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ORGANIZATION AND TRANSCRIPTION OF B1 HORDEIN GENES IN HIGH LYSINE MUTANTS OF BARLEY

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A library of double stranded cDNA clones was constructed using mRNA isolated from developing barley endosperms as template. The cDNA clones were classified by restriction endonuclease mapping and by hybridization to single stranded cDNA prepared from mRNA of two hordein deficient mutants of barley. This classification and nucleotide sequence analysis identified cDNAs coding for B1 hordein polypeptides. Hybridization of selected cDNA sequences to a B1 hordein cDNA probe at different conditions of stringency demonstrated the presence of a group of related but not identical sequences. Hybridization of the B1 hordein cDNA probe to restriction endonuclease fragments of barley nuclear DNA suggests that the B1 hordein polypeptides are encoded by a multigene family. Abundant mRNA sequences ranging in size from 1,200 to 1,400 nucleotides were detected by hybridization to the B1 hordein cDNA probe. This size is sufficient to code for the B1 hordein polypeptide precursor, which is estimated to have a molecular weight in the range of 29,000 to 35,000.

Mutant Risø 56 (*hor2ca*), which is defective in the synthesis of B hordein polypeptides, lacked B1 hordein mRNAs, thus strongly indicating that the mutation prevents the transcription of these genes. Mutant Risø 1508 (*lys3a*), which is deficient in the synthesis of all hordein polypeptides, contained detectable amounts of RNA sequences homologous to the B1 hordein cDNA, although the hybridization level was reduced in comparison to the wild type. Failure of these RNA sequences to be translated into B1 hordein polypeptides in a cell free protein synthesizing system hints that the *lys3a* gene is involved in the post-transcriptional modification of the messenger RNA. Major deletions were not detected in the gene cluster coding for B hordein polypeptides by hybridization of the B1 hordein cDNA to restriction endonuclease fragments of the nuclear DNA from the two mutants Risø 56 and Risø 1508.

Abbreviations: bp = base pairs; cDNA = DNA complementary to mRNA; kbp = kilobase pairs; SDS = sodium dodecyl sulphate; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; SSC = 0.15 M-NaCl, 15 mM-Na citrate; TCA = trichloroacetic acid; Tm = melting temperature of the DNA.

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1. INTRODUCTION

The prolamin is the most abundant protein component of many cereal grains but is particularly deficient in the amino acid lysine. This abundance and the resultant poor nutritional quality of these proteins, has led to intensive breeding programs for increased lysine levels in the grain. In this context mutations that result in a reduction of the amount of prolamin are of particular interest since they usually are accompanied by a parallel increase of other grain proteins that have a higher amount of essential amino acids. In barley several high lysine mutants have been described (5, 19, 21, 31, 37, 38, 45, 50).

Barley prolamin is termed hordein and comprises a group of at least ten related hydrophobic polypeptides differing in molecular weight, charge and peptide maps (20, 22, 29, 54). Hordein polypeptides are synthesized on the rough endoplasmic reticulum of the endosperm cell (7, 11, 25) as larger sized precursor polypeptides (8, 42), which are vectorially discharged into the lumen of the endoplasmic reticulum (11, 12) and then deposited in protein bodies (13, 30). Considerable varietal polymorphisms of hordein polypeptide sizes have been reported (20, 22, 54). Genetic analysis revealed that this polymorphism is controlled by three loci on chromosome 5 (3, 22, 32, 50, 55, 56, 61) each of which probably is a cluster of closely linked genes, coding for different groups of polypeptides. The designation Hor-1, Hor-2 and Hor-3 have been given to these three loci specifying respectively the C hordein polypeptides (molecular weights from 48,000 to 67,000 daltons), the B hordein polypeptides (molecular weights from 27,000 to 38,000 daltons) and the D hordein polypeptides (molecular weights from 83,000 to 91,000 daltons).

The 7S storage proteins of soybean (58) and the zein polypeptides of maize (9, 28, 51, 64) are encoded in multigene families. It is of interest to find out if each of the three groups of hordein polypeptides is determined by a multigene family.

One high lysine mutant in barley, Risø 1508 derived from the cultivar Bomi contains the mutant gene *lys3a* on chromosome 7 (37) which results in a major reduction of all hordein polypeptides (5, 45). Another mutant, Risø 56 derived from the cultivar Carlsberg II, contains the mutant gene *hor2ca* located in chromosome 5 which prevents the synthesis of the B hordein polypeptides (19). DNA cloning techniques have been applied to prepare double-stranded DNA complementary to the messenger RNAs coding for barley endosperm proteins (6, 24). The cloned cDNA is in the present study used to analyze the organization and transcription of the B1 hordein genes and the nature of the mutations resulting in a depressed hordein biosynthesis.

2. MATERIALS AND METHODS

2.1. Chemicals

Avian myeloblastosis virus reverse transcriptase was obtained from Life Science, USA. *Escherichia coli* DNA polymerase I and polyadenylic acid were from Boehringer Mannheim, calf thymus terminal transferase from P-L Biochemicals, USA.; Poly(U)-Sepharose 4B and SP-Sephadex C-50 from Pharmacia, Sweden; and Dowex 50W from Sigma Chemicals, USA. Radioactively labelled nucleotides, amino acids and rabbit reticulocyte lysate were obtained either from New England Nuclear, USA or from Amersham International, UK.

Restriction endonucleases Eco RI, Pst I, Hpa II, Taq I, Alu I, Bgl I, and Bam HI were from Boehringer Mannheim, West Germany; Hinf I, Sau 3A, Sau 96I, and Xba I from Bethesda, USA.; Rsa I and Acc I from New England Biolabs, USA.

Gene Screen Hybridization Transfer Membranes were purchased from New England Nuclear, USA and nitrocellulose filters from Millipore, USA. Agarose for DNA and RNA gel electrophoresis was obtained from Sigma, USA and Seakem, USA, respectively.

2.2. Plant material

Barley plants (*Hordeum vulgare* L.) cv. Bomi, cv. Carlsberg II, mutants Risø 1508 and Risø 56 were grown, and 20 days old endosperms isolated as previously described in (8).

2.3. RNA isolation

The RNA extractions were performed essentially as described in (8). Endosperms isolated

from 20 spikes were homogenized in 40 ml 0.1M-Tris-HCl, 0.01 M-EDTA, 0.1 M-NaCl pH 9.0 at 0 °C with mortar and pestle. The supernatant was further homogenized three times in a Potter-Elvehjem homogenizer operated at 3,000 rpm, SDS was then added to a final concentration of 1% and the homogenate was centrifuged at 10,000x g for 2-3 minutes at 2 °C. The supernatant was extracted with one volume of phenol: chloroform: 3-methyl-1-butanol (50:50:1, v/v/v) and the mixture shaken for ten minutes at room temperature. The phases were separated by centrifugation at 10,000x g for 10 minutes and the resulting aqueous phase was extracted twice with chloroform: 3-methyl-1-butanol (24:1, v/v). $CsCl_2$ was then added to the aqueous phase to a final concentration of 1 mg · ml^{-1} and the solution was layered over 5.7 M-CsCl in 0.01 M-EDTA pH 8.0 (26). Following 18 hours of centrifugation at 100,000x g_{AV} in a Beckman 70 Ti rotor the resulting RNA pellet was redissolved in distilled water. Polyadenylated RNA was isolated as previously described in (8).

2.4. Barley DNA isolation

Endosperms isolated from 20 spikes were homogenized in 40 ml 0.1 M-Tris-HCl, 0.1 M-NaCl, 0.01 M-EDTA pH 8.0 and centrifuged at 10,000x g for 10 minutes. The resulting supernatant was extracted with phenol, chloroform and 3-methyl-1-butanol and the aqueous phase was made 0.95 g · ml^{-1} of CsCl and 1 mg · ml^{-1} of ethidium bromide. Ultracentrifugation was for 72 hours at 100,000x g_{AV} in a Beckman 70 Ti rotor at 20 °C. The resulting DNA band was recovered by side puncturing the tube. Ethidium bromide was removed by passing the DNA solution through a Dowex 50W column equilibrated in 10 mM-Tris-HCl, 1 mM-EDTA pH 7.5 and the solution dialyzed for 18 hours against the same buffer.

2.5. Cloning of cDNA

cDNA synthesis using polyadenylated RNA as template, was performed as described in (6). Tailing of plasmid pBR322 DNA digested with the restriction endonuclease Pst I was performed in a 20 µl mixture containing 140 mM-cacodylic acid, 30 mM-Tris base, 1 mM-$CoCl_2$, 0.1 mM-dithiotreitol, 20 µCi α-^{32}P-deoxyguanosine triphosphate (400 Ci · mmole^{-1}) and 26 units of calf thymus terminal transferase (18). Double-stranded cDNA was tailed in a similar way except that the 20 µl reaction mixture contained 20 µCi α-^{32}P-deoxycytidine triphosphate (400 Ci · mmole^{-1}). The tailing reactions were carried out for 5-10 minutes at 37 °C allowing 10-15 residues to be added per 3'-end. SDS and EDTA were added to a final concentration of 0.1% and 5 mM, respectively. The two reaction mixtures were pooled and extracted with one volume phenol: chloroform: 3-methyl-1-butanol (50:50:1, v/v/v). After ethanol precipitation the sample was annealed and used for transformation of *Escherichia coli* HB101 cells as described in (40).

2.6. Isolation of plasmid DNA

Cultures of recombinant colonies were grown to OD_{600} = 1.2 before amplification of the plasmid DNA by addition of 150 µg · ml^{-1} chloramphenicol (15). Plasmid DNA was isolated by $CsCl_2$ gradient centrifugation as described in (16). Small scale plasmid DNA preparations were performed as described by Birnboim and Doly (2).

2.7. DNA and RNA gel electrophoresis

DNA was separated on horizontal agarose slab gels in 90 mM-Tris-borate, 2.5 mM-EDTA buffer pH 8.4 at 2 V · cm^{-1} for 18 hours. Wild type λ phage DNA digested with the restriction endonuclease Hind III was included as size marker. The gels were visualized after the electrophoresis by staining with 1 µg · ml^{-1} ethidium bromide in the electrophoresis buffer, and photographed under long wave ultraviolet irradiation using UV and orange filters. For restriction endonuclease analysis 6% polyacrylamide gel electrophoresis was performed. Buffer and running conditions as well as staining and photographing were the same as for agarose gel electrophoresis. Plasmid pBR322 DNA digested with the restriction endonuclease Hpa II was included as size marker. RNA samples were subjected to electrophoresis in horizontal 1.5% agarose gels containing 6% formaldehyde, 30 mM-MOPS (morpholinopropanesulfonic acid), 5 mM-sodium acetate and 1 mM-EDTA at 2 V · cm^{-1} for 18 hours. After electrophoresis, the gels were washed with distilled water to remove the formaldehyde. Staining and photography were as described for DNA gel electrophoresis.

2.8. DNA and RNA transfers

DNA blotting to nitrocellulose filters was performed as described by SOUTHERN (62). RNA transfer to Gene Screen Hybridization Transfer Membranes was carried out as recommended by the supplier with slight modifications. The RNA gel was incubated for 30 minutes in the presence of 50 mM-NaOH, neutralized for another 30 minutes with 0.1 M-Tris-HCl pH 7.0, and finally equilibrated for 20 minutes in 1x SSC pH 7.0. Transfer buffer was 1x SSC. After approximately 18 hours the RNA was fixed to the membrane by baking at 80 °C for two hours.

2.9. Hybridizations

DNA hybridizations were carried out in the presence of DENHARDT solution (17) (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) and 2xSSC containing 200 μg · ml^{-1} of sheared and heat denatured herring sperm DNA and 200 μg · ml^{-1} polyadenylic acid and the heat denatured radioactively labelled hybridization probe. The hybridization was carried out in a slowly rotating sealed 40 ml tube at 68 °C for 18 hours. Prehybridizations were performed for two hours at similar conditions without the probe. Following hybridization the filters were extensively washed at 68 °C in 2x SSC and finally in 0.1x SSC containing 0.1% SDS. The air dried filters were autoradiographed at -70 °C using Kodak X-omat film and intensifying screens.

RNA hybridizations were performed at 42 °C in 50% deionized formamide, DENHARDT solution (17), 5x SSC, 1% SDS, 0.2 mg · ml^{-1} of heat denatured herring sperm DNA and 0.2 mg · ml^{-1} polyadenylic acid and the heat denatured radioactively labelled probe. Prehybridization was carried out overnight at the same conditions without the probe. The rest of the hybridization procedure, washings and autoradiography were as described for the DNA hybridizations. The probes were radioactively-labelled by nick-translation (53) of 1 μg plasmid DNA containing cDNA coding for B1 hordein polypeptides in the presence of 0.1 mCi α-^{32}P-deoxyadenosine triphosphate (400 Ci · mmole^{-1}) for two hours yielding 3x 10^7 to 10^8 cpm · μg^{-1}.

2.10. In vitro translations of barley messenger RNA

The reaction mixture, conditions of incubation and extraction of alcohol soluble proteins were performed as previously described (8). The hordein polypeptides were extracted (57) and separated in 12.5% SDS-PAGE according to CHUA and BENNOUN (14). The gels were processed for fluorography by the procedure of BONNER and LASKEY (4).

2.11. Restriction endonuclease digestions

The DNA was cleaved with the restriction endonucleases as recommended by the respective purchasers.

2.12. Containment

The experiments were registered with the Committee on Genetic Engineering of the Danish National Research Councils, and when required, carried out under P1 laboratory conditions (27).

3.RESULTS

3.1.Hordein polypeptide composition of wild type and mutant barley endosperms

When barley seeds are ground and extracted with 55% 2-propanol, hordein is solubilized in the supernatant. The hordein can be separated into at least ten molecular weight species by SDS-PAGE. The C hordein polypeptides range in molecular weights from 67,000 to 48,000 daltons and the B hordein polypeptides from 38,000 to 27,000 daltons. The C hordein polypeptide pattern of Bomi and Carlsberg II barley endosperms is identical, whereas these two barley varieties differ in the pattern of the B hordein polypeptides (Figure 1, tracks 1,2). The mutant Risø 56 pattern does not contain the prominent B hordein polypeptide bands (Figure 1, track 3). The pattern of the C hordein polypeptides is comparable to that of its wild type, Carlsberg II barley. On the other hand, the hordein composition of mutant Risø 1508 shows a drastic reduction of all hordein polypeptides (Figure 1, track 4).

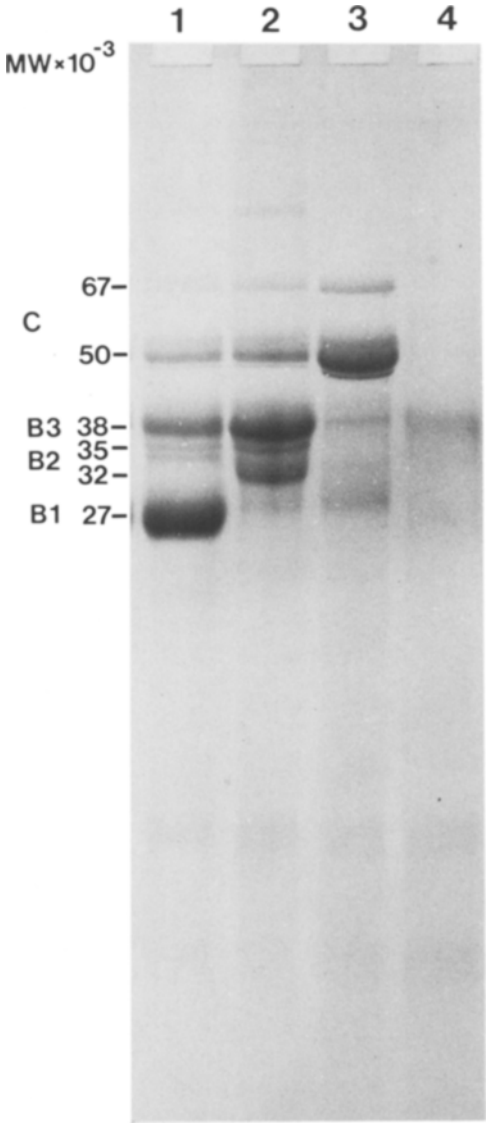

Figure 1. Hordein polypeptide composition of mutant and wild type barley endosperms.

Hordein polypeptides were extracted from mature barley endosperms with 55% 2-propanol and 2% 2-mercaptoethanol. Samples were prepared for SDS-PAGE, separated in a 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. Track 1: Bomi. Track 2: Carlsberg II. Track 3: Mutant Risø 56. Track 4: Mutant Risø 1508.

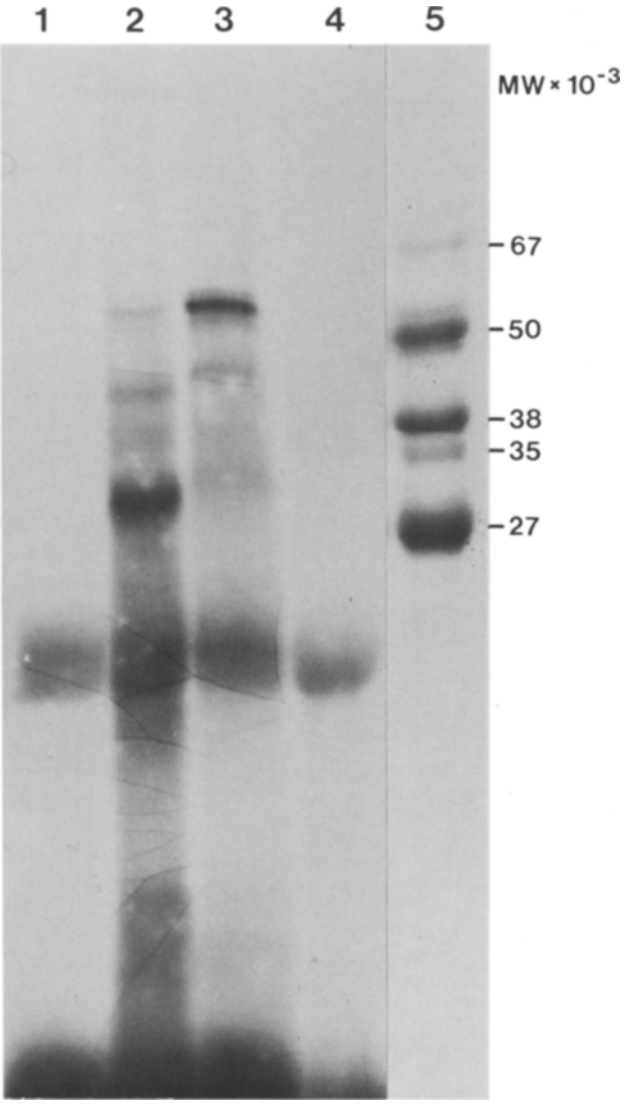

Figure 2: Fluorogram of the alcohol-soluble translation products of messenger RNA from mutant and Bomi barley endosperms, separated by SDS-PAGE.

The 55% 2-propanol soluble translation products were analyzed by electrophoresis and fluorography as described under 2.10. Track 1: ^{3}H-proline labelled polypeptides translated without added RNA (control). Track 2: ^{3}H-proline labelled polypeptides translated using polyadenylated RNA isolated from Bomi endosperm as template. Track 3: ^{3}H-proline labelled polypeptides translated using polyadenylated RNA isolated from mutant Risø 56 endosperms as template. Track 4: ^{3}H-proline labelled polypeptides translated using polyadenylated RNA isolated from mutant Risø 1508 endosperms as template. Track 5: In vivo ^{14}C-labelled hordein marker polypeptides extracted from Bomi endosperms.

3.2. Polypeptides encoded by messenger RNA isolated from wild type and mutant barley endosperms

Polyadenylated RNA was obtained by Poly(U)-Sepharose 4B affinity chromatography, and translated in the reticulocyte lysate cell free protein synthesizing system containing ^{3}H-labelled proline. The newly synthesized hordein polypeptides were analyzed by autoradiography of the polypeptides after separation by SDS-PAGE.

Hordein polypeptides were selectively extracted from the translation mixture prior to electrophoresis using 55% 2-propanol and 2% 2-mercaptoethanol. The ability of the different mutants and wild type endosperm RNAs to direct hordein synthesis is shown in Figure 2. The polypeptide composition of the in vivo labelled hordein (Figure 2, track 5) is presented for comparison with the translation products. Track 2 in Figure 2 shows that the messenger RNA isolated from Bomi barley endosperms translates into alcohol-soluble polypeptides with a higher apparent molecular weight than those of the native hordein. These proteins have been characterized as hordein polypeptide precursors (8, 42).

When RNA of the mutant Risø 56 was used as template (Figure 2, track 3) the major B hordein polypeptide precursor bands, and especially the B1 hordein precursor band, were absent. On the other hand, the synthesis of the C hordein polypeptides appears to be relatively more prominent. When polyadenylated RNA of mutant Risø 1508 endosperms was translated no 2-propanol soluble polypeptides were obtained (Figure 2, track 4).

3.3. Preliminary analysis of the barley endosperm cDNA library

Polyadenylated RNA was purified from total Bomi endosperm RNA and used as template for the synthesis of cDNA that was then cloned in plasmid pBR322. More than 1,000 recombinant clones were obtained containing cDNA inserts of variable lenghts. Figure 3 shows the size of the cDNA inserts of 34 randomly chosen recombinant plasmids. The average size was approximately 250 base pairs, with 20% of the inserts larger than 500 base pairs and 5% larger than 800 base pairs.

Plasmids were purified from 150 randomly chosen cDNA clones and after 0.7% agarose gel electrophoresis blotted to nitrocellulose filters. Messenger RNAs isolated from the endosperms of the mutants Risø 56 and 1508 were used as templates for the synthesis of ^{32}P-labelled single

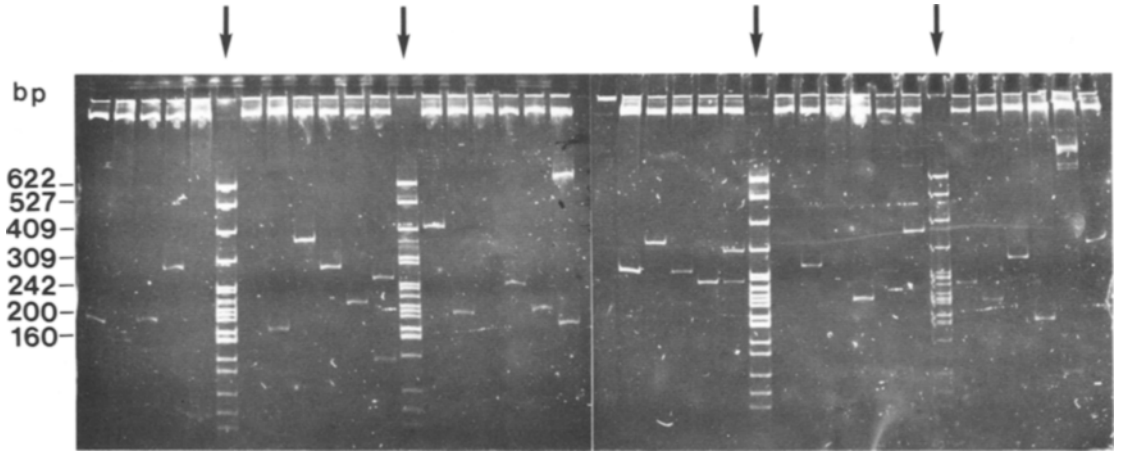

Figure 3: Example of molecular size determinations of the cDNA fragments cloned in the plasmid pBR322.

Randomly selected recombinants from the cDNA library were prepared according to Birnboim and Doly (2) and the cDNA inserts excised by digestion with the restriction endonuclease Pst I. The DNA was submitted to electrophoresis in 6% polyacrylamide gels and stained with ethidium bromide. Arrows indicate the tracks containing Hpa II restriction endonuclease fragments of plasmid DNA as molecular size markers. Molecular sizes (in base pairs) are indicated.

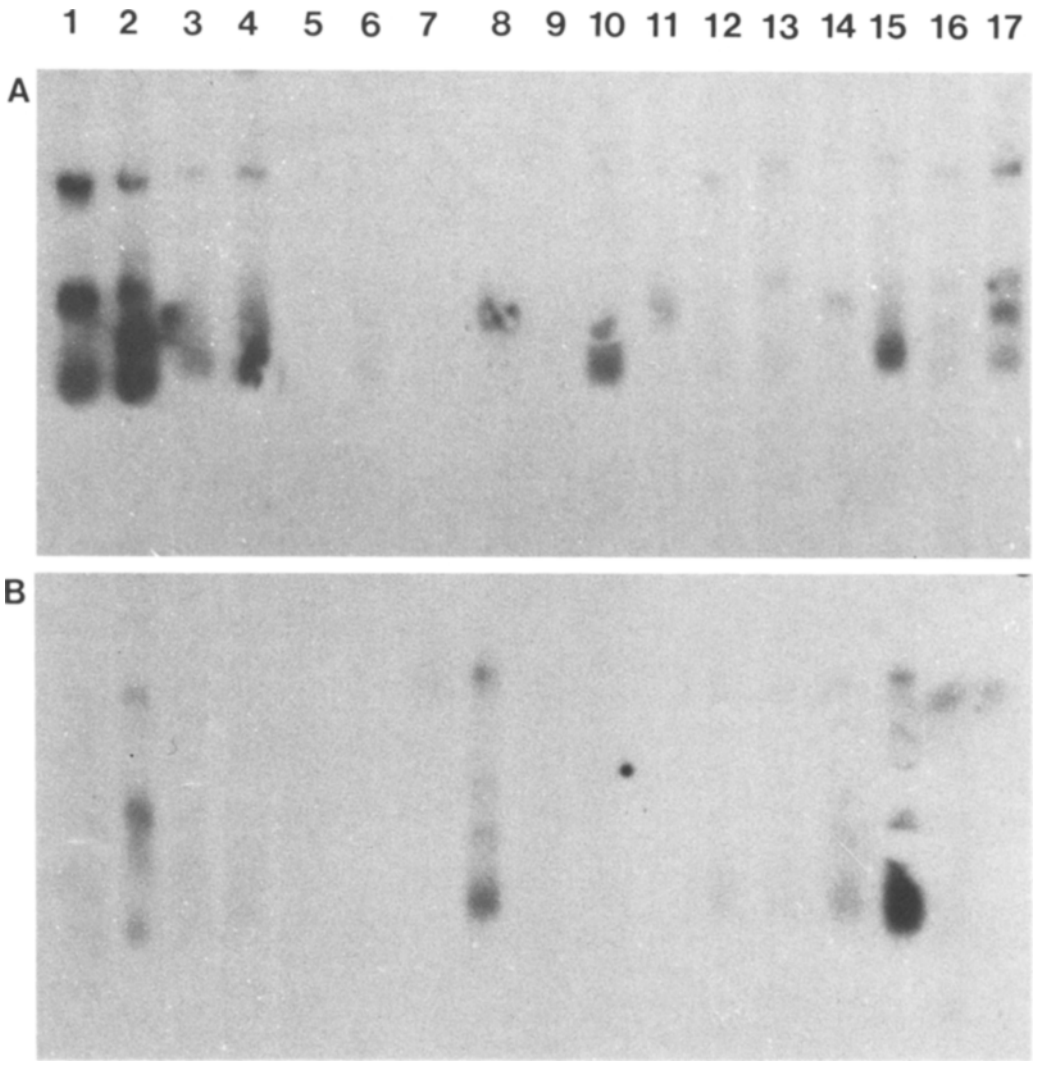

Figure 4. Hybridization of 17 cDNA plasmids to ^{32}P-labelled single stranded cDNA of messenger RNA from mutant Risø 56 (A) and mutant Risø 1508 (B).

Plasmid DNA was prepared according to Birnboim and Doly (2), separated in 0.7% agarose gels and transferred to nitrocellulose filters according to Southern (62). The filters were hybridized to ^{32}P-labelled single stranded cDNA made by reverse transcription of polyadenylated RNA from mutant Risø 56 endosperms (A) or polyadenylated RNA from mutant Risø 1508 endosperms (B). Tracks 2, 8, and 15 contain plasmids hybridizing to both probes; tracks 1, 3, 4, 10 and 17 are examples of plasmids hybridizing to the mutant Risø 56 probe while tracks 5, 6, 7, 9, 11, 12, 13, 14 and 16 contain plasmids hybridizing weakly or not hybridizing to the probes.

stranded cDNA hybridization probes. Figure 4 shows an autoradiogram of such a hybridization experiment which permitted the classification of the cDNA library into four groups: a) About 40% of the cDNA clones hybridized only to mutant Risø 56 single stranded cDNA. Under the assumption that mutant 56 does not produce B hordein messenger RNA, these clones are expected to encode C hordein polypeptides. b) About 35% of the cDNA clones hybridized weakly or not at all to both mutant probes. Under the assumption of absence of B hordein mes-

senger RNA in mutant Risø 56 and reduced amounts of B and C hordein messenger RNA in mutant Risø 1508, these clones are expected to encode B hordein polypeptides. c) About 20% of the cDNA clones hybridized to both mutant probes. These clones probably encode proteins not related to the hordein polypeptides. d) A small group (less than 2%) hybridized to cDNA derived from mutant Risø 1508 RNA only.

3.4. Restriction endonuclease mapping of the cDNA clones

Representative members of the cDNA inserts belonging to the above mentioned hybridization groups were mapped with restriction endonucleases. Structural heterogeneity was apparent when enzymes that recognize frequently occurring restriction sites in the DNA were used. Four clones which did not hybridize to the cDNA probe of mutant Risø 56 (group b) gave the restriction maps shown in Figure 5. Subsequently the DNA of these clones was sequenced (52) and proved to encode at least the carboxy-terminal portion of a B1 hordein polypeptide. The Xba I restriction site of the cDNA pc hor2-4 has been located 15 nucleotides downstream from the third nucleotide of the termination codon (52). The cDNA insert of clone pc hor2-4 covers the coding region of a B1 hordein messenger RNA. The cDNA insert of clone pc hor2-1 contains the entire 3' non coding region including a polyadenylic tail of 34 nucleotides and a small part of the coding region of a different B1 hordein messenger RNA (52). The cDNA inserts of pc hor2-2 and pc hor2-3 contain small parts of the coding region which are identical to that of pc hor2-1. The 3' non coding regions of these two clones are identical to the other two clones but terminate at different positions, clone pc hor2-2 with and clone pc hor2-3 without a polyadenylic tail (52).

In order to estimate the number of related sequences among the large sized cDNA inserts of the library, the cDNA insert of pc hor2-3 was hybridized at conditions of different stringency with 17 members of the cDNA library (Figure 6). At high stringency the probe hybridized only to itself (Figure 6, Tm -10). Lowering of the stringency resulted in hybridization with 10 of the clones. When the cDNA insert of pc hor2-1 was hybridized to a large sample of clones at medium stringency, approximately 10% of the cDNAs cross hybridized. Specifically, pc hor2-3 cross hybridized with pc hor2-1 and pc hor2-2 under stringent conditions, whereas pc hor2-3 and pc

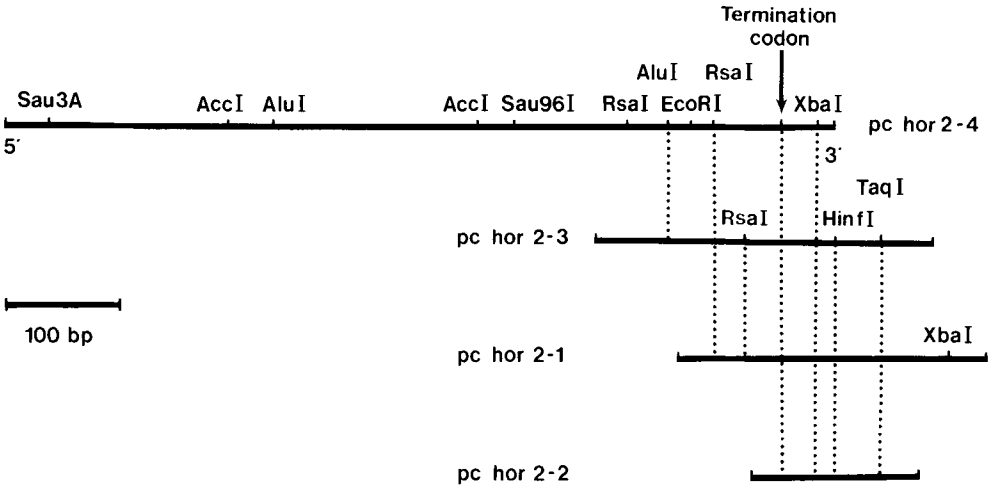

Figure 5: Restriction endonuclease maps of four B1 hordein cDNA clones.

The restriction endonuclease sites were mapped by single and double digestions of the recombinant plasmids with the restriction endonucleases indicated, and confirmed by DNA sequence analysis (52). All the restriction endonuclease cleavage sites in the 3' non coding region are conserved while the coding regions displayed polymorphisms.

hor2-4 (Figure 5) cross hybridized only under conditions of low stringency in spite of the high degree of sequence homology between these two clones. Nucleotide sequence analysis thus established that cross hybridization was not a useful procedure to distinguish clones with homologous from those with identical hordein messenger RNA sequences. Indeed, the nucleotide sequence of one clone selected for its cross hybridization with pc hor2-1 at conditions of medium stringency coded for a sequence of amino acids present in the carboxy-terminus (57) of a C hordein polypeptide.

3.5. Hybridization of B1 hordein cDNA to wild type and mutant endosperm RNA

In order to probe for the presence of messenger RNA coding for B hordein polypeptides in mutant barley endosperms, total RNA from wild type and mutant endosperms was isolated, electrophoretically separated on agarose gels containing formaldehyde and transferred to nitrocellulose filters. Hybridization was carried out to the radioactively-labelled plasmid pc hor2-3 (Figure 7). Bomi barley endosperms contained abundant RNA sequences hybridizing to the probe and these had an estimated lenght of 1,400 to 1,200 bases (Figure 7, track 3). The diffuse band indicates considerable heterogeneity in sizes of the messenger RNAs. This heterogeneity is due either to differences in the length of the polyadenylic tails or to the presence of messenger RNA coding for polypeptides of different lengths.

Carlsberg II barley endosperms likewise contained significant amounts of RNA sequences in the same region of the gel (Figure 7, track 5). In other experiments it was shown that the hybridization intensity was similar to that of Bomi RNA. Mutant Risø 1508 endosperm RNA gave consistently a weak hybridization of two diffuse bands (Figure 7, track 2). Mutant Risø 56 endosperm RNA consistently did not hybridize to the probe (Figure 7, track 4). When the hybridization was carried out with the plasmids pc hor2-1 or pc hor2-4 identical results were obtained. These results reveal that mutant Risø 56 lacks in its grain B1 hordein polypeptides because of its inability to transcribe stable messenger RNA.

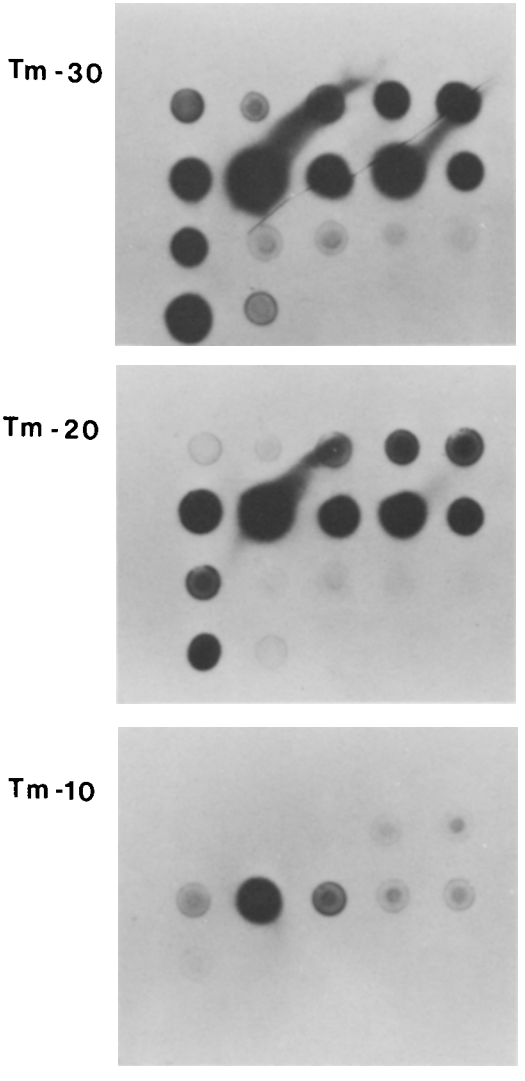

Figure 6: Dot-hybridization of different recombinant plasmids using ^{32}P-labelled pc hor2-3 as a probe.

The cDNA insert of pc hor2-3 was excised from the recombinant plasmid by cleavage with the restriction endonuclease Pst I and isolated by polyacrylamide gel electrophoresis. The cDNA fragment was eluted from the gel and labelled by nick-translation (53). Recombinant plasmids of the cDNA library were prepared (16) and approximately 0.5 µg spotted on three different nitrocellulose filters, denatured and fixed by baking. Hybridization temperature was 76 °C (Tm: -10 °C), 66 °C (Tm: -20 °C) and 56 °C (Tm: -30 °C) in the presence of 1x SSC. Otherwise the hybidization conditions were as described in 2.9. The differences in stringency of hybridization are evident from the autoradiograms.

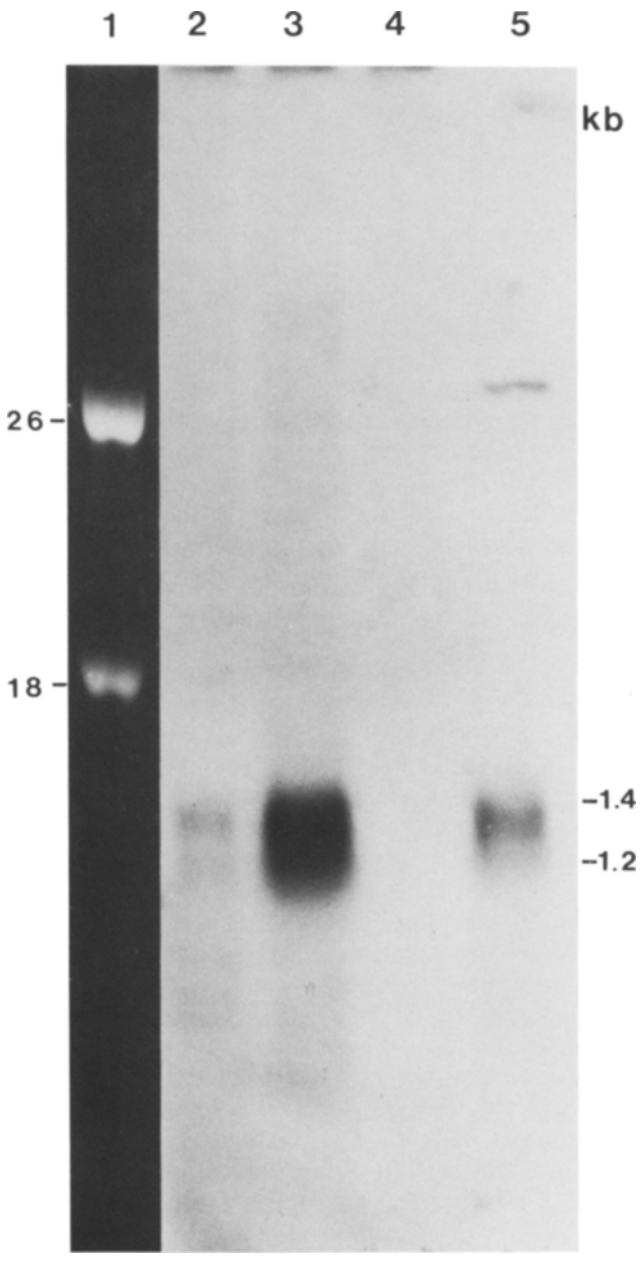

Figure 7: Detection of messenger RNA encoding B hordein polypeptides in wild type and mutant endosperms.

Ten µg of total barley endosperm RNA were separated by agarose gel electrophoresis in the presence of formaldehyde, transferred to nitrocellulose filters and hybridized to ^{32}P-labelled pc hor2-3 DNA at Tm -28 °C. The RNA complementary to the probe was then detected by autoradiography. The apparent molecular size was estimated by comparison with *Escherichia coli* ribosomal RNAs as markers. Track 1: Total RNA isolated from Bomi endosperms stained with ethidium bromide. The sedimentation coefficients of the ribosomal RNA are indicated. Tracks 2-5: Autoradiogram showing the hybridization of the B1 cDNA hordein sequence pc hor2-3 to endosperm RNA. Track 2: RNA isolated from mutant Risø 1508. Track 3: RNA isolated from Bomi. Track 4: RNA isolated from mutant Risø 56. Track 5: RNA isolated from Carlsberg II.

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3.6. Hybridization of B1 hordein cDNA to nuclear DNA of wild type and mutant endosperms

DNA from mutant and wild type barley endosperms was isolated by cesium chloride equilibrium density gradient ultracentrifugation and digested with Eco RI, Bam HI, and Hpa II restriction endonucleases. The resulting fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized to the labelled pc hor2-3 plasmid at conditions of medium stringency. Two very strongly hybridizing Eco RI restriction endonuclease fragments with estimated molecular sizes of 5.8 and 5.2 kilobase pairs were observed in Bomi DNA (Figure 8 A, track 3). In addition, a fragment of 2 kilobase pairs hybridizes with weak intensity and some four other fragments hybridize very weakly. The same was observed with DNA from mutant Risø 1508 (Figure 8 A, track 4). The hybridization pattern of DNA from mutant Risø 56 was identical to that of Bomi and mutant Risø 1508 except that the weakly hybridizing fragment of 2 kilobase pairs was absent (Figure 8 A, track 2). The DNA of Carlsberg II was only partially digested as evidenced by the presence of some hybridizing bands of higher molecular size (Figure 8 A, track 1). The same intensively hybridizing fragments of 5.8 and 5.2 kilobase pairs were present and the 2 kilobase pairs fragment absent like in mutant Risø 56. Experiments using the plasmids pc hor2-2 and pc hor2-4 as hybridization probes also yielded the two intensively hybridizing bands of 5.8 and 5.2 kilobase pairs with wild type and mutant preparations.

Bomi barley DNA contained two strongly hybridizing Bam HI restriction fragments with an estimated molecular size of 5.3 and 4.0 kilobase pairs. These same two prominently

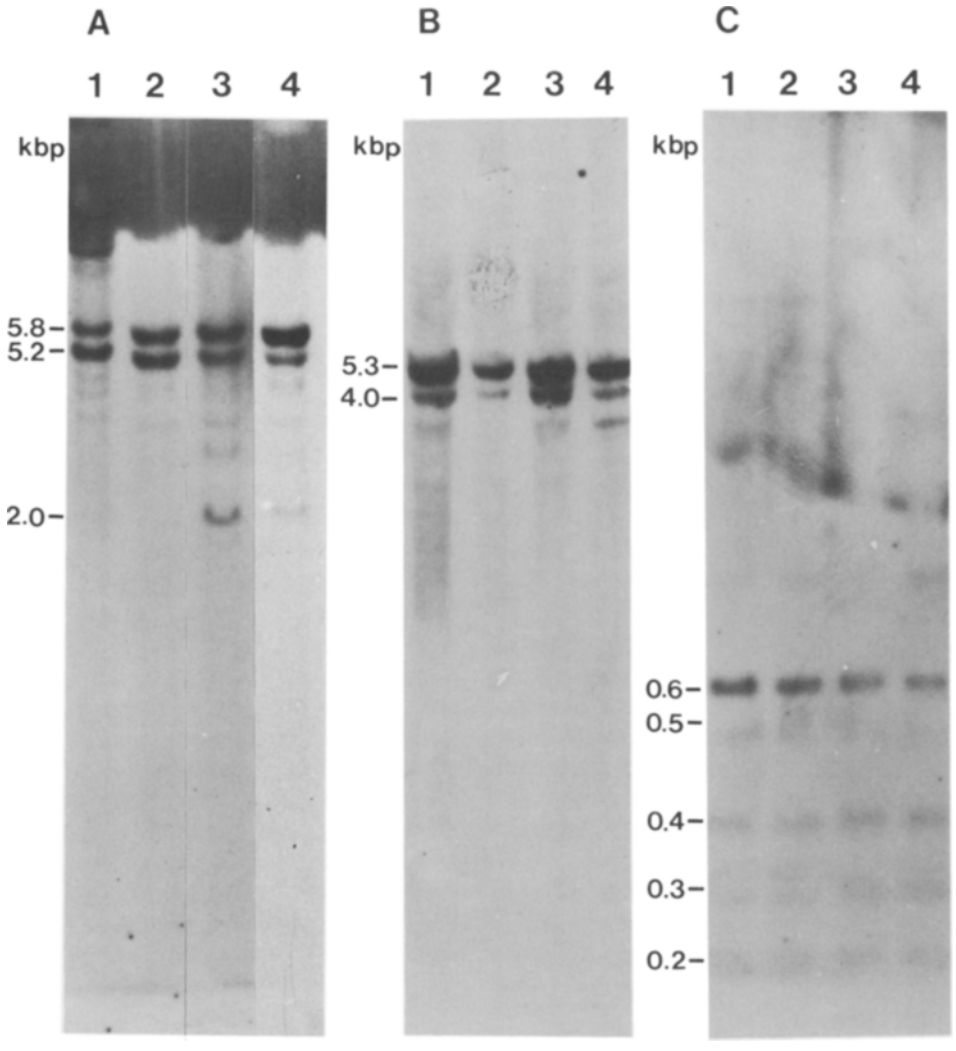

Figure 8: Organization of the B hordein genes in the nuclear DNA of wild type and mutant barleys.

Twenty μg of barley endosperm DNA were cleaved with the restriction endonucleases Eco RI, Bam HI and Hpa II, separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized to ^{32}P-labelled pc hor2-3 at Tm -28 °C. A: Autoradiogram of the Eco RI restriction endonuclease fragments hybridizing to the probe. B: Autoradiogram of the Bam HI restriction endonuclease fragments hybridizing to the probe. C: Autoradiogram of the Hpa II restriction endonuclease fragments hybridizing to the probe. Track 1: Carlsberg II endosperm DNA fragments. Track 2: Mutant Risø 56 endosperm DNA fragments. Track 3: Bomi endosperm DNA fragments. Track 4: Mutant Risø 1508 endosperm DNA fragments. The sizes of the fragments in kilobase pairs were estimated with size markers consisting of Hind III restriction endonuclease fragments of wild type λ DNA as well as Hinf I restriction endonuclease fragments of pBR322 plasmid DNA.

hybridizing Bam HI fragments were present in the DNA of mutant Risø 1508, Carlsberg II and mutant Risø 56 (Figure 8 B, tracks 1,2,4). Digestion with the restriction endonuclease Hpa II gave rise to much smaller fragments (Figure 8 C). The same five clearly recognizable fragments ranging from 200 to 600 base pairs in length were observed to hybridize in the DNA of all four genotypes. Some additional larger sized fragments gave a weak hybridization response. In

conclusion, the defect in hordein synthesis displayed by the two mutants cannot be attributed to sizable deletions or insertions in the chromosomal sequences hybridizing to the B1 hordein probes.

A gene titration was carried out for the 5.8 + 5.2 kilobase pair Eco RI restriction fragments according to HAGEN and RUBINSTEIN (28). A known amount of barley endosperm DNA was separated by 0.7% agarose gel electrophoresis side by side with a dilution series of known amounts of the plasmid pc hor 2-3. After blotting of the gel and hybridization with the radioactive pc hor2-3 insert the hybridization intensity was compared. The hybridization with 100 copies of the plasmid corresponded to the intensity observed with the restricted barley DNA. This gives a rough estimate of 50 copies of each strongly hybridizing Eco RI restriction fragment per haploid genome.

4. DISCUSSION

Comparison of the nucleotide sequences from cDNA clones with amino acid sequence data have identified 6 to 8 unique B1 hordein polypeptides (52). The close homology of these polypeptides and the strict conservation of the 3' non coding regions of their messenger RNAs make understandable the extensive cross hybridizations achievable among the cDNA clones. Extensive cross hybridizations have also been reported between cDNA clones of wheat and polyadenylated RNA of *Triticum monococcum*, rye, barley as well as the barley cDNA clone pC 179 (1). From the experiences gained with cross hybridization of the cDNA clones studied in the present paper it cannot be judged whether the interspecies hybridizations are due to conserved 3' non coding regions, prolamin coding regions or both. Conservation of 3' non coding messenger RNA regions have been reported for α and α' subunits of soybean 7S storage protein (58), the β-globin genes within and among closely related primate species (41) and different γ-immunoglobulin genes in mice (59). The present analysis supports the notion that the B1 hordein polypeptides are encoded by a multigene family. The evolutionary origin of such multigene families has been discussed by several authors (23, 45, 49).

The high lysine mutant Risø 1508 in barley is analogous to the mutants opaque-7 and floury-2 of maize. The former causes a reduction in all hordein polypeptides (5, 45, Figure 1) and the latter two in both types of zein polypeptides (43, 46, 47, 48). Mutant Risø 1508 produces low levels of messenger RNA coding for B1 hordein polypeptides. Similarily THOMPSON and BARTELS observed low levels of messenger RNA for B hordein polypeptides in mutant Risø 1508 when compared to the variety Sundance (63). It is likely that also the presence of messenger RNA for the C hordein polypeptides is limited in the mutant. When the maize mutants opaque-7 and floury-2 were examined significant amounts of zein messenger RNA were observed (10). Increasing the numbers of the floury-2 allele in the maize endosperm, reduced zein template activity of isolated membrane bound polysomes in a stepwise fashion (33). CAMERON-MILLS and INGVERSEN reported that functional microsomes could not be reconstituted in vitro using stripped microsomes isolated from mutant Risø 1508 endosperms and initially membrane-bound polyribosomes isolated from wild type barley endosperms (11). The presence in mutant Risø 1508 endosperms of B1 hordein messenger RNA sequences (Figure 7) and the fact that these sequences are not translated in vitro (Figure 2) may suggest a post-transcriptional defect of hordein polypeptide synthesis. In this case an increased degradation of the hordein messenger RNA may be due to its untranslatability or by an impaired binding of the initiation complex to the membrane of the endoplasmic reticulum. However, the possibility that this mutation decreases the transcription of hordein genes cannot be ruled out.

The mutation Risø 56 results in a specific elimination of the B hordein polypeptides encoded by Hor-2 (19, Figure 1). In the present paper it has been demonstrated that the mutant does not produce messenger RNA encoding B1 hordein polypeptides. It is likely that also transcription of messenger RNA for the other B hordein polypeptides is inhibited by the mutation, which has been mapped (19) at or close to the Hor-2 locus. In maize the opaque-2 mutation eliminates the synthesis of the 22,500 molecular weight zein components (10, 34, 35, 36, 65). Also

in this mutant messenger RNA is not produced (10, 34, 39, 65). According to Soave et al. (60) and Viotti et al. (65) the opaque-2 mutation does not map at any of the several sites implicated to contain structural genes for zein polypeptides.

The B1 hordein cDNA was used to study the DNA structure of the barley varieties Bomi and Carlsberg II as well as the mutants Risø 1508 and 56 (Figure 8). The probe hybridized prominently to 5.8 and 5.2 kilobase pair Eco RI restriction endonuclease fragments in all four genotypes. This result was expected for mutant 1508, since this mutation maps on chromosome 7 (37) and not on chromosome 5 where the genes coding for the B1 hordein polypeptides are located. The failure of mutant 56 to produce messenger RNA for B1 hordein polypeptides appears to be due to a point mutation or a deletion too small to significantly change the restriction endonuclease fragment patterns of the B1 hordein genes. Analogous observations have been reported for the opaque-2 mutation in maize (10, 64). The absence of a major deletion in mutant Risø 56 of barley is in contrast to the interpretation of the Hind III restriction fragment pattern of this mutant probed with the cDNA clone pC 179 (44). In comparison with a hybridization pattern of Carlsberg II DNA the absence of 8 Hind III fragments adding up to more than 100 kilobase pairs was reported (44). From the in vitro translation pattern of the mutant Risø 56 line presented in the same paper it is apparent that the mutant Risø 56 line investigated by these authors is not the same as the one studied here. Nucleotide sequencing of appropriate genomic clones are required to determine the nature of the mutation that abolishes messenger RNA formation from the genes encoding the B hordein polypeptides in mutant Risø 56 (*hor2ca*).

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REFERENCES

1. Bartels, D. & R.D. Thompson: The endosperms of common cereals contain related poly A+ RNA sequences. Theor. Appl. Genet. 64, 269-273 (1983)
2. Birnboim, H.C. & J. Doly: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. 7, 1513-1523 (1979)
3. Blake, T.K., S.E. Ullrich & R.A. Nilan: Mapping of the *Hor-3* locus encoding D hordein in barley. Theor. Appl. Genet. 63, 367-372 (1982)
4. Bonner, W.M. & R.A. Laskey: A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46, 83-88 (1974)
5. Brandt, A.: Endosperm protein formation during kernel development of wild type and high lysine barley. Cereal Chem. 53, 890-901 (1976)
6. Brandt, A.: Cloning of double stranded DNA coding for hordein polypeptides. Carlsberg Res. Commun. 44, 255-267 (1979)
7. Brandt, A. & J. Ingversen: In vitro synthesis of barley endosperm proteins on wild type and mutant templates. Carlsberg Res. Commun. 41, 311-320 (1976)
8. Brandt, A. & J. Ingversen: Isolation and translation of hordein messenger RNA from wild type and mutant endosperms in barley. Carlsberg Res. Commun. 43, 451-469 (1978)
9. Burr, B., F.A. Burr, T. P. St. John, M. Thomas & R. W. Davis: Zein storage protein gene family of maize: An assessment of heterogeneity with cloned messenger RNA sequences. J. Mol. Biol. 154, 33-49 (1982)
10. Burr, F.A. & B. Burr: Three mutations in *Zea mays* affecting zein accumulation: a comparison of zein polypeptides, in vitro synthesis and processing, mRNA levels and genomic organization. J. Cell Biol. 94, 201-206 (1982)

11. CAMERON-MILLS, V. & J. INGVERSEN: In vitro synthesis and transport of barley endosperm proteins: Reconstitution of functional rough microsomes from polyribosomes and stripped microsomes. Carlsberg Res. Commun. 43, 471-489 (1978)
12. CAMERON-MILLS, V., J. INGVERSEN & A. BRANDT: Transfer of in vitro synthesized barley endosperm proteins into the lumen of the endoplasmic reticulum. Carlsberg Res. Commun. 43, 91-102 (1978)
13. CAMERON-MILLS, V. & D. VON WETTSTEIN: Protein body formation in the developing barley endosperm. Carlsberg Res. Commun. 45, 577-594 (1980)
14. CHUA, N.-H. & P. BENNOUN: Thylakoid membrane polypeptides of *Chlamydomonas reinhardtii* wild type and mutant strains deficient in photosystem II reaction center. Proc. Natl. Acad. Sci. U.S.A. 72, 2175-2179 (1975)
15. CLEWELL, D.B.: Nature of colE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. 110, 667-676 (1972)
16. CLEWELL, D.B. & D.K. HELINSKI: Supercoiled circular DNA protein complex in *Escherichia coli*: Purification and induced convertion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. 62, 1159-1166 (1969)
17. DENHARDT, D.T.: A membrane filter technique for the detection of complementary DNA. Bio chem. Biophys. Res. Commun. 23, 641-644 (1966)
18. DEVOS, R., J. VAN EMMELO, R. CONTRERUS & W. FIERS: Construction and characterization of a plasmid containing nearly full-size DNA copy of bacteriophage M52 RNA. J. Mol. Biol. 128, 595-619 (1979)
19. DOLL, H.: A nearly non-functional mutant allele of the storage protein locus *Hor 2* in barley. Hereditas 93, 217-222 (1980)
20. DOLL, H. & A.D.H. BROWN: Hordein variation in wild (*Hordeum spontaneum*) and cultivated (*Hordeum vulgare*) barley. Can. J. Genet. Cytol. 21, 391-404 (1979)
21. DOLL, H., B. KØIE & B.O. EGGUM: Induced high lysine mutants in barley. Rad. Bot. 14, 73-80 (1974)
22. FAULKS, A.J., P.R. SHEWRY & B.J. MIFLIN: The polymorphism and structural homology of storage polypeptides (hordein) coded by the *Hor 2* locus in barley (*Hordeum vulgare* L.) Biochem. Genet. 19, 841-858 (1981)
23. FAVRET, E.A., R.M. SOLARI & L.E. MANGHERS: On the genetics of prolamin synthesis in cereal endosperms and its implication in plant breeding. In: The use of nuclear techniques for cereal grain protein improvement. Proc. of a symposium. FAO/IAEA/GSF/SIDA Vienna (in press)
24. FORDE, B.G., M. KREIS, M.B. BAHRAMIAN, J.A. MATTHEWS, B.J. MIFLIN, R.D. THOMPSON, D. BARTELS & R.B. FLAVELL: Molecular cloning and analysis of cDNA sequences derived from poly A+ RNA from barley endosperm: identification of B hordein related clones. Nucl. Acids Res. 9, 6689-6707 (1981)
25. FOX, I.E., H.M. PRATT, P.R. SHEWRY & B.J. MIFLIN: The in vitro synthesis of hordeins with polysomes from normal and high-lysine varieties of barley. In: Nucleic acid and protein synthesis in plants. Paris: Center National de la Recherche Scientifique pp. 501-509 (1977)
26. GLISIN, V., R. CRKVENJAKOV & C. BUYS: Ribonucleic acid isolated by cesium chloride centrifugation. Biochem. 13, 2633-2637 (1974)
27. Guidelines for research involving DNA molecules. Federal Register U.S.A., 47 (77), 17180-17198 (1982)
28. HAGEN, G. & I. RUBINSTEIN: Complex organization of zein genes in maize. Gene 13, 239-249 (1981)
29. HOLDER, A.A. & J. INGVERSEN: Peptide mapping of the major components of in vitro synthesized barley hordein: Evidence for structural homology. Carlsberg Res. Commun. 43, 177-184 (1978)
30. INGVERSEN, J.: Structure and composition of protein bodies from wild type and high lysine barley endosperm. Hereditas 81, 69-76 (1975)
31. INGVERSEN, J., B. KØIE & H. DOLL: Induced seed protein of barley. Experientia 29, 1151-1152 (1973)
32. JENSEN, J., J.H. JØRGENSEN, H.P. JENSEN, H. GIESE & H. DOLL: Linkage of the hordein loci *Hor 1* and *Hor 2* with the powdery mildew resistance loci *Ml-k* and *Ml-a* on barley chromosome 5. Theor. Appl. Genet. 58, 27-31 (1980)
33. JONES, R.A.: Effects of *Floury-2* locus on zein accumulation and RNA metabolism during maize endosperm development. Biochem. Genet. 16, 27-38 (1978)
34. JONES, R.A., B.A. LARKINS & C.Y. TSAI: Reduced synthesis of zein in vitro by a high lysine mutant in maize. Biochem. Biophys. Res. Commun. 69, 404-410 (1976)
35. JONES, R.A., B.A. LARKINS & C.Y. TSAI: Storage protein synthesis in maize: II. Reduced synthesis of a major zein component by the *opaque-2* mutant of maize. Plant Physiol. 59, 525-529 (1977)

36. Jones, R.A., B.A. Larkins & C.Y. Tsai: Storage protein synthesis in maize: III. Developmental changes in membrane-bound polyribosome composition and in vitro protein synthesis of normal and *opaque-2* maize. Plant Physiol. 59, 733-737 (1977)
37. Karlsson, K.-E.: Linkage studies in a gene for high lysine content in Risø barley mutant 1508. Barley Genet. News Lett. 7, 40 (1977)
38. Køie, B. & H. Doll: Protein and carbohydrate components in the Risø high lysine barley mutants. In: Seed protein improvements in cereals and grain legumes, Vol I, IAEA, Vienna pp. 205-215 (1979)
39. Langridge, P., J.A. Pintor Toro & G. Feix: Zein precursor mRNAs from maize endosperm. Mol. Gen. Genet. 187, 432-438 (1982)
40. Lederberg, E.M. & S.N. Cohen: Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. 119, 1072-1074 (1974)
41. Martin, S.L., E.A. Zimmer, W.S. Davidson & A.C. Wilson: The untranslated regions of β-globin mRNA evolve at a functional rate in higher primates. Cell 25, 737-741 (1981)
42. Matthews, J.A. & B.J. Miflin: In vitro synthesis of barley storage proteins. Planta 149, 262-268 (1980)
43. Mertz, E.T., L.S. Bates & O.E. Nelson: Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145, 279-280 (1964)
44. Miflin, B.J., S. Rahman, M. Kreiss, B.G. Forde, L. Blanco & P.R. Shewry: The hordeins of barley: Developmentally and nutritionally regulated multigene families of storage proteins. Proc. of Symp. Structure and function of plant genomes. NATO/FEBS meeting (in press)
45. Miflin, B.J. & P.R. Shewry: The synthesis of proteins in normal and high lysine barley seeds. In: Recent advances in the biochemistry of cereals. Eds. D. L. Laiman & R. G. Wyn Jones, Academic Press, London pp. 239-273 (1979)
46. Misra, P.S., R. Jambunathan, E.T. Mertz, D.V. Glover, H.M. Barbosa & K. McWhirter: Endosperm protein synthesis in maize mutants with increased lysine content. Science 176, 1425-1426 (1972)
47. Nelson, O.E. & B. Burr: Mutations that affect the synthesis of storage proteins in maize. Ann. Rev. Plant Physiol. 24, 504-506 (1973)
48. Nelson, O.E., E.T. Mertz & L.S. Bates: Second mutant gene affecting the amino acid pattern of maize endosperm protein. Science 150, 1469-1470 (1965)
49. Ohta, T.: An extension of a model for the evolution of multigene families by unequal crossing over. Genetics 91, 591-607 (1979)
50. Oram, R.N., H. Doll & B. Køie: Genetics of two storage protein variants in barley. Hereditas 80, 53-58 (1975)
51. Pedersen, K., S.K. Bloom, J.N. Anderson, D.V. Glover & B.A. Larkins: Analysis of the complexity and frequency of zein genes in the maize genome. Biochem. 19, 1644-1650 (1980)
52. Rasmussen, S.K., H.E. Hopp & A. Brandt: Nucleotide sequences of cDNA clones for B1 hordein polypeptides. Carlsberg Res. Commun. 48, 187-199 (1983)
53. Rigby, P.W., M. Dieckmann, C. Rhodes & P. Berg: Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113, 237-251 (1977)
54. Shewry, P.R., J.K.S. Ellis, H.M. Pratt & B.J. Miflin: A comparison of methods for the extraction and separation of hordein fractions from 29 barley varieties. J. Sci. Food Agric. 29, 433-441 (1978)
55. Shewry, P.R., A.J. Faulks, R.A. Pickering, I.T. Jones, R.A. Finch & B.J. Miflin: The genetic analysis of barley storage proteins. Heredity 44,383-389 (1980)
56. Shewry, P.R., H.M. Pratt, R.A. Finch & B.J. Miflin: Genetic analysis of hordein polypeptides from single seeds of barley. Heredity 40, 463-466 (1978)
57. Schmitt, J.M.: Purification of hordein polypeptides by column chromatography using volatile solvents. Carlsberg Res. Commun. 44, 431-438 (1979)
58. Schuler, M.A., E.S. Schmitt & R.N. Bachy: Closely related families of genes code for the α and α' subunits of the soybean 7S storage protein complex. Nucl. Acids Res. 10, 8225-8244 (1982)
59. Sikorav, J.L., C. Auffray & F. Rougeon: Structure of the constant and 3' untranslated regions of the murine Balb/c γ2a heavy chain messenger RNA. Nucl. Acids Res. 8, 3143-3155 (1980)
60. Soave, C., N. Suman, A. Viotti & F. Salamini: Linkage relationship between regulatory and structural gene loci involved in zein synthesis in maize. Theor. Appl. Genet. 52, 263-267 (1978)
61. Solari, R.M. & E.A. Favret: Polymorphism in

endosperm proteins of barley and its genetic control. In: Proc. 2. Int. Barley Genet. Symp. Ed. R. A. Nilan, Washington State Univ. Press, pp. 23-31 (1971)

62. SOUTHERN, E.M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517 (1975)
63. THOMPSON, R.D. & D. BARTELS: Hordein messenger RNA levels in wild type and mutant barley endosperms. Plant Science Letters (in press)
64. VIOTTI, A., E. SALA, R. MAROTTA, P. ALBERI, C. BALDUCCI & C. SOAVE: Genes and mRNAs coding for zein polypeptides in Zea mays. Eur. J. Biochem. 102, 211-222 (1979)
65. VIOTTI, A., D. ABILDSTEN, N. POGNA, E. SALA & V. PIRROTTA: Multiplicity and diversity of cloned zein cDNA sequences and their chromosomal localisation. The EMBO Journal 1, 53-58 (1982)