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Molecular biology of the grain storage proteins of the Triticeae

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Chemical studies show that there are close relationships between the storage proteins of the Triticeae. We have investigated these relationships by the study of the synthesis of the proteins *in vivo* and *in vitro*, and by making libraries of double-stranded complementary DNA (cDNA) derived from poly A$^+$ RNA isolated from developing endosperms of barley, wheat and rye.

These cDNA clones have been used to probe the organization and regulation of expression of the *Hor* loci in barley. The results suggest that regulation of synthesis is generally achieved by changes in the amounts of mRNA for the different proteins, both in response to time of development and the relative supply of sulphur and nitrogen, although there may also be differences in the relative amounts of mRNA translated. The sequencing of the cDNA clones has shown the importance of repeated sequences in the evolution of prolamin genes.

Introduction

The chemical nature of the endosperm storage proteins of the Triticeae has been described earlier in this volume (Shewry *et al.* 1983, this symposium). These proteins are tissue-specific in that they have only been reported as being present in the endosperm and they are deposited relatively late during seed development (Bishop 1930; Rahman *et al.* 1982). The proteins are synthesized on the rough endoplasmic reticulum, pass through into the lumen and aggregate into protein deposits (see Miflin *et al.* 1983*a*; Shewry & Miflin 1983).

A number of structural loci for prolamin storage proteins have been described and mapped using conventional genetic analysis (Shewry *et al.* 1983*b*, this symposium; Payne *et al.*, this symposium). Each of these encodes a number of polymorphic proteins and is probably composed of a number of structural genes. In this paper we will review some of our recent studies on the organization of these genes and the control of their expression.

Isolation and identification of cDNA clones related to the major storage proteins

The starting point for RNA preparations has been the isolation of membrane-bound polysomes from barley, wheat and rye endosperms harvested midway through development. When these polysomes and the poly A$^+$ RNA derived from them are translated *in vitro* a high proportion of the products are storage proteins (Brandt & Ingversen 1976; Fox *et al.* 1977; Matthews & Miflin 1980; Greene 1981; J. Forde & Miflin 1983; Shewry *et al.* 1983*a*). The poly A$^+$ RNA isolated from the polysomes by affinity chromatography on oligo-dT cellulose

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was used to make complementary DNA (cDNA) by use of conventional procedures. The cDNA was made double stranded and inserted into a plasmid that was used to transform *Escherichia coli*. The detailed methods used by us are given in B. G. Forde *et al.* (1981) and J. Forde *et al.* (1983).

To date we have generated several thousand clones containing sequences derived from poly A$^+$ RNA obtained from the membrane-bound polysomes of barley, wheat and rye. Many of these clones have been characterized by means of hybrid-selection translation, cross-hybridization and DNA sequence analysis (B. G. Forde *et al.* 1981; Kreis *et al.* 1983*a*; Miflin *et al.* 1983*b*; J. Forde *et al.* 1983). In this paper we will particularly concentrate on clones that contain sequences related to the B-, C- and D-hordeins of barley and the high molecular mass prolamins of wheat. We have used these clones to deduce amino acid sequences for the proteins, to indicate details of gene organization and to study the control of hordein gene expression in response to different nutrient regimes, high-lysine mutant genes and time of endosperm development.

Gene organization
B-Hordein genes

The first clones identified contained sequences related to B-hordeins (B. G. Forde *et al.* 1981). Previous studies had suggested that B-hordein polypeptides could be grouped into three classes on the basis of their cyanogen bromide (CNBr) cleavage patterns. This indicates the position of methionine residues within the polypeptides. Different cultivars of barley contain different alleles at the *Hor 2* locus (the structural locus for B-hordein), and comparison of the CNBr cleavage patterns of the B-hordein polypeptides showed that each cultivar contained polypeptides of two out of the three classes, either class I and class III or class II and class III (Faulks *et al.* 1981). The cDNA clones were prepared by using poly A$^+$ RNA from cultivar Sundance. B-hordein from Sundance is separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS p.a.g.e.) into three fractions called B1, B2 and B3. B1-hordein is largely composed of class I polypeptides and B3 of class III polypeptides. From our library of cDNA clones we have selected some that are related to B1-hordein and some related to B3-hordein.

We have used the B1 and B3 related clones to investigate mRNA populations in other cultivars (Kreis *et al.* 1983*a*). Translation of the poly A$^+$ RNA fractions of the different cultivars showed that they directed the synthesis of products that could be clearly related to known classes of hordein polypeptides, also indicating that hordein polymorphism was not due to post-translational modifications. Hybrid-selection translation showed that the B1 related (i.e. class I related) clones selected mRNAs that directed the synthesis of either class I or class II polypeptides according to the cultivar used whereas B3 (class III) related clones always selected mRNAs that when translated gave class III polypeptides. We therefore concluded that class I and class II polypeptides are generally similar in sequence whereas there are significant sequence differences between polypeptides of these two classes and those of class III. From this we propose there are two major subfamilies of mRNAs, and thus of genes, associated with the *Hor 2* locus.

Subsequently we have identified two longer cDNA clones, one related to B1-hordein and one to B3-hordein. These are being sequenced at the moment and although the information is not yet complete some interesting features have been found. Firstly, the amino acid sequences predicted from the cDNAs differ in a number of positions (they are approximately 80 %

homologous over the common areas sequenced so far) that would result in changes in the isoelectric point and size of the polypeptides specified, and in the position of methionine residues. All these changes are in accord with predictions based on our knowledge of the polypeptides. Secondly the sequence appears to consist of two domains. The partial sequence of one of these has been published (B. G. Forde *et al.* 1981) and shows sequence homology with a subsequently isolated wheat-gliadin cDNA clone (Bartels & Thompson 1983). The second domain has a series of well conserved octapeptide repeats based on a consensus amino acid sequence of Pro-Gln-Gln-Pro-x-Pro-Gln-Gln (B. G. Forde, R. Fry, M. Kreis & M. Williamson, unpublished). Intriguingly this repeat motif is the same as that present in C-hordein peptides and specified by a C-hordein related cDNA clone (see below). This suggests the possibility of a common evolutionary origin for the C-hordein and at least part of the B-hordein gene.

C-Hordein genes

The C-hordein group of polypeptides, encoded by the *Hor 1* locus, are rich in glutamine, proline and phenylalanine. These residues together make up about 80 % of the total in the appropriate proportions of 4:3:1. Amino-terminal amino acid sequencing of position of C-hordein polypeptides (Shewry *et al.* 1980 *a, b*, 1981; Schmitt & Svendsen 1980; Kasarda *et al.* 1983) indicates the presence of a short N-terminal domain followed by several repeats of a pentapeptide with the consensus sequence Pro-Gln-Gln-Pro-Tyr. Subsequent analysis of unordered chymotryptic peptides showed the presence of repeated octapeptides with the consensus sequence Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln (P. R. Shewry & J. F. March, unpublished results).

A cDNA clone selecting mRNAs that direct the synthesis of products with identical mobilities to C-hordein has been identified (Miflin *et al.* 1983 *b*). This clone has been partially sequenced and found to specify a sequence of amino acids at the carboxyl terminus of the protein (B. G. Forde, M. Kreis, R. Fry & M. Williamson, unpublished). The three amino acids at the carboxy terminus of the predicted sequence agree with those found by direct sequencing of C-hordein using carboxypeptidase Y (Schmitt & Svendsen 1980; Shewry *et al.* 1981). Further into the molecule repeated octapeptides are present that also have a consensus sequence of Pro-Gln-Gln-Pro-Phe-Pro-Gln-Gln. This sequence specifies an amino acid ratio of 4 glutamine: 3 proline: 1 phenylalanine. Comparison of the amino acid composition of this peptide with that of the whole protein suggests that this repeat motif occurs through much of the molecule.

High molecular mass prolamin genes

D-Hordein is specified by the *Hor 3* locus in barley and the high molecular mass (h.m.m.)† gluten polypeptides by the *Glu-1* locus in wheat (Shewry *et al.* 1983 *b*; Payne *et al.*, this symposium). These loci are in similar positions on homoeologous chromosomes 5 of barley and 1A, 1B and 1D of wheat and the proteins have many similar properties (Field *et al.* 1982). In screening our barley cDNA library we identified a D-hordein-related clone by hybrid-select translation. We than used this clone to probe a cDNA library derived from wheat endosperm poly A$^+$ RNA and identified five cross-hybridizing clones. These clones have been further characterized in a number of ways and found to have sequences related to the h.m.m. gluten polypeptides (J. Forde *et al.* 1983). Once again sequence analysis shows the presence of repeated sequences and we have deduced tandem repeats based on a consensus sequence of x-Gly-Gln-Gly-Gln-Gln with an interspersed repeat of Gly-Tyr-Tyr-Pro-Thr-Ser-Pro-Gln-Gln.

† These correspond to HMW polypeptides described by the author in previous papers.

From the sequence information available to us at present (see Shewry *et al.*, this symposium; Field *et al.* 1982; J. Forde *et al.* 1983) we have identified two factors that we think might be important in terms of the role of the h.m.m. gluten proteins in breadmaking (see Miflin *et al.* 1983*a* for a discussion). Firstly, there are cysteine residues present at the amino and carboxyl terminii that would allow the polypeptides to be joined end to end to form long linear polymers according to the proposed 'linear-glutelin' hypothesis of Ewart (1977) and, secondly, there is a strong tendency for every third amino acid to be either a glycine or a proline; this suggests that the protein might assume a conformation similar to the polyproline helix (Isemura *et al.* 1983).

CONTROL OF GENE EXPRESSION

Seed development

We have measured the rates of accumulation of the different hordein fractions (Rahman *et al.* 1982) and, if we assume that there is little or no turnover of hordeins (Shewry *et al.* 1979), we can suggest that these reflect synthesis. Although the synthesis of all hordeins appears to start at the same time, the different groups accumulate at different rates. C- and B3-hordein form a greater proportion at the earlier stages of development whereas B1 polypeptides make up an increasing proportion of the total as development proceeds.

We have used the characterized cDNA clones to estimate the amounts of RNA sequences related to the different hordeins that are present during different stages of development and we have also estimated relative mRNA levels by measuring amounts of the different translation products of polysomes, polysomal poly A^+ RNA and total RNA. These results suggest that, in broad terms, the amounts of B1-, B3- and C-hordein mRNA vary during development in the same way as the rate of accumulation of the proteins (Rahman *et al.* 1983*a*). The only discrepancy appears to be that the estimated amounts of mRNA stop increasing, and perhaps even fall slightly, some days before there is any change in the rate of hordein accumulation. This implies that there may be more rapid translation of the hordein mRNAs during the later stages of seed development.

Nutrient status

Increases in nitrogen supply (Kirkman *et al.* 1982) or the imposition of sulphur stress (Shewry *et al.* 1983*c*) have been found to change the proportions of the different hordeins accumulated. When the ratio of nitrogen to sulphur supply was high, a high proportion of C-hordein was accumulated but under extreme conditions of sulphur stress the ratio of B- to C-hordein fell from 4 to 0.25. In terms of absolute amounts per endosperm the actual amount of C-hordein remained unaffected but that of B-hordein decreased. In particular there was a greater effect of sulphur stress on the B1- as compared with the B3-hordeins (Rahman *et al.* 1983*b*).

Estimation of the different mRNA populations obtained by translation of RNA fractions and hybridization of cDNA probes suggested that there were changes in the ratios of the mRNAs present in sulphur-poor as compared to sulphur-rich endosperms. However, these changes were far less marked than those that occur in the proteins; thus whereas the amount of B-hordein fell to about one quarter under sulphur stress, the amount of mRNA was only decreased by around one half. There were also changes in the relative amounts of B1- and B3-hordein mRNAs with B1 mRNA being more affected by sulphur stress (Rahman *et al.* 1983*b*).

High-lysine mutants

We have studied the high-lysine mutants Risø 56 and Risø 1508, both of which accumulate much less hordein than normal cultivars. In Risø 56 the mutant locus has been mapped near to or at the *Hor 2* locus. It results in a preferential decrease in B-hordein accumulation and a compensatory increase in the amounts of C- and D-hordein (Miflin & Shewry 1979). We have found that the major B-hordein polypeptides and the mRNAs that encode them are absent from the developing endosperms of the mutant. Analysis of genomic DNA by Southern blotting by using a B-hordein related cDNA clone suggests that a major deletion of a least 85 kilobases has occurred at the *Hor 2* locus (Kreis *et al.* 1983*b*).

The mutant gene in Risø 1508 has been designated *lys 3a* and mapped to chromosome 7 (Jensen 1979). This mutation results in decreases in the amounts of B- and C-hordeins to about 20% and 7% respectively of the amount in the parental cultivar (Bomi). There is a greater effect on B1 relative to B3 polypeptides. In contrast the amount of D-hordein polypeptides increases fourfold. Detailed analyses of the populations of mRNAs showed that only traces of C-hordein mRNAs were present in developing endosperms of Risø 1508, while the abundances of mRNAs for the B1 and B3 hordeins were reduced to 40% and 5% of those in Bomi. However, the mRNA for D-hordein increased twofold (Kreis *et al.* 1983*c*).

Conclusion

Gene expression

Hordein genes appear to be expressed only in the endosperm; all attempts to find evidence of hordein mRNA in other tissues have failed. This absolute control on expression does not lead to completely coordinated control, rather the relative amounts of mRNAs for the different hordeins vary according to the time of development, the nutritional status and the presence of a mutant gene on another chromosome. This modulation of expression occurs between the products of the different loci and also between the two major subdivisions of the *Hor 2* locus. How the modulation is achieved is not clear, although in broad terms changes in the amounts of proteins reflect changes in amounts of mRNAs, this is by no means absolute. Any conclusions as to the mechanisms involved will depend on more detailed assessments of rates of transcription and mRNA turnover.

Gene organization

Classical genetic analysis has identified loci for the major prolamin storage proteins of barley and wheat. Each locus specifies a polymorphic series of proteins. Chemical analyses have shown that microheterogeneity exists between different polypeptides encoded by one locus and have identified the differences in composition of the proteins specified by different loci. This approach has also emphasized the homology of the different groups of proteins in different members of the Triticeae (Shewry *et al.*, this symposium; Miflin *et al.* 1983*a*). In one particular case, the B-hordeins, polypeptide analysis has suggested that there are at least two subfamilies of proteins specified by one locus (*Hor 2*).

In general terms these results have been confirmed by studies of the cDNA clones that have been identified as being related to the different polypeptides. These have confirmed the subdivision of the *Hor 2* locus and have shown that the expression of the two subfamilies is

modulated separately. The sequencing of the clones has provided at least three interesting pieces of information. Firstly, it has confirmed that microheterogeneity of amino acid sequence exists within the polypeptides encoded by one locus. Secondly, it has shown that each type of polypeptide is composed of a number of different structural domains. In the simplest form this consists of an NH_2 terminal domain, a COOH terminal domain and an internal section that contains a number of repeated blocks of residues that are variants of one or more consensus sequences. The consensus sequences for C-hordeins and h.m.m. gluten polypeptides differ from each other and from that previously reported for zein (Larkins 1983; Messing *et al.* 1983). Thirdly, the amino acid sequences deduced from the B-hordein clones show at least two internal domains, one of these contains only vestiges of repeats while the other contains repeats of a consensus sequence that matches that found in C-hordein.

These observations suggest that the prolamin genes have arisen by cycles of internal sequence duplication in much the same way that has been suggested for the longer reiterated sequences that are generally non-transcribed (Flavell 1980). These genes have then, it is presumed, also undergone cycles of duplication to build up complex loci. Other events such as insertions, deletions, unequal crossing over and point mutations, have probably occurred interspersed into both duplication cycles, thereby giving rise to the polymorphism observed at present. The exact significance of the homology between the repeated sequence in C-hordein cDNAs and the sequence that is repeated in one of the domains of the B-hordein cDNAs is not clear. It is presumed that it reflects either a gene fusion or the derivation of the *Hor 1* locus from *Hor 2*. In either event these studies should provide a fascinating insight into the evolution of prolamin genes in the Triticeae.

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