NUCLEOTIDE SEQUENCES OF cDNA CLONES FOR C-HORDEIN POLYPEPTIDES

by

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Keywords: Barley, endosperm, homology, gene family, polyadenylation signal, storage protein.

Two partial cDNA clones, pc hor1-3 and pc-919, encoding the carboxyterminal portion of two different C-hordein polypeptides have been sequenced. pc hor1-3 can be translated into a frame of 74 amino acids, part of which is identical to the amino acid sequence of a C-hordein chymotryptic peptide. The 3' non-coding region is 174 nucleotides in length and extends to the polyadenylation site. The region has two putative polyadenylation signals, AATAAA, located 27 and 80 nucleotides upstream from the poly(A)-tail. Three RNA species, 1800, 2100 and 4300 bases in length, can be detected in the endosperm using pc hor1-3 as probe. These could code for C-hordein polypeptides of 48, 67 and 144 kD, respectively. Hybridization of pc hor1-3 to restriction endonuclease fragments of nuclear barley DNA suggests that 6-8 genes code for the C-hordein polypeptides.

The nucleotide sequence of the second cDNA clone, pc-919, can be translated into 64 amino acids, of which the 22 carboxyterminal amino acid residues are identical to the carboxyterminal reading frame encoded by pc hor1-3. The adjacent 42 amino acid long reading frame shows homology to that deduced from pc hor1-3, but the nucleotide sequence is different and highly homologous to the 5' coding sequence found in a B-hordein genomic clone. Hybridization using this part of pc-919 as a probe detects RNAs of 1200 and 1400 bases only. Thus pc-919 represents a gene containing sequences related to both Hor1 and the Hor2 locus, and is the first nucleotide sequence data giving evidence that the two loci are related.

1. INTRODUCTION

The storage proteins of the barley endosperm, B-, C- and D-hordein are coded for by three separate loci on chromosome 5, Hor2, Hor1 and Hor3, respectively (2, 15, 27, 28). C-hordein can be separated by SDS-PAGE into 5 polypeptides, C1 to C5, with molecular sizes from 48 to 67 kD. Two dimensional electrophoresis resolves these into 10 polypeptides indicating charge polymorphism within each molecular size class (12, 16).

The sequence of the six amino terminal amino acids of purified C2 and C3 polypeptides are identical and polymorphisms have only been detected in 4 positions among the 25 amino terminal residues analysable from a total hordein preparation (5, 16). This indicates a high conservation of the N-terminal regions within the C-hordein group. Amino acid sequences of two chymotryptic peptides from total C-hordein (31) showed that C-hordein contain

Abbreviations: kb = kilobase; kbp = kilobase pair; kD = kilodalton; PAGE = polyacrylamide gel electrophoresis; SDS = sodium dodecyl sulphate.

the repeated sequence motif PQQPQQP. Recently, FORDE et al. (11) identified two partial cDNA clones encoding C-hordein polypeptides and showed the sequence motif to be present close to the carboxyterminal. The sequence motif PQQP and modifications thereof are also found in the amino terminal region of B-hordein polypeptides as deduced from two genomic clones (6, 10) and peptide mapping suggests homologies as well as differences among B- and C-hordein polypeptides (12).

In order to gain more information on the primary structure of hordein polypeptides and to provide molecular probes for the study of storage protein gene expression in the developing endosperm, cDNA clones from endosperm mRNAs have been constructed and cDNA encoding B-hordein and protein Z polypeptides characterized (3, 21, 22). In this paper nucleotide sequences of two different C-hordein clones are presented. They provide the first evidence of nucleotide sequence homology between the *Hor1* and the *Hor2* loci and hint at their common genetic origin.

2. MATERIALS AND METHODS

2.1. Chemicals, strain and media

All chemicals were obtained from the suppliers listed in (6, 21). *E. coli* HB 101 cells were used for transformation with recombinant pBR 322 plasmids and *E. coli* JM 103 cells served for propagation of phage M13 mp8. Media and stock solutions were prepared as described in (19).

2.2. Cloning of cDNA

Polyadenylated RNA isolated from 20 day old barley endosperms was used as template for cDNA synthesis. The cDNA was inserted into the Pst I site of pBR322 by G-C tailing and cloned in *E. coli* HB 101 cells as described in (3, 13).

2.3. Nucleotide sequence determination

The recombinant plasmids were isolated either from 5 ml cultures according to BIRNBOIM

and DOLY (1) or from 1 l cultures according to (7). The plasmids were cleaved by the restriction endonuclease Pst I and the resulting DNA fragments separated by electrophoresis in low melting point agarose, extracted and ethanol precipitated as described in (18). The isolated cDNA fragments were ligated into the replicative form of M13 mp8 (18), and *E. coli* JM 103 cells were transformed (17) and plated on indicator plates (18). Harvesting of phages, purification of single stranded phage DNA and the chain termination reactions were essentially as described in (18, 21, 24).

2.4. Isolation of barley endosperm DNA and RNA

Total DNA was isolated from 20 day old endosperms and purified by CsCl gradient centrifugation as described in (13). RNA was isolated as described in (4, 13). DNA was separated by agarose gel electrophoresis and transferred to nitrocellulose filters according to SOUTHERN (30). Hybridization was performed in 2×SSC at 68 °C using C-hordein cDNA fragments as a probe (23). RNA was electrophoretically separated on agarose gels containing formaldehyde, transferred to nitrocellulose filters and hybridized as described in (13). Single stranded M13 phage DNA containing the strand complementary to the C-hordein messenger RNA was radioactively labelled according to HU and MESSING (14) and used as a probe. The hybridizations were carried out in 3×SSC, 50% (v/v) formamide at 42 °C.

2.5. Nucleotide data analysis

The nucleotide sequence data were analyzed using a computer programme obtained from R. STADEN, Medical Research Center, Cambridge, UK. The programme was extended and adapted to fit a Honeywell Bull DPS-8 computer.

3. RESULTS AND DISCUSSION

3.1. Nucleotide sequences of pc hor1-3 and pc-919

The cDNA clone pc hor1-3 is 362 nucleotides in length (Figure 1A), contains an internal Pst I

A

CHYMOTRYPTIC PEPTIDE 1 48

TTT CCC CAG CCC CAG GAA CCA TTT CCC CAG CAA CCA CAA CAA CCA TTC
 phe pro gln pro gln glu pro phe pro gln gln pro gln gln pro phe

96

CCA CTG CAA CCG CAA CAA CCA TTC CCC CAA CAA CCA CAA CAA CCA TTC
 pro leu gln pro gln gln pro phe pro gln gln pro gln gln pro phe

144

CCC CAG CCC CAA CAA CCA TTC CGC CAA CAA GCA GAA CTA ATA ATT CCC
 pro gln pro gln gln pro phe arg gln gln ala glu leu ile ile pro

Pst I

192

CAG CAA CCT CAA CAA CCA TTC CCT CTG CAG CCA CAC CAA CCA TAT ACA
 gln gln pro gln gln pro phe pro leu gln pro his gln pro tyr thr

247

CAA CAA ACC ATC TGG AGT ATG GTC TAG TCATCAGGGGCCTATGAAGCGACAAGT
 gln gln thr ile trp ser met val *

310

TGTAATACTAAATGGGTGGATCATCATCCTTTAGTCAACGGAGTGTTTAATGTAATGATGATA

373

AATAAAGTGATGTGGACCATCATGTGTAACCCCGGCCTATACTAGTTCAAATG
 AGAATAAAAG

ACCAAAGAAAGTTCCTGTGC (A)_n

B

48

TAC CCA CAA CAA CCA CAA CCA TTT CCA CAA CAG CCC ATC CCA CAA CAA
 tyr pro gln gln pro gln pro phe pro gln gln pro ile pro gln gln

96

CCA CAA CCA TAC CCA CAA CAA CCA CAA CCA TTT TCA CAA CAG CCC ATC
 pro gln pro tyr pro gln gln pro gln pro phe ser gln gln pro ile

144

CCA CAA CAA CCA CAA CCA TAC CCA CAA CAA CCA CAA CCA TTT CCA CAA
 pro gln gln pro gln pro tyr pro gln gln pro gln pro phe pro gln

Pst I

192

CAA CCC ATC CCT CTG CAG CCA CAC CAA CCA TAT ACA CAA CAA ACC ATC
 gln pro ile pro leu gln pro his gln pro tyr thr gln gln thr ile

247

TGG AGT ATG GTC TAG TCATCAGGGGCCTATGAAGCGACAAGTTGTAATACTAAATGGG
 trp ser met val *

TGGAT

Figure 1. The nucleotide sequence of pc hor1-3 (A) and pc-919 (B) coding for the carboxyterminal amino acids of two C-hordein polypeptides, and the entire 3' noncoding sequence including additional 28 nucleotides of pc hor1-1 (A). Putative polyadenylation signals are underlined. The repeated blocks of proline and glutamine are boxed. The colinear amino acid sequence determined for chymotryptic peptide 1 of a C-hordein (31) is indicated. Also given is the PstI site, which separates two characteristic regions in the two clones.

restriction site which divides the cDNA into two fragments of 171 and 191 nucleotides. The nucleotide sequence preceding the termination codon, TAG, can be translated into 74 amino acids. The carboxyterminal sequence ser-met-val is identical to that determined for a C2-hordein polypeptide (25, 26). The 25 amino acid long sequence of chymotryptic peptide 1 (31) is co-linear with the amino acid sequence deduced by the codons from nucleotide 19 to 93, except that the last amino acid in peptide 1 is ile instead of pro. It requires two nucleotide substitutions to change the codon from ATA (ile) to CCA (pro). The alignments unequivocally show that pc hor1-3 encodes the carboxyterminal portion of a C-hordein polypeptide.

The clone extends 146 nucleotides into the 3' non-coding region of the mRNA. Alignment with another colinear cDNA clone, pc hor1-1, allow the 3' non-coding region to be extended up to 174 nucleotides. Two putative polyadenylation signals, AATAAA (20), are localized 27 and 81 nucleotides upstream from the polyadenylation site. Although no sequence homology is found, the 3' non-coding regions of B- (6, 22) and C-hordein cDNAs are similar in length and have the two polyadenylation signals spaced by 48 to 52 nucleotides. Analyses of B-hordein cDNA clones have shown that B-hordein messenger RNAs are polyadenylated at two sites (22) suggesting that both signals can be recognized by the poly(A)-adding enzymes. Multiple polyadenylation patterns have also been observed in the mRNAs coding for ribulose biphosphate carboxylase and chlorophyll a/b-binding proteins of petunia (8).

The cDNA clone pc hor1-3 presented in this paper is different from the C-hordein cDNA clones, pcP387 and pHvE-c21, described by FORDE et al. (11). The 3' non-coding regions of three clones are nearly identical but pc hor1-3 is 17 nucleotides longer than pHvE-c25 and pc P387. pc hor1-3 encodes 22 amino acids more than pHvE-c25. The overlapping regions encode an identical 50 amino acid long peptide. One A at position 183 in pc hor1-3 is changed to a G in pHvE-C25. Whether the cDNA clones represent different C-hordein genes or different alleles of the same gene in the two varieties remains to be analysed. pc P387 encodes 33

additional amino acids than pc hor1-3. The regions encoding the 51 carboxyterminal amino acids are identical except that a T at position 96 and a C at 165 in pc hor1-3 are changed to a T and a G, respectively, in pc P387. This results in two phenylalanines in pc hor1-3 are changed to leucine residues in the deduced amino acid sequence of pc P387. The region encoding the remaining 23 amino acids of pc hor1-3 is different from the sequence found in pc P387. Only pc hor1-3 encodes the chymotryptic peptide of TATHAM et al. (31). By taking the available sequence information into account (5, 11, 25, 26, 31) and by comparison to the amino acid composition of a purified C2-hordein polypeptide (25) it may be calculated that the main body of the protein, except for the amino and carboxy terminal regions, is made up of blocks of proline + glutamine (PQQPQP) separated by one amino acid which either is isoleucine, leucine or phenylalanine.

The cDNA clone pc-919 is 252 nucleotides long. An internal Pst I restriction site divides the sequence into two fragments of 159 and 96 nucleotides, respectively. The nucleotide sequence and the deduced amino acid sequence are presented in Figure 1 B. The 204 nucleotides preceding the termination codon, TAG, can be translated into 68 amino acids. The cDNA clone extends 51 nucleotides into the 3' noncoding region, but does not include a poly(A)-tail. The nucleotide sequence encoding the 16 carboxyterminal amino acids, and the 3' sequence downstream of the stopcodon is identical to the sequence of pc hor1-3. The nucleotide sequence encoding the 52 amino acids in the proline + glutamine rich region of pc-919 have very little homology to pc hor1-3. The identity of the 3' end to pc hor1-3 suggests that pc-919 is encoded by the Hor1 locus. The proline + glutamine rich region is organized in repeated blocks of PQQP and PQQPQP separated by one or two amino acids. The composition of the blocks is more similar to an N-terminal region of a B-hordein than to a C-hordein polypeptide.

Codon usage for proline and glutamine in the two clones is biased. The codons CAA for glutamine and CCA for proline are preferred in pc-919, as is the case in the B-hordein genes (6, 10, 21), whereas the CCC and CCA codons for

Figure 2. Southern blot analysis of Carlsberg II barley DNA. Endosperm DNA was digested with three restriction endonucleases, the fragments separated by agarose gel electrophoresis, transferred to nitrocellulose filters and hybridized to ^{32}P -labelled pc hor1-3 DNA. Lane 1: Bam HI, lane 2: Eco RI, lane 3: Hind III. Sizes of fragments were estimated using Hind III fragments of $\lambda\text{WES}/\lambda\text{b}$ as markers.

proline are equally used in pc hor1-3. Whether this bias in codon usage is of importance for the differential expression of these genes in the developing endosperm remains to be seen.

3.2. Probing for C-hordein mRNAs and genes

DNA from developing barley endosperms was digested with the restriction endonucleases Bam HI, Eco RI and Hind III. The resulting fragments were separated by agarose gel electrophoresis and transferred to a nitrocellulose filter. Hybridization was carried out at medium stringency ($T_m -20^\circ\text{C}$) with the nicktranslated pc hor1-3 (Figure 2). The clone hybridizes strongly to an 8.5 kbp Bam HI fragment and weaker to three fragments of 5.2, 11 and 15 kbp (lane 1), it hybridises strongly to a 4.6 kbp Eco RI fragment and to a lesser extent with fragments of 6.6, 13 and 16 kbp (lane 2). Strong hybridisation occurs to a Hind III fragment of 2.2 kbp and weak hybridisation to at least two fragments of 1.8 and 2.5 kbp (lane 3). Thus the fragment pattern obtained using three different restriction endonucleases suggests that the Hor1 locus encoding the C-hordein polypeptides is complex. Assuming that the weaker hybridizing fragment corresponds to one copy we estimate that the sequence is present in 6 to 8 copies per haploid genome. The Hor1 locus is therefore less complex than the 15-20 members of the Hor2 locus encoding the B-hordein polypeptides (6, 10).

Messenger RNA from Carlsberg II barley endosperms was isolated, separated by electrophoresis on a denaturing agarose gel and transferred to a nitrocellulose filter. Hybridization was carried out with the radioactively labelled pc hor1-3 (Figure 3A). The barley en-

dosperm contains three RNA species of 1800, 2100 and 4300 nucleotides in length hybridizing to the probe. Based on the hybridization intensity, the 1800 nucleotide RNA is far the most abundant whereas the 4300 nucleotide RNA is only present in small amounts. The C-hordein polypeptides can be resolved into at least 5 molecular weight species by SDS-PAGE. Three range in size from 48 to 54 kD and two are approximately 67 kD. The 1800 nucleotide RNA could encode a 54 kD protein and the 2100 nucleotide RNA a 67 kD protein leaving approximately 250 nucleotides for non-coding regions and the poly(A)-tail. Based on similar calculations the 4300 nucleotide RNA could encode a 144 kD polypeptide, but no C-hordein polypeptide of that size has so far been reported. The relatively strong hybridizations of both the 1800 and the 2100 nucleotide RNA indicate that the 3' ends of genes encoding different C-hordein polypeptides are conserved.

3.3. Comparison of pc hor1-3 and pc-919 by northern blot analysis

Both pc hor1-3 and pc-919 can be divided by PstI digestion into two regions. The first region is characterized for its coding capacity for proline + glutamine. Repeated blocks of proline + glutamine are found in the deduced amino acids from both clones. Very little homology is apparent on the nucleotide level and it is to be expected that these parts of the clones will not cross hybridize. On the other hand, the second regions are identical. Hybridization to barley messenger RNA using the differential 5' regions of pc hor1-3 and pc-919 is shown in Figure 3B. The region encoding the proline + glutamine rich region of pc hor1-3 hybridized to the same sized messenger RNAs as did the entire insert

(Figure 3B, lane 1). In contrast, the proline + glutamine coding region of pc-919 hybridizes to messenger RNAs of 1200 to 1400 nucleotides in length (Figure 3B, lane 2). These sizes are identical to those determined for the B-hordein messenger RNAs (13). Computer analysis and visual inspection showed that the sequence encoding the proline + glutamine rich region in pc-919 are very similar to the region encoding the amino terminal region of two B-hordein polypeptides (6, 10). Thus, pc-919 represents a gene which contains 3' sequences which are characteristic for the Hor1 locus and 5' sequences typical for the Hor2 locus. It remains to be determined whether the gene encodes a polypeptide in the B-, C- or the recently identified γ -hordein group (29). It is not likely that pc-919 originates from the Hor3 locus encoding D-hordein polypeptides, since pc-919 shows no homology to a cDNA encoding a D-hordein polypeptide (9). The homology claimed by FORDE et al. (11) between B- and C-hordein encoding cDNAs is weak and the clone pc-919 reported in this study is the first nucleotide sequence that gives evidence that some hordein genes may be a mosaic of genetic material typical for the Hor1 and Hor2 loci. This may either be due to a common evolutionary origin of the two loci or gene pc-919 is a result of unequal crossing over between the two loci.

Probing hordein polypeptides with monoclonal antibodies (32) as well as peptide mapping (12) have demonstrated significant homologies and differences among B- and C-hordein polypeptides. The results described in this paper suggest that the homologies lie in regions of the polypeptides rich in proline and glutamine. Primary structure determination of a complete C-hordein polypeptide is required to determine the nature of the unique regions.

Figure 3. Detection of mRNA species encoding C-hordein polypeptides. Carlsberg II barley endosperm RNA was separated by agarose gel electrophoresis in the presence of formaldehyde, blotted over to nitrocellulose and hybridized to ³²P-labelled pc hor1-3 DNA (A). Detection on mRNA species using the 5' PstI fragments of pc hor1-3 (1) and pc-919 (2) encoding the proline + glutamine rich portion of the peptides (B).

Hybridization was carried out in 50% (v/v) formamide, 2×SSC at 42 °C. The length of the hybridizing RNAs was estimated using barley ribosomal RNAs as markers.

ACKNOWLEDGEMENTS

This work was supported by the Biotechnological Action Programme of the Commission of the European Communities with contract No. BAP-0091-DK(B) to Mr. H.G. SARX and Professor D. VON WETTSTEIN.

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Accepted by H. KLENOW