texas, Austin, TX, U.S.A.), at a 1:1000 dilution. The membrane was then incubated for 2 h with anti-mouse horseradish peroxidase-labelled conjugate antibody at a 1:3000 dilution. Immunodetection was performed by enhanced chemoluminescence (ECL) reagents (Amersham Pharmacia Biotech, Uppsala, Sweden) and autoradiography. The $^7\text{mGTP}$ -eluted proteins, as well as the total cytoplasmic proteins from maize extracts, were also blotted to PVDF membrane and tested against human 4E-BP1 and 4E-BP2 antiserum (a gift from Dr N. Sonenberg, University of Quebec, Montreal, Canada), at a 1:1000 dilution similarly as described above.

Polysomal and total RNA analysis by Northern blots

Total RNA was isolated from 0.4 g of embryonic axes by a procedure modified for tissues enriched in polysaccharides [19]. The method was essentially the same as described previously by Chomczynski and Sacchi [20], except for an additional step after the phenol extraction. The last step consisted of 1 h of polysaccharide precipitation on ice by addition of an equal volume of 2 M potassium acetate, followed by centrifugation at $44\,000 \text{ g}$ for 30 min. Polysomal fractionation and further RNA isolation were achieved by the method described previously [21]. Polysomal and total RNAs were resolved on 1.2% agarose/6% formaldehyde gels. Either $20 \mu\text{g}$ of denatured polysomal RNA or $30 \mu\text{g}$ of total RNA was used per lane. The RNAs were blotted on to a nylon membrane (Amersham Pharmacia Biotech), UV-

crosslinked (UV Stratalinker, Stratagene, La Jolla, CA, U.S.A.) and hybridized with specific ^{32}P -labelled cDNAs.

Molecular probes

The cDNA probes used in the Northern-blot analysis were purified as fragments from the corresponding plasmids as follows: from R0456, a 1.04-kb *SalI*-*NotI* fragment containing rice eIF4E cDNA, and from S4954 a 1.1-kb *SalI*-*NotI* fragment containing rice eIFiso4E cDNA, donated by the MAFF DNA Bank; and from 01A12 a 0.55-kb *XbaI*-*SalI* fragment containing maize S6 rp cDNA [22], donated by Theresa Musket (University of Missouri-Columbia, Columbia, MO, U.S.A.). The cDNA inserts were labelled with [^{32}P]dCTP by random priming (Random Primer Extension Kit; NEN, Boston, MA, U.S.A.) according to manufacturer's instructions.

In vitro translation experiments

Rabbit reticulocyte lysate (RRL) from the *in vitro* translation system (Roche Diagnostics and Promega) was ribosome-depleted by 1 h of centrifugation at $250\,000 \text{ g}$. Translation reactions were set with this lysate (40% of total volume) supplemented with maize ribosomes at $400 \mu\text{g} \cdot \text{ml}^{-1}$ (measured by A_{260}). The ribosomes were obtained from 3-h-germinated embryonic axes (ribosomes not phosphorylated at the S6 rp), 24-h-germinated axes (phosphorylated ribosomes) or insulin-stimulated 24-h-germinated axes (highly phosphorylated ribosomes; see below) [13,23,24]. In control experiments, these ribosomes were treated previously with puromycin and GTP in buffer D (20 mM Tris/HCl, pH 7.6, 400 mM KCl, 3 mM MgCl_2 , 5 mM 2-mercaptoethanol and 5% sucrose) at a final concentration of 0.5 mM each, for 45 min, at 37°C to dissociate mRNA-ribosome complexes, centrifuged, and the subunits used for translation at the same concentration as the native ribosomes. The translation system was also supplemented with [^{35}S]methionine ($3.7 \times 10^6 \text{ Bq} \cdot \text{ml}^{-1}$) and $8 \mu\text{g}$ of poly(A) $^+$ RNA obtained from the total RNA pool of 24-h-germinated axes, in a total volume of $100 \mu\text{l}$. The translation reaction was allowed to proceed at 30°C for 30 min. At the end of incubation, eIFiso4E or eIF4E proteins were purified from the *in vitro*-translated mixture by $^7\text{mGTP}$ -affinity chromatography and detected by immunoblotting as described above. After identification of the corresponding bands, they were excised and the label incorporated into each 4E isoform was determined by liquid-scintillation counting (Packard scintillator). Results were corrected with the value of [^{35}S]methionine incorporated into total proteins per reaction. An *in vitro*-translation control reaction for each maize ribosome set (treated or not with puromycin) was performed with $2 \mu\text{g}$ of β -globin mRNA and incubated for 30 min under the stated conditions.

Stability of phosphorylated S6 rp on the ribosomes during translation

Maize ribosomes were phosphorylated *in vivo* with [^{32}P]P $_i$ by pulse labelling during the last 2 h of germination of the 24-h-germinated axes, as described previously [13]. The purified ribosomes were incubated for 30 min at 30°C (*in vitro*-translation reaction conditions) or for 45 min at 37°C (puromycin-treatment conditions) and the ribosomal proteins isolated, resolved by SDS/PAGE and autoradiographed as described in [13]. The phosphorylation at the S6 rp on the ribosomes incubated at these temperatures proved to be stable under the experimental conditions as compared with the non-incubated ribosomes (results not shown).

RESULTS

Maize eIFiso4E, but not eIF4E, is dependent on *de novo* synthesis early during germination

The eIF4E and eIFiso4E mRNAs and proteins have already been demonstrated to be present in quiescent mature maize axes [5]. To investigate the mechanism regulating the expression of these factors during germination, the levels of eIF4E and eIFiso4E proteins were tested at 6, 9, 12 and 24 h of germination in the absence or presence of CHX, an inhibitor of translation. The factor levels were estimated in the ⁷mGTP-bound fraction of total cytoplasmic proteins obtained from 100 embryonic axes. In the presence of the inhibitor, the eIFiso4E protein progressively declined from 12 to 24 h until its content was negligible, in contrast with the steady protein level found under control conditions (Figure 1). The accumulation of the eIF4E protein was also affected by the presence of CHX; however, this isoform displayed constant levels under the conditions of translation inhibition at 9, 12 and 24 h. These results suggest high eIFiso4E turnover during germination, and need for active translation to keep up the eIFiso4E protein level. In contrast, the eIF4E isoform displays higher stability and lower dependence on translation than eIFiso4E during the same period.

De novo synthesis of the two eIF4E isoforms was analysed in axes at early (6 h) and late (24 h) germination periods. After exposing axes to [³⁵S]methionine for 2 h, the translation-initiation factors were purified and resolved by SDS/PAGE. The corresponding fluorography showed that both the eIFiso4E and eIF4E proteins were synthesized *de novo* since early germination (6 h), and this synthesis increased towards the end of the period (24 h; Figure 2a). On the other hand, eIF4E showed lower ³⁵S incorporation than eIFiso4E at each germination period. It should be pointed out that both isoforms contain an equal number of methionines per molecule [25]. Insulin addition to the germinating axes induced a large increase in eIFiso4E [³⁵S]-methionine incorporation, particularly at 24 h of germination (Figure 2a, lane 4). For eIF4E synthesis, however, the insulin stimulation was poor (Figure 2a, lane 3). Densitometric analysis of the insulin-stimulated label incorporation at 24 h from three replicates confirmed a significant increment ($P < 0.01$) for [³⁵S]-methionine incorporation of about 3.5-fold above the control in the eIFiso4E protein, whereas the label was incorporated into eIF4E to a lower extent (less than 1.5-fold), which was statistically non-significant (Figure 2b).

Figure 2 Insulin stimulation of eIFiso4E *de novo* protein synthesis during maize germination

(a) Maize seeds were germinated for 6 or 24 h. Embryonic axes were excised and during the last 2 h incubated with 11.1×10^6 Bq of [³⁵S]methionine and exposed (lanes 3 and 4) or not (lanes 1 and 2) to $200 \mu\text{units} \cdot \text{ml}^{-1}$ insulin. At the end of the period the axes were washed with water, and the eIF4E and eIFiso4E factors were purified and analysed by electrophoresis and fluorography. (b) Evaluation of insulin stimulation was performed by densitometric analysis of the corresponding bands of either eIF4E or eIFiso4E from 100 24-h-germinated axes in three replicates. Label incorporated into controls was considered as 100% for each isoform. C, Control seeds. I, Insulin-treated seeds. Error bars indicate S.D.

Negative immunodetection of 4E-BPs in maize tissue extracts

To analyse whether 4E-BPs are present or not in maize extracts, immunoblotting of total cytoplasmic and ⁷mGTP-specifically eluted proteins from maize axes was performed against human 4E-BP antibodies (Figure 3). Wheat-germ extract and RRL were also used as negative and positive controls, respectively. Results

Figure 1 Translation-inhibition effect on eIF4E and eIFiso4E protein levels during maize germination

Maize seeds (100 in total) were germinated for 6, 9, 12 and 24 h. During the last 3 h the embryonic axes were excised and incubated in MS medium in the presence or not of $70 \mu\text{M}$ CHX. Protein extracts were obtained from the axes at each germination period, the ⁷mGTP-binding proteins were purified, quantified (approx. $2.5 \mu\text{g}$ each), electrophoresed, and the levels of eIF4E and eIFiso4E factors were assessed by Western-blot analysis with antibodies against the corresponding wheat proteins. Numbers refer to periods of germination in hours.

Figure 3 Western-blot analysis of 4E-BPs

Total cytoplasmic proteins (T) and specifically $^7\text{mGTP}$ -Sepharose-eluted proteins (E) obtained from maize embryonic axes, wheat-germ or rabbit reticulocytes were resolved by SDS/PAGE (13% gels), transferred on to a PVDF membrane and assayed by immunoblotting against human 4E-BP antibodies (1:1000 dilution). Arrows indicate the 4E-BP1 peptides recognized in RRL as control.

Figure 4 Insulin-induced enhancement of eIFiso4E expression by transcript recruitment into polysomes: effect of insulin signal-transduction-pathway inhibitors

Maize axes were germinated for 24 h under control conditions (C) or were stimulated with insulin ($200 \mu\text{units} \cdot \text{ml}^{-1}$) stimulated for the last 2 h (I). To test the effect of insulin inhibitors, the axes were exposed to either $0.1 \mu\text{M}$ wortmannin (I + W) or $0.1 \mu\text{M}$ rapamycin (I + R) for 1 h prior to addition of insulin. (a) Polysomal RNA was isolated from the indicated axes and analysed by Northern blot with cDNA probes for eIFiso4E, eIF4E and S6 rp. (b) Total RNA was extracted from the same sets of maize axes and analysed by Northern blot with the corresponding probes as in (a).

revealed the presence of two main cross-reacting peptides for 4E-BP1 in the RRL, as well as in its $^7\text{mGTP}$ -bound fraction (Figure 3, lanes 5 and 6), in accordance with results in the literature [7,9]. However, peptides cross-reacting with 4E-BP1 antibody in both the $^7\text{mGTP}$ -bound fraction and the total cytoplasmic proteins were not found for either maize or wheat-germ (Figure 3, lanes 1–4). Similar results were obtained with the 4E-BP2 antibody (results not shown). These data most probably indicate the absence of eIF4E or eIFiso4E binding-protein homologues to mammalian 4E-BPs in maize or wheat tissues, in agreement with a previous report [7].

eIFiso4E synthesis *de novo* is regulated by selective mRNA recruitment into polysomes

Insulin and insulin-like growth factors are known to stimulate 5'TOP mRNA translation in several eukaryotes by transcript-selective recruitment into polysomes [16]. A similar effect has been documented in germinating maize axes [13]. In the present study we addressed whether insulin had any effect on either eIFiso4E or eIF4E transcript recruitment into polysomes for *de novo* synthesis of these proteins in maize axes.

The insulin-stimulated [^{35}S]methionine incorporation into eIFiso4E observed in Figure 2 could be either the result of increased translation by selective transcript mobilization into polysomes or due to an increase in the total amount of the corresponding mRNA in the tissues. To discriminate between these possibilities, Northern blots were performed for both factors in polysomal and total RNAs. S6 rp mRNA was used as a positive control for mRNA mobilization into polysomes, since specific recruitment of this transcript into polysomes has been demonstrated in insulin-stimulated axes [13]. The amount of 18 S rRNA was used as a loading control. Results indicate increased eIFiso4E transcript in the polysomal RNA fraction in insulin-stimulated axes at 24 h of germination (Figure 4a, lanes 1 and 2), similar to the S6 rp transcript. In contrast with this observation, insulin did not affect the eIF4E transcript recruitment into polysomes (Figure 4a). Interestingly, addition of either wortmannin or rapamycin (inhibitors of the insulin signal-transduction pathway [10]) to the axes before addition of insulin blocked the insulin-stimulated increment. Notice that eIFiso4E and S6 rp transcripts in the polysomal RNA fraction decreased to levels even below the control (Figure 4a, lanes 3 and 4). The wortmannin treatment also showed a slight decrease in eIF4E transcript recruitment. This suggests that wortmannin might cause a certain overall reduction in transcript recruitment into polysomes.

To test whether the insulin effect on eIFiso4E was exerted specifically at the level of polysomal recruitment and was not due to mRNA accumulation, a Northern-blot analysis was also performed with total RNA extracts. Total transcript levels of eIFiso4E as well as S6 rp did not show increase after insulin addition to the 24-h-germinating axes (Figure 4b). On the contrary, a slight decrease in total mRNA levels for these transcripts could be detected in the presence of insulin. These data allow us to propose that the main insulin effect in the axes is to up-regulate the recruitment of specific mRNAs, such as the eIFiso4E and S6 rp transcripts, into polysomes.

S6 rp phosphorylation and eIFiso4E translation *in vitro*

Insulin stimulation of 5'TOP transcript recruitment into polysomes is known to correlate with S6 rp phosphorylation increment on the 40 S ribosomal subunit [14,15]. This mechanism is also known to work in plants, as demonstrated in embryonic

Figure 5 *In vitro*-translation dependence on phosphorylated S6 rp ribosome

An *in vitro* protein-synthesizing system was set up with ribosome-depleted RRL supplemented with S6 rp-non-phosphorylated (np), -phosphorylated (p) or -highly phosphorylated (hp) maize ribosomes. Poly(A)⁺ mRNA from 24-h-germinated axes was used as a source of eIFiso4E and eIF4E mRNAs for translation in each system. Other translation conditions were as recommended by the manufacturer, including 3.7×10^6 Bq·ml⁻¹ [³⁵S]methionine (Roche, Molecular Diagnostics, GmbH). Translation was allowed to proceed for 30 min at 30 °C. (a) The products of the reaction mixture were purified by ⁷mGTP-affinity chromatography and analysed by electrophoresis and Western blotting with eIFiso4E and eIF4E antibodies. (b) The bands corresponding to eIFiso4E and eIF4E were excised, counted for label incorporation by liquid-scintillation counting, and the values are represented graphically after correction by total label incorporation in each reaction mixture. The results are the means $\pm$ S.D. of three independent *in vitro*-translation reactions.

axes during maize germination [13]. To learn whether S6 rp phosphorylation is part of the mechanism regulating eIFiso4E mRNA recruitment into polysomes by insulin stimulation, the following experiment was performed. Poly(A)⁺ mRNAs obtained from 24-h-germinated axes were translated *in vitro* in a ribosome-depleted RRL system supplemented with different sets of maize

ribosomes. The endogenous ribosomes of the RRL system were replaced by S6 rp-non-phosphorylated, -phosphorylated or -highly phosphorylated maize ribosomes (see the Experimental procedures section). After 30 min of synthesis, eIF4E and eIFiso4E proteins were purified by affinity chromatography from the translation mixture and identified by Western-blot analysis among the translation products (Figure 5a). The [³⁵S]methionine incorporation into each 4E isoform was quantified by liquid-scintillation counting. The results showed that the synthesis of eIF4E and eIFiso4E had distinct dependence on S6 rp phosphorylation in the ribosome (Figure 5b). The eIF4E isoform was translated similarly by all of the translation systems tested, regardless of the phosphorylation status of their ribosomes. On the other hand, a clear difference was found for eIFiso4E synthesis, depending on the phosphorylation status of the ribosomes. The system with maize non-phosphorylated ribosomes translated eIFiso4E poorly, whereas the best translation of this protein was obtained in the system with the highly phosphorylated ribosomes (Figure 5b). It should be pointed out that incubation of phosphorylated ribosomes for 30 min at 30 °C (*in vitro* translation conditions) did not cause dephosphorylation of these ribosomes (see the Experimental procedures section).

As a control for the translation-specific efficiency of the maize ribosome sets, alternative mRNAs were tested in the same translation systems: β -globin mRNA or whole maize poly(A)⁺ RNAs (Table 1). Each of the mRNA sources was almost equally well translated by the reconstituted systems with any of the maize ribosomes, although the maize non-phosphorylated ribosome set in general showed slightly lower *in vitro*-translation efficiency than the other two (Table 1). Thus the phosphorylated status of the ribosomes showed no distinctive difference on translation efficiency when tested with β -globin, eIF4E mRNA or total maize poly(A)⁺ mRNAs. However, a clear positive correlation can be established between the level of S6 rp phosphorylation and the amount of [³⁵S]methionine incorporated into the eIFiso4E protein, indicating a selective recognition of this mRNA by S6 rp-phosphorylated ribosomes (Figure 5b and Table 1). Notice that, in these experiments, only one product was found for the maize eIF4E, in contrast with the doublet observed by Western blotting in the native maize extracts (Figure 1). This finding suggests that the doublet corresponds to only one peptide, the eIF4E and its post-translationally phosphorylated form, as described previously [25].

To ensure that the differences between the amounts of eIFiso4E translated by non-phosphorylated, phosphorylated and highly

Table 1 Translation *in vitro* of different mRNA sets by maize ribosomes with different S6 rp-phosphorylation levels

Ribosomes were from maize embryonic axes obtained under different germination conditions, treated or not (native) with puromycin (see the Experimental procedures section). Protein-synthesis values were calculated as the ratio between trichloroacetic acid-precipitable radioactivity in ribosome-supplemented and ribosome-depleted RRLs, after the translation reaction had been allowed to proceed for 30 min (see the Experimental procedures section). The percent of radioactivity incorporated into either eIF4E or eIFiso4E protein was calculated from total c.p.m. incorporated into trichloroacetic acid-precipitable material in 100 μ l of translation reaction. Results represent the means of three (native) or two (puromycin treatment) independent experiments. np, non-phosphorylated; p, phosphorylated; hp, highly phosphorylated.

	Type of ribosome...			Puromycin-treated		
	np	p	hp	np	p	hp
mRNA source						
β -Globin	8.5	9.7	10.6	6.86	10.69	10.21
Maize poly(A) ⁺ (24 h of germination)	5.2	7.4	7.2	7.56	7.81	8.1
Radioactivity (% of total)						
eIF4E	0.05	0.08	0.08	0.065	0.05	0.06
eIFiso4E	0.08	0.17	0.26	0.045	0.127	0.165

Figure 6 *In vitro* translation of eIFiso4E and eIF4E in puromycin-treated maize ribosomes

Maize non-phosphorylated (np), phosphorylated (p) and highly phosphorylated (hp) ribosomes were dissociated with puromycin and GTP by 45 min of incubation at 37 °C as described in the Experimental procedures section. The *in vitro*-translation reactions and further eIFiso4E and eIF4E purification were performed as described in Figure 5. (a) Coomassie Brilliant Blue-stained membrane of eIF4F complexes purified from *in vitro*-translation products. Arrows indicate eIF4E and eIFiso4E peptides. (b) The bands corresponding to eIFiso4E and eIF4E were excised, counted for label incorporation by liquid-scintillation counting, and the values represented graphically after correction by total label incorporation in each reaction mixture. The results are representative of two independent *in vitro*-translation reactions.

phosphorylated maize ribosomes were not only due to the mRNAs previously recruited *in vivo* into these ribosomes (see Figure 4a), the native ribosomes were dissociated into ribosomal subunits by puromycin and GTP, prior to translation *in vitro*, to allow disruption of mRNA–ribosomal-subunit complexes (Figure 6 and Table 1). The stability of S6 rp phosphorylation was not appreciably altered during puromycin treatment, as measured using *in vivo*-labelled ribosomes (see the Experimental procedures section). The results of translation *in vitro* with the puromycin-treated ribosomes reproduced the pattern observed previously (Figure 5b). In this case, the overall eIFiso4E translation stimulus was lower than that with the native ribosomes, as observed for phosphorylated and highly phosphorylated ribosomes (Figure 6b). This effect might indicate that the native ribosomes were already differentially charged *in vivo* with eIFiso4E transcripts, and thus the effect observed with puromycin-treated ribosomes corresponds to the values of mRNA recruitment during the *in vitro*-translation process. Translation of other mRNAs, such as eIF4E, β -globin or maize poly(A)⁺, in the puromycin-treated ribosomes showed again the pattern observed with the native ribosomes (Figure 6 and Table 1); that is, similar translation efficiency regardless of the phosphorylation status of the ribosomes. These results confirm the role of S6 rp phosphorylation in the selective translation of target mRNAs.

DISCUSSION

During the first hour of germination a switch from negligible to very active metabolism has been observed in embryonic axes [26]. In particular, the translation process is re-initiated and synthesis of new proteins based on stored and later on newly synthesized

transcripts is detected [27]. Thus mechanisms of translational control are expected to be at least partially responsible for producing the precise types and amounts of the proteins required in the axes to reach successful germination.

Distinctive expression patterns for the two translation-initiation factors, eIF4E and eIFiso4E, have been reported previously in maize axes during germination [5]. The levels of these factors are determinant for translation of cap-dependent transcripts [23], probably by discriminating among different mRNA cap structures, based on different affinity constants [28]. Hence, regulation of maize eIF4E and eIFiso4E expression must be relevant for the production of the precise newly synthesized proteins required for seed germination. Considering the apparent lack of 4E-BP proteins in plants (Figure 3 and [7]), regulation of free eIF4E levels in maize most probably does not follow the mechanism reported for other non-plant organisms [1]. It is likely that here the mechanism would greatly rely on *de novo*-synthesis regulation of each 4E isoform. Indeed, eIFiso4E level in axes largely depends on its synthesis during germination, whereas eIF4E does not (Figure 1). Also, eIFiso4E synthesis, but not eIF4E synthesis, is selectively enhanced under insulin stimulation (Figure 2).

Insulin-mediated mRNA mobilization into polysomes has been reported for specific mRNAs carrying the 5'TOP sequence [16], among which rp and elongation-translation-factor mRNAs have been found [14–16]. A similar regulatory mechanism has also been proposed to exist in plants [29,30] and demonstrated particularly in maize for rp mRNA translation [13]. Further support for the presence of this mechanism in maize axes is provided by the effect of the insulin signal-transduction-pathway inhibitors, wortmannin and rapamycin, which inhibited the stimulation of insulin recruitment into polysomes of both the eIFiso4E and S6 rp mRNAs (Figure 4a, lanes 3 and 4). Thus the selective mobilization of eIFiso4E mRNA into polysomes, after insulin addition, is most probably due, at least in part, to the activation in maize of a signal-transduction pathway similar to the one reported to be activated by insulin in other eukaryotes [10,31]. An important component of this transduction pathway is the phosphorylation status of the S6 rp on the 40 S ribosomal subunit [14,32]. Indeed, insulin stimulation causes increased S6 rp phosphorylation in maize ribosomes of the target tissues [13]. For eIFiso4E mRNA, a consistent correlation was observed between its translation efficiency, both *in vivo* and *in vitro*, and the phosphorylation level of S6 rp in ribosomes (Figures 2, 5b and 6b). This correlation, however, was not observed for eIF4E mRNA translation or for other mRNAs in the same systems (Figures 5 and 6, and Table 1).

It is proposed that the expression of the eIFiso4E isoform is regulated at the translational level by a mechanism similar to the one described for translation of 5'TOP mRNAs in other eukaryotes. In this mechanism, the S6 rp phosphorylation status contributes to the enhanced eIFiso4E synthesis observed under insulin-stimulated conditions [14]. These findings suggest that the maize eIFiso4E transcript might be a member of the 5'TOP mRNA family, and encourage further analysis of the 5'-untranslated nucleotide region of this mRNA in the search for the consensus oligopyrimidine tract.

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