Regulated Heterogeneity in 12-kDa P-protein Phosphorylation and Composition of Ribosomes in Maize (*Zea mays* L.)*

Kathleen Szick-Miranda and Julia Bailey-Serres‡

From the Department of Botany and Plant Sciences, University of California, Riverside, California 92521-0124

Maize (*Zea mays* L.) possesses four distinct ~12-kDa P-proteins (P1, P2a, P2b, P3) that form the tip of a lateral stalk on the 60 S ribosomal subunit. RNA blot analyses suggested that the expression of these proteins was developmentally regulated. Western blot analysis of ribosomal proteins isolated from various organs, kernel tissues during seed development, and root tips deprived of oxygen (anoxia) revealed significant heterogeneity in the levels of these proteins. P1 and P3 were detected in ribosomes of all samples at similar levels relative to ribosomal protein S6, whereas P2a and P2b levels showed considerable developmental regulation. Both forms of P2 were present in ribosomes of some organs, whereas only one form was detected in other organs. Considerable tissue-specific variation was observed in levels of monomeric and multimeric forms of P2a. P2b was not detected in root tips, accumulated late in seed embryo and endosperm development, and was detected in soluble ribosomes but not in membrane-associated ribosomes that copurified with zein protein bodies of the kernel endosperm. The phosphorylation of the 12-kDa P-proteins was also developmentally and environmentally regulated. The potential role of P2 heterogeneity in P-protein composition in the regulation of translation is discussed.

A complex of acidic ribosomal proteins (r-proteins)[1] forms a universally conserved lateral stalk on the large ribosomal subunit that facilitates the translocation phase of protein synthesis (1). In eukaryotes the structure is formed by a complex of acidic phosphoproteins. P0 (~35 kDa), homologous to prokaryotic L10, interacts with 28 S rRNA to form the base of the stalk, and P1 and P2 (~12 kDa), homologous to prokayotic L7/L12, are tethered as dimers to the stalk (2–5). P1 and P2 are structurally similar; each protein has three domains that include an α-helical N-terminal region and a central, flexible acidic hinge region followed by a highly conserved C terminus (E/KSD/EDMGFG/SLD). The C-terminal region of P0 is structurally similar to 12-kDa P-proteins because it possesses the three domains of P1 and P2 (for review, see Ref. 6).

The 12-kDa P-proteins are the only r-proteins found in multiple copies within the ribosome. They do not assemble onto preribosomes in the nucleolus but cycle between ribosomes and a cytosolic pool in numerous species including, *Artemia salina*, *Saccharomyces cerevisiae* (yeast), humans, and rats (7–11). Saenz-Robles *et al*. (12) demonstrated quantitatively that exponentially growing yeast cells contain more 12-kDa P-proteins/ribosome than cells in the stationary phase of growth. This suggests that the level of P-proteins in yeast ribosomes is affected by the metabolic state of the cell and possibly reflects the translational activity of the ribosome. The presence of these proteins in ribosomes has been shown to stimulate the eEF2-dependent GTPase activity of ribosomes (13–17), poly(U)-directed phenylalanine synthesis (14, 18), and eEF1A binding (19). Hence, modulation of the 12-kDa P-protein component of ribosomes may impart eukaryotes with a means of ribosome-regulated translational control.

Higher eukaryotes possess one type of P1 and P2, whereas lower eukaryotes possess multiple forms of P1 and P2 (20). *S. cerevisiae* ribosomes, for example, possess two forms of P1 (P1α and P1β) and P2 (P2α and P2β) (21). Mutant yeast strains in which one to four of the 12-kDa P-protein genes were disrupted remained viable but had decreased rates of cell growth (20, 22, 23). Ribosomes isolated from strains in which two or three genes were disrupted showed reduced eEF2-dependent GTPase activity and levels of protein synthesis *in vitro* (23). The strain in which all four genes were disrupted was more severely impaired, unable to produce spores, and cold-sensitive. Remarkably, the profiles of proteins synthesized from the same poly(A) mRNA sample with ribosomes from this strain and a wild-type strain were distinct, suggesting that the presence of P1 and P2 influences the efficiency of translation of individual mRNAs.

P1 and P2 and their phosphorylated forms appear to be functionally distinct. In yeast, the presence of P1 was required for the assembly of P2 into the ribosome (23, 24), and the ability to dephosphorylate one or more of these proteins was necessary for an adaptive response to osmotic stress (25). Studies with recombinant P1 and P2 from rat suggested that phosphorylation of P2 more effectively stimulates eEF2 activity *in vitro* than phosphorylation of P1 (17). Further analyses indicated that P1 has a higher binding affinity for eEF2 but that phosphorylation of both proteins stabilizes the interaction of eEF2 with the ribosome (16). These data suggest that the presence and phosphorylation of the 12-kDa P-proteins is involved in ribosome-mediated translational regulation.

We reported that maize (*Zea mays* L.) possesses one form of P1, two forms of P2 (P2a and P2b), and a third, plant-specific P1/P2-type protein designated P3 (26, 27). Here we examined whether maize ribosomes vary with respect to the composition

[1] The abbreviations used are: r-protein(s), ribosomal protein(s); DPP, days postpollination; TEMED, *N*,*N*,*N'*,*N'*-tetramethylethylenediamine; PBS, phosphate-buffered saline; PB, protein body; PAGE, polyacrylamide gel electrophoresis.

Heterogeneity in the Composition of the Ribosomal Stalk

and phosphorylation of these proteins. Antisera that specifically recognize the four 12-kDa P-proteins (P1, P2a, P2b, P3) were used to examine levels of these proteins in ribosomes isolated from a number of plant organs, including kernel tissues during seed maturation. Levels were also examined in ribosomes of root tips following flooding (anoxia), an environmental stress condition known to promote selective mRNA translation (28). We observed considerable developmentally and environmentally regulated heterogeneity in the levels and phosphorylation of these proteins.

EXPERIMENTAL PROCEDURES

Plant Material and Oxygen-deprivation Treatment—Maize (*Z. mays* L.) (inbred B73, gift of Pioneer Hi-Bred International, Johnston, IA) plants were grown in the field, and leaves of ear husks, ears, and silks were harvested at silk emergence. Ears were hand pollinated and harvested at 10, 15, 20, 25, 30, and 40 days postpollination (DPP) and after complete desiccation to isolate embryos (including the scutellum and embryonic axis), aleurone (tissue included the aleurone layer and attached pericarp), and endosperm (refers to tissue within the pericarp and aleurone, excluding the embryo).

For seedling tissue, kernels were surface sterilized with 0.25% (v/v) sodium hypochlorite, imbibed for 8 h, and germinated in the dark for 4–5 days at room temperature. Previously described methods were used for oxygen deprivation (anoxia) of intact seedlings by submergence in an aqueous solution that was continuously sparged with 99.995% argon (28). The apical 1 cm of the primary root and the entire coleoptile were harvested. All samples were frozen directly in liquid N_2 and stored at -80 °C.

RNA Isolation and Northern Hybridization—Total RNA was extracted following a CsCl gradient method (29), and RNA blots (20 μg of RNA/sample) were prepared (30). Hybridization was with [α-^{32}P]dATP-labeled cDNAs (GenBank U62752, maize P1; T18290, maize P2a; U62753, maize P2b; U62751, maize P3; tomato 18 S rRNA (gift of Dr. D. Bird)) separately, overnight at 42 °C in 6 × SSC, 5 × Denhardt's, 0.5% SDS (w/v), 100 μg/ml denatured calf thymus DNA, and 50% formamide (v/v) (31), washed twice in 2 × SSC and 0.1% SDS (w/v), once in 0.2 × SSC, and 0.1% SDS (w/v) for 20 min each at 65 °C, and exposed to autoradiographic film (Hyperfilm; Amersham Pharmacia Biotech) overnight at -80 °C with an intensifying screen. Prior to reuse, membranes were washed in 0.05 × SSC, 0.01 M EDTA, pH 8.0, and 0.1% SDS (w/v) for 20 min at 100 °C.

Isolation of Ribosomes and Soluble Proteins and Purification of Acidic Ribosomal Proteins—1–10 g of tissue was ground to a fine powder under liquid N_2 with a mortar and pestle and hydrated in 2–20 ml of extraction buffer A (0.2 M Tris, pH 7.5, 0.2 M KCl, 0.025 M EGTA, 0.036 M $MgCl_2$, 0.001 M Na_2MoO_4, 0.001 M dithiothreitol, 50 μg/ml cycloheximide, 50 μg/ml chloramphenicol) containing a detergent mix (1% Triton X-100 (v/v), 1% Brij 35 (w/v), 1% Tween 40 (v/v), 1% IGEPAL CA-630 (tert-octylphenoxy poly(oxyethylene) ethanol) (v/v)). The homogenate was centrifuged at 4 °C for 20 min at 7,740 × *g*, and the resulting supernatant (S-8 extract) was filtered (Miracloth; Calbiochem). Ribosomes were isolated by centrifugation of the S-8 extract through a 1.3 M sucrose cushion (0.4 M Tris, pH 7.5, 0.2 M KCl, 0.005 M EGTA, 0.036 M $MgCl_2$, 0.001 M Na_2MoO_4, 0.001 M dithiothreitol, 50 μg/ml cycloheximide, 50 μg/ml chloramphenicol) at 4 °C for 18–20 h at 135,000 × *g*. Ribosome pellets were resuspended in 0.02 M Tris, pH 7.5, 0.1 M KCl, 0.005 M $MgCl_2$, 0.001 M Na_2MoO_4, and 0.001 M dithiothreitol. To analyze zeins, S-8 extracts were prepared from endosperm tissue in the same manner, except that cycloheximide and chloramphenicol were omitted from the extraction buffer. Protein concentration was determined by the Bradford method using a protein determination reagent (United States Biochemical Corp.).

Acidic ribosomal proteins were purified from coleoptile ribosomes as previously described by Bailey-Serres *et al.* (26) in the absence of sodium molybdate.

Isolation of Soluble and Membrane-bound Ribosomes—Ribosomes were isolated following the protocol outlined by Mösinger and Schopfer (32). 1–10 g of tissue was ground to a fine powder under liquid N_2 with a mortar and pestle and hydrated in 2–20 ml of extraction buffer B (0.2 M Tris, pH 7.5, 0.06 M KCl, 0.05 M $MgCl_2$, 0.005 M dithiothreitol, 50 μg/ml cycloheximide). The homogenate was filtered through Miracloth and centrifuged at 4 °C for 10 min at 37,000 × *g*. Detergents (as described above) were added to the supernatant, which contained soluble ribosomes. The pellet, which contained membrane-bound ribosomes, was resuspended in extraction buffer containing detergents.

Ribosomes were isolated from each fraction by centrifugation through a 1.3 M sucrose cushion in extraction buffer B at 4 °C for 18–20 h at 135,000 × *g* and resuspended in extraction buffer B minus cycloheximide.

SDS-Polyacrylamide Gel Electrophoresis of Proteins and Immunoblot Analysis—Ribosomes (2.5 μg) and S-8 extract protein (75 μg) were diluted with SDS-sample buffer to a final concentration of 0.005 M Tris, pH 6.8, 5% glycerol (v/v), 2% SDS (w/v), 0.5% β-mercaptoethanol, and 0.125% bromphenol blue (w/v). Samples were heated at 100 °C for 5 min, and insoluble material was removed by centrifugation. Ribosomal proteins were fractionated in resolving gels (15% acrylamide (w/v), 0.5% (w/v) *N,N'*-methylene-bisacrylamide, 0.375 M Tris, pH 8.8, 0.1% SDS (w/v), 0.5% ammonium persulfate (w/v), and 0.5% TEMED). To examine zein levels, the S-8 extract was fractionated in 12% acrylamide (w/v), 0.5% (w/v) *N,N'*-methylene-bisacrylamide gels. Proteins were electrophoresed in SDS running buffer (25 mM Tris, 250 mM glycine, 0.1% SDS (w/v) and electrophoretically transferred to nitrocellulose membranes (0.22 μm; NitroBind; Micron Separations Inc.) in 0.025 M Tris, 0.250 M glycine, 20% methanol (v/v), and 0.01% SDS (w/v).

Membranes were blocked for 1 h in PBST (PBS (31), 0.1% Tween 20 (v/v)) that contained 5% nonfat dry milk (w/v) and incubated with rabbit antiserum against maize S6 (1:5,000 dilution) (kindly provided by A. Williams), rabbit antisera against maize 12-kDa P-protein peptides (P1 (1:250 dilution), P2a (1:250 dilution), P2b (1:250 dilution), P3 (1:1,000 dilution)), or rabbit antiserum against maize γ-zein (1:10,000 dilution) (kindly provided by A. Esen, North Carolina State University) in PBST, 1% nonfat dry milk (w/v) for 1 h. Membranes were washed three times for 5 min in PBST and incubated for 1.5 h in PBST, 1% nonfat dry milk (w/v) with goat anti-rabbit IgG horseradish peroxidase conjugate (1:15,000 dilution; Bio-Rad) or ^{35}S-labeled donkey anti-rabbit IgG (2 × 10^5 cpm/ml; Amersham Pharmacia Biotech). Antibody-antigen interaction was detected by chemilumenscence using the ECL reagent (Amersham Pharmacia Biotech). Membranes that were incubated with ^{35}S-labeled anti-rabbit IgG were exposed to a PhosphorImager screen (Molecular Dynamics) and were quantified using QuantityOne software (Bio-Rad).

Production of Polyclonal Antibodies—Peptides specific to maize P-proteins ([44]ALFAKLLEKRNVED[57] for P1, [40]ELLLSQLSGKD[50] for P2a, [39]LEFLLTELKDKDI[51] for P2b, and [9]RNNGGEWTAKQHSGEI[24] for P3) were synthesized, conjugated with a carrier protein (keyhole limpet hemocyanin for P2a, P2b, and P3; tetanus toxoid for P1) and injected into rabbits. Antisera against P2a and P3 were purified by affinity chromatography using the specific peptide bound to a Sepharose column (Quality Controlled Biochemicals Inc., Brighton, MA).

RESULTS

Maize P-protein Transcript Accumulation Is Developmentally Regulated—We examined the abundance of P1, P2a, P2b, and P3 mRNA transcripts in total RNA from several organs of maize and in kernel tissues during seed maturation and found that the accumulation of individual P-protein transcripts is developmentally regulated (Fig. 1). Among the organs and tissues examined, transcript accumulation for all four 12-kDa P-proteins was highest in coleoptiles and immature ears, relative to 18 S rRNA levels, as expected for organs undergoing rapid cell division and differentiation (33). P1, P2a, and P3 mRNA accumulation patterns were very similar. These transcripts were detected at very low levels in leaf, silk, and pollen, where, by contrast, P2b transcripts accumulated to moderately high levels. In all of the kernel tissues examined, P1, P2a, and P3 mRNAs were abundant, with the highest levels at 15 DPP, whereas P2b mRNA was present at low levels. This variation in mRNA accumulation led us to examine the possibility of developmental differences in P-protein composition of ribosomes.

Specific Antisera against the 12-kDa P-proteins of Maize Detect Phosphorylation Variants and Protein Complexes—Peptides specific to each of the four types of 12-kDa P-proteins of maize were synthesized and used to prepare antisera in rabbits. To evaluate the specificity of the antisera, ribosomes were isolated from coleoptiles, washed with high salt (0.8 M NH_4Cl and 50% ethanol) under conditions that release the 12-kDa P-proteins (26), and immunoblot analyses were performed. Preimmune sera showed no detectable cross-reaction to r-proteins (data not shown). P1, P2a, P2b, and P3 antisera detected
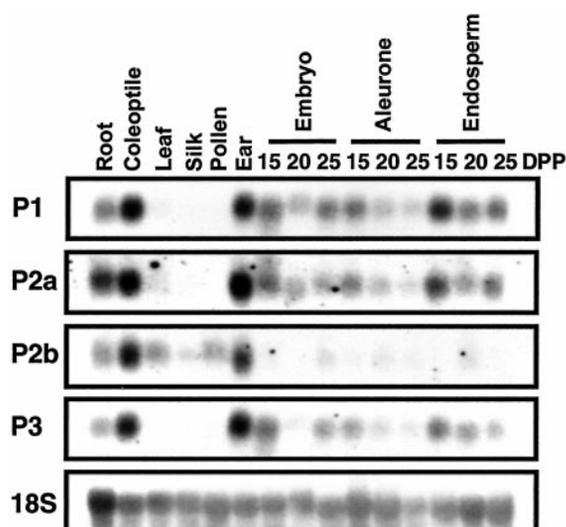
FIG. 1. **Developmental regulation of 12-kDa P-protein mRNA accumulation.** Total RNA was isolated from various tissues, separated (20 μg/lane), blotted, and sequentially hybridized at high stringency with ^{32}P-labeled cDNAs encoding P1, P2a, P2b, P3, and 18 S rRNA.

polypeptides of distinct molecular mass from coleoptile ribosomes, confirming that the antisera were specific (Fig. 2*A*, *lane 1* in each *panel*).

Maize P1 is encoded by a single-copy gene, has a predicted molecular mass of 11.0 kDa and three putative phosphorylation sites (26, 27). P1 antisera recognized polypeptides with apparent molecular masses of ~14.5 and 50 kDa in coleoptile ribosomes (Fig. 2*A*, *P1 panel*, *lane 1*). A low level of P1 remained in the salt-washed ribosomes (*lane 2*). Following release from ribosomes with 0.8 M NH$_4$Cl, P1 was detected primarily as 15 and 14.5 kDa (*lane 3*). The 50-kDa protein detected with the P1 antiserum was not released from ribosomes (*lane 3*). This protein was identified as r-protein L4 by matrix-assisted laser absorption ionization-time of flight mass spectrometry (data not shown), indicating that the detection of the 50-kDa protein is most likely caused by nonspecific binding of the P1 antiserum.

Maize P2a is encoded by a gene family of approximately four members, has a predicted molecular mass of 11.5 kDa, and five or six putative phosphorylation sites (26, 27). The P2a antiserum detected a group of ~12-kDa polypeptides in coleoptile ribosomes (Fig. 2*A*, *P2a panel*, *lane 1*). The detection of multiple 12-kDa forms of P2a is most likely caused by the expression of more than one *rpp2a* gene. The P2a antiserum also detected groups of ~26-, ~42–48-, and ~60-kDa polypeptides that were not detected with the other antisera. Following elution from ribosomes with high salt these polypeptides had slightly altered electrophoretic mobility (Fig. 2*A*, *lane 3*, *white*, *gray*, and *stippled arrows*, respectively). However, not all of the 26-kDa form was released from the salt-washed ribosomes (*lane 2*). We were unable to promote or inhibit formation of the P2a complexes in ribosomes or eluate samples by manipulation of SDS, urea, sulfhydryl-reducing agents, or heat, indicating that they involve strong hydrophobic interactions (data not shown). These results suggest that P2a is present in dimeric (24–26 kDa) and additional multimeric complexes (42–48 kDa, 60 kDa) in coleoptile ribosomes.

Maize P2b is encoded by a single gene, has a predicted molecular mass of 11.8 kDa, and three putative phosphorylation sites (26, 27). P2b was detected as a 14-kDa polypeptide in coleoptile ribosomes (Fig. 2*A*, *P2b panel*, *lane 1*) and was efficiently released from ribosomes by 0.8 M NH$_4$Cl (*lane 2*). Sim-

ilar to P1, P2b was detected after elution as 14- and 14.5-kDa forms (*lane 3*).

Maize P3 is encoded by one to two genes, has a predicted molecular mass of 12.2 kDa, and three putative phosphorylation sites (26, 27). The P3 antiserum detected a 15-kDa polypeptide in coleoptile ribosomes (Fig. 2*A*, *P3 panel*, *lane 1*). When P3 was released with 0.8 M NH$_4$Cl two forms of 15.5 and 15 kDa were detected (*lane 3*). Much of the P3 protein remained in the salt-washed ribosomes (*lane 2*), indicating that the noncovalent interactions between P3 and the ribosome are stronger than that observed for P1, P2a, and P2b.

Electrophoretic variants of the different P-proteins and P2a complexes were observed between different tissues (Fig. 3, discussed below) and following release from coleoptile ribosomes with 0.8 M NH$_4$Cl (Fig. 2*A*). More slowly migrating forms of all of the P-proteins were observed following extraction from ribosomes with high salt. We determined that the *in vivo* phosphorylation status of these proteins was maintained if ribosomes were isolated in the presence of the nonspecific phosphatase and kinase inhibitor sodium molybdate. The effect of the presence or absence of sodium molybdate in the extraction and resuspension buffers is shown for root tip ribosomes in Fig. 2*B*. To confirm loading of similar quantities of r-proteins in each sample, immunoblots were coincubated with an antiserum prepared against r-protein S6 (30 kDa). In all cases, the presence of sodium molybdate increased the level of the more slowly migrating forms of the different P-proteins (Fig. 2*B*). A panel for P2b is not shown because this form was not detected in root tip ribosomes (see Fig. 3). These results indicate that root extracts contain a phosphatase activity that is inhibited by sodium molybdate and that P1, P2a, and P3 of root ribosomes are predominantly phosphorylated. By contrast, the P-proteins of coleoptile ribosomes had faster electrophoretic mobility (Fig. 3, compare *root tip* and *coleoptile lanes*), indicating that these proteins are either dephosphorylated in coleoptile ribosomes or a phosphatase was not inhibited during the extraction. The ability of a ribosome-associated kinase to phosphorylate coleoptile P-proteins was evidenced by the change in electrophoretic mobility following resuspension in a buffer lacking sodium molybdate (Fig. 2*A*, *panel P3*) or release from ribosomes with high salt (*panels P1*, *P2a*, and *P2b*). These results suggest that the phosphorylation status of these proteins may be regulated by ribosome-associated kinases.

12-kDa P-protein Phosphorylation Is Reduced in Response to Anoxia—Our previous investigations indicated that the phosphorylation status of the 12-kDa P-proteins is altered in response to anoxia (26). The immunoblot shown in Fig. 2*C* demonstrates that when intact seedlings were deprived of oxygen for up to 24 h, an increase was observed in the amount of the faster migrating, dephosphorylated forms of P1, P2a, and P3. This result demonstrates unambiguously that 12-kDa P-proteins are dephosphorylated under anoxia.

Developmental Distinctions in the 12-kDa P-protein Composition of Ribosomes—Levels of the 12-kDa P-proteins in ribosomes from several organs (root tip, coleoptile, leaf, silk, and ear) and kernel tissues (embryo, aleurone, and endosperm) were surveyed relative to levels of r-protein S6. Ribosomes were isolated in the presence of sodium molybdate to control protein phosphorylation status during extraction. The immunoblots shown in Fig. 3 demonstrate considerable developmental differences in quantity and electrophoretic mobility of the 12-kDa P-proteins relative to the level of S6 (shown in *P2 panel*) in ribosomes.

P1 was detected in ribosomes of all of the samples but in different amounts and in several electrophoretic variants (Fig. 3, *panel P1*). P1 was less abundant in leaf, silk, and ear ribo-
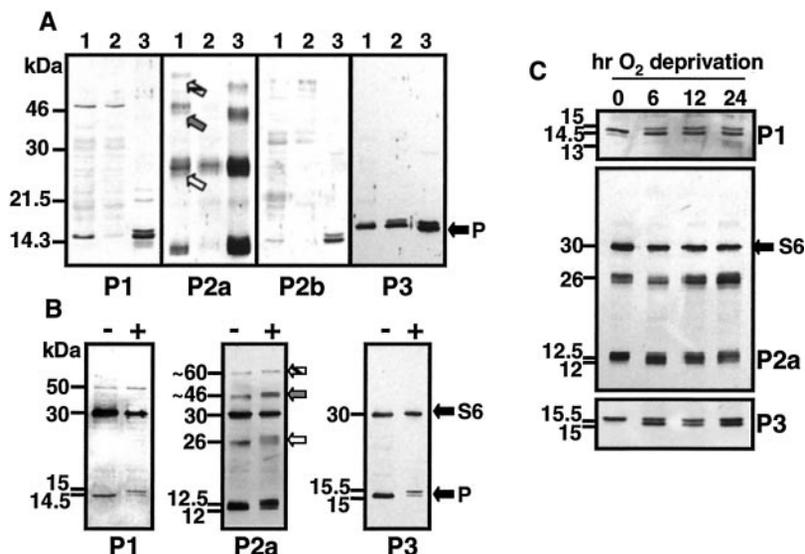
FIG. 2. **Detection of maize P1, P2a, P2b, and P3 with specific antisera.** *Panel A*, ribosomes isolated from coleoptiles (*lane 1*), acidic proteins released from coleoptiles ribosomes with 0.8 M NH₄Cl (*lane 3*), and corresponding salt-washed ribosomes (*lane 2*) were resolved by SDS-PAGE, transferred to nitrocellulose, and were incubated with P1, P2a, P2b, or P3 antisera. The *black arrow* indicates the position of the monomeric proteins. *White*, *gray*, and *stippled arrows* indicate the P2a dimeric and multimeric complexes, respectively. *Panel B*, ribosomes isolated from root tips in the presence (+) or absence (−) of sodium molybdate were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated simultaneously with antisera against S6 (as indicated) and P1, P2a, or P3. *White*, *gray*, and *stippled arrows* indicate the P2a dimeric and multimeric complexes, respectively. *Panel C*, ribosomes isolated from aerobic, 6-h, 12-h, or 24-h oxygen-deprived root tips in the presence of sodium molybdate were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated simultaneously with antisera against S6 (as indicated) and P1, P2a, or P3. The higher molecular mass forms of P2a were detected in all samples after longer exposure (data not shown)
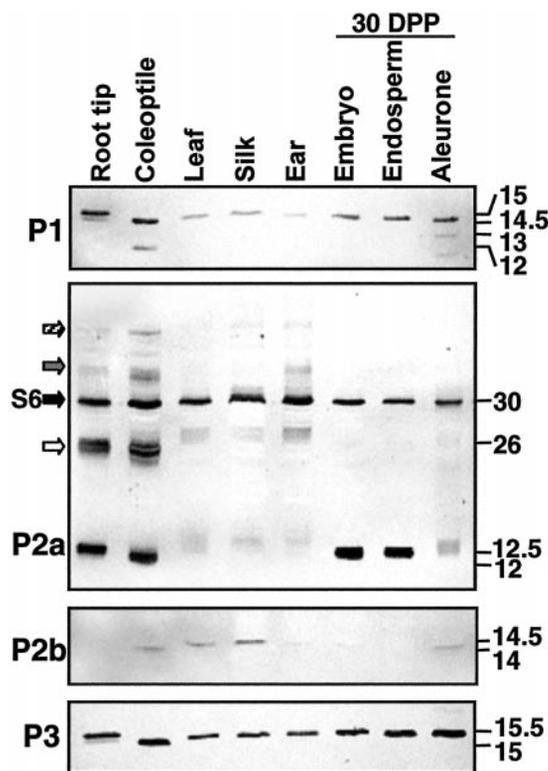

FIG. 3. **Detection of P1, P2a, P2b, P3, and S6 in ribosomes from various tissues of maize.** Ribosomes isolated from various developmental tissues and organs (see "Experimental Procedures") were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated simultaneously with antisera against S6 (indicated by *black arrow*) and P1, P2a, P2b, or P3. *White*, *gray*, and *stippled arrows* indicate the P2a dimeric and multimeric complexes, respectively. The apparent molecular mass of each protein (kDa) is indicated on the *right* as determined by the migration of molecular mass markers.

somes, compared with root, coleoptile, and kernel tissues. Variations in P1 included the presence of a single form (15 kDa) in leaf, silk, ear, embryo, and endosperm, two forms in root tip (15 and 14.5 kDa) and coleoptile (14.5 and 12 kDa), and three forms (15, 13, and 12 kDa) in aleurone ribosomes. As demonstrated in Fig. 2*B*, the 15- and 14.5-kDa forms differ in phosphorylation status. The 13- and 12-kDa forms detected in coleoptiles and aleurone were present at varying levels between preparations and are most likely degradation products.

The P2a antiserum detected polypeptides that varied considerably in abundance and electrophoretic mobility between the different organ and tissue samples (Fig. 3, *panel P2a*). The 12.5-kDa forms of P2a were abundant in root tip, embryo, and endosperm ribosomes, whereas a 12-kDa form was detected in coleoptile ribosomes. Consistent with mRNA accumulation data, lower levels of P2a were detected in leaf, silk, ear, and aleurone ribosomes. Levels of dimeric (24–26 kDa) and multimeric P2a (48 and 60 kDa) were proportional to levels of the 12- and 12.5-kDa P2a in all samples except ribosomes of embryo and endosperm. P2a was only detected as a monomer in these kernel samples. As seen for P1, the apparent molecular mass of P2a in root ribosomes was higher than that of coleoptiles. The apparent molecular mass of P2a in leaf, silk, and ear ribosomes was even higher than that of root tips. The observed variations in electrophoretic mobilty could reflect differential expression of the *rpp2a* genes and/or distinctions in protein phosphorylation.

P2b was detected at dramatically different levels in ribosomes isolated from various organs and tissues (Fig. 3, *P2b panel*). A 14-kDa form of P2b was detected in coleoptile ribosomes, whereas a 14.5-kDa form was detected in leaf, silk, and 30-DPP aleurone ribosomes. P2b was not detected in root tip ribosomes and was present at very low levels in ear, embryo, and endosperm ribosomes. Higher levels of P2b in coleoptile, leaf, and silk ribosomes correlated with the mRNA accumulation data (Fig. 1). P2b levels were low to undetectable in ear and root tip ribosomes despite the detection of P2b mRNA in these organs, whereas P2b levels in aleurone ribosomes were higher than predicted from the RNA blot data. The faster migration of P2b of coleoptile ribosomes was consistent with that observed for the other P-proteins.
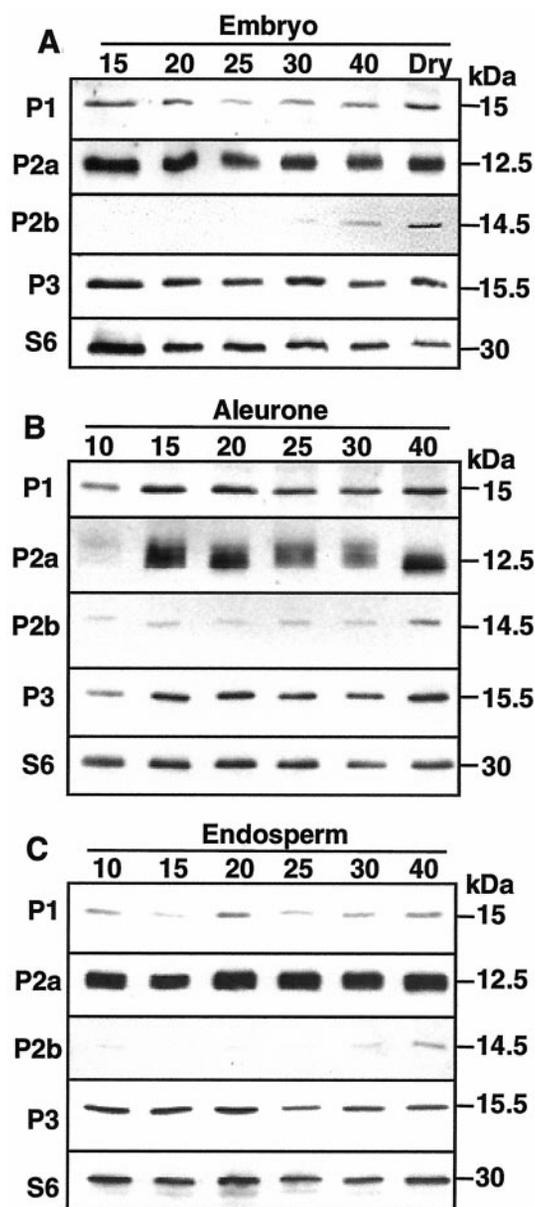
Fig. 4. **Detection of P1, P2a, P2b, P3, and S6 in ribosomes during maize kernel maturation.** Ribosomes isolated from (*panel A*) 15, 20, 25, 30, and 40 DPP and dry embryos; (*panel B*) 10-, 15-, 20-, 25-, 30-, and 40-DPP aleurone; and (*panel C*) 10-, 15-, 20-, 25-, 30-, and 40-DPP endosperm were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated simultaneously with antisera against S6 and P1, P2a, P2b, or P3. The apparent molecular mass (kDa) of each protein is indicated on the *right*.

The lack of variability in the abundance of P3 associated with ribosomes was in marked contrast to that observed for the two forms of P2. P3 was detected as a 15.5-kDa polypeptide at similar levels in all ribosome samples with the exception of coleoptile ribosomes where it was detected as a 15-kDa polypeptide, evidently because of reduced phosphorylation (Fig. 3, *P3 panel*). A small amount of dephosphorylated P3 was detected consistently in root tip ribosomes. The levels of P1 and P3 in leaf, silk, and ear ribosomes (Fig. 3) are higher than predicted from the transcript accumulation data (Fig. 1).

Regulation of 12-kDa P-protein Levels Occurs during Kernel Development—Given the considerable developmental regulation in accumulation of the 12-kDa proteins in ribosomes in the maize organs and tissues surveyed, we decided to monitor P-protein levels during the temporal development and maturation of the kernel. Fig. 4,*A–C*, compares the P-protein content

of ribosomes over the time course of kernel development and maturation, relative to levels of r-protein S6. The maize embryo consists of the embryonic axis surrounded by the scutellum, a modified cotyledon. In embryos monitored from 15 to 40 DPP, levels of P1, P2a, and P3 were not altered dramatically (Fig. 4*A*). P1 was detected as a 15-kDa polypeptide, P2a was detected only in the 12.5-kDa monomeric form, and P3 was detected as a 15.5-kDa polypeptide. By contrast, levels of P2b increased dramatically in embryo ribosomes after 25 DPP. All four P-proteins were detected in ribosomes isolated from dry embryos of mature kernels, indicating that ribosomes stored in the seed embryo possess these proteins. There was no indication that phosphorylation status was modulated during embryo maturation.

The kernel aleurone, the outermost cell layer of the endosperm, develops until about 40 DPP, at which time it has become quiescent and desiccated; upon seed imbibition the stored ribosomes actively synthesize the starch hydrolases required to mobilize nutrients. Levels of P1, P2a, P2b, and P3 in ribosomes increased during early aleurone development (10 and 15 DPP) and again at the late maturation stage (30 and 40 DPP) (Fig. 4*B*). An increase in the electrophoretic mobility of P2a that was consistent with dephosphorylation was observed at 40 DPP. The level of P2b was modulated less dramatically in the aleurone than in the kernel embryo or endosperm.

The endosperm is the triploid, nutritive organ of the kernel that stores carbohydrate, lipid, and protein reserves for the embryo. The endosperm develops from fertilization until 12–15 DPP, at which time grain filling begins and proceeds until ~40–50 DPP, when a spatial progression in programmed cell death occurs (34). We observed that levels of P1, P2a, and P3 were not regulated markedly during endosperm development (Fig. 4*C*). By contrast, levels of P2b increased dramatically after 25 DPP, as observed in embryo ribosomes. Ribosomes could not be isolated in sufficient quantities from dry endosperm to perform immunoblot analyses, most likely because of a reduction in ribosome levels at the end of endosperm maturation (data not shown).

Distinctions in Ribosomes Associated with Storage Protein Bodies of Endosperm—Large quantities of storage proteins are synthesized during endosperm maturation. Prolamines (α, β, γ, δ-zeins) are alcohol-soluble proteins that are synthesized on rough endoplasmic reticulum and are assembled into protein bodies (PBs) in the lumen. Zein accumulation begins at about 10 DPP but accelerates only after 25 DPP (Fig. 5; 35), despite a peak in zein mRNA levels at 15-DPP (35, 36), indicative of post-transcriptional regulation of expression. We observed that ribosomes isolated from 25-, 30-, and 40-DPP endosperm were contaminated with zeins. This was not unexpected because prolamine PBs are found tightly associated with the cytoskeleton and rough endoplasmic reticulum in maize and rice (37, 38). Cell fractionation was performed to examine the 12-kDa P-proteins of soluble and membrane-associated ribosomes of 40-DPP endosperm and coleoptile. Cell extracts were prepared in the absence of detergent and centrifuged to produce a supernatant that contained soluble ribosomes and a pellet that contained membrane-associated ribosomes. The pellet was resuspended in a detergent-containing buffer and recentrifuged to obtain a clarified supernatant of detergent-solubilized membrane-associated ribosomes. In the endosperm sample some of the ribosomes were not released by this detergent treatment and were pelleted again. This pellet contained high levels of zeins and r-proteins, confirming the purification of a fraction enriched in PB-associated ribosomes (Fig. 6, γ-*zein panel*). A similar detergent-resistant fraction was not obtained from coleoptiles. The P-proteins levels in soluble and membrane-asso-
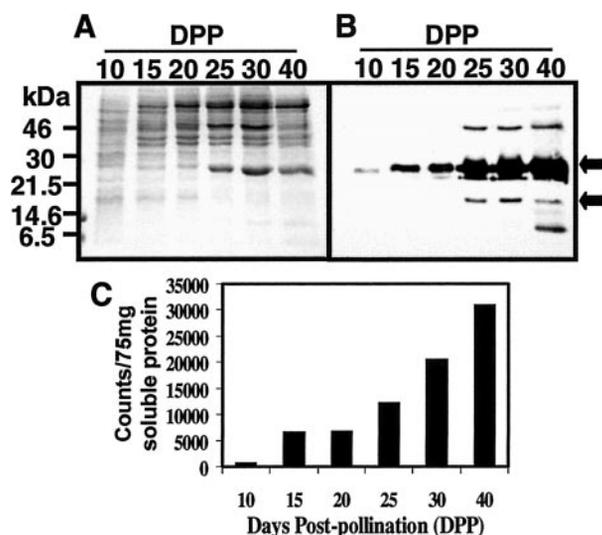
FIG. 5. **Accumulation of γ-zein seed storage proteins during endosperm development.** *Panel A*, soluble protein isolated from 10-, 15-, 20-, 25-, 30-, and 40-DPP endosperm was resolved by SDS-PAGE. Proteins were visualized by Coomassie Blue staining. The positions of molecular mass markers (kDa) are indicated on the *left*. *Panel B*, soluble protein isolated from 10-, 15-, 20-, 25-, 30-, and 40-DPP endosperm was resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with maize γ-zein antiserum (*black arrows*). *Panel C*, relative abundance of γ-zein in endosperm development. Soluble protein isolated from 10-, 15-, 20-, 25-, 30-, and 40-DPP endosperm was resolved by SDS-PAGE, transferred to nitrocellulose, and detected with maize γ-zein antiserum. The signals of the ^{35}S-labeled cross-reacting anti-rabbit IgG secondary antibody were quantified with a PhosphorImager.
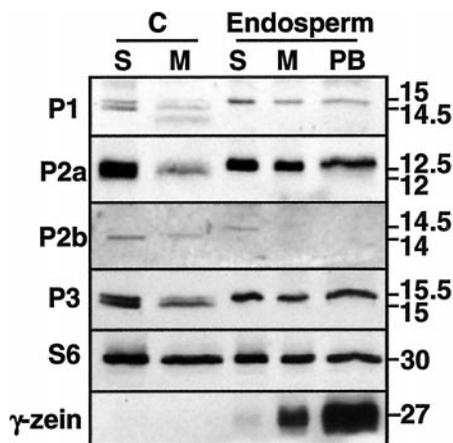

FIG. 6. **Detection of P1, P2a, P2b, P3, and S6 in membrane-associated and soluble ribosomes.** Soluble (*S*), membrane-associated (*M*), and γ−zein protein body-associated (*PB*) ribosomes isolated from coleoptiles or 40-DPP endosperm were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated simultaneously with S6, P1, P2a, P2b, P3, or zein antiserum. The apparent molecular mass of each protein (kDa) is indicated on the *right* as determined by the migration of molecular mass markers.

ciated ribosomes, relative to r-protein S6, were not identical in coleoptile or endosperm (Fig. 6). In coleoptiles, slightly reduced levels of these proteins were reproducibly detected in membrane-associated ribosomes. This was especially evident for the P2a monomer (Fig. 6) and dimer (data not shown). In the 40-DPP endosperm, the presence and abundance of P1, P2a, and P3 showed little variation between soluble, membrane ribosomes and PB-associated ribosomes. In contrast, P2b levels were reduced in membrane-associated ribosomes compared with soluble ribosomes, and P2b was undetectable in PB-associated ribosomes. These results provide evidence that the 12-kDa P-protein composition of ribosomes surrounding PBs and

translating zein mRNAs is distinct from ribosomes translating soluble proteins.

DISCUSSION

Heterogeneity in Ribosomal P-protein Composition—The results presented here clearly demonstrate that maize ribosomal protein composition is variable with respect to the four ~12-kDa P-proteins, P1, P2a, P2b, and P3. Ribosome heterogeneity is caused by differences in 1) phosphorylation of the 12 kDa P-proteins; 2) the presence and abundance of distinct 12-kDa P-proteins at the tissue and subcellular (membrane *versus* soluble) levels; and 3) the presence and abundance of multimeric complexes of P2a.

Phosphorylation of the P-proteins of Root Ribosomes Is Developmentally and Environmentally Regulated—Our previous studies indicated that the maize 12 kDa P-proteins are phosphoproteins and that their phosphorylation is modulated in response to anoxia (26). Our current analyses reveal several examples of modulation of phosphorylation of the 12 kDa P-proteins in response to cues from the environment and during development. The isolation of ribosomes from root tips in the presence of sodium molybdate, a nonspecific phosphatase and kinase inhibitor, revealed that the phosphorylation of P1, P2a, and P3 (P2b was not detected in root tip ribosomes) was reduced in response to anoxia, consistent with earlier predictions. P1, P2, and P3 of coleoptiles migrated at a rate similar to the dephosphorylated forms of root ribosomes, suggesting that these proteins are either dephosphorylated in coleoptiles or a phosphatase was not inhibited during extraction. Also, the phosphorylation status of P2a was dephosphorylated in late aleurone development.

Several kinases that phosphorylate the P-proteins have been isolated from yeast; however, very little is known about the phosphatases that act on these proteins. Recently, a protein phosphatase, isolated from a ribosome-free extract from yeast, was shown to dephosphorylate P1, P2, and P0 *in vitro* (39). Protein kinase 60 S (PK60S) (40), casein kinase II (CK II) (41, 42), ribosome acidic protein kinase I (RAP I) (43), and RAP II (44) have been shown to *in vitro* phosphorylate P-proteins from several organisms. In yeast, Bou *et al.* (44) demonstrated that RAP II and PK60S preferentially phosphorylate P1β and P2α, whereas RAP I and CK II modify all of the P-proteins. The distinct phosphorylating activities of the various P-protein kinases suggest that the differential phosphorylation of the P-proteins is of functional significance. Because all of the maize P-proteins were modified during anoxia in the same manner, we predict that this modulation is caused by the inactivation of a general P-protein kinase and/or activation of a general P-protein phosphatase. In contrast to the global P-protein dephosphorylation observed in response to anoxia, the developmentally regulated dephosphorylation observed in late aleurone maturation was P2a-specific. This modulation could be the result of the inactivation of a kinase and/or activation of a phosphatase that specifically modifies P2a.

Yeast strains in which the phosphorylated C-terminal serine of P1α was mutated to an alanine were less sensitive to osmotic stress than wild-type or mutant strains in which this serine was mutated to threonine, leading to the prediction that dephosphorylation of P1 is required for an adaptive response to stress (25). Dephosphorylation is plausibly a global down-regulator of protein synthesis because dephosphorylation of the C-terminal serine of rat P2 reduced translational activity *in vitro* (17). Additional studies are needed to determine whether the dephosphorylation of P1, P2a, and P3 in response to anoxia in maize is involved in some aspect of the translational control observed under this stress.

Ribosome Heterogeneity Is Caused Mainly by Variations in

P2—Examination of the ribosomal P-proteins in a range of organs revealed striking heterogeneity, especially in P2 composition. The novel plant P-protein, P3, showed little variation in accumulation and was less efficiently eluted from salt-washed ribosomes than the other stalk proteins. P1 accumulation patterns were slightly more complex. P1 was detected as a monomer with varying electrophoretic mobility. P1 was less efficiently released from ribosomes with high salt than P2a or P2b. By contrast, considerable tissue-specific, and developmental regulation was observed in the levels of the two forms of P2. P2a levels were higher in root tip, coleoptile, embryo, and endosperm ribosomes compared with leaf, silk, immature ear, and kernel aleurone ribosomes. The low levels of P2a were reciprocated by higher accumulation of P2b in ribosomes of leaf, silk, and aleurone, but apparently not in the ear at silk emergence, or aleurone during early development (10 DPP).

Variations in P2 also included the accumulation of P2a complexes (dimers and other multimeric forms). P2a dimers were observed in all samples except in ribosomes of kernel tissues. The N-terminal regions of the yeast 12-kDa P-proteins are predicted to form α-helices with hydrophobic residues spaced at intervals that would promote coiled-coil interactions (45). Similar to yeast, maize P2a is predicted to form an α-helix with a strong hydrophobic edge that may result in the observed dimers and multimers.[2] The absence of P2a dimers in kernel tissue ribosomes is enigmatic, but may reflect differences in phosphorylation at N-terminal sites or differential expression of P2a gene family members.

The most striking distinctions we observed were in the levels of P2b. This protein was not detected in ribosomes from roots and showed a dramatic developmentally programmed increase in embryo and endosperm. P2b was also undetectable in the PB-associated ribosomes from 40-DPP endosperm but was present in the soluble ribosomes from the same sample. Although the functional significance of the observation is unclear, these distinctions could be involved in the translational control of zein protein synthesis if P2b-containing ribosomes do not translate zein mRNAs.

Recently, Zurdo *et al*. (24) demonstrated in yeast that binding of P1 to the ribosome must proceed binding of P2, suggesting that assembly of the P-protein stalk is an ordered process. We observed that both P2a and P2b were efficiently eluted from ribosomes washed with high salt, whereas P1 and especially P3 were eluted less efficiently. This, together with the limited variation in the amounts of P1 and P3, suggests that P1 and P3 precede and may be required for P2 assembly. Given the low levels of P2 proteins in leaf, silk, ear, and early aleurone ribosomes, the presence of P1 and P3 may be sufficient for translation. The extensive level of P2 modulation and their efficient removal from salt-washed ribosomes may indicate that these proteins are not necessary for protein synthesis but impart some mode of translational regulation.

Heterogeneity in P-protein Composition and Its Possible Functional Significance—Our results illustrate clear distinctions in the presence, abundance, and phosphorylation of the 12-kDa P-proteins of maize ribosomes and that these distinctions are affected by cell identity, growth conditions, and stage of development. These results raise several intriguing questions: 1) Do maize ribosomes possess each of the three types of 12-kDa P-proteins (P1, P2, and P3) at similar or distinct levels? 2) Is there a heterogeneous population of ribosomes within a plant cell (*i.e.* ribosomes with different levels of the 12-kDa P-proteins)? 3) Are differences in abundance and phosphorylation of the P-proteins involved in translational regulation?
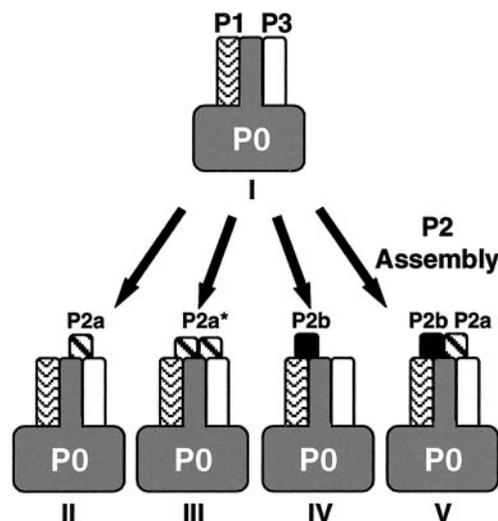

FIG. 7. **Model of ribosome heterogeneity due to variation in P2 protein composition of the acidic stalk.** Type I ribosomes possess a minimal P-protein complex with the stalk formed by P0, P1, and P3 and exist during early aleurone development and in ears prior to pollination. Variations in P2 assembly onto the minimal P-protein complex result in ribosome types II–V. Type II ribosomal stalks possess the minimal complex with the addition of the monomeric form of P2a and predominate in kernel tissues. Type III ribosomal stalks possess the minimal complex and dimers or multimers of P2a (P2a*) and predominate in root and coleoptile. Type IV ribosomal stalks possess the minimal complex and P2b and predominate in leaf and silk, whereas type V ribosomal stalks possess the minimal complex and both P2a and P2b and may exist in cells of coleoptile and other tissues that contain P2a and P2b. Variations in stalk protein composition could influence elongation rates (eEF2-dependent GTPase activity). Regulated differences in phosphorylation of the 12-kDa P-proteins provide additional ribosomal heterogeneity and potential ramifications on translation.

Ribosome heterogeneity in yeast was considered by Ballesta *et al*. (46) when they examined the P-protein complex by affinity purification of ribosomes with His-tagged P2α. They determined that 95% of the ribosomes contained all four of the 12-kDa P-proteins (P1α, P1β, P2α, and P2β) and concluded that the yeast ribosomes are homogeneous with respect to P-protein composition. By contrast, our results indicated that P1 and P3 levels were fairly constant and coordinate, but levels of P2a and P2b were dramatically variable in maize ribosomes even when levels of both P2a and P2b were considered. Phylogenetic studies indicate that P2a and P2b are present in maize and rice, indicating that these two forms arose from a gene duplication event prior to the divergence of a common ancestor and that two classes of P2 are present in *Arabidopsis*.[2] The significant heterogeneity in P2 levels and prevalence of multiple P2 forms in plants may be indicative of a functional role of the distinct P2 forms.

Our results show that distinctions in the 12-kDa P-proteins of maize ribosomes are determined by development and environment. To account for our data and to consider the functional significance of ribosome heterogeneity on translational activity we present a model based on the structure depicted by Ballesta *et al*. (6, 46). We propose that ribosomes with distinct 12-kDa P-proteins composition exist in maize (Fig. 7). Ribosomes may possess a minimal acidic protein complex of P0, P1, and P3 (ribosome type I) or a minimal complex distinguished by quantitative differences in P2 composition (ribosome types II–V). Type I ribosomes predominate in aleurone at 10 DPP and ears at silk emergence. These ribosomes may have a basal level of eEF2-dependent GTPase activity and hence a basal rate of translational elongation. Type II ribosomes contain the minimal complex with the addition of P2a and predominate in kernel tissues. Type III ribosomes contain the minimal complex

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and multimeric forms of P2a and predominate in roots and coleoptiles. Type IV ribosomes contain the minimal complex with the addition of P2b and predominate in leaf and silk. Finally, type V ribosomes, possessing all four 12-kDa P-proteins, may exist in cells that express both P2a and P2b. Ribosomes possessing stalks with distinct P2 composition (types II–IV) may have distinct levels of eEF2-dependent GTPase activity (higher or lower than the basal level) and/or differential efficiency in translation of specific mRNAs. Because P1, P2, and P3 phosphorylation levels are modulated in response to environmental stress (anoxia) and during development, we propose that C-terminal phosphorylation further regulates translational elongation. This ribosome heterogeneity, largely caused by variations in P2 composition, strongly indicates a regulatory role of the acidic stalk of the large subunit in translation at the global or message specific level.

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