Nucleotide sequence and endosperm-specific expression of the structural gene for the toxin α-hordothionin in barley (*Hordeum vulgare* L.)

(Recombinant DNA; genomic DNA clones; plant toxin; transcriptional start point; oligodeoxynucleotide primer; promoter; phage λ; genomic library)

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SUMMARY

A barley genomic library, obtained by cloning in the vector λEMBL-4, was screened with a cDNA probe encoding the α-hordothionin toxin. A positive clone, designated λTH1, was selected for further characterization. The coding and flanking regions of the α-hordothionin gene (*Hth-1*) were sequenced. *Hth-1* has two introns of 420 and 91 nucleotides (nt), respectively. The promoter region has the following main features: one TATA box; three CATC boxes; an enhancer-like sequence, starting at nt position −282 from the first ATG codon, which is homologous to sequences appearing at similar positions in other endosperm genes; two versions of an 18-nt sequence that is more highly repeated in structural domains of several prolamin genes; two extensive regions close to the first ATG codon that are homologous to a sequence located much further upstream in the B-hordein promoter. The transcription start point was determined at nt positions −46 to −47, both by the S1 nuclease-protection and by the primer-extension assays. A maximum of 2–4 copies of the *Hth-1* gene per haploid genome was determined by Southern-blot hybridization. Expression of the *Hth-1* gene was detected during the cell proliferation stage of endosperm development (maximum at 13–16 days after pollinization) and was not detected in either etiolated or green coleoptiles.

Abbreviations: aa, amino acid(s); bp, base pair(s); BSA, bovine serum albumin; cv., cultivar; dap, days after pollinization; Denhardt's solution, see MATERIALS AND METHODS, section g; Hth, hordothionin; *Hth-1*, gene coding for αHth; kb, kilobase(s) or 1000 bp; MOPS, morpholinopropane sulfonic acid; nt, nucleotide(s); P-box, nt sequence that is repeated in structural regions of some prolamin genes; pfu, plaque-forming units; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; Rubisco, ribulose-1,5-biphosphate carboxylase; SDS, sodium dodecyl sulfate; SSPE, see MATERIALS AND METHODS, section g.

Thionins and their homologues constitute a well-characterized polypeptide family that includes the endosperm thionins from cereals (Balls et al., 1940; Carbonero and García-Olmedo, 1969; Redman and Fisher, 1968; Hernández-Lucas et al., 1978; Békés and Lasztity, 1981), the viscotoxins from the mistletoes (Samuelsson et al., 1968), the crambin from the abyssinian cabbage (Teeter et al., 1981), the thionins from *Pyrularia pubera* (Vernon et al., 1985) and, as reported quite recently, the thionins from barley leaves (Gausing, 1987; Böhlmann et al., 1987). Although their biological function is as yet unknown, they have received considerable attention because of their toxicity to laboratory animals (intraperitoneally), cultured mammalian cells, yeasts and phytopathogenic bacteria (see García-Olmedo et al., 1982) and because their small size (45–47 aa residues) and high cysteine content make them good model systems for a variety of structural studies (Lecomte et al., 1982; Hendrickson and Teeter, 1981; Clore et al., 1986). Both the endosperm thionins and the viscotoxins have been found to mimic in vitro the chloroplast-specific thioredoxin in its ability to activate photosynthetic enzymes (Wada and Buchanan, 1981), but the concentrations required are probably incompatible with such a role in vivo.

Studies of their in vitro and in vivo synthesis, carried out in developing barley endosperm (Ponz et al., 1983), as well as the molecular cloning of barley cDNAs corresponding to α- and β-hordothionins (Ponz et al., 1986; Hernández-Lucas et al., 1986), indicate that they are synthesized as much larger precursors that undergo at least two processing steps: the co-translational excision of a signal peptide and the posttranslational elimination of a C-terminal acidic sequence. More recently, thionin cDNAs from barley leaves have been found to have the same general structure (Gausing, 1987; Böhlmann et al., 1987).

We report here the nucleotide sequence of a barley gene that corresponds to α-hordothionin and show that it is specifically expressed in endosperm.

(a) Biological material

Diploid barley *Hordeum vulgare* L. cv. Bomi was used as a source of RNA. Endosperms at different stages of development (10 to 20 dap) and seven-day-old etiolated or green coleoptiles, obtained from seeds germinated in vermiculite, were collected into liquid nitrogen, prior to extraction.

(b) Reagents

Chemicals used were of analytical grade. Restriction enzymes, T4 DNA ligase, PolIk, S1 nuclease and other enzymes were obtained from Boehringer, Amersham, or New England Biolabs. The [α-^{32}P]dATP or [γ-^{32}P]ATP were from Amersham; oligodeoxynucleotides used as primers for sequencing and transcription initiation experiments were synthesized in an Applied Biosystems apparatus (Centro de Biología Molecular, Madrid).

(c) Screening of a barley genomic library

A genomic library from *Hordeum vulgare* cv. Villa was the kind gift of W. Rohde (Köln, FRG). The library was constructed by inserting DNA fragments in the 14–22 kb size range, obtained by partial digestion with *Mbo*I, into the λEMBL-4 vector restricted with *Bam*HI. Packaged recombinant DNA was used to infect *E. coli* K803. The library was screened by the in situ plaque hybridization technique of Benton and Davis (1977), using as a probe the insert of the cDNA clone pTH1 (Ponz et al., 1986). The screening was carried out in duplicate and positive plaques appearing at matching positions in the duplicate filters were purified by multiple rounds of plating and screening. Large-scale preparation of purified recombinant phages was carried out according to Yamamoto et al. (1970). Restriction maps of cloned DNA were deduced by standard procedures (Maniatis et al., 1982).

(d) Nucleotide sequencing

The appropriate fragment from the genomic clone was subcloned in the plasmid pUC19 (Norrander et al., 1983). The relevant part of the fragment, which

contained the *Hth-1* gene, was sequenced by the chemical modification method of Maxam and Gilbert (1980) and by the dideoxy chain-termination method of Sanger et al. (1977). The latter was carried out with the M13mp18 and M13mp19 vectors, using both universal and specific synthetic oligodeoxynucleotide primers (Norrander et al., 1983; Sanchez-Pescador and Urdea, 1984). Over 95% of the sequence was determined twice or more. Computer-assisted analysis of nucleotide and deduced amino acid sequences was performed using the Micro-Genie program from Beckman (Queen and Korn, 1984).

(e) S1 nuclease protection analysis

This analysis was carried out by standard procedures (see Maniatis et al., 1982). Poly(A)$^+$ RNA from developing endosperm (20 dap) was prepared as described by Ponz et al. (1983). The *Bam*HI-*Pst*I DNA fragment used included the sequence coding for the signal peptide and the first three amino acids of the mature protein, as well as over 1 kb of the promoter region, and was labeled in the 5′ end at the *Pst*I site using polynucleotide kinase and [γ-^{32}P]ATP. The labeled DNA (100000 cpm) and 12 μg of poly(A)$^+$ RNA were precipitated and redissolved in 50 μl of 40 mM MOPS, pH 6.8, 400 mM NaCl, 1 mM EDTA, and 80% formamide. After denaturation for 2 min at 70°C, the mixture was incubated for 12 h at 40°C and then diluted with 500 μl of 30 mM Na · acetate, pH 4.6, 50 mM NaCl, 4 mM $ZnCl_2$ and 10% glycerol. Treatment with 500 units of mung-bean nuclease I was carried out for 90 min at 37°C.

(f) Primer extension

A 17-mer synthetic oligodeoxynucleotide, complementary to the *Hth-1* mRNA, was used to prime the reverse trancriptase reaction, using 1 μg of poly-(A)$^+$ RNA which had been heated at 100°C for 10 min and rapidly cooled. Reaction conditions and other details were as previously reported (Ponz et al., 1986).

(g) DNA blots, RNA blots and hybridization

Genomic DNA was isolated from seven-day-old dark-grown barley seedlings essentially as described by Murray and Thompson (1980). Restriction digestion, agarose gel electrophoresis and Southern blotting to nylon membranes (Hybond N, Amersham) were performed according to Maniatis et al. (1982) and to the manufacturer's instructions. Hybridization to nick-translated inserts of appropriate cDNA clones was in 5 × SSPE (0.9 M NaCl, 0.05 M NaH_2PO_4, pH 7.4, 0.005 M EDTA), 2 × Denhardt's (0.04% polyvinylpyrrolidone, 0.04% BSA, 0.04% Ficoll), 0.2% SDS, 100 μg/ml of herring sperm DNA, at 65°C or at 58°C, according to the stringency required in each case. The RNA for Northern blots was isolated from developing endosperms and from seven-day-old etiolated or green coleoptiles. Glyoxal-denatured RNAs (Thomas, 1983) were fractionated by electrophoresis in 1.2% agarose gels, transferred to nitrocellulose filters (Hybond C, Amersham) and hybridized as previously described (Ponz et al., 1986). Hybridization of Southern-blotted DNA with partially degraded RNA labeled with T4 polynucleotide kinase was carried out as described by Domoney and Casey (1983; 1987).

RESULTS AND DISCUSSION

(a) Isolation and characterization of an α-hordothionin genomic clone

About 400000 pfu from the genomic library were screened with a cDNA radioactive probe corresponding to barley *Hth-1*. Two clones λTH1 and λTH2, were thus detected and purified. The cloned DNAs in the two recombinant phages had identical restriction maps, so clone λTH1 was chosen for further characterization (Fig. 1). The *Eco*RI-*Sal*I terminal fragment of the insert in clone λTH1 was the only one giving a positive hybridization signal with the probe. A clone of this fragment (pTHG1) was obtained in the plasmid vector pUC19, and a more detailed restriction map was drawn to facilitate election of a sequencing strategy.

(b) Structure and nucleotide sequence of the *Hth-1* gene

The *Hth-1* gene was sequenced following the strategy indicated in Fig. 1. The nucleotide sequence

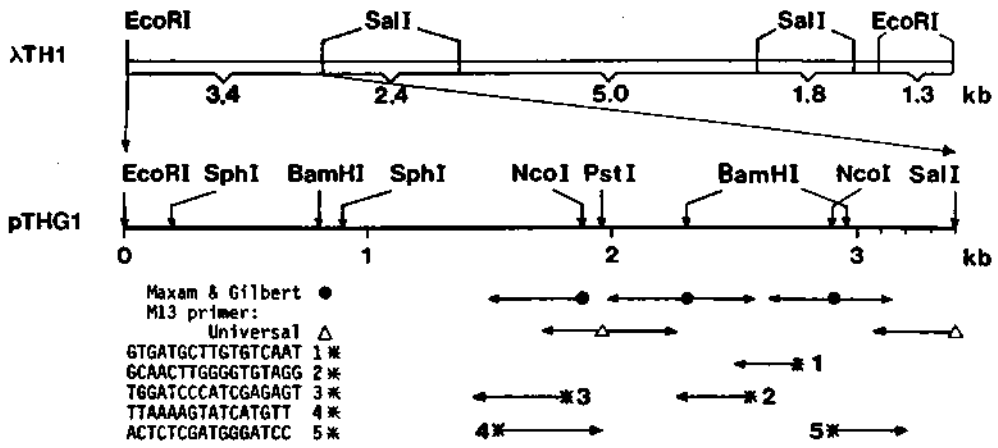


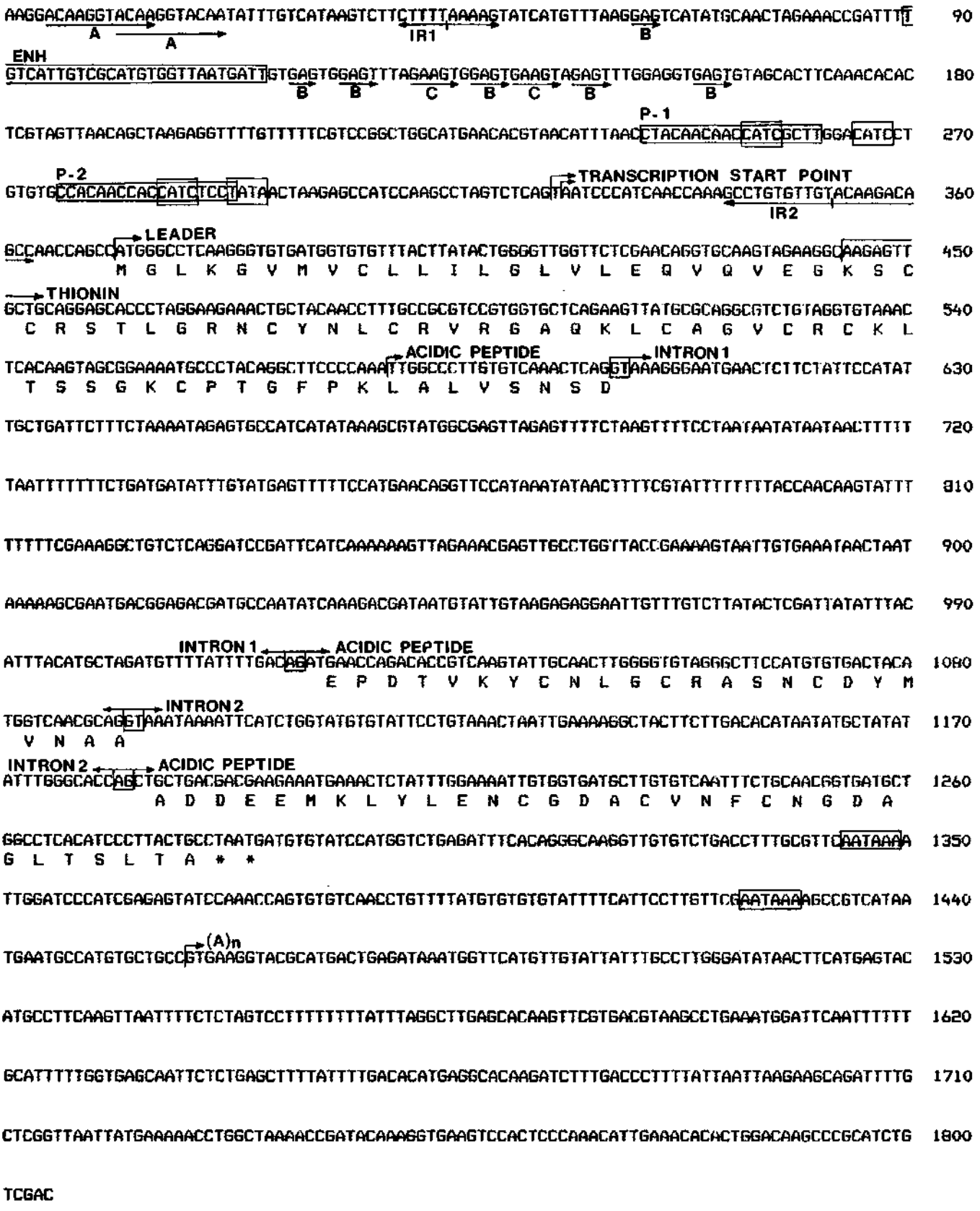
Fig. 1. Partial restriction maps of the inserts in clones λTH1 and pTHG1, and sequencing strategy for the *Hth-1* gene. The *Eco*RI sites at the extremes of the λTH1 map are from the λ EMBL-4 vector and are adjacent to the cloned insert. The 3.4-kb *Eco*RI-*Sal*I fragment was the only one hybridizing with the cDNA probe in a Southern-blot hybridization experiment and was subcloned (pTHG1) in the plasmid vector pUC19. Sequencing was carried out by the methods of Maxam and Gilbert (1980) and Sanger et al. (1977). The sequencing strategy and the primers used for the second method are indicated.

of the gene, including the 5′ and 3′ regions, respectively, adjacent to the start and stop codons, is presented in Fig. 2. Comparison of the previously determined sequence of the cDNA with that of the genomic clone indicates the presence of two introns, 420 and 91 nt in length, which interrupt the sequence in that part coding for the C-terminal acidic peptide (Fig. 2). The intron sequences are typically A + T-rich (70% and 61%, respectively) and are flanked by GT (at +1 and +2) in the 5′ ends and by AG (at −2 and −1) in the 3′ ends, as reported for many other genes (see Shapiro and Senapathy, 1987). Those parts of the sequence common to the cDNA and the genomic DNA are identical with exception of nt position 593, where a C → A substitution does not lead to a change in the coded amino acid, and of nt position 1452, where an extra T appears in the cDNA, 6 nt upstream from the poly(A) tail. These minor differences might be due to the fact that the cDNA had been synthesized from barley cv. Bomi, whereas the genomic clone was from cv. Villa.

(c) Characterization of the initiation site of the *Hth-1* gene

To determine the 5′ terminus of the gene both the S1 nuclease-protection and the primer-extension methods were used. The *Bam*HI-*Pst*I fragment was labeled at the 5′ end and protected from the nuclease treatment with poly(A)$^+$RNA obtained from developing endosperm (approx. 20 dap). Two prominent protected fragments, respectively extending to nt positions 325 and 326 (−47 and −46), plus several minor components were detected (Fig. 3A).

To confirm that the fragments protected from S1 nuclease digestion represent the transcriptional start points, primer extension analysis was performed, using the 17-mer synthetic oligodeoxynucleotide 5′–CTCCTGCAGCAACTCTT–3′, as primer. This primer was complementary to the mRNA between nt positions 444 and 461 of the sequence in Fig. 2. Using the same poly(A)$^+$RNA as template, the primer was extended with reverse transcriptase, yielding again two main fragments which extended precisely to nt positions 325 and 326 of the sequence, respectively, plus several additional ones at lower mobilities (Fig. 3B). The consensus of the two experiments indicates the existence of two apparent transcriptional start points, which probably means that both in the protection and in the extension experiments the mRNA for βHth, which is highly homologous to αHth (Hernández-Lucas et al., 1986), has been acting as an effective complement or, alternatively, that the heterogeneity could be due to melting at the end of the hybrid formed. Since 87% of the reported plant genes have an A at the transcription start, position 326 (A) is more likely the true start point for the *Hth-1* gene than position 325 (T).



TCGAC

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the *Hth-1* gene. Short direct repeats are indicated by horizontal arrows and identified by letters (A–C). Inverted repeats (IR) are indicated by divergent horizontal arrows. TATA, CATC and AATAAA boxes are indicated. An enhancer-like sequence (ENH) and a repeated sequence (P-1/P-2), which is homologous to the coding sequence of some prolamins, are also boxed. The sequence of the posttranslationally excised, C-terminal peptide is interrupted by two introns as indicated. The one-letter amino acid symbols have been aligned with the first base of each codon.

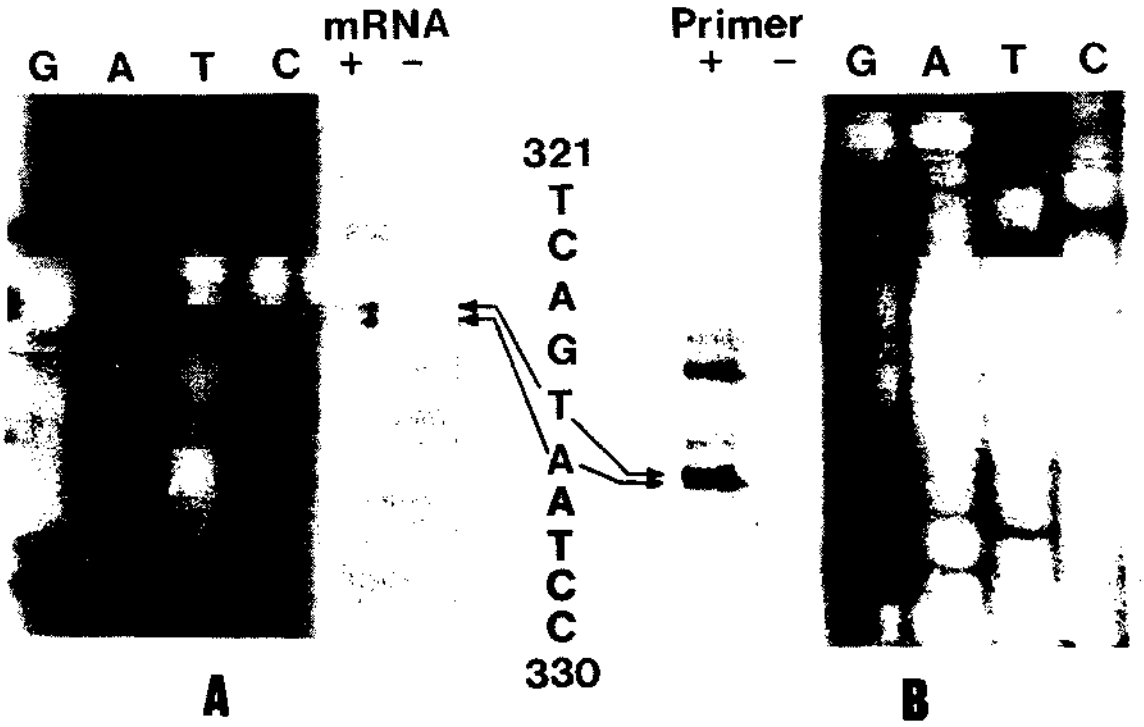


Fig. 3. Determination of the transcription start point for the *Hth-1* gene. (A) S1 nuclease protection experiment carried out with poly(A)$^+$ RNA from developing endosperm (20 dap) and the *Bam*HI-*Pst*I fragment from the insert in clone pTHG1 (see Fig. 1). Lanes marked with + and − correspond to reaction mixtures with and without poly(A)$^+$ RNA, respectively. (B) Primer extension experiment carried out with the same poly(A)$^+$ RNA and a 17-mer synthetic oligodeoxynucleotide which was complementary to the Hth mRNA in the region corresponding to nt positions 444–461 of Fig. 2. Lanes marked + and − correspond to reaction mixtures with and without primer, respectively. For details of experiments, see MATERIALS AND METHODS, sections e and f. Protected and extended fragments were sized in 8% polyacrylamide sequencing gels. A DNA fragment of known sequence was run in parallel.

(d) Location of the translational start codon

The sequence from the genomic DNA around the first two Met codons that could act as translational starts has been aligned with those of the cDNAs from α- and βHth (Ponz et al., 1986; Hernández-Lucas et al., 1986) and from the leaf thionins (Gausing, 1987; Böhlmann et al., 1987) in Fig. 4. Since the sequences around both Met codons are compatible with the start function, according to the rules of Kozak (1987), the first one, which is upstream from the 5′ end of the published αHth cDNA, is the more likely candidate. However, it should be pointed out that in the βHth cDNA the second Met codon is conserved at the same position, while the first is not, and in the cDNAs from the leaf thionins a single Met codon is present four positions upstream from the first one from αHth. Furthermore, in wheat, a cDNA clone from developing endosperm has two Met codons (C. Maraña, unpublished), the second of which is conserved at the same position as the second in barley, while the first one is at nt position −3 with respect to the first αHth Met codon (+1 with respect to the leaf one). This extreme heterogeneity around the start codon is in contrast with the high conservation of the rest of the leader sequence within the leaf or endosperm groups of thionin precursors.

(e) Number of copies of the cloned gene

To estimate the number of copies of the cloned gene in the barley haploid genome, amounts of the *Eco*RI-*Sal*I fragment (3.4 kb) corresponding to 1, 2, 4 and 8 copies were treated with *Bam*HI (Fig. 5A) and electrophoresed in parallel with appropriate amounts of genomic DNA treated with the same enzyme. After Southern blotting, the filters were hybridized with the *Eco*RI–*Sal*I fragment, labeled by nick translation. An expected *Bam*HI fragment of about 1.5 kb was detected in the genomic DNA with a signal intensity corresponding to approximately

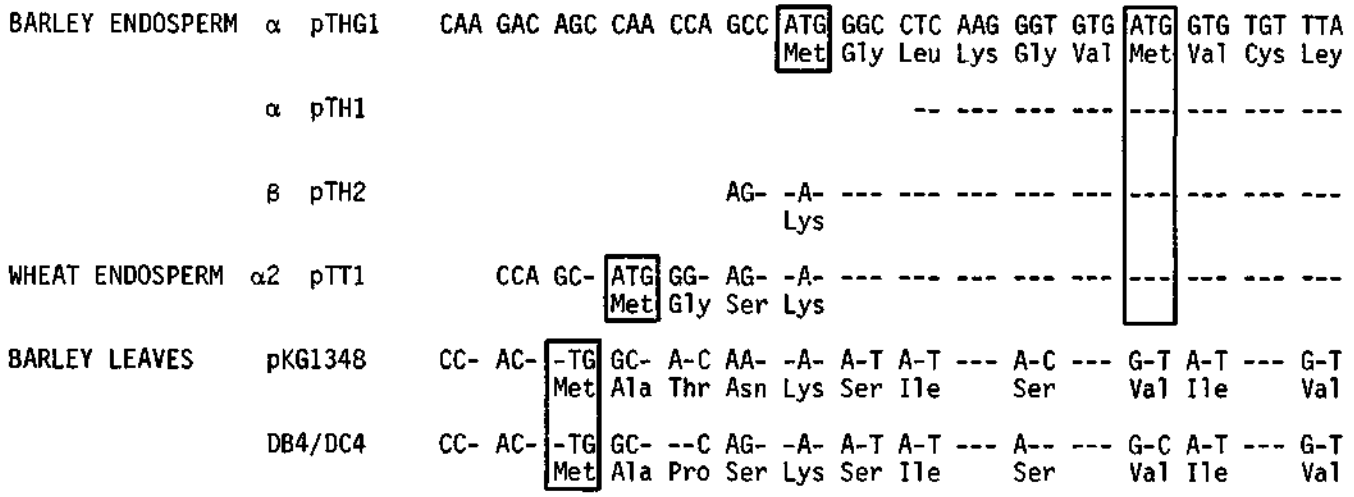


Fig. 4. Translation start codon in the *Hth-1* gene. The sequence around the putative first ATG codon in the *Hth-1* gene (pTHG1) has been aligned with the corresponding regions of the α- and β-thionin cDNAs from barley (Ponz et al., 1986; Hernández-Lucas et al., 1986), the cDNA of α2-thionin from wheat (C. Maraña, unpublished), and the cDNAs of leaf thionins (pKG1348, Gausing, 1987; DB4 DC4 Böhlmann et al., 1987). Dashes indicate identity. Met codons are boxed.

two copies (Fig. 5A). Strong signals at lower electrophoretic mobilities seemed to indicate that sequences within the 3.4-kb *Eco*RI–*Sal*I fragment were highly repeated elsewhere in the genome. These highly repeated sequences were found to be outside the coding and intron sequences by using three subprobes: the *Eco*RI-*Nco*I, *Nco*I-*Nco*I, and *Nco*I-*Sal*I fragments, which, respectively, represent the 5′ region up to the translation start, the coding and intron regions from the translation start to close to the stop codon, and the 3′ region (Figs. 1 and 2). Only the second probe gave a clean two-band pattern against *Hin*dIII-digested DNA (Fig. 5B), indicating that the highly repeated sequences were outside the coding and intron regions.

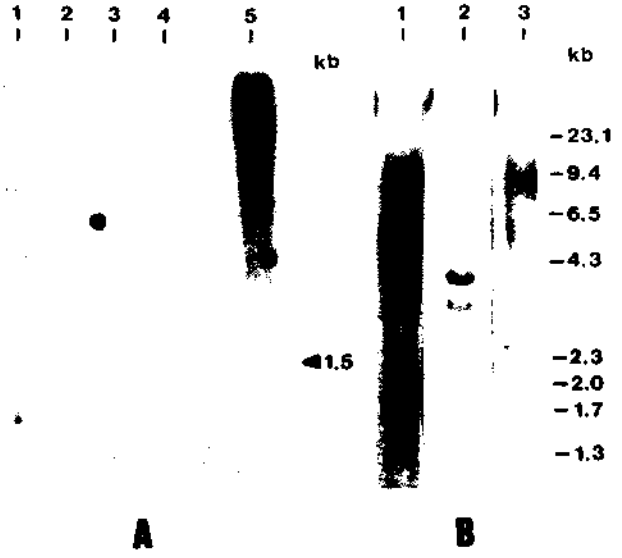


Fig. 5. Number of copies of the *Hth-1* gene. (A) Hybridization of nick-translated pTHG1 insert to 10 μg of barley DNA (lane 5) and amounts of the same fragment corresponding to 8, 4, 2 and 1 copy equivalents per haploid genome (lanes 1–4), all digested with *Bam*HI endonuclease. (B) *Hin*dIII-digested barley DNA (15 μg/lane) hybridized with the following nick-translated probes derived from pTHG1: *Eco*RI-*Nco*I (lane 1), *Nco*I-*Nco*I (lane 2), and *Nco*I-*Sal*I (lane 3).

(f) Relevant features of the 5′-flanking region

Besides usual features of promoter regions, such as one TATA box, three CATC boxes, and tandem and inverted repeats (Fig. 2), the following features of the 5′-flanking region deserve attention: a putative enhancer sequence, starting at position −282 with respect to the first ATG codon (Fig. 6A); two 18-nt-long sequences (P-boxes) that appear upstream from the TATA box and are homologous to sequences that are repeated several times in a structural domain of certain storage prolamins (Fig. 6B); and an extensive homology with the B-hordein promoter that includes an inverted repeat (Fig. 6C). In Fig. 6A, a stretch of 27 nt (nt positions 90 to 116 in Fig. 2) has been aligned with homologous sequences appearing at similar positions in genes expressed in the endosperms of maize, wheat and barley. The maize (zein) sequence includes a 22-nt specific binding-site

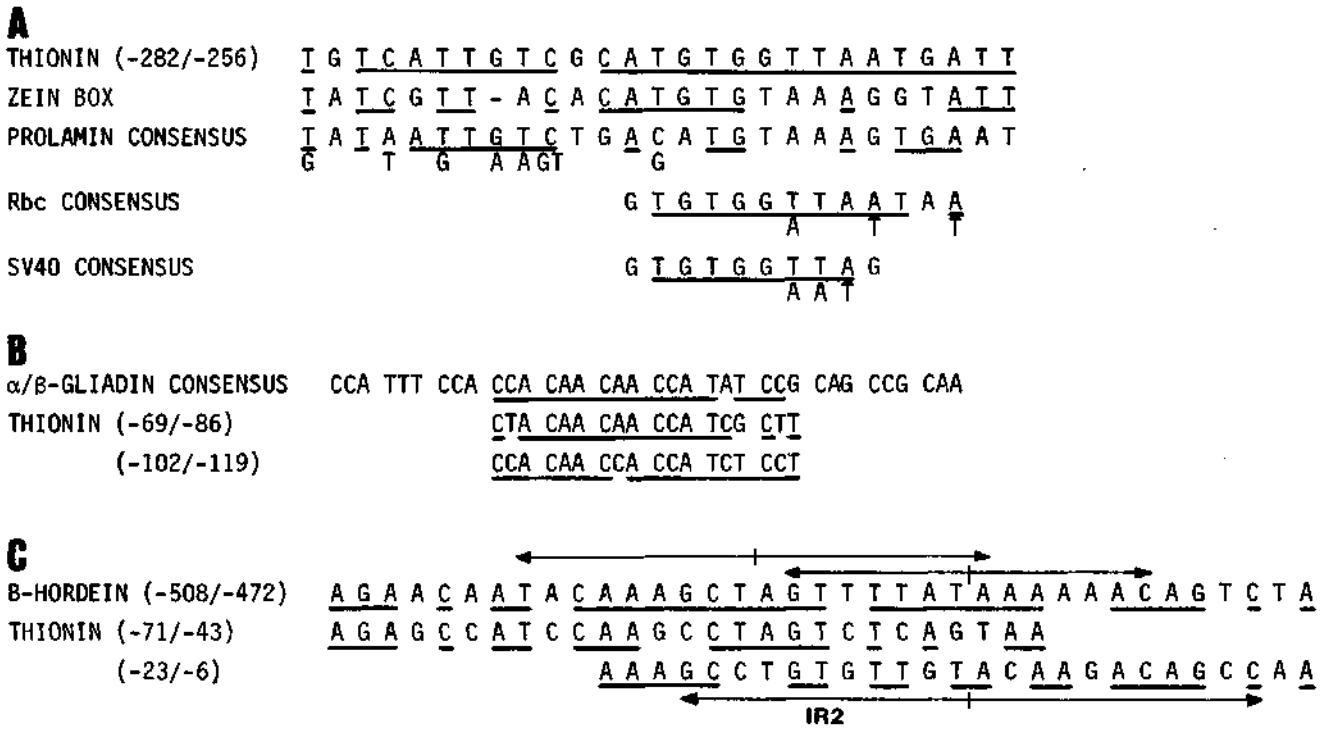


Fig. 6. Homologies of the 5'-flanking region of the *Hth-1* gene with other genes. **(A)** The sequence between nt positions −282 and −256 has been aligned with the putative enhancer sequences appearing in similar positions in zein genes from maize (Maier et al., 1987), a consensus sequence derived from other prolamin genes (wheat α-gliadin, Rafalski et al., 1984; α/β-gliadin, Reeves et al., 1987; γ-gliadin A and B, Rafalski, 1986; barley B-hordein, Forde et al., 1985; wheat LMW glutelin, Colot et al., 1987), a consensus sequence from Rubisco genes (Kuhlemeier et al., 1987) and the consensus core sequence from the enhancers of the virus SV40 (Sassone-Corsi and Borrelli, 1986). **(B)** The P-1 and P-2 sequences have been aligned with a consensus sequence coding for the repetitive motive in the α/β-gliadin repetitive structural domain (Summer-Smith et al., 1985). **(C)** A sequence from the B-hordein gene (Forde et al., 1985) starting at position −508 with respect to the first ATG codon has been aligned with the indicated thionin sequences.

for a nuclear factor, which has been determined by filter binding, gel retention, and DNase I footprinting assays (Maier et al., 1987). This region also overlaps the −300 box described for cereal storage proteins by Forde et al. (1985) and has homology with a regulatory sequence from Rubisco genes, tested by plant transformation (Kuhlemeier et al., 1987; Strittmatter and Chua, 1987), and with the core sequence of many viral and animal enhancers (Sassone-Corsi and Borrelli, 1986). In Fig. 6B, the two P-boxes are compared with a consensus nucleotide sequence corresponding to a repetitive motive in the N-terminal domain of an α/β-gliadin gene (Summer-Smith et al., 1985). In Fig. 6C a 36-nt sequence from the promoter of B-hordein (nt positions −508 to −472) can be aligned with two sequences from the *Hth-1* promoter which are much closer to the first ATG codon (nt positions −71 to −43 and −23 to −6, respectively). Both in the B-hordein gene sequence and in the second *Hth-1*, imperfect inverted repeats can be discerned (Fig. 6C).

(g) Endosperm-specific expression of the *Hth-1* gene

As expected from previous experiments in which Hth was pulse-labeled at different stages of endosperm development (Ponz et al., 1983), mRNA levels, as estimated by dot-blot assays (Fig. 7A), indicated that the *Hth-1* gene was expressed in the cell-proliferation phase, with a maximum at 13–16 dap. No *Hth-1* mRNA was detected in either etiolated or green coleoptiles, indicating that this gene is not among those thionin genes that are highly expressed in developing leaves (Gausing, 1987; Böhlmann et al., 1987). This observation was confirmed by using radioactive RNA from the different tissues to hybridize to Southern-blotted DNA from

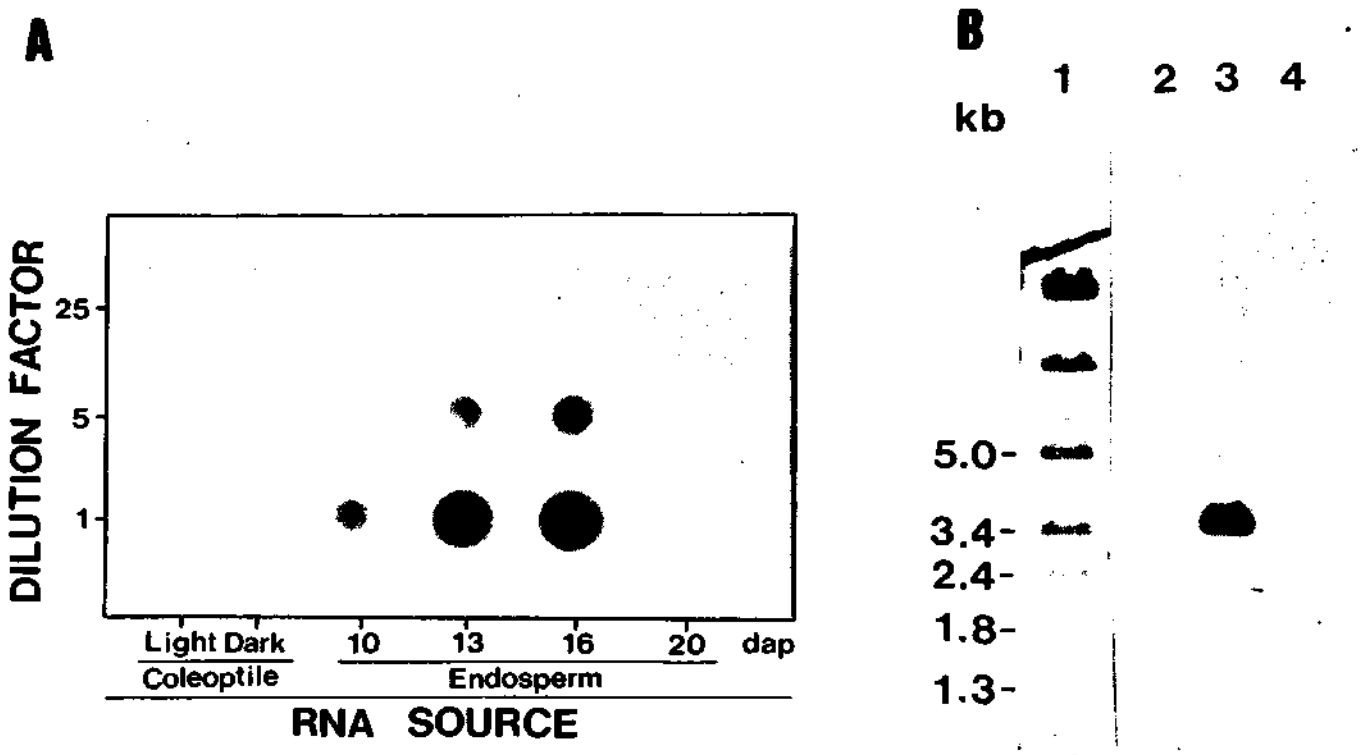


Fig. 7. Endosperm-specific expression of the *Hth-1* gene. (**A**) Dot-blot hybridization of RNAs from the indicated sources with the nick-translated insert in clone pTHG1 (10^7 cpm/μg). Equal amounts (2 μg) of each RNA were spotted. (**B**) DNA from clone λTH1 was digested with *Eco*RI + *Sal*I, fractionated by electrophoresis in a 5% agarose gel and stained with ethidium bromide (lane 1) or transferred to a Hybond N (Amersham) nylon filter (lanes 2–4) and hybridized with partially degraded, radioactively labeled RNAs from the following sources: etiolated coleoptiles (lane 2), 20 dap endosperm (lane 3) and green coleoptiles (lane 4).

the λTH1 clone (Fig. 7B). This experiment further showed that none of the DNA fragments, other than the expected 3.4 kb one containing the *Hth-1* gene, was expressed at a significant level in these tissues.

Although expressed in the same tissue, the *Hth-1* gene is switched on and off at earlier moments than the B-hordein genes, so the homologies in the putative enhancer sequences (Fig. 6A) and in the regions represented in Fig. 6C are more likely to be involved in the tissue specificity than in determining expression at a given developmental stage.

(h) Conclusions

The gene for the barley α-hordothionin toxin (*Hth-1*) has the following features: (*i*) Two introns of 420 and 91 nt respectively, that interrupt the sequence coding for the precursor protein in the C-terminal acidic region that is not retained in the mature protein. (*ii*) One TATA box. (*iii*) Three CATC boxes. (*iv*) An enhancer-like sequence that is homologous to sequences appearing at similar positions in the promoters of other endosperm genes, and that includes shorter regions of homology to regulatory sequences in Rubisco promoters and to enhancers from the SV40 virus. (*v*) Two versions of an 18-nt sequence (nt −69 to −86 and −102 to −119) that is repeated in coding sequences of some storage prolamins. (*vi*) Two extensive regions (nt −71 to −43 and −23 to −6) which are homologous to one region of the promoter of the B-hordein gene that is quite distant from the translation start (−508 to −472).

The *Hth-1* gene, for which there is a maximum of 2–4 copies per haploid genome, is specifically expressed in developing endosperm during the cell proliferation phase, with a maximum mRNA level at 13–16 dap.

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