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Hordein-gene expression during development of the barley (*Hordeum vulgare*) endosperm

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A previous study [Rahman, Shewry & Miflin (1982) *J. Exp. Bot.* **33**, 717–728] showed differential accumulation of the major storage proteins (called B and C hordeins) in developing endosperms of barley (*Hordeum vulgare*). To determine how this accumulation is regulated, we have studied mRNA fractions prepared from similar endosperms. Hordein-related mRNA species were detected some days before the deposition of hordeins *in vivo*. The translation products *in vivo* directed by polyribosomes, polysomal RNA and total cellular RNA showed similar changes in the proportions of the hordein products to those observed in the accumulations of the proteins *in vivo*. There was a relative increase in one of the subfamilies of B hordeins (called B1 hordein) and a decrease in the second subfamily of B hordeins (B3 hordein) and in C hordeins. The populations of RNA species related to these three groups of hordeins were studied by 'dot hybridization', with specific complementary-DNA probes for B1-, B3- and C-hordein-related sequences. This showed a 10–15-fold increase in sequences related to the B1 hordein during endosperm development, but only a 4-fold increase in sequences related to B3 and C hordeins. These results indicate that the rates of synthesis of hordeins are related to the abundance of their respective mRNA species. The different results observed for the two subfamilies of B hordeins are of interest, since they indicate differential expression of two subfamilies of genes present at a single multigenic locus.

Hordeins are alcohol-soluble storage proteins present in the barley endosperm. They are rich in glutamine and proline and poor in charged amino acids, notably the essential basic amino acid lysine (Shewry *et al.*, 1980*a*; Miflin *et al.*, 1982). Hordeins can be separated into about ten bands by SDS/polyacrylamide-gel electrophoresis amd 40 components by two-dimensional isoelectric focusing–SDS/polyacrylamide-gel electrophoresis (Rahman *et al.*, 1982); the exact number and electrophoretic mobilities of the polypeptides varies between genotypes (Shewry *et al.*, 1978). Despite this polymorphism, the components can be classified into a small number of groups, the members of which have a high degree of structural homology (Faulks *et al.*, 1981; Shewry *et al.*, 1981). Two of these groups, called B and C hordein, together account for over 95% of the total hordein and are coded for by linked structural loci, designated *Hor 2* and *Hor 1* respectively, located on the short arm of chromosome 5 (Doll & Brown, 1979; Shewry *et al.*, 1980*b*; Jensen *et al.*, 1980). SDS/polyacrylamide-gel electrophoresis of B hordein from cultivars containing the *Hor 2*Ze allele shows two major double bands with apparent M_r values of about 35000 and 46000 (called B1 and B3 hordein respectively) and two minor bands of intermediate M_r (B2). Similarly, SDS/polyacrylamide-gel electrophoresis of C hordein from cultivars with the *Hor 1*Pr allele shows a group of bands of apparent M_r 59000 (C1) and a single band of apparent M_r 72000 (C2) (Faulks *et al.*, 1981). A further group of hordein polypeptides, called D hordein, account for a small proportion of the total fraction (2–4%) (Miflin *et al.*, 1982) and are encoded by a single structural locus (*Hor 3*) on the long arm of chromosome 5 (Shewry *et al.*, 1983).

Abbreviations used: cDNA, complementary DNA; SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; poly(A)$^+$, polyadenylated.

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Hordeins are accumulated relatively late in grain development, first being observed about 22 days after anthesis (when the grain weighs about 33% of its final dry weight) and increasing in amount until maximum dry weight is reached (Rahman *et al.*, 1982), when they account for about 35–50% of the grain nitrogen, depending on nutritional status (Andersen & Køie, 1975; Kirkman *et al.*, 1982). We have shown that in the cultivated variety (cv). Sundance (*Hor 1*Pr, *Hor 2*Ze) the B1 polypeptides make up an increasing proportion of the total hordein fraction during development, with decreases in the relative amounts of B2, B3 and C1 (Rahman *et al.*, 1982).

mRNA species for hordeins have been extracted from membrane-bound polysomes and used to construct a library of cDNA clones (Forde *et al.*, 1981). Analysis of this library has identified clones for each of the major groups of hordeins and suggested that the polymorphism at the protein level is due to the presence of multiple mRNA species, and thus that the *Hor* loci consist of multigene families (Forde *et al.*, 1981; Kreis *et al.*, 1983). The *Hor 2*Ze locus probably consists of two subfamilies of genes, which broadly code for either the B1 or B2 and B3 hordein polypeptides (Faulks *et al.*, 1981; Kreis *et al.*, 1983).

The mechanisms of developmental control of *Hor*-gene expression are not known. In order to provide more information at the molecular level, we have studied the changes in the population of hordein mRNA species during development by using both translation *in vitro* and hybridization to characterized cDNA clones.

Materials and methods

Materials

The plant material used was described previously by Rahman *et al.* (1982). Barley [*Hordeum vulgare* L., cv. Sundance (*Hor 1*Pr, *Hor 2*Ze)] was grown in the field at this Station, and individual ears were labelled at anthesis. The endosperms obtained at indicated times thereafter were frozen in liquid N_2 and stored at −80°C until required. General chemicals were obtained from Sigma, BDH or Fisons. L-[2,3,4,5-^{3}H]Proline (100–130 Ci/mmol) and L-[4,5-^{3}H]leucine (130–190 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Nitrocellulose and restriction enzymes were obtained from Bethesda Research Laboratories and medical-grade X-ray film from Fujimex, Dorcan, Swindon, Wilts., U.K.

Extraction and measurement of hordeins

Hordein was extracted from milled freeze-dried endosperms as described previously (Rahman *et al.*, 1982), with propan-1-ol/2-mercaptoethanol/water (25:1:24, by vol.) as solvent. The different hordein fractions were separated by SDS/polyacrylamide-gel electrophoresis by the method of Forde *et al.* (1981), and the proteins fixed by placing the gels in 10% (w/v) trichloroacetic acid for 16 h. They were then washed in 1.0 M-NaCl and scanned in the same solution at 300 nm in a Beckman gel scanner (Rahman *et al.*, 1982).

Preparation of membrane-bound polyribosomes

This essentially followed the method described previously (Matthews & Miflin, 1980). Polyribosomes, obtained by pelleting through a 54% (w/v) sucrose cushion at 250000$g_{av.}$ for 4 h, were briefly surface-washed with ice-cold sterile distilled water and stored at −80°C in 10 mM-Hepes/KOH (pH 7.6)/1 mM-KCl/4 mM-magnesium acetate/1 mM-dithiothreitol.

Preparation of RNA

Polyribosomal cellular RNA was prepared from polyribosomes by phenol extraction (Wienand & Feix, 1978). Poly(A)$^+$ RNA was obtained from polyribosomal RNA (Matthews & Miflin, 1980) by affinity chromatography using oligo(dT)–cellulose (Bantle *et al.*, 1976).

Total cellular RNA was prepared from 5–10 g of material by the use of methoxyethanol (Ralph & Bellamy, 1964) to remove contaminants after initial deproteinization with phenol/chloroform.

Translation in vitro and quantification of products

A wheat-germ S-30 cell-free extract was prepared by the method of Roberts & Paterson (1973), but omitting the preincubation step. Translations were carried out in 20 μl volumes and samples were treated as described previously (Forde *et al.*, 1981). The products were separated by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and detected by fluorography (Chamberlain, 1979) using preflashed film (Laskey & Mills, 1975). For quantification, tracks were cut out of the fluorographs and scanned at 540 nm with a Beckman gel scanner.

Purification of plasmid DNA

The construction of the cDNA library was described previously by Forde *et al.* (1981). The clones were derived from poly(A)$^+$ mRNA prepared from membrane-bound polyribosomes of endosperms of cv. Sundance and designated pHvE-c1–pHvE-c300 (here abbreviated to pc1–pc300). Plasmid DNA species were purified from cells of chloroamphenicol-amplified transformants, which were lysed with Triton X-100 (Forde *et al.*, 1981).

Dot hybridization

Plasmid DNA was nick-translated as described by Rigby *et al.* (1977). Usually 30–60% of the [^{32}P]-dATP was incorporated, to give DNA of specific radioactivity (1–2) × 10^7 c.p.m./μg.

Nitrocellulose sheets were pretreated as described by Thomas (1980) and used to bind RNA. The RNA was diluted in 20 × SSC (20 × SSC is 3M-NaCl/0.3M-sodium citrate), heated to 100°C for 60s, chilled immediately in liquid N_2 and applied as 200μl samples using a Bethesda Research Laboratories Hybridot apparatus. After application of the samples, the wells were washed with 3vol. of 20 × SSC. The filter paper was then dried *in vacuo* at 80°C for 2h.

Conditions used for hybridization were moderately stringent [T_m (the 'melting' point of DNA) −15°C] and correspond closely to those used for hybrid selection of RNA (Forde *et al.*, 1981), except that the final wash in EDTA was not performed. The dot hybridization took place under conditions in which the reaction was far from completion (Kafatos *et al.*, 1979) (~10% completion for an error of about 5%). Under these conditions the concentration of hybridizable sequences is related to the extent of hybridization. No difference in the results was found when two different concentrations of RNA were applied. After drying, the nitrocellulose filters were exposed at −80°C to preflashed film in cassettes using intensifying screens. The intensities and areas of the spots were measured with a Quantimet image-analysing computer.

Results

Hordein deposition

Nitrogen was present in the hordein fraction 18 days after anthesis (Fig. 1*a*), although at this stage it was not possible to recognize hordein polypeptides on SDS/polyacrylamide electrophoresis gels (Rahman *et al.*, 1982); these were first observed at 22 days. From 22 days onwards, changes were observed in the relative amounts of the different hordein fractions, with decreases in the proportion of B2, B3 and C1 polypeptides and an increase in B1 (Fig. 1*a*).

Yield of polyribosomes and polyribosomal RNA

Membrane-bound polyribosomes were prepared from barley endosperms at 14, 18, 22, 26 and 34 days after anthesis. The yield of polyribosomes per endosperm increased over 20-fold between 14 and 24 days and thereafter declined almost as rapidly (Fig. 1*b*). A similar curve was observed for the yield of polyribosomal RNA (Fig. 1*b*). Surprisingly, the maxima for both occurred about 12 days before the period of most rapid storage-protein accumulation (cf. Fig. 1*a*). Similar results for total RNA have been reported for maize (*Zea mays*) and wheat (*Triticum*) (Ingle *et al.*, 1965; Donovon, 1979; Viotti *et al.*, 1975). Unfortunately we were unable to obtain quantitatively reliable yields of total RNA throughout all stages of development (results not shown).

Synthesis in vitro of hordein polypeptides directed by mRNA from developing endosperms

Polyribosomes, polyribosomal RNA, polyribosomal poly(A)$^+$ RNA and total RNA were translated in a wheat-germ system and the products separated by SDS/polyacrylamide-gel electrophoresis and detected by fluorography (Figs. 2*a*–2*d*). The major polypeptides present in the B- and C-hordein regions of the gels have been previously identified as hordeins on the basis of their mobilities, tissue-specificity, characteristic band patterns, low incorporation of [^{14}C]lysine compared with [^{3}H]proline or [^{3}H]leucine, and by hybridization of their mRNA species to hordein-related cDNA clones (Matthews & Miflin, 1980; Forde *et al.*, 1981; Kreis *et al.*, 1983; M. Kreis & S. Rahman, unpublished work). Hordein was first detected in the products of the polysomal poly(A)$^+$ fraction at about 14 days after anthesis. This was some 8 days earlier than the detection of hordein polypeptides *in vivo* (Fig. 1*a*).

The relative amounts of B1, B3 and C hordeins synthesized *in vitro* from the various mRNA preparations were quantified by densitometric scanning of the fluorographs. The results show (Figs. 2*e*–2*h*) that the relative amount of B1 hordein synthesized increased and those of B3 and C hordeins decreased as endosperm development proceeded. The ratio of B1 to B3 hordeins in the translation products of the different RNA fractions increased from 1.8 for those isolated from 18-day endosperms to about 2.5 for mRNA from endosperms 35 days after anthesis. These ratios were very similar for the products of polyribosomes and total RNA products (compare Fig. 2*e* with Fig. 2*h*) and were not affected by increasing the concentration of RNA in the translation mixture (results not shown). We therefore infer that there was no translational competition between B1- and B3-hordein mRNA. The ratio of B1 to C hordeins in the translation products of polyribosomes increased from 1.5 at 18 days to 2.5 at 34 days; this differed from the products of total RNA, when the ratio increased from 1.6 to 5.0 over the same period. The ratio of B3- to C-hordein products from polyribosomes remained almost constant (at about 1), but the ratio of B3 to C hordeins in the products of total RNA increased from 1.1 at 18 days to 2.2 at 30 days after anthesis. The proportions of C hordein in the products of translation of polyribo-

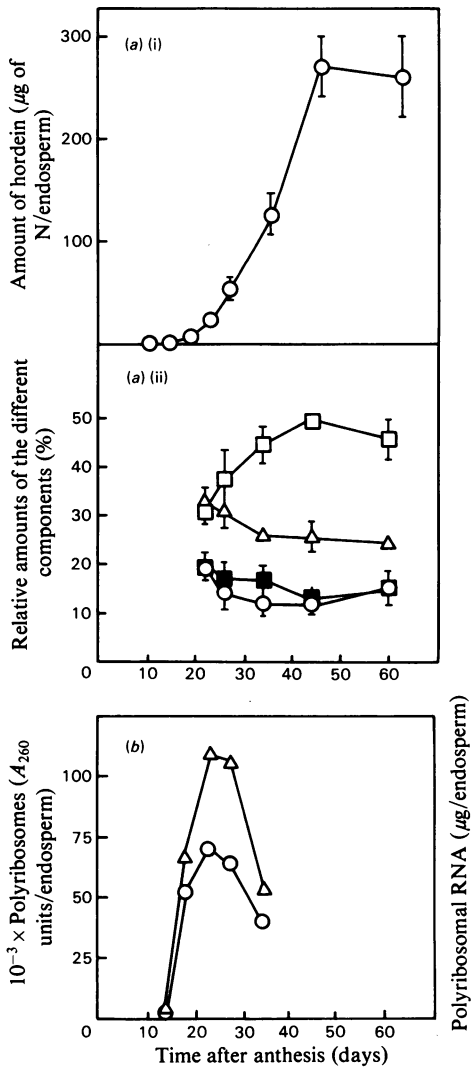

Fig. 1. *(a) Amount of hordein (i) and the proportions of the different components (ii) at different stages of development and (b) yields per endosperm of membrane-bound polyribosomes (△) and polyribosomal RNA (○) from endosperms at different stages of development*

(a) Endosperms were harvested from field-grown barley at various days after anthesis, and hordeins were extracted in propan-1-ol/2-mercaptoethanol/water (25:1:24, by vol.). The amount of hordein (○) was determined by Kjeldahl analysis. The results are the means of four determinations. The hordeins were separated by SDS/polyacrylamide-gel electrophoresis and the relative amounts of the different B and C hordeins (□, B1; ■, B2; △, B3; ○, C1) were determined by gel scanning. Results are the mean of at least two scans of each sample from at least three gels. The error bars represent 2S.D.. The results are taken from the experiments described in detail by Rahman *et al.* (1982). *(b)* Polyribosomes were isolated from the membrane fraction of developing endosperms harvested at various days after anthesis. The yields quoted are the averages for two extractions. RNA was extracted from the polyribosomes with phenol and the results given are based on duplicate extractions. The S.D. was about 10% of the mean value in each case.

somal RNA and polyribosomal poly(A)$^+$ RNA were very low at all stages, and no significant changes could be observed. The difference in the ratio of B to C hordein between the polyribosomal and total RNA products could indicate that there is competition for initiation between B- and C-hordein mRNA species in the wheat-germ system *in vitro* used in these experiments. To confirm the above results in a system independent of mRNA competition, the concentrations of hordein-related RNA sequences were measured by 'dot' hybridization.

Relative abundances of hordein-related RNA sequences during endosperm development

The construction and partial characterization of a cDNA library from barley endosperm polyribosomal poly(A)$^+$ RNA has already been reported (Forde *et al.*, 1981; Kreis *et al.*, 1983). Three cDNA probes were selected from this library as specific for C hordein (pc251), B3 hordein (pc179) and B1 hordein (pc35). Evidence for the identification of these clones has been obtained by translation of hybrid-selected RNA *in vitro* (Fig. 3), and partial sequencing (Forde *et al.*, 1981; Miflin *et al.*, 1983). Plasmid DNA from each of the three clones was labelled by nick translation and hybridized to polyribosomal RNA or polyribosomal poly(A)$^+$ RNA extracted from endosperms of different developmental stages and dotted on to nitrocellulose paper (Thomas, 1980). The stringency of hybridization was the same as that used for the hybrid selection of mRNA.

After hybridization and washing, the relative concentrations of RNA sequences related to B1, B3 and C hordeins were measured from the autoradiographs with a Quantimet image-analysing computer (Figs. 4*a* and 4*d*). The results show that there was a 10–15-fold increase in the concentration of sequences complementary to pc35 (B1-hordein-related) and a 3–4-fold increase in sequences hybridizing to pc179 (B3-hordein-related) and to pc251 (C-hordein-related) during endosperm development. By multiplying the arbitrary values for the concentrations of the different hordein sequences in the polyribosomal RNA by the amounts of polyribosomal RNA per endosperm (Fig. 1) the relative amounts of RNA species related to given sequences can be calculated (Fig. 4*c*). The results show that the increases

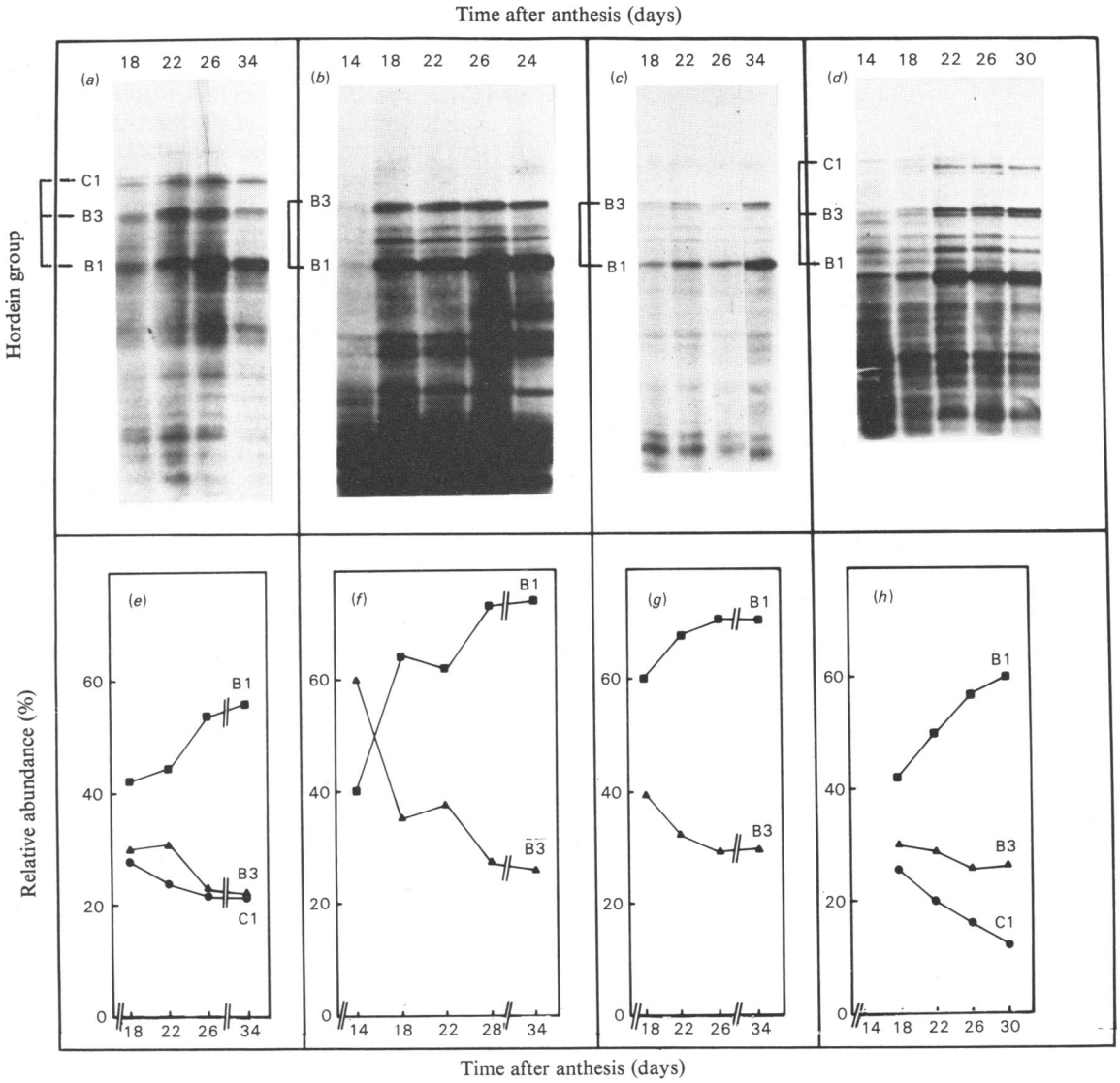

Fig. 2. *Synthesis in vitro of hordeins with RNA fractions prepared from endosperms at different stages of development*
Membrane-bound polyribosomes (*a*), polyribosomal poly(A)$^+$ RNA (*b*), polyribosomal RNA (*c*) and total RNA (*d*) were prepared from endosperms at various days after anthesis and translated in a wheat-germ cell-free system using [^{3}H]proline (polyribosomes) or [^{3}H]proline and [^{3}H]leucine. The products were separated by SDS/polyacrylamide-gel electrophoresis and detected by fluorography. The age of the endosperm is indicated as 14, 18, 22, 26, 30 or 34 days after anthesis. The relative incorporation of the labelled amino acids into the products was determined by densitometric scanning of the fluorographs. Figs. (*e*)–(*h*) refer to the relative proportions in the products of polyribosomes, poly(A)$^+$ RNA, polyribosomal RNA and total RNA respectively.

in the relative amounts of polyribosomal RNA sequences related to B3 and C1 hordein are approximately the same, but about 3-fold less than the increases in sequences related to B1 hordein. Very similar results (Fig. 4*d*) were obtained when the calculations were made for the polyribosomal poly(A)$^+$ RNA, assuming that the poly(A)$^+$ RNA formed a constant proportion of the polyribosomal RNA. Figs. 4(*c*) and 4(*f*) also show that the amounts of hordein-related RNA sequences (i.e. those hybridizing to pc35, pc179 and pc251) per endosperm decreased from about 26 days after anthesis. However, this decrease was much less than that for total polyribosomal RNA (Fig. 1*b*).

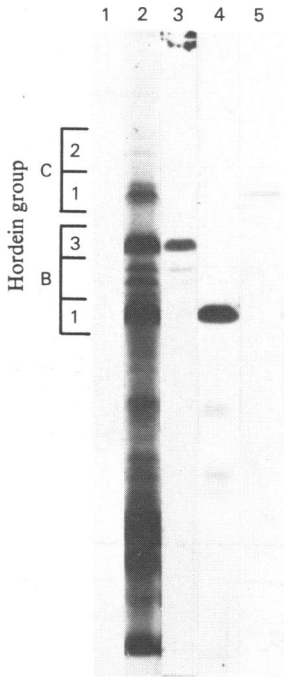

Fig. 3. *Identification of the products of translation in vitro of hybrid-selected RNA*
Plasmid DNA from transformed clones were bound to nitrocellulose and hybridized to poly(A)$^+$ RNA from barley endosperms. The RNA selected was released and translated in a wheat-germ system *in vitro* with [^{3}H]proline and [^{3}H]leucine. The translation products were separated by SDS/polyacrylamide-gel electrophoresis and detected by fluorography. Lanes 1–5 are respectively translation products of no RNA added, unselected RNA, RNA selected by pc179 (related to B3 hordein), RNA selected by pc35 (related to B1 hordein), and RNA selected by pc251 (related to C hordein).

Discussion

The results presented here provide further evidence for the differential expression of the *Hor* loci during barley endosperm development. The changes in the relative abundances of the mRNA species for B1, B3 and C hordeins as measured by translation *in vitro* (Fig. 2), or as inferred from dot hybridization (Fig. 4), agree in broad terms with the changes in the relative amounts of B1, B3 and C hordein synthesized *in vivo*. The major differences between the results observed *in vitro* and *in vivo* are: (1) mRNA species for specific hordein were detected some days before the detection of hordeins *in vivo* (Figs. 1 and 2); (2) the ratio of B1- to B3-hordein mRNA increased little from 26 days after anthesis, whereas the ratio of the protein accumulated continued to increase; (3) the amount of hordein mRNA decreased from 26 days onwards, although the rate of hordein accumulation did not. Some of these differences, particularly (1) and (2), are probably due to the greater sensitivity of the mRNA techniques and to the fact that in the present study we have measured the potential for synthesis, whereas previously we measured actual accumulation (presumed to be due to cumulative synthesis). Thus at day 14 the cumulative amount of hordeins may represent an unmeasurably small amount of cumulative protein synthesized, but nevertheless represent a significant proportion of current protein synthesis, particularly of the membrane-bound polyribosomes. Similarly, changes in the ratios of different hordein polypeptides being synthesized are only gradually reflected in the ratios of the hordeins accumulated during development. The results obtained up to 26 days are therefore in reasonable agreement with the hypothesis that the rates of synthesis *in vivo* of the different hordeins reflect the relative abundances of their mRNA species.

The differences between the changes in the estimated amounts of hordein mRNA from day 26 to 34 after anthesis compared with the rates of hordein accumulated may be real or an artefact. Although all reasonable care was taken, the experiments are technically difficult and the interpretations made rest on the assumptions that: (1) the dot hybridizations took place under conditions in which the reaction was far from completion. Only under these conditions is the concentration of hybridizable sequences related to the extent of hybridization. We found no difference in the results when two different concentrations of applied RNA were used, suggesting that the required conditions had been met. (2) The activity of ribonuclease or the integrity of the polyribosomes did not change during development. However, it is known that the activity of ribonuclease in wheat and maize endosperms is greatest early in development, and then decreases (Dalby & Cagampang, 1970; Donovon *et al.*, 1977). We used extraction conditions that are inhibitory and observed no indications that ribonuclease was active in our preparations. (3) We have assumed that a constant proportion of the total membrane-bound polyribosomes has been extracted at all the stages. One method of verifying this would be by electron-microscopic examination of the extracted tissue. However, Briarty *et al.* (1979) reported a large increase in rough endoplasmic reticulum of 14 days after anthesis in wheat, and in the present study there was a large increase in membrane-bound polyribosomal RNA at 18 days. This suggests that the extraction conditions were efficient. If the results are real, then they imply that a change in the mechanism of the control of hordein synthesis occurs during development.

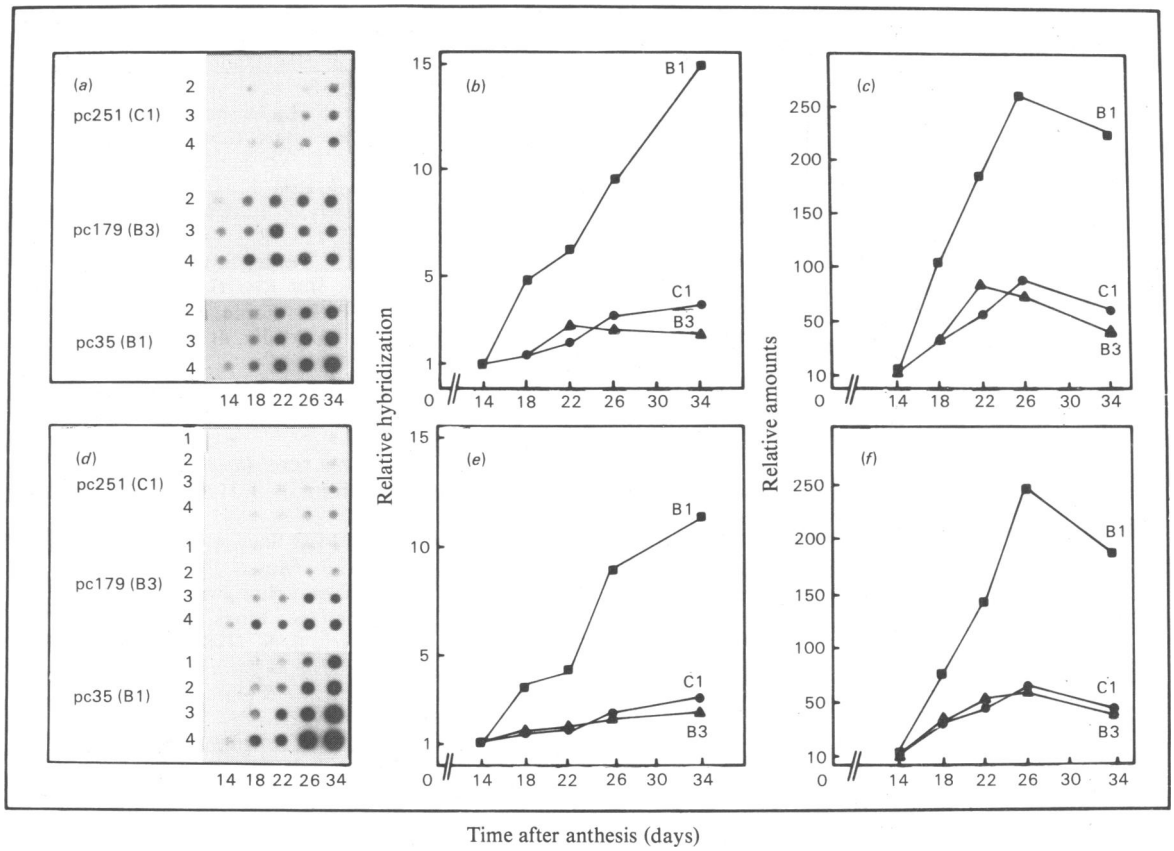

Fig. 4. *Determination of the relative abundances of RNA sequences during endosperm development by dot hybridization* Membrane-bound polyribosomal RNA (*a*) and poly(A)$^+$ RNA (*d*) were prepared from endosperms at different stages of development and applied to nitrocellulose filters at several different concentrations. The filters were hybridized to the indicated plasmid DNA species, which were labelled by nick translation. The hybridization was detected by autoradiography with pre-flashed film. For polyribosomal RNA, 2, 3 and 4 refer to 2, 4 and 8 μg of RNA applied to filters; for poly(A)$^+$ RNA, 1, 2, 3 and 4 refer to 100, 200, 400 and 800 ng. The age of the endosperm is indicated as 14, 18, 22, 26 or 34 days after anthesis. The intensity of hybridization was measured for polyribosomal RNA (*b*) and poly(A)$^+$ RNA (*e*) by using an image-analysing computer to integrate the size and intensity of the 'dots' on autoradiographs. The value at 14 days was taken to be 1.0 for each of the plasmid clones and the values at the later ages are expressed relative to this. The results are the mean of determinations at the two highest concentrations from one (for polyribosomal RNA) or two [for poly(A)$^+$ RNA] autoradiographs. The S.D. was about 20%. By combining the intensity of the hybridization with the amount of polyribosomal RNA (Fig. 1*b*) per endosperm, the relative amount of RNA of a given sequence type during development can be estimated. This is shown for polyribosomal RNA in (*c*) and poly(A)$^+$ RNA in (*f*). For poly(A)$^+$ RNA the assumption is made that it forms a relatively small proportion of the polyribosomal RNA. The amount for 14 days was taken to be 1.0 in each case, and the values at other stages are expressed relative to this.

Control of gene expression may occur at a number of steps (Darnell, 1982). The general agreement between the relative abundancies of the different hordein mRNA species and the estimated rates of synthesis of hordeins *in vivo* during endosperm development up to 26 days implies that translational and post-translational controls are of little importance and that the primary determinant of the amount of hordein synthesized is the amount of mRNA present. The amount of hordein mRNA present is likely to be a function of the rate of hordein-gene transcription and hordein-mRNA turnover. As we have not measured either of these, we cannot present any evidence as to which might be the major factor, although it is tempting to assume, as has been suggested for legume seed storage proteins (Goldberg *et al.*, 1981; Boulter, 1984), that the major control is at the level of transcription.

The discrepancy between the amount of hordein

mRNA and the rate of hordein synthesis in the later stages of development may suggest that the hordein mRNA species are translated preferentially to other RNA species during this period. Changes in the ability of different mRNA species to compete for translation have been observed in other systems (see Lodish, 1976), but further work is required to see if this occurs in the barley endosperm.

Finally, the results presented here provide further evidence for the differential behaviour of the two subfamilies of mRNA species specified by the *Hor 2* locus. Other results have shown that the amounts of these mRNA species are differentially affected by allelic variants (Kreis *et al.*, 1983), mutant high-lysine genes (Kreis *et al.*, 1984) and sulphur stress (Rahman *et al.*, 1983). This suggests that, although the multigene family at this locus is subject to overall developmental controls, the expression of different groups of genes within the family may be modulated according to time of development, nutrient availability or *trans*-acting mutations in other genes.

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