

# The Phosphorylation State of Translation Initiation Factors Is Regulated Developmentally and following Heat Shock in Wheat\*

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Several translation initiation factors in mammals and yeast are regulated by phosphorylation. The phosphorylation state of these factors is subject to alteration during development, environmental stress (heat shock, starvation, or heme deprivation), or viral infection. The phosphorylation state and the effect of changes in phosphorylation of the translation initiation factors of higher plants have not been previously investigated. We have determined the isoelectric states for the wheat translation initiation factors eIF-4A, eIF-4B, eIF-4F, eIF-iso4F, and eIF-2 and the poly(A)-binding protein in the seed, during germination, and following heat shock of wheat seedlings using two-dimensional gel electrophoresis and Western analysis. We found that the developmentally induced changes in isoelectric state observed during germination or the stress-induced changes were consistent with changes in phosphorylation. Treatment of the phosphorylated forms of the factors with phosphatases confirmed that the nature of the modification was due to phosphorylation. The isoelectric states of eIF-4B, eIF-4F (eIF-4E, p26), eIF-iso4F (eIF-iso4E, p28), and eIF-2 $\alpha$  (p42) were altered during germination, suggesting that phosphorylation of these factors is developmentally regulated and correlates with the resumption of protein synthesis that occurs during germination. The phosphorylation of eIF-2 $\beta$  (p38) or poly(A)-binding protein did not change either during germination or following a thermal stress. Only the phosphorylation state of two factors, eIF-4A and eIF-4B, changed following a heat shock, suggesting that plants may differ significantly from animals in the way in which their translational machinery is modified in response to a thermal stress.

Exposure to heat shock results in profound changes at almost every level of gene expression including transcription, splicing, nucleocytoplasmic transport, translation, and protein turnover (for reviews, see Refs. 1–3). Although many of the molecular events involved in heat shock gene induction are remarkably conserved in eukaryotes, the control of translation following heat shock varies considerably among species. In yeast, the heat shock response appears to involve only transcriptional mechanisms, whereas in *Xenopus* oocytes, the heat

shock response is mediated entirely at the translational level (4, 5). As with mammals, the heat shock response in plants lies between these extremes, with gene regulation involving both transcriptional and translational mechanisms. In addition to a specific set of heat-induced genes, *i.e.* those encoding the heat shock proteins, a low level of non-heat shock mRNA translation continues in plants following heat shock (6–9). We have shown that heat shock causes a reduction in translational efficiency and an increase in the mRNA half-life of non-heat shock mRNAs in plants that are proportional to the severity of the stress (10). Under these conditions, the translational machinery loses its ability to discriminate between capped and uncapped mRNAs. As a consequence, translation becomes less cap-dependent, and the functional co-dependence between the cap and the poly(A) tail is reduced (10, 11).

In animals, the reprogramming of translation following thermal stress correlates with changes in phosphorylation for several initiation factors (12). Two of the best studied examples are 1) the dephosphorylation of the cap-binding protein subunit of eukaryotic initiation factor (eIF)<sup>1</sup> 4F (also known as eIF-4E) (13) and 2) the phosphorylation of eIF-2 $\alpha$  (reviewed in Refs. 14–17). eIF-4F binds to the cap structure at the 5'-terminus of the mRNA and stimulates the binding of eIF-4A and eIF-4B. eIF-4A is an RNA-dependent RNA helicase that, together with eIF-4B and eIF-4F, unwinds any secondary structure present in the 5'-untranslated leader, thereby preparing the mRNA for binding to the 40 S ribosomal subunit. The smallest of the three subunits that constitute mammalian eIF-4F is eIF-4E (p25). The site of phosphorylation in eIF-4E is at serine 209, near the C terminus (18, 19). Dephosphorylation of mammalian eIF-4E occurs following serum starvation (20), mitosis (21), viral infection (22), or heat shock (12) and correlates with reduced eIF-4F binding to the cap and protein synthesis activity (23–26).

eIF-2 is a three-subunit complex that is responsible for bringing Met-tRNA<sup>Met</sup> to the 40 S subunit in both plants and animals (reviewed in Ref. 27). The  $\alpha$ -subunit is subject to phosphorylation in animal cells by a number of physiological events, including heat shock (13), viral infection, and heme deprivation (reviewed in Ref. 28), or in yeast following amino acid starvation (reviewed in Refs. 27–29). Phosphorylation of the  $\alpha$ -subunit prevents GDP/GTP exchange by eIF-2B and consequently inhibits eIF-2 activity (30). Overexpression of an eIF-2 $\alpha$  mutant that is resistant to phosphorylation partially protected Chinese hamster ovary cells from inhibition of protein synthesis following a heat shock (30), suggesting that the control of this initiation factor constitutes an important regulatory point following a heat shock in animal cells.

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<sup>1</sup> The abbreviations used are: eIF, eukaryotic initiation factor; PAB, poly(A)-binding protein; IEF, isoelectric focusing.

Additional eIFs that are modified following a stress include the dephosphorylation of mammalian eIF-4B during heat shock (12) and the phosphorylation of eIF-4A in hypoxic maize roots. Both of these events correlate with reduced translational activity (31). In addition, heat shock causes an increase in the synthesis of the poly(A)-binding protein in HeLa cells (32), a protein essential for efficient translation (33). Although heat shock profoundly impacts translation in plants, the modifications that initiation factors undergo following this stress have not been investigated. We demonstrate that plants differ from other eukaryotes in the way that several key components of the translational machinery are modified following thermal stress. In addition, we show that modification of several initiation factors occurs during seed germination, a developmental process unique to plants.

#### EXPERIMENTAL PROCEDURES

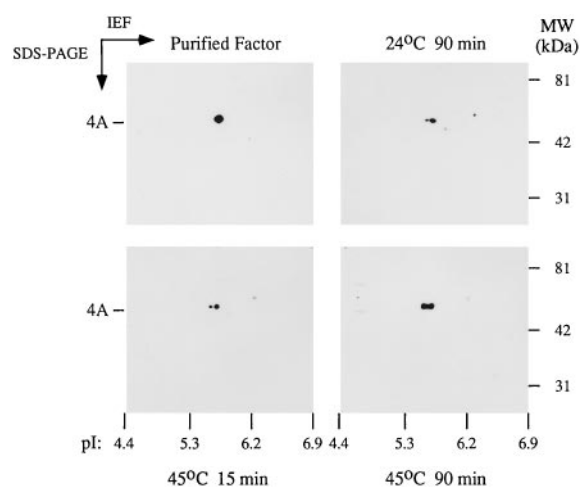
**Antibody Preparation**—eIF-4F and eIF-iso4F (34), eIF-4B (35), and eIF-4A and eIF-2 (36) were purified from wheat germ (commercially prepared wheat embryos) as described. The recombinant p86 (eIF-iso4G) and p28 (eIF-iso4E) subunits of eIF-iso4F were purified as described (37). Polyclonal antibodies to the initiation factors were produced in rabbits or mice and purified as described (38, 39).

PAB was purified essentially as described by Yang and Hunt (40), except that KAc was substituted for KCl, and fractionation on a Mono Q column immediately followed Affi-Gel blue chromatography. Antibodies raised against purified PAB were purified as described previously (41).

**Plant Extract Preparation and Two-dimensional Gel Electrophoresis/Western Blot Analysis**—Wheat seeds were germinated aseptically for 4 days. Wheat seedling leaves were excised and placed in 3 ml of MS medium (42) in a plastic 60-mm Petri dish. The leaves were heat-shocked by placing the dish in a 45 °C shaking water bath for 15 or 90 min. Control leaves were kept at 24 °C for 90 min. Total soluble protein extracts were prepared by grinding the tissue in a mortar first with liquid nitrogen, followed by 10% trichloroacetic acid, 0.07%  $\beta$ -mercaptoethanol at -20 °C. The samples were pelleted by centrifugation at 12,000  $\times$  *g* for 15 min, thoroughly washed in acetone with 0.07%  $\beta$ -mercaptoethanol, and resuspended in 9.5 M urea with 0.5% dithiothreitol. Cell debris was then pelleted by centrifugation, and the protein concentration was determined (43). 20–30  $\mu$ g of protein was loaded on IEF tube gels (3.6% acrylamide, 9 M urea, 2% ampholytes, 2% Nonidet P-40) and run at 400 V for 4.5 h, followed by 0.5 h at 500 V. The protein was then resolved in the second dimension using standard SDS-PAGE cooled to 4 °C during electrophoresis, and the protein was transferred to 0.22- $\mu$ m nitrocellulose membrane by electroblotting. Equal protein loading was confirmed by staining replicate gels with Coomassie Brilliant Blue. Following transfer, the nitrocellulose membranes were blocked overnight in 5% milk, 0.01% thimerosal in TPBS (0.1% Tween 20, 13.7 mM NaCl, 0.27 mM KCl, 1 mM  $\text{Na}_2\text{HPO}_4$ , 0.14 mM  $\text{KH}_2\text{PO}_4$ ), followed by incubation with primary antibodies diluted typically from 1:500 to 1:2000 in TPBS with 1% milk for 1.5 h. The blots were then washed twice with TPBS and incubated with goat anti-rabbit (used for those antibodies raised against eIF-4A, eIF-4B, eIF-4F, eIF-iso4F, eIF-2 $\alpha$ , eIF-2 $\beta$ , or PAB) or anti-mouse (used for those antibodies raised against recombinant p28, p86, or p26) horseradish peroxidase-conjugated antibodies (Southern Biotechnology Associates, Inc.) for 1 h. The blots were washed twice with TPBS, and the signal was detected typically between 1 and 15 min using chemiluminescence (Amersham Corp). In some cases, purified protein was resolved using two-dimensional gel electrophoresis and stained with Coomassie Brilliant Blue. The range of ampholytes used is indicated in the legend to each figure. The pH range of the IEF tube following isoelectric focusing was determined from the measurements of 5-mm sections of a control tube gel soaked in 1 ml of 15 mM NaCl. Phosphatase treatments were performed with the phosphorylated form of each factor prior to analysis on two-dimensional Western blots. Phosphorylation studies of eIF-4B were carried out using 30–50 units of casein kinase II for 1 h.

#### RESULTS AND DISCUSSION

**eIF-4A**—Wheat eIF-4A, a single polypeptide (46,932 Da) (44), is an ATP-dependent RNA helicase that, in conjunction with eIF-4B and eIF-4F, is thought to remove secondary structure present within a 5'-leader (45, 46). It is also an RNA-de-

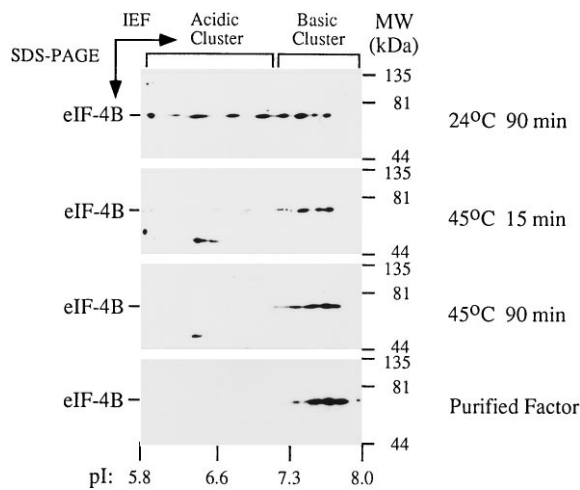


**FIG. 1. Two-dimensional gel electrophoresis/Western analysis of eIF-4A in control and heat-shocked wheat seedlings.** 1  $\mu$ g of eIF-4A purified from wheat embryos (*Purified factor*; top left panel) and 20  $\mu$ g of total wheat leaf extract (*remaining panels*) were resolved in the first dimension using IEF with 75% pH 5–8 and 25% pH 3–10 ampholytes and on a 10% SDS-polyacrylamide gel for the second dimension. Extracts were prepared from leaves treated at 24 °C for 90 min or at 45 °C for either 15 or 90 min. The isoelectric point was determined following IEF and is shown at the bottom of the gels. PAGE, polyacrylamide gel electrophoresis.

pendent ATPase in plants, animals, and yeast (47, 48). Although there is no evidence that eIF-4A is phosphorylated in mammalian cells, a phosphorylated form has been demonstrated in *Drosophila* (49) and in maize (31). Phosphorylation of maize eIF-4A occurs in roots following oxygen deprivation (31); consequently, phosphorylation of eIF-4A is part of the hypoxic stress response. To examine whether heat shock also results in the appearance of phosphorylated eIF-4A, 4-day-old wheat leaves were treated at 45 °C for 15 or 90 min. Control tissue was maintained at 24 °C for 90 min. The tissue was frozen in liquid nitrogen immediately following the treatment. Extracts made from the tissue were displayed on two-dimensional gels, transferred to nitrocellulose membrane, and probed with anti-wheat eIF-4A antibodies. eIF-4A purified from wheat embryos was also analyzed using the same approach.

A single species (pI 5.6) was observed for eIF-4A purified from wheat embryos (Fig. 1). In control wheat leaves, a second, more acidic form (pI 5.5) was present at ~10% the level of the basic form. The acidic and basic isoforms correspond well with the monophosphorylated (pI 5.75) and nonphosphorylated (pI 5.9) forms, respectively, of eIF-4A observed in maize that had been shown previously to be modified by phosphorylation (31). The nonphosphorylated and phosphorylated isoforms were present in an approximate 9:1 ratio (nonphosphorylated to phosphorylated) in wheat leaves subject to a 15-min treatment at 45 °C. When leaves were treated for 90 min at 45 °C, however, the amount of phosphorylated eIF-4A increased such that both isoforms were present in equal amounts. Therefore, as in hypoxically treated maize roots, heat shock can result in the phosphorylation of wheat eIF-4A, suggesting that phosphorylation of this initiation factor may be a characteristic response to stress in plants.

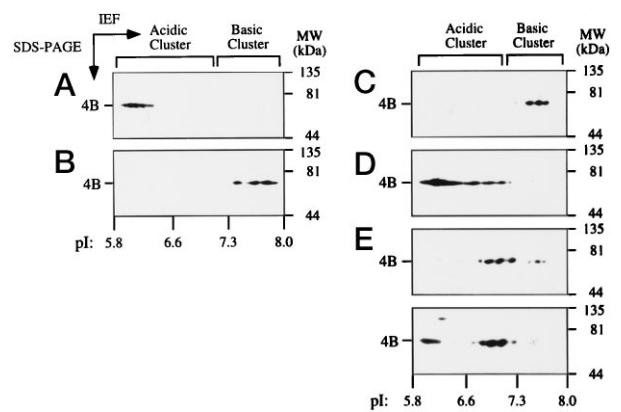
We have shown previously that translational efficiency was severely reduced following only a 15-min heat shock at 45 °C (10). Our observation that a 15-min heat treatment at 45 °C was insufficient to induce phosphorylation of eIF-4A and that a far longer heat shock (90 min) was required suggests that phosphorylation of eIF-4A is not responsible for the immediate reduction in translation following a heat shock. Instead of being a part of the initial response to heat shock, phosphoryl-



**FIG. 2. Two-dimensional gel electrophoresis/Western analysis of eIF-4B in control and heat-shocked wheat seedlings.** 2  $\mu$ g of eIF-4B purified from wheat embryos (*Purified factor*; bottom panel) and 20  $\mu$ g of total wheat leaf extract (*top three panels*) were resolved in the first dimension using IEF with 75% pH 8–10 and 25% pH 3–10 ampholytes and on a 10% SDS-polyacrylamide gel for the second dimension. Extracts were prepared from leaves treated at 24 °C for 90 min or at 45 °C for either 15 or 90 min. The isoelectric point was determined following IEF and is shown at the bottom of the gels. The acidic and basic clusters are indicated at the top of the gels. Cross-reaction with additional proteins or degradation products of eIF-4B can be seen in the two middle panels. PAGE, polyacrylamide gel electrophoresis.

ation of eIF-4A might be part of an adaptive response to prolonged stress.

**eIF-4B**—Mammalian eIF-4B is a single subunit of 69,843 kDa (deduced from the amino acid sequence) present in 8–10 isoforms (12). The acidic cluster (four to six isoforms) is composed of phosphorylated isoforms, whereas the basic cluster (approximately four isoforms) results from an as yet undetermined modification (50). Although the actual eIF-4B phosphorylation sites have not been identified, five serine phosphorylation sites have been reported (51). The dephosphorylation of mammalian eIF-4B that is observed following heat shock (51), serum depletion (52), or mitosis (21) correlates with a reduction in translation, whereas the stimulation of eIF-4B phosphorylation by insulin treatment (53) correlates with an increase in translation. The addition of phosphorylated eIF-4B partially restored translation to an *in vitro* translation lysate prepared from heat-shocked HeLa cells (12, 51). eIF-4B, a 59-kDa polypeptide in wheat (35), is present in wheat leaves in several isoelectric states similar to those observed in mammalian cells (Fig. 2). By analogy with animal eIF-4B, the acidic cluster (pI 5.9–7.0) is consistent with a phosphorylated form of the basic cluster (pI 7.2–8.0). The acidic cluster is completely absent when eIF-4B is purified from commercially prepared wheat embryos (wheat germ), suggesting that either eIF-4B is developmentally regulated or the modifications to eIF-4B are lost during purification. We have observed that the acidic cluster is also missing when crude extracts from freshly excised embryos are analyzed,<sup>2</sup> supporting the possibility that the modification of eIF-4B is developmentally regulated. Upon even a brief exposure to heat shock (15 min at 45 °C), the acidic cluster of eIF-4B is lost in leaves (Fig. 2). Similar observations were made for leaves treated for 90 min at 45 °C (Fig. 2). The appearance of the acidic cluster of eIF-4B correlates with the resumption of active translation during germination, and its loss correlates with the repression of translation following heat shock.

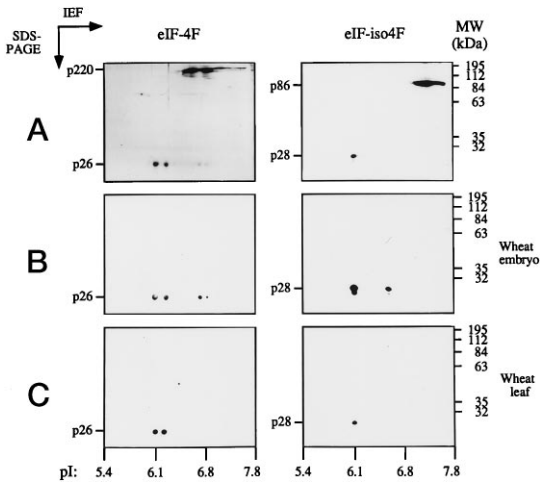


**FIG. 3. Determination of the modification of eIF-4B.** 20  $\mu$ g of total wheat leaf extract was resolved on a two-dimensional gel, transferred to a membrane, and probed with eIF-4B antibodies in A and B. A, control; B, alkaline phosphatase-treated eIF-4B. In C–F, the isoforms of eIF-4B purified from embryos were determined by two-dimensional gel electrophoresis/Western analysis. 2  $\mu$ g of eIF-4B was used as a control (C) or for treatment with a high (D) or low (E and F) level of casein kinase II. The isoforms in A–E were detected using Western analysis. The phosphorylation of eIF-4B (F) was carried out using [ $\gamma$ -<sup>32</sup>P]ATP, and the radiolabeled isoforms (F) were detected using autoradiography. PAGE, polyacrylamide gel electrophoresis.

The acidic isoforms of eIF-4B are modified by phosphorylation (Fig. 3). eIF-4B from wheat leaf extract, in which the acidic cluster of eIF-4B isoforms is predominant (Fig. 3A), was treated with alkaline phosphatase and resolved on a two-dimensional gel, followed by Western analysis. Treatment with the phosphatase resulted in the complete conversion of the acidic isoforms to the basic isoforms (Fig. 3B), suggesting that eIF-4B is multiply phosphorylated. No dephosphorylation of eIF-4B was observed when potato acid phosphatase was used (data not shown). eIF-4B purified from embryos (*i.e.* hypophosphorylated) was treated with casein kinase II to determine whether the acidic isoforms could be generated. Treatment with a high level of the kinase resulted in complete conversion (Fig. 3D) to the acidic isoforms, whereas a lower level of the enzyme resulted in a partial conversion of the basic cluster to the acidic isoforms (Fig. 3E). As [ $\gamma$ -<sup>32</sup>P]ATP was used in the phosphorylation of eIF-4B in Fig. 3E, the membranes could also be exposed to film to detect which eIF-4B isoforms were radiolabeled (Fig. 3F). The basic isoforms observed in Fig. 3E were not radiolabeled, as expected for isoforms that are not phosphoproteins. The intermediate isoforms resulting from the phosphorylation of the basic eIF-4B isoforms were observed as radiolabeled phosphoproteins in Fig. 3F. In addition, a small amount of the most acidic isoforms was detected as radiolabeled phosphoproteins in Fig. 3F that had not been detected by Western analysis in Fig. 3E.

**eIF-4F and eIF-iso4F**—Mammalian eIF-4E (the 25-kDa cap-binding protein subunit of eIF-4F) is normally phosphorylated in animal cells and is required for activity (reviewed in Refs. 14–17). Dephosphorylation of mammalian eIF-4E occurs following serum starvation (20), mitosis (21), and viral infection (22). As dephosphorylation of eIF-4E correlates with a reduced rate of translation, the small subunit of eIF-4E is thought to function as a key control point in the regulation of translation in animal cells. Heat shock also causes dephosphorylation of eIF-4E (12, 23, 24, 51), resulting in a reduced interaction between eIF-4F and the 5'-cap structure (25, 54) and reduced eIF-4F complex formation (26). Reduced eIF-4F activity would mean that the 5'-cap would offer less of a translational advantage to an mRNA, and consequently, translation would become less cap-dependent. This prediction was borne out in serum-starved 3T3-L1 cells, in which eIF-4E underwent dephospho-

<sup>2</sup> H. Le, unpublished data.

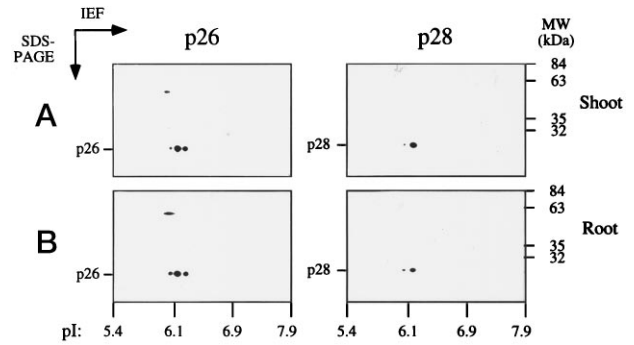


**FIG. 4. Two-dimensional gel electrophoresis/Western analysis of eIF-4F and eIF-iso4F from wheat embryos and leaves.** 2.5  $\mu$ g of eIF-4F and eIF-iso4F from embryos (A and B) and 30  $\mu$ g of total leaf extract (C) were resolved in the first dimension using IEF with 75% pH 8–10 and 25% pH 3–10 ampholytes and on a 12% SDS-polyacrylamide gel for the second dimension. A, purified eIF-4F and eIF-iso4F resolved on two-dimensional gels and stained with Coomassie Brilliant Blue; B, two-dimensional gel electrophoresis/Western analysis of eIF-4E (p26) (left panel) and eIF-iso4E (p28) (right panel) from embryos using antibodies raised against recombinant p26 (left panel) or p28 (right panel), respectively; C, two-dimensional gel electrophoresis/Western analysis of eIF-4E (left panel) and eIF-iso4E (right panel) from leaves using antibodies raised against recombinant p26 (left panel) or p28 (right panel), respectively. The p220, p26, and p28 subunits are indicated to the left of the gels. The isoelectric point was determined following IEF and is shown at the bottom of the gels. PAGE, polyacrylamide gel electrophoresis.

rylation (20) and translation became less cap-dependent (55).

In plants, heat shock causes a loss in cap-dependent translation that is proportional to the severity of the stress such that a severe heat shock can result in the complete loss of cap function (10). Such observations suggest that the activity of one or more of the cap-associated initiation factors, *e.g.* eIF-4B or eIF-4F, may be altered following thermal stress. The translational machinery of plants differs significantly from that of animals and yeast in that plants contain not only eIF-4F, but also an isoform, designated eIF-iso4F (34, 38), which are encoded by separate genes (56, 57). Like mammalian eIF-4F, wheat eIF-4F and eIF-iso4F can be purified as a three-subunit (containing eIF-4A) or two-subunit (no eIF-4A) complex depending on the purification scheme employed (31, 38). eIF-4E (p26) and eIF-iso4E (p28) share homology and are functionally analogous (38, 56). The same is true for the large subunits of eIF-4F (*i.e.* eIF-4G) and eIF-iso4F (*i.e.* eIF-iso4G), which are also referred to as p220 and p86, respectively. As in mammalian cells, eIF-4F and eIF-iso4F are the least abundant of the initiation factors (39). Whether they play a key role in the regulation of translation in plants remains unknown.

The large and small subunits of eIF-4F and eIF-iso4F can be seen when these factors purified from wheat embryos are displayed on two-dimensional gels and stained with Coomassie Brilliant Blue (Fig. 4A). p220 and p86 are large basic proteins that do not resolve well on a two-dimensional gel. p220 has a pI range of  $\sim$ 6.5–7.0 (Fig. 4A, left panel), whereas p86 has a pI range of 7.1–7.7 (right panel). eIF-4E (p26) from eIF-4F in embryos is present as two pairs (Fig. 4A, left panel), whereas two isoforms of eIF-iso4E (p28) from eIF-iso4F are observed (right panel). Note that the basic p26 and p28 isoforms are very faint. When antibodies raised against recombinant p26 and p28 were used to probe eIF-4E and eIF-iso4E from embryos, the two pairs of p26 isoforms (pI values of 6.15/6.25 and 6.75/6.85) and

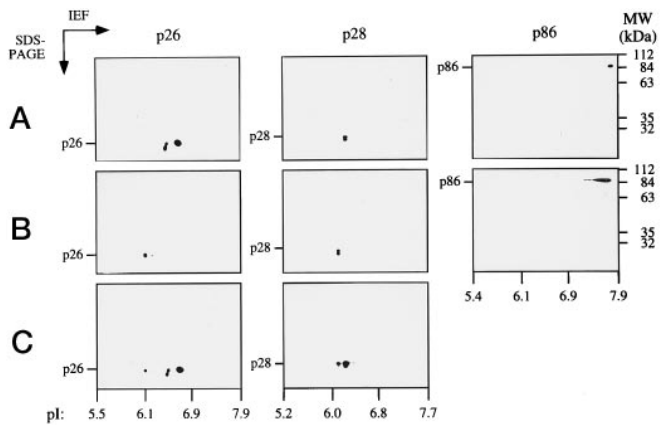


**FIG. 5. Two-dimensional gel electrophoresis/Western analysis of eIF-4E (p26) and eIF-iso4E (p28) from young wheat shoots and roots.** 50  $\mu$ g of total leaf extract was resolved as described in the legend of Fig. 4. Anti-p26 antibodies (left panels) were used to detect the eIF-4E (p26) isoforms present in shoots (A) and roots (B) of 3-day-old germinated seed. Anti-p28 antibodies (right panels) were used to detect the eIF-iso4E (p28) isoforms present in shoots (A) and roots (B) of 3-day-old germinated seed. PAGE, polyacrylamide gel electrophoresis.

the two p28 isoforms (pI values of 6.1 and 6.6) could be more clearly seen (Fig. 4B). The basic pair of p26 and the basic isoform of p28 were observed in only some fractions following the final purification of eIF-4F or eIF-iso4F on  $m^7$ GTP-Sepharose, suggesting a differential affinity for  $m^7$ GTP or a reduced abundance for the basic forms of these subunits. In contrast to the observations in embryos, only the acidic pair of p26 isoforms and the acidic isoform of p28 were observed when soluble protein extracts from leaves were probed with the anti-p26 and anti-p28 antibodies (Fig. 4C). The presence of the basic isoforms of p26 and p28 in embryos and their absence in leaves suggests that eIF-4E and eIF-iso4E may undergo modification that is developmentally regulated.

An additional eIF-4E isoform could be detected in young shoots and roots as a minor isoform (Fig. 5, left panels) that exhibited a more acidic pI than the two more predominant isoforms observed in Fig. 4. This isoform was detected only in shoots and roots of 3-day-old seedlings, which are composed of rapidly dividing cells in which translation is highly active. It was not observed in later stages of leaf development (Fig. 4C, left panel) even upon long exposure of the chemiluminescent signal (data not shown). The basic p26 isoforms observed in wheat embryos (Fig. 4B, left panel) were not detected in the 3-day-old shoots and roots, suggesting that these basic isoforms disappear early during germination. As with eIF-4E, an additional acidic eIF-iso4E (p28) isoform was also observed in young shoots and roots (Fig. 5, right panels). Like the most acidic isoform of eIF-4E, this additional eIF-iso4E isoform was not detected in older leaves (Fig. 4C, right panel), and the basic eIF-iso4E isoform observed in embryos (Fig. 4B, right panel) was not detected in young shoots and roots (Fig. 5, right panels).

To examine further whether eIF-4E and eIF-iso4E are subject to modification in wheat, recombinant p26 and p28 were overexpressed in *Escherichia coli* and purified (37), and their isoelectric points were compared with those of p26 and p28 isolated from wheat (Fig. 6). Recombinant p26 was composed of a major species and a minor, more acidic species (Fig. 6A, left panel). The nature of the difference between these two recombinant isoforms is unknown. An additional minor species of slightly smaller size than the acidic species was also observed and may represent a degradation product of recombinant p26. Recombinant p26 (Fig. 6A, left panel) exhibited a more basic isoelectric point than did wheat-purified p26 (Fig. 6B, left panel). This is most clearly seen when the recombinant p26 and wheat-purified p26 proteins were analyzed together (Fig. 6C,

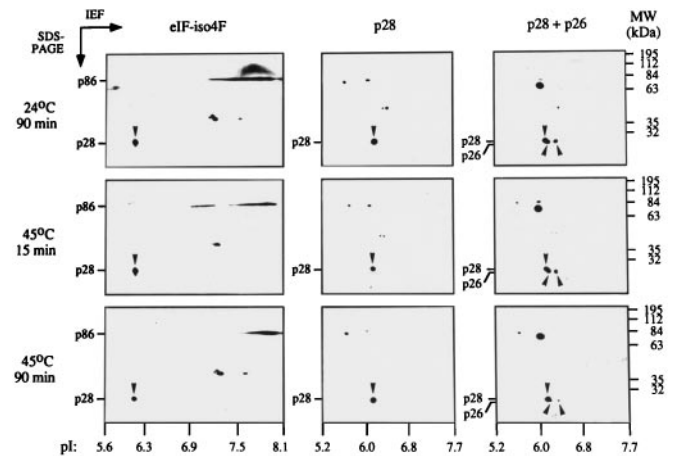


**FIG. 6. Two-dimensional gel electrophoresis/Western analysis of recombinant and wheat-purified eIF-4E (p26), eIF-iso4E (p28), and eIF-iso4G (p86).** The proteins were resolved as described in the legend of Fig. 4. Anti-p26 antibodies (*left panels*), anti-p28 antibodies (*middle panels*), and anti-eIF-iso4F antibodies (*right panels*) were used to detect eIF-4E, eIF-iso4E, and eIF-iso4G isoforms, respectively. For p26 and p28, recombinant (A), wheat-purified (B), and a mixture of recombinant and wheat-purified (C) factors were analyzed. For p86, recombinant (A) and wheat-purified (B) factors were analyzed. PAGE, polyacrylamide gel electrophoresis.

*left panel*). Similar results were observed for eIF-iso4E (p28) (Fig. 6, *middle panels*). A single species was observed for recombinant p28 with a smaller species of identical pI that may be a degradation product (Fig. 6A, *middle panel*). Recombinant p28 was more basic than the acidic isoform of wheat-purified p28 (Fig. 6B, *middle panel*). By resolving both recombinant p28 and wheat-purified p28 on the same two-dimensional gel, the difference in their isoelectric points can be clearly seen (Fig. 6C, *middle panel*).

The cDNA for eIF-iso4G (the p86 subunit of eIF-iso4F) has also been isolated (56), and recombinant p86 can be overexpressed and purified from *E. coli* (37). Recombinant p86 ran as a single, highly basic isoform (Fig. 6A, *right panel*) compared with the smear of wheat-purified p86 isoforms (Fig. 6B, *right panel*). These observations suggest that eIF-4E, eIF-iso4E, and eIF-iso4G are modified in wheat. However, no alteration in the isoelectric point of the acidic isoforms of wheat-purified p26 or p28 was observed following treatment with potato acid phosphatase, alkaline phosphatase,  $\lambda$ -protein phosphatase, or phosphatase 2A. Moreover, the isoelectric point of recombinant p26 or p28 was not altered following treatment with casein kinase II (data not shown). Although *in vivo* labeling of phosphoproteins with  $^{32}\text{P}$  also failed to establish that the acidic p26 or p28 isoforms are phosphoproteins (data not shown), wheat leaves take up  $^{32}\text{P}$  poorly, and consequently, the possibility that eIF-4E and eIF-iso4E are modified by phosphorylation in wheat cannot be excluded.

When eIF-iso4F was examined in control and heat-shocked leaves using antibodies raised against purified eIF-iso4F, no substantial change was observed in either eIF-iso4G (p86) or eIF-iso4E (p28) (Fig. 7, *left panels*). Only the acidic p28 isoform was present in both control and heat-shocked leaves, suggesting that a short or long heat shock does not result in the generation of detectable levels of the basic isoform. As eIF-iso4G (p86) is a basic protein and consequently does not resolve well, it is difficult to conclude whether heat shock has an effect on this factor. Antibodies raised against recombinant p28 confirmed that only the acidic isoform of eIF-iso4E (pI 6.1) was present in leaves and that the heat shock treatment employed had no detectable effect (Fig. 7, *middle panels*). These same membranes were then probed with anti-p26 antibodies without stripping the anti-p28 antibodies from the membrane. With



**FIG. 7. Two-dimensional gel electrophoresis/Western analysis of eIF-iso4E (p28) and eIF-4E (p26) in control and heat-shocked wheat seedlings.** 30  $\mu\text{g}$  of total leaf extract was resolved in the first dimension using IEF with 75% pH 8–10 and 25% pH 3–10 ampholytes and on a 12% SDS-polyacrylamide gel for the second dimension. Extracts were prepared from leaves treated at 24  $^{\circ}\text{C}$  for 90 min or at 45  $^{\circ}\text{C}$  for either 15 or 90 min. *Left panels*, anti-eIF-iso4F antibodies were used to probe the membranes. *Middle panels*, antibodies to recombinant p28 were used to probe for eIF-iso4E. *Right panels*, antibodies to recombinant p26 were used to reprobe the *middle panels* of membranes for eIF-4E. The membranes were not stripped before reprobing, so p28 is visible in addition to the p26 isoforms. The p86, p28, and p26 subunits are indicated to the left of the gels. Note that the most acidic isoform of p26 partially overlaps with p28. The p26 isoforms are indicated by upward-pointing arrowheads, whereas p28 is indicated by downward-pointing arrowheads. Cross-reaction with additional proteins can be seen and serves as a reference point for comparison of the different panels. PAGE, polyacrylamide gel electrophoresis.

this approach, residual p28 can be detected along with p26, and their relative pI values directly compared (Fig. 7, *right panels*). Only the acidic pair of p26 isoforms (pI 6.15/6.25) was present in leaves, and as with p28, heat shock had no impact on their isoelectric point. Note that the most acidic isoform of p26 partially overlaps with p28, making it somewhat difficult to distinguish p28 from the most acidic p26 isoform (Fig. 7, *right panels*). These data suggest that, unlike mammalian eIF-4E, wheat eIF-4E and eIF-iso4E do not undergo modification following heat shock.

**eIF-2**—eIF-2 is a three-subunit complex that is responsible for initiator Met-tRNA binding to the 40 S subunit. Phosphorylation of the  $\alpha$ -subunit prevents GDP/GTP exchange in eIF-2 $\alpha$  in animal cells and yeast, resulting in its inhibition (reviewed in Refs. 15 and 58). Phosphorylation of eIF-2 $\alpha$  occurs following amino acid starvation in yeast (reviewed in Refs. 27–29) or viral infection (reviewed in Ref. 28), heme deprivation, and heat shock (13) in animal cells and therefore represents another key regulatory component of the translational machinery.

Like its animal counterpart, wheat eIF-2 is also a three-subunit complex composed of an  $\alpha$ -subunit (42 kDa), a  $\beta$ -subunit (38 kDa), and a  $\gamma$ -subunit (50 kDa) (59–61). Although the  $\alpha$ -subunit of mammalian eIF-2 is smaller than the  $\beta$ -subunit, the recent isolation of the subunit cDNAs for wheat eIF-2 has clearly established the identity of the 42-kDa polypeptide as the  $\alpha$ -subunit and the 38-kDa polypeptide as the  $\beta$ -subunit.<sup>3</sup>

eIF-2 purified from wheat embryos was displayed using two-dimensional gel electrophoresis and stained with Coomassie Brilliant Blue (Fig. 8). Multiple (five to six) isoforms for the  $\beta$ -subunit were observed over a pI range of 6.1–6.6 (Figs. 8 and 9B, *right panel*). An acidic doublet representing the  $\alpha$ -subunit

<sup>3</sup> A. M. Metz and K. S. Browning, manuscript in preparation.

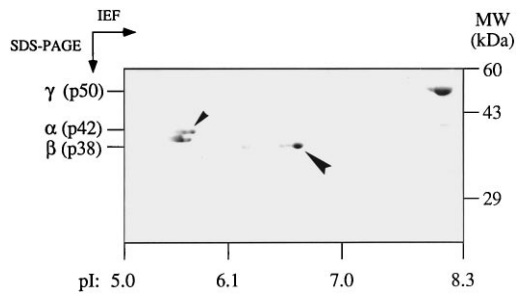


FIG. 8. **Two-dimensional gel electrophoresis/Western analysis of eIF-2 purified from wheat embryos.** 10  $\mu$ g of eIF-2 was resolved in the first dimension using IEF with 75% pH 5–8 and 25% pH 3–10 ampholytes and on a 10% SDS-polyacrylamide gel for the second dimension, and the gel was stained with Coomassie Brilliant Blue. The *large arrowhead* points to the most abundant isoform of the  $\beta$ -subunit, and the *small arrowhead* points to the  $\alpha$ -subunit doublet. The  $\gamma$ -subunit is seen in the upper right-hand corner of the gel. PAGE, polyacrylamide gel electrophoresis.

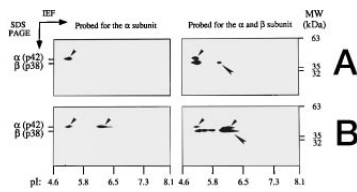


FIG. 9. **Determination of the modification of eIF-2 $\alpha$ .** 2  $\mu$ g of eIF-2 $\alpha$  purified from wheat embryos was resolved using two-dimensional gel electrophoresis, transferred to a membrane, and probed with anti- $\alpha$ -subunit (*left panels*) or anti- $\beta$ -subunit (*right panels*) antibodies. A, control; B, phosphatase 2A-treated eIF-2 $\alpha$ . The membranes probed with anti- $\alpha$ -subunit antibodies were not stripped before reprobing with the anti- $\beta$ -subunit antibodies, so the  $\alpha$ -subunit is visible in addition to the  $\beta$ -subunit. The *large arrowheads* point to the most abundant isoform of the  $\beta$ -subunit, and the *small arrowheads* point to the  $\alpha$ -subunit isoforms. PAGE, polyacrylamide gel electrophoresis.

was observed at a pI of 5.4–5.6, and the  $\gamma$ -subunit is a highly basic protein (Fig. 8). Western analysis of eIF-2 from embryos using affinity-purified anti- $\alpha$ - or anti- $\beta$ -subunit antibodies confirmed the assignment of these two subunits (Fig. 9). To determine the nature of the modification of the  $\alpha$ -subunit, eIF-2 from embryos was treated with phosphatase 2A and resolved using two-dimensional gel electrophoresis for subsequent Western analysis. Treatment with the phosphatase substantially dephosphorylated the eIF-2 $\alpha$  subunit (Fig. 9, compare *left panels* in A and B), resulting in a shift in pI for this subunit that corresponded to that observed for the  $\alpha$ -subunit present in leaves (see the following paragraph). No other phosphatase tested (potato acid phosphatase, alkaline phosphatase, or  $\lambda$ -protein phosphatase) resulted in the dephosphorylation of the  $\alpha$ -subunit (data not shown). The  $\beta$ -subunit also underwent some extent of dephosphorylation following treatment with phosphatase 2A (Fig. 9, compare *right panels* in A and B).

Membranes containing protein extracted from leaves that had been resolved using two-dimensional gel electrophoresis were also probed individually with affinity-purified anti- $\alpha$ - and anti- $\beta$ -subunit antibodies (Fig. 10, *left* and *middle panels*). Only the most abundant  $\beta$ -subunit isoforms were observed, although a long exposure of membranes (from a separate experiment) that were probed with both anti- $\alpha$ - and anti- $\beta$ -subunit antibodies revealed the less abundant  $\beta$ -subunit isoforms (Fig. 10, *right panels*). The  $\alpha$ -subunit was observed when the membranes were probed with anti- $\alpha$ -subunit antibodies (Fig. 10, *left panels*), and as the anti- $\alpha$ -subunit antibodies contain a small amount of anti- $\beta$ -subunit antibodies, a weak signal representing the most abundant  $\beta$ -subunit isoform can also be seen, which serves as a point of reference. The  $\alpha$ -subunit can also be seen in the panels representing a long exposure of

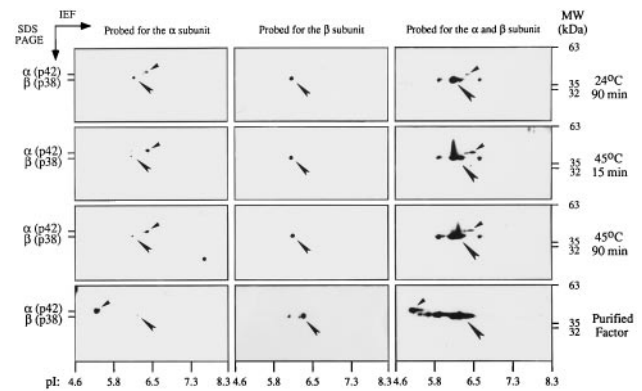


FIG. 10. **Two-dimensional gel electrophoresis/Western analysis of eIF-2 in control and heat-shocked wheat seedlings.** 2  $\mu$ g of eIF-2 purified from embryos (*Purified factor*; *bottom row of panels*) and 20  $\mu$ g of total leaf extract (*top three rows of panels*) were resolved in the first dimension using IEF with 75% pH 5–8 and 25% pH 3–10 ampholytes and on a 10% SDS-polyacrylamide gel for the second dimension. Extracts were prepared from leaves treated at 24  $^{\circ}$ C for 90 min or at 45  $^{\circ}$ C for either 15 or 90 min. *Left panels*, antibodies to the  $\alpha$ -subunit were used to probe the membranes. The affinity-purified anti- $\alpha$ -subunit antibodies contain a small amount of anti- $\beta$ -subunit antibodies, which results in the faint detection of the  $\beta$ -subunit. *Middle panels*, antibodies to the  $\beta$ -subunit were used to probe the membranes. *Right panels*, antibodies to both  $\alpha$ - and  $\beta$ -subunits were used to probe the membranes, and a long exposure was used to show the minor  $\beta$ -subunit isoforms. The  $\alpha$ - and  $\beta$ -subunits of eIF-2 are indicated to the left of the gels. The *large arrowheads* point to the most abundant isoform of the  $\beta$ -subunit, and the *small arrowheads* point to the  $\alpha$ -subunit isoforms. PAGE, polyacrylamide gel electrophoresis.

membranes probed with both anti- $\alpha$ - and anti- $\beta$ -subunit antibodies (Fig. 10, *right panels*). Comparison of eIF-2 from leaves and embryos revealed that the  $\alpha$ -subunit exists in a phosphorylated state in embryos and is converted to a hypophosphorylated or dephosphorylated state in leaves (compare the purified factor from embryos with the factor present in 24  $^{\circ}$ C-treated leaves; Fig. 10, *left panels*). There appears to be at least two basic isoforms of eIF-2 $\alpha$  in leaves. A heat shock, whether short or long in duration, had little detectable impact on the  $\alpha$ -subunit (Fig. 10, *left panels*). If phosphorylation of wheat eIF-2 $\alpha$  results in its inactivation, as it does in mammalian cells and yeast, then wheat eIF-2 $\alpha$  may be maintained in an inactive (phosphorylated) state in the seed and shifted to an active (dephosphorylated) state upon germination. The dephosphorylation of eIF-2 $\alpha$  in leaves does correlate with the activation of translation that occurs during germination. Following a heat shock, however, the lack of phosphorylation of the  $\alpha$ -subunit and only minor changes in the  $\beta$ -subunit suggest that the response to heat stress by the translational apparatus may differ substantially in plants compared with yeast and animal cells.

**Poly(A)-binding Protein**—The cap and the poly(A) tail function in a cooperative manner to establish an efficient level of translation (11). This means that neither element functions well in the absence of the other and suggests communication between these two regulatory elements. Heat shock causes not only a loss in cap function, but a loss also in the co-dependence between the cap and the poly(A) tail (10), which could be a consequence of a heat shock-induced inactivation of PAB or a reduction in the communication between this protein and the 5'-terminus. PAB can therefore be considered as a participant in the translation initiation process.

PAB is present in multiple isoforms in yeast and sea urchin (62), although the nature of the modification has not been determined. PAB was purified from wheat embryos, and following its resolution using two-dimensional gel electrophoresis and transfer to a membrane, it was probed with anti-wheat

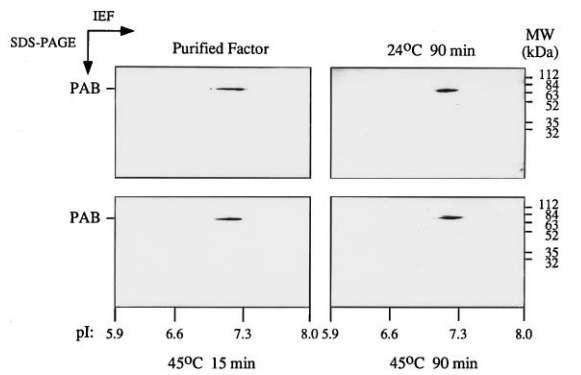


FIG. 11. Two-dimensional gel electrophoresis/Western analysis of PAB in control and heat-shocked wheat seedlings. 1  $\mu$ g of PAB purified from embryos (*Purified factor*; top left panel) and 30  $\mu$ g of total leaf extract (*remaining panels*) were resolved in the first dimension using IEF with 75% pH 8–10 and 25% pH 3–10 ampholytes and on a 10% SDS-polyacrylamide gel for the second dimension. Extracts were prepared from leaves treated at 24  $^{\circ}$ C for 90 min or at 45  $^{\circ}$ C for either 15 or 90 min. The isoelectric point was determined following IEF and is shown at the bottom of the gels. PAGE, polyacrylamide gel electrophoresis.

PAB antibodies. As in yeast and sea urchin, multiple isoforms were observed for wheat PAB that exhibited a pI range of 6.9–7.5 (Fig. 11), although upon longer exposure, additional isoforms with a pI range of 6.1–7.5 can be seen (data not shown). No change in the distribution of the PAB isoforms was observed in leaves or following a heat shock (Fig. 11), suggesting that the PAB isoforms are not regulated developmentally or following heat shock. This suggests also that the loss in co-dependence between the cap and the poly(A) tail following a heat shock may be a consequence of the observed heat-induced modifications of the initiation factors.

To determine the nature of the post-translational modification of PAB, the most acidic isoforms (pI 6.1–6.5) of PAB were isolated following the separation of purified PAB on a preparative isoelectric focusing apparatus (Fig. 12A). Treatment of the acidic PAB isoforms with alkaline phosphatase resulted in the dephosphorylation of the acidic isoforms and shifted their pI to that of the most basic isoforms (pI 7.0–7.5) (Fig. 12B). These data suggest that PAB exists as a multiply phosphorylated protein in wheat and is the first elucidation of a modification of PAB from any species.

**Conclusions**—The changes in phosphorylation of both transcription and translation factors following thermal stress provide the basis for a rapid response in reprogramming gene expression (2, 63). We have determined the number of isoforms and the isoelectric states of several of the translation initiation factors in wheat and established that several of these are modified by phosphorylation. We have also examined whether their phosphorylation state changes during development or following a heat shock. eIF-4A, eIF-4B, the  $\alpha$ - and  $\beta$ -subunits of eIF-2, and PAB can exist as phosphoproteins *in vivo* depending on the developmental stage and environmental conditions. Moreover, eIF-4E (p26), eIF-iso4E (p28), and eIF-iso4G (p86) are post-translationally modified in wheat in a way that is consistent with their phosphorylation. Although the translational machinery of plants is functionally similar to that of yeast and animals, several differences were observed that suggest that the stress response at the translational level may differ significantly in plants. These differences may underlie the unique challenge that environmental stress presents to plants compared with other eukaryotes. As plants are sessile, they cannot avoid environmental changes and use a variety of methods, including changes in physiology and morphology, to minimize the deleterious effects of abiotic stress. Whereas eIF-

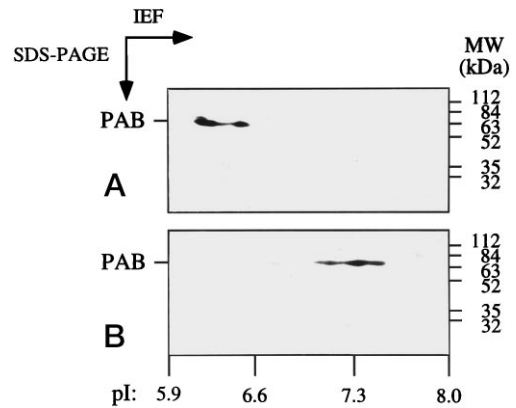


FIG. 12. Determination of the modification of PAB. Following purification from wheat embryos and preparative isoelectric focusing, 1  $\mu$ g of the acidic PAB isoforms was resolved using two-dimensional gel electrophoresis, transferred to a membrane, and probed with anti-PAB antibodies. A, control; B, alkaline phosphatase-treated PAB. PAGE, polyacrylamide gel electrophoresis.

4E, eIF-4B, and eIF-2 $\alpha$  are modified in mammals following a heat shock, in wheat, only eIF-4A and eIF-4B are subject to heat-induced modifications. Although phosphorylation of eIF-4A following a prolonged stress may not account for the rapid changes in translation observed in plants following a short heat shock, eIF-4B does undergo rapid dephosphorylation following thermal stress as well as during germination. Heat shock had little impact on wheat eIF-4E, eIF-iso4E, or eIF-2 $\alpha$ . Thus, in their response to thermal stress, plants may differ from animals in two key points: in the regulation of eIF-4E/eIF-iso4E and of eIF-2 $\alpha$ . It should be noted, however, that the developmental regulation of the isoelectric state of eIF-4E, eIF-iso4E, and eIF-2 $\alpha$  during germination suggests that the activity of these initiation factors may indeed be regulated developmentally in plants, but that the translational response of plants to a heat shock may be limited to other components of the translational machinery, such as eIF-4B and eIF-4A.

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