Gene Expression in Developing Wheat Endosperm[1]

ACCUMULATION OF GLIADIN AND ADPGLUCOSE PYROPHOSPHORYLASE MESSENGER RNAS AND POLYPEPTIDES

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ABSTRACT

The developmental accumulation pattern of messenger RNA transcripts and polypeptides for wheat gliadins and ADPglucose pyrophosphorylase was determined using cDNA and antibody probes. Gliadin mRNA was detected on Northern and RNA dot blots at 3 days after flowering, it increased 100-fold by 10 days and decreased subsequent to 14 days. The abundant mRNAs encoding α/β- and γ-type gliadins and mRNA for ADPglucose pyrophosphorylase, a key regulatory enzyme of starch biosynthesis, accumulated coordinately. Despite the coordinate accumulation of their mRNA transcripts, the accumulation of gliadin and ADPglucose pyrophosphorylase polypeptides, as determined by Western blot, differed significantly. The time at which gliadin and ADPglucose pyrophosphorylase mRNAs began accumulating was also the time when the overall pattern of gene expression, as seen by two-dimensional gel electrophoresis of *in vitro* translation products, changed most significantly. However, the accumulation of a number of other mRNAs or polypeptides having unknown function occurred at other times during endosperm development. The pattern of expression in the earliest stages of development was strikingly similar to that of coleoptile, another rapidly growing, nonphotosynthetic tissue. Thus, the pattern of gene expression reflects the program of development observed cytologically.

Wheat endosperm is a relatively homogeneous tissue and a good model system for studying developmental gene regulation in plants. The cytological events of endosperm development are divided into three distinct phases (3, 5). In the first few DAF[2] endosperm tissue is formed as a multinucleated syncytium, followed by cellularization and multiple rounds of mitotic division. During the middle phase, between about 10 to 25 DAF, large amounts of storage protein, starch, and other seed reserves accumulate. The late phase (26–40 DAF) is a period of dehydration and seed maturation.

The wheat seed storage proteins that accumulate during the middle phase consist primarily of the alcohol-soluble gliadins, which are encoded by a family of at least 40 genes (27) on separate genetic loci located on two different chromosomes (21). Although gliadin mRNAs accumulate early in the middle phase of endosperm development (8), it is not clear whether the individual genes are coordinately or differentially expressed. We have recently characterized the sequence diversity of the α/β- and γ-

type gliadin gene subfamilies which encode most of the gliadin polypeptides (19). Individual genes within each subfamily are highly homologous, but can be resolved into additional homology groups by the presence of unique restriction sites, cross-hybridization analysis at high stringency, and direct DNA sequence comparison. With probes specific for various homology groups within these subfamilies in hand, a study of the developmental regulation of this large gene family has now been initiated.

Starch also accumulates at approximately the same time as the gliadins during the middle phase of endosperm development (26). ADPglucose pyrophosphorylase, a key regulatory enzyme in starch biosynthesis (23), has a much higher activity in endosperm than other wheat tissues (26) and therefore provides a useful developmental marker in addition to the gliadins. Before attempting to answer the fundamental question of what cis- and trans-acting factors determine the tissue and developmental specificity of gene expression in wheat endosperm, it is important to determine the temporal pattern of expression of the different endosperm-specific genes.

In this study we have quantified the developmental accumulation of protein and mRNA for α/β- and γ-type gliadins as well as for ADPglucose pyrophosphorylase. The timing of accumulation of these mRNAs and polypeptides was compared to the overall pattern of mRNA accumulation as revealed by two-dimensional gel electrophoresis of *in vitro* translation products. The temporal pattern of mRNA and polypeptide accumulation conforms to the cytological picture of early rapid cell proliferation followed by a shift to the accumulation of storage reserves.

MATERIALS AND METHODS

Plant Material. *Triticum aestivum* L. var. Cheyenne was germinated, vernalized for 6 weeks at 7°C, grown in a greenhouse. Heads at various stages after anthesis were collected and stored at −80°C. Seeds were separated from pericarp and other maternal tissue under liquid N_2. Three day seed was purified further by removing seeds retained on a No. 8 Tyler screen (2.4 mm pores).

Isolation and Gel Electrophoresis of Endosperm Proteins. Gliadins were purified by 70% ethanol extraction followed by electrophoresis on pH 3.1 aluminum lactate (ICN Pharmaceuticals) polyacrylamide gels (15). The two predominant Coomassie-stained bands corresponding to the α/β- and γ-gliadins were excised and the gel slices were washed with 100 mM Tris-HCl (pH 8), macerated, and soak-eluted at 37°C with 1% SDS, 0.05 M Tris-HCl (pH 7.9), 0.1 mM EDTA, 0.15 M NaCl. Eluted protein was precipitated with five volumes of cold acetone and sedimented at 28,000g for 15 min. The pellet was dried *in vacuo* and resuspended in 1% SDS to 1 mg/ml. Alcohol extracted proteins were analyzed using a two-dimensional gel system with the first dimension being a 20 mM sodium lactate gel (pH 3.1) (25). Thin strips were cut from the first dimension gels, equilibrated in SDS sample buffer, and applied to SDS polyacrylamide gels (12).

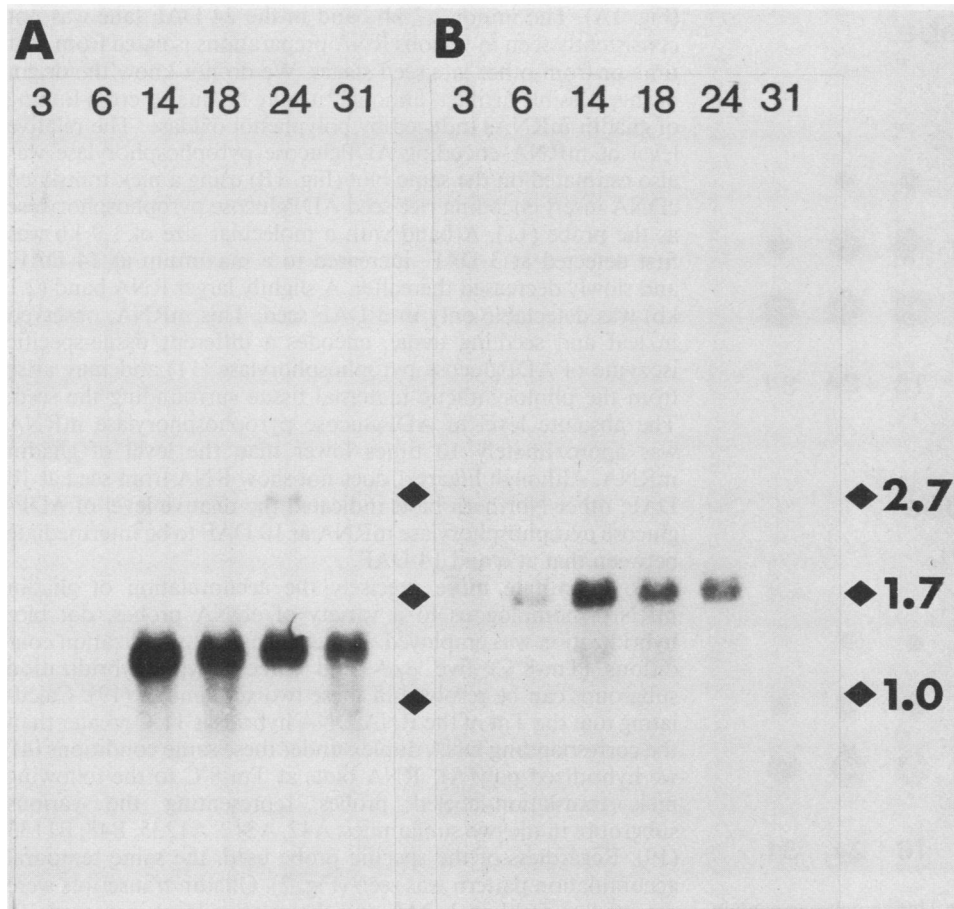[2] Abbreviations: DAF, days after flowering; kb, kilobases.

FIG. 1. Northern blot analysis of mRNAs during development. One μg of poly(A)⁺ RNA was applied to each lane and resolved on a 1% agarose-formaldehyde gel alongside identically treated EcoR1/ScaI cut pUC19 fragments. The gel was blotted to GeneScreenPlus and the blot was hybridized to cDNA inserts labeled by nick translation. In panel A the cDNA probe was from clone A212 (19), an α/β-gliadin sequence. In panel B, the cDNA probe was AGP-1 (11), an ADPglucose pyrophosphorylase sequence. The developmental stage from which the RNA was isolated is indicated at the top and the position of the size markers is indicated at the right.

Two-dimensional (O'Farrell) gel electrophoresis was carried out as described (17) with sample loading at the acidic (anodic) end of the first dimension gels. Gels were visualized by autofluorography and densitometry was performed on a Beckman DU-8 spectrophotometer with a gel scanner attachment.

Antibodies. Female albino rabbits were subcutaneously injected at 2 week intervals with 0.5 mg of purified gliadin protein in complete Freund's adjuvant and serum was collected 1 week after the third injection. Antiserum to spinach leaf ADPglucose pyrophosphorylase was a generous gift from Dr. Jack Preiss (Michigan State University, East Lansing). IgG was purified by chromatography on DEAE-Sephadex or protein A Sepharose (10).

Protein Blotting and Immunodetection. Western blotting to nitrocellulose sheets was essentially as described (6). The sheets were incubated in heat-sealable bags with 36 μg/ml purified IgG and then washed with Tris-saline. One μCi of ^{125}I-labeled *Staphylococcus aureus* protein A (New England Nuclear) was added and incubation continued for 30 min. After extensive washing, immunoreactive bands were visualized by autoradiography using a Cronex (DuPont) intensifying screen.

RNA Isolation. All labware and solutions, where appropriate, were sterilized to eliminate ribonuclease. The following methods were adapted from Lizzardi (14). Each g of seed pulverized under liquid N_2 was suspended in 10 ml of buffer containing 4 M guanidine HCl, 10 mM Tris-HCl (pH 8), 10 mM EDTA, 0.5% (w/v) Sarkosyl, and 3% (w/v) insoluble polyvinylpolypyrrolidone. After phenol:chloroform extraction, nucleic acid was precipitated at −20°C for 2 h by addition of 1/100 vol of 3 M sodium acetate (pH 4.8), and 1 vol ethanol. After centrifugation at 12,000g for 10 min, the pellet was resuspended in 1% Sarkosyl containing 100 μg/ml proteinase K using a glass-Teflon homog-

enizer and warming to 50°C. For each 2.5 ml, 1 g CsCl was added and this solution was centrifuged at 200,000g for 16 h. The RNA pellet was dissolved in 10 mM Tris (pH 7), 100 mM NaCl, 0.5% SDS, and ethanol precipitated. Poly(A)⁺ RNA was obtained by oligo(dT)-cellulose chromatography.

In Vitro **Translation and Immunoprecipitation.** One microgram of poly(A)⁺ RNA and 25 μCi of [^{35}S]methionine (1100 Ci/mol, NEN) were added per 25 μl wheat germ translation reaction (1). Aliquots containing 1 × 10⁶ hot TCA-precipitable cpm were incubated with specific antisera and protein A-Sepharose as described (10). Total translation products were acetone precipitated and dried *in vacuo*. Acetone pellets or dried protein A-Sepharose with immunoconjugated translation products were prepared for electrophoresis by suspending in SDS sample buffer and heating to 100°C for 2 min.

Cloning of ADPglucose Pyrophosphorylase mRNA. A cDNA library of rice seed poly(A)⁺ RNA was constructed in λgt11 and clones were selected by screening with purified anti-ADPglucose pyrophosphorylase antibody as previously reported (9). Details of this cloning are presented elsewhere (11).

RNA Blotting and Hybridization. Poly(A)⁺ RNA (1–2 μg) was resolved on 1% agarose-formaldehyde gels (16), transferred by capillary blot to GeneScreen Plus (New England Nuclear) and hybridized according to the manufacturer's recommendations. Formaldehyde-denatured poly(A)⁺ RNA samples or alkali-denatured plasmid DNA was applied to nitrocellulose in a Schleicher and Schuell manifold as recommended by the manufacturer. RNA blots were probed with cDNA inserts labeled by nick translation (16). The cDNA inserts used for probes were all near full length and included representative clones from different hybridization subgroups within both the α/β- and γ-type gliadin gene subfamilies as described previously (19). DNA blots were
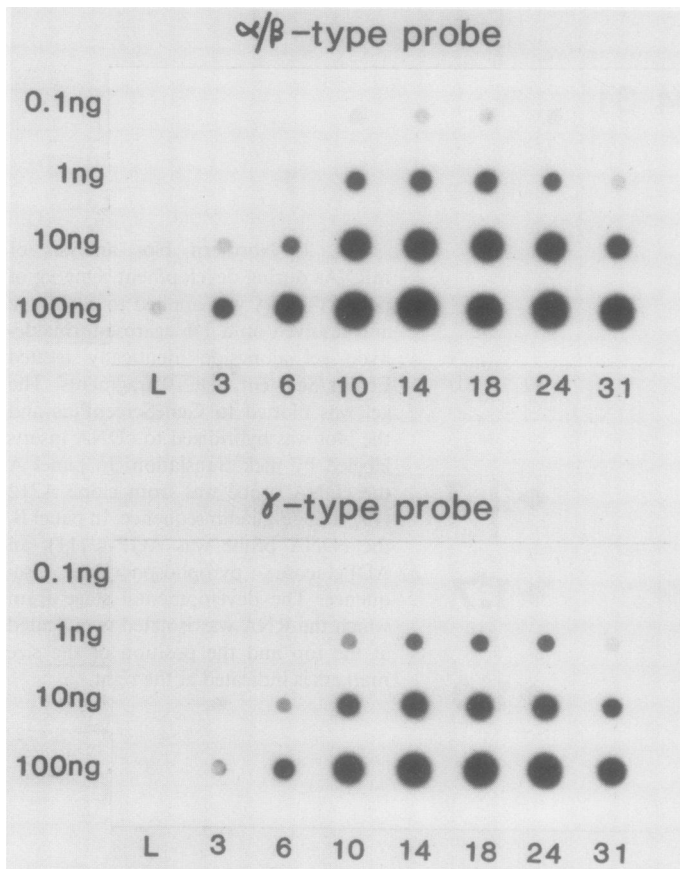
FIG. 2. RNA dot blot hybridization analysis. Horizontal rows contain equal amounts of poly(A)$^+$ RNA as indicated on the left. Vertical columns contain RNA from leaf (L) and various stages of seed development (in DAF) as indicated at the bottom. Hybridization was for 20 h at Tm-8°C using the cDNA inserts A212 (upper blot) and B1133 (lower blot) labeled by nick translation.

probed with ^{32}P-labeled cDNA reverse transcribed from oligo(dT)-primed poly(A)$^+$ RNA (16). The hybridization buffer contained 50% formamide, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 2× Denhart's solution, 10% dextran sulfate, 100 µg/ml denatured salmon sperm DNA, and 10 µg/ml poly(A). The Tm of gliadin cDNA duplex was determined experimentally to be 56°C under these conditions (19); the T_m of the corresponding RNA:DNA hybrid was calculated to be about 67°C (4).

RESULTS

Accumulation of the mRNAs for Gliadin and ADPglucose Pyrophosphorylase. Poly(A)$^+$ RNA was isolated from different stages of developing endosperm and analyzed by *in vitro* translation. The translational activity per µg of poly(A)$^+$ RNA isolated from any of these stages was always at least equal to that of purified cowpea chlorotic mottle virus RNA (gift of Dr. S. D. Wyatt, WSU). SDS-PAGE of the *in vitro* translation products gave results similar to those of Greene (8). The putative gliadin bands appeared coordinately, were just detectable at 6 DAF, peaked at 14 DAF, and remained high up to 31 DAF.

The physical intactness, size, and relative amount of gliadin mRNA at different stages of development was determined by Northern blot analysis (Fig. 1A). Messenger RNA for the α/β-gliadins could be detected at 3 DAF, though at very low levels, and increased most dramatically between 6 and 14 DAF. Similar results were obtained using the γ-gliadin probe B48 (18), except that the size of the RNA transcripts was 1.4 kb instead of 1.2 kb

(Fig. 1A). The minor 2.7 kb band in the 24 DAF lane was not consistently seen in various RNA preparations isolated from that time or from other late seed stages. We do not know the origin of this possible artifact, although it may be due to cross-linking of gliadin mRNAs induced by polyphenol oxidase. The relative level of mRNA encoding ADPglucose pyrophosphorylase was also estimated on the same blot (Fig. 1B) using a nick translated cDNA insert encoding rice seed ADPglucose pyrophosphorylase as the probe (11). A band with a molecular size of 1.9 kb was first detected at 3 DAF, increased to a maximum at 14 DAF, and slowly decreased thereafter. A slightly larger RNA band (2.1 kb) was detectable only in 3 DAF seed. This mRNA, observed in leaf and seedling tissue, encodes a different tissue-specific isozyme of ADPglucose pyrophosphorylase (11) and may arise from the photosynthetic maternal tissue surrounding the seed. The absolute level of ADPglucose pyrophosphorylase mRNA was approximately 10 times lower than the level of gliadin mRNA. Although Figure 1 does not show RNA from seed at 10 DAF, other Northern blots indicated the relative level of ADP-glucose pyrophosphorylase mRNA at 10 DAF to be intermediate between that at 6 and 14 DAF.

To quantitate more precisely the accumulation of gliadin mRNAs homologous to a variety of cDNA probes, dot blot hybridization was employed. Under stringent hybridization conditions (Tm-8°C) five α/β- and three γ-type hybridization subgroups can be resolved in these two subfamilies (19). Calculating that the Tm of the RNA:DNA hybrids is 11°C greater than the corresponding DNA duplex under these same conditions (4), we hybridized poly(A)$^+$ RNA blots at Tm-8°C to the following nick translation-labeled probes representing the various subgroups in the two subfamilies: A42, A212, A1235, B48, B1133 (19). Regardless of the specific probe used, the same temporal accumulation pattern was seen (Fig. 2). Gliadin transcripts were detected as early as 3 DAF and the relative level increased 10-fold by 6 DAF and 100-fold by 10 DAF. As an alternate approach, cDNA plasmids representing the various hybridization subgroups were bound to nitrocellulose, and these filters were probed with ^{32}P-labeled cDNA made from poly(A)$^+$ RNA at 6 and 18 DAF (not shown). When the blots were exposed to film to the same overall intensity, the relative intensity of hybridization between the various clones was the same using 6 or 18 DAF cDNA probe. These experiments indicate that at least the more abundant mRNAs within the α/β- and γ-gliadin subfamilies accumulate coordinately.

Accumulation of the Polypeptides. Proteins extracted with 70% ethanol (the classical gliadin fraction) were resolved on two-dimensional gels allowing about 35 individual spots to be unambiguously identified (not shown). Between 10 DAF, the earliest time at which gliadins could be detected, and 31 DAF there was no significant difference in the gel pattern indicating coordinate accumulation of the polypeptides. To quantitate gliadin accumulation more precisely and analyze antigenic differences between α/β- and γ-type gliadins, antibodies were raised to these two gliadin components as described in "Materials and Methods." On SDS polyacrylamide gels, the purified γ-gliadin fraction gave two bands with mol wt of 41 and 37 kD, while the α/β-gliadin fraction gave major and minor bands of 34 and 31 kD, respectively. Rabbit antibodies obtained from these two fractions were shown by Ouchterlony double diffusion to cross-react, indicating either extensive antigenic similarity or cross-contamination of the purified fractions. Antigenic similarity is expected in view of the sequence homology between these two gliadin subfamilies (19).

A Western blot of total endosperm proteins probed with γ-gliadin antibodies is shown in Figure 3A. Both antibodies recognized both the α/β- and γ-gliadin bands at approximately 32 and 40 kD, respectively. This confirmed the cross-reactivity.
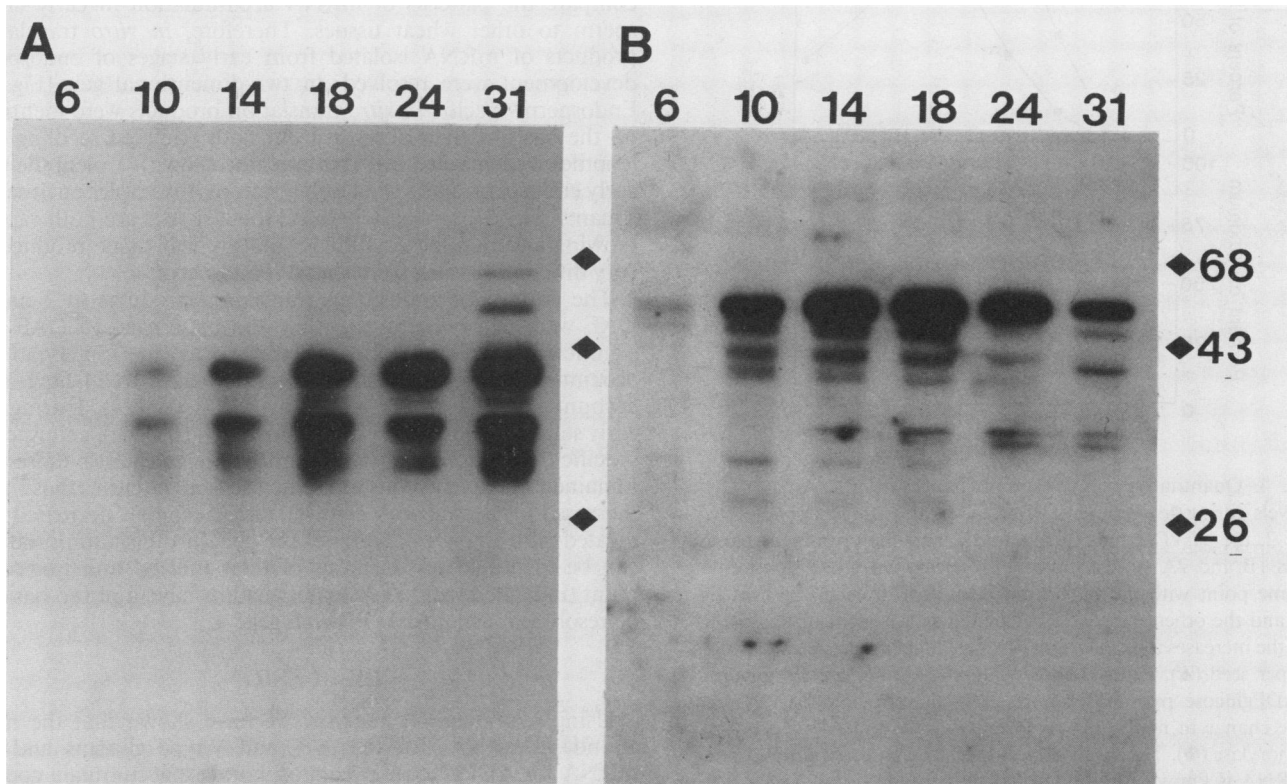
FIG. 3. Western blot analysis of polypeptide accumulation. Endosperm proteins were extracted with SDS sample buffer, resolved on a 12% polyacrylamide gel, and electrophoretically transferred to nitrocellulose. In panel A the blot was probed with γ-gliadin antibody and in panel B the blot was probed with ADPglucose pyrophosphorylase antibody. After incubation with ^{125}I-labeled protein A, the blots were exposed to film for 8 h using an intensifying screen. The developmental stages are indicated at the top and the mol wt markers are indicated at the right.
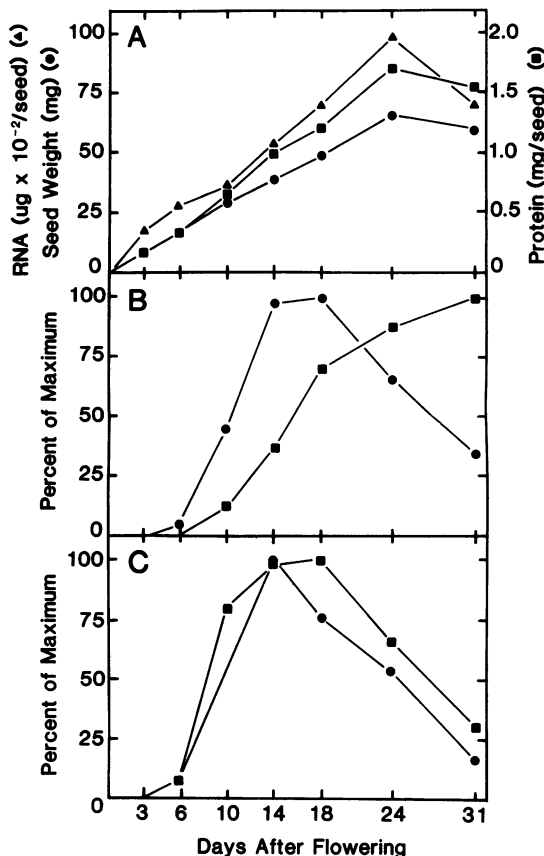
FIG. 4. Quantitative comparison of changes in mRNA and polypeptide levels during development. Rows for 10 and 100 μg applied RNA in Figure 2, the 47 kD bands of Figure 2B, and the prominent bands of Figures 1B and 2A were scanned on a Beckman DU-8 densitometer. The time point with the maximum peak area in each case was set to 100% and the other areas were calculated as a percent of this. Panel A shows the increases in seed weight (●), protein per seed (■), and poly(A)⁺ RNA per seed (▲). Panel B shows the relative increases in gliadin (■) and ADPglucose pyrophosphorylase (●) protein. Panel C shows the relative change in mRNA levels for gliadin (■) and ADPglucose pyrophosphorylase (●). The 10 DAF point for ADPglucose pyrophosphorylase mRNA is known to be intermediate between 6 and 14 DAF from other Northern blots (see text).

Gliadin was not detectable at 6 DAF or earlier, even when the blots were overexposed three times longer than shown in Figure 3A. The two minor higher mol wt bands that appeared at 31 DAF in Figure 3A could not be detected using α/β-gliadin antibody even when the blots were 10-fold overexposed. Thus, there was at least some difference in the specificity of the antibodies. The two bands could also be detected on Coomassie-stained SDS gels and clearly did not accumulate coordinately with the bulk of the gliadins.

The developmental changes in ADPglucose pyrophosphorylase polypeptide levels were also quantitated by Western blot (Fig. 3B). The size of the mature polypeptide was 50 kD; it was first detected at 6 DAF, increased to a maximum at 14 to 18 DAF, and decreased thereafter.

Comparison of the Kinetics of Accumulation. Densitometer values for each time point on the Northern and Western blots were plotted as a percent of the maximum value for each blot and these plots are shown along with the increases in seed weight, protein, and RNA content in Figure 4. Since both protein and poly(A)⁺ RNA content increased in parallel with seed weight, changes in the percent of maximum values approximately reflect changes in the absolute amount per unit weight of seed. The

profiles for gliadin and ADPglucose pyrophosphorylase mRNA are very similar, both with respect to the rate of increase and the subsequent decrease. The respective changes in polypeptide levels, on the other hand, are not similar. ADPglucose pyrophosphorylase polypeptide follows approximately the accumulation profile of its mRNA, while that of gliadin polypeptide lags considerably behind. Furthermore, the level of ADPglucose pyrophosphorylase decreases after 18 DAF, whereas the gliadin level continues to increase through 31 DAF.

Two-Dimensional Gel Survey of the Major Changes in mRNA Transcripts during Early Endosperm Development. In view of the coordinate accumulation of gliadin and ADPglucose pyrophosphorylase mRNAs, it was of interest to survey the overall changes during this same time. Furthermore, it was of interest to compare the patterns of mRNA accumulation in early endosperm to other wheat tissues. Therefore, *in vitro* translation products of mRNA isolated from early stages of endosperm development were resolved on two-dimensional gels (Fig. 5). Endosperm-specific *in vitro* translation products were identified on the basis of their absence from both etiolated seedling (coleoptile) and mature leaf (pattern not shown). Coleoptile and early endosperm had very similar patterns (examples enclosed in squares, Fig. 5), probably because these tissues are both rapidly growing and nonphotosynthetic. Mature leaf tissue produced a very different pattern from that of endosperm.

The pattern of endosperm translation products at 3 and 6 DAF were nearly identical, while the pattern at 10 DAF was significantly different. The difference was due largely to the accumulation of gliadin mRNAs, which at 14 to 24 DAF may account for 60% of the total poly(A)⁺ RNA (22). During the early stages of endosperm development (3–10 DAF), endosperm-specific translation products fell into three categories: those that remained relatively constant (enclosed in circles), those that increased (indicated with arrows), and those that decreased (indicated with triangles). Some of the gliadin translation products can be identified on the basis of their mol wt and isoelectric point (indicated with a G); many gliadins have a pI too basic to be resolved on standard O'Farrell gels.

DISCUSSION

Using hybridization analysis, we have shown that the most abundant mRNAs for the α/β- and γ-type gliadins and the mRNA for ADPglucose pyrophosphorylase accumulate coordinately during endosperm development. This is consistent with the fact that the timing of starch accumulation (26) roughly parallels storage protein accumulation and seed weight increase. Gliadin mRNA was detected at about 1% of the maximum level as early as 3 DAF and since these same gliadin sequences were not detected in seedling or leaf tissue, transcription of gliadin genes is tissue-specific and begins very early in development. This is also true for ADPglucose pyrophosphorylase mRNA, which has been shown to have tissue-specific forms in cereals (11).

Gliadin and ADPglucose pyrophosphorylase polypeptides had quite different accumulation patterns in contrast to the coordinate accumulation of their mRNAs. The fact that gliadin polypeptides were not observed until several days after gliadin mRNA was detected suggests that there may be additional control at the translational level, as has been demonstrated for the oat globulins (7). Alternatively, gliadins may turn over rapidly in the early stages, but not at later stages of endosperm development. More rapid turnover in early *versus* late stages has been reported for β-conglycinin in developing soybean cotyledons (24), as well as in developing embryos of transgenic petunia (2). The continued increase in gliadins compared to the decrease in ADPglucose pyrophosphorylase at the later stages indicates that gliadin is a more stable protein during the later stages, suggesting that glia-
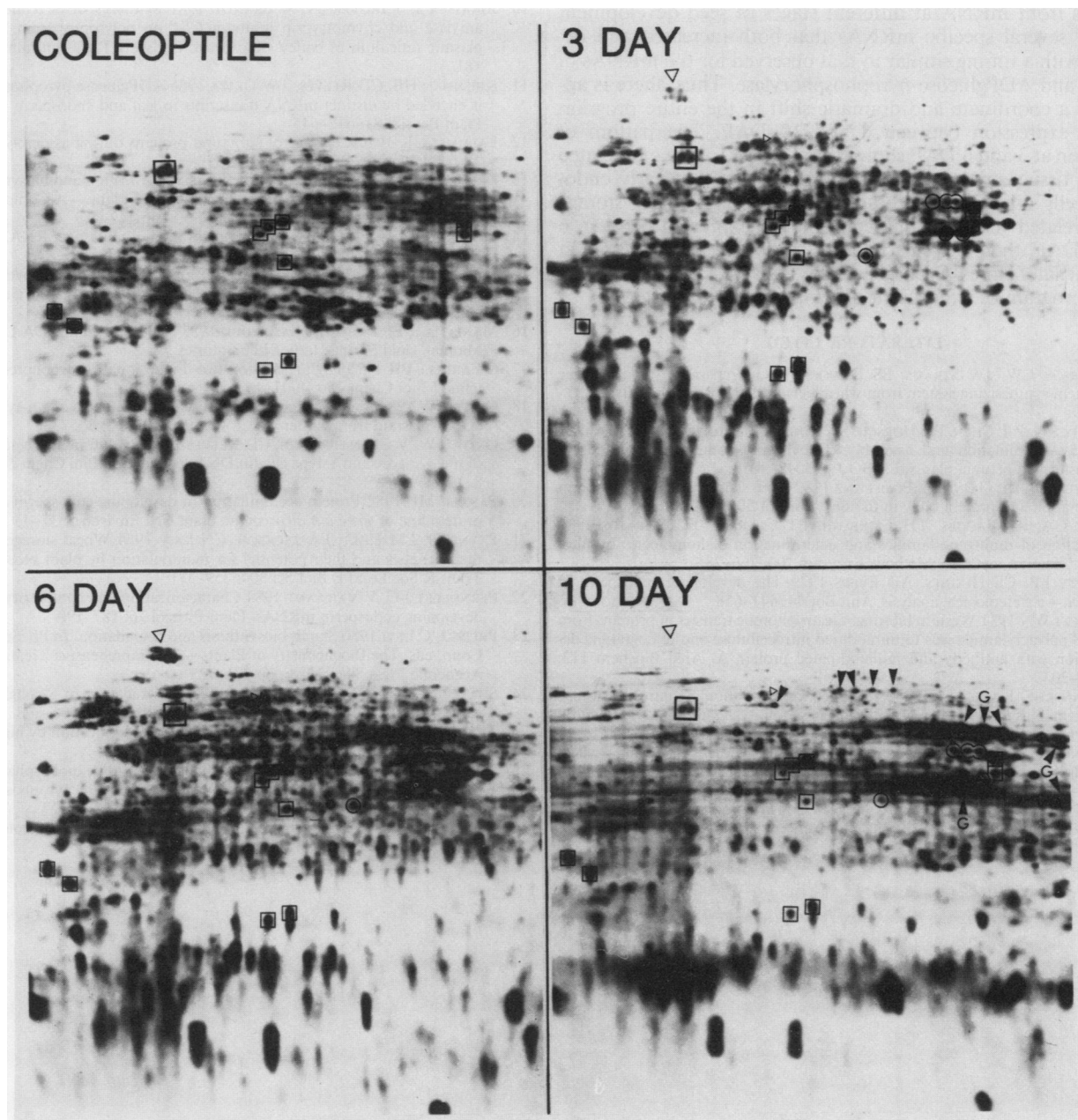
FIG. 5. Patterns of *in vitro* translation products during early endosperm development. Shown are two-dimensional gel separations of translation products programmed by poly(A)$^+$ RNA from coleoptile, 3, 6, and 10 DAF seed as indicated at top left. Photographs are oriented with pI≈4 on the left and pI≈7 on the right. Separation in the second (SDS) dimension is from top to bottom. Squares indicate spots present in all four cases. Circles indicate spots present only in endosperm but relatively constant with respect to development. Endosperm-specific spots which increased between 3 and 10 DAF are indicated with arrows; gliadins are identified with a G. Spots which decreased are indicated with triangles. See text for details.

dins become increasingly protected within the dense protein bodies. It is also possible that ADPglucose is specifically degraded during this later stage. It is not yet clear whether the pathway of gliadin deposition into protein bodies is golgi-mediated as for β-conglycinin, as suggested by Parker (20), or whether gliadins are deposited directly into RER vesicles as are the zeins of maize (13).

The detection of gliadin mRNA at 3 DAF indicates, as mentioned above, that transcription begins very early in endosperm development, yet the mRNA does not begin to accumulate rapidly for 3 to 4 d. This may be simply because various trans-acting factors required for efficient transcription of gliadin genes must accumulate, but may also reflect yet other levels of regulation.

Additional polypeptide bands related immunologically to γ-, but not α/β-gliadins, were seen to accumulate much later than did the gliadins (31 DAF as opposed to 10 DAF). The intensity of their interaction with γ-gliadin antibody on Western blots was much less than expected from their relative prominence on Coomassie-stained gels, suggesting that they share only some immunological epitopes with the γ-gliadins. Since these two proteins were not detected in the 70% ethanol-soluble fraction and had sizes in the range of 36 to 52 kD, they could be low mol wt glutenins which are known to be related to γ-gliadins (19). As they do not accumulate coordinately with the bulk of the gliadins, it will be interesting to identify these proteins and determine how their mRNAs accumulate during development.

Two-dimensional gel electrophoresis of *in vitro* translation

products from mRNA at different stages of seed development revealed several specific mRNAs that both increased and decreased with a timing similar to that observed for the mRNAs of gliadins and ADPglucose pyrophosphorylase. Thus, there is apparently a coordinate and dramatic shift in the entire program of gene expression between 6 and 10 DAF. The pattern of expression at 3 and 6 DAF showed similarities to that of etiolated seedling tissue, presumably reflecting the fact that early endosperm cells are rapidly proliferating. Although many storage reserve-related genes may be coordinately expressed between 6 and 10 DAF, there are clearly other genes that are expressed at earlier or later times. Such genes may be very important in the overall program of endosperm development.

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