

## Primary structure and differential expression of $\beta$ -amylase in normal and mutant barleys

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The primary structure of barley endosperm  $\beta$ -amylase, an enzyme which catalyses the liberation of maltose from 1,4- $\alpha$ -D-glucans, has been deduced from the nucleotide sequence of a cloned full-length cDNA. The mRNA is 1754 nucleotides long [excluding the poly(A) tail] and codes for a polypeptide of 535 amino acids with a relative molecular mass of 59 663. The deduced amino acid sequence was compared with the sequences of ten peptides obtained from the purified enzyme and unambiguous identification was obtained. The N-terminal region of the deduced sequence was identical to a 12-residue cyanogen-bromide-peptide sequence, indicating that  $\beta$ -amylase is synthesized as the mature protein. A graphic matrix homology plot shows four glycine-rich repeats, each of 11 residues, preceding the C-terminus. Southern blotting of genomic DNA demonstrates that  $\beta$ -amylase is encoded by a small gene family, while cDNA sequence analysis indicates the presence of at least two types of mRNA in the endosperm. Dot and northern blot analysis show that Hiproly barley contains greatly increased levels of  $\beta$ -amylase mRNA compared to the normal cultivar Sundance, whereas Risø mutant 1508 contains only trace amounts. These results correlate well with the deposition of  $\beta$ -amylase during endosperm development in these lines. Low but similar amounts of  $\beta$ -amylase mRNAs sequences were detected in leaves and shoots from normal and mutant barleys, demonstrating that the mutant *lys3a* (1508) and *lys1* (Hiproly) genes do not affect the expression of  $\beta$ -amylase in these tissues.

$\beta$ -Amylase catalyses the liberation of maltose from the non-reducing end of 1,4- $\alpha$ -D-glucans. The enzyme is found in the embryo and the endosperm of the barley seed [1, 2]. It is, however, most abundant in the endosperm where it accounts for 1–2% of the total protein [3].  $\beta$ -Amylase occurs in two forms: free and latent or bound [4]. The two forms are associated with the salt-soluble fraction and the latent form can only be extracted in the presence of a reducing agent. Pure crystalline  $\beta$ -amylase has been prepared from mature barley grains [5] and it was shown by sedimentation equilibrium centrifugation that the protein has a relative molecular mass of 57 200. Little is known about the subcellular localization of  $\beta$ -amylase in the barley endosperm, but it seems to be restricted to the cytoplasm of the pea leaves [6]. Jonassen et al. [7] have shown that barley endosperm  $\beta$ -amylase is synthesized on membrane-bound polysomes and therefore likely to be deposited in membrane-bound compartments.

Several mutant genes of barley affect the deposition of  $\beta$ -amylase dramatically in the endosperm. These include the high-lysine mutant genes of Hiproly and Risø mutant 1508. Hiproly is a spontaneous mutant found by screening the world barley collection [8], and Risø mutant 1508 was induced using ethyleneimine [9]. Whereas the amount of  $\beta$ -amylase is increased substantially in Hiproly seeds [3], it is very low in Risø

mutant 1508 [10]. The mutant genes in Hiproly (*lys1*) and Risø mutant 1508 (*lys3a*) are 'regulatory' and are both located on chromosome 7, although not linked [11].

$\beta$ -Amylase is encoded by a locus which has been mapped on barley chromosome 4 by linkage analysis [12]. At present nothing is known of how the genes on chromosome 7 affect the expression of the  $\beta$ -amylase gene(s) on chromosome 4.

The present paper describes the isolation of two  $\beta$ -amylase cDNA clones from a barley endosperm cDNA library. Sequence analysis of these two clones together with peptide sequence analysis have enabled us to obtain the complete amino acid sequence of  $\beta$ -amylase. Furthermore we have studied the expression of  $\beta$ -amylase, both at the protein and RNA levels in developing endosperms and other tissues of normal and mutant barleys. The amino acid sequence will facilitate structural analysis of the protein and the cDNAs will provide useful probes to study the organization and the control of expression of the genes in cereals.

### MATERIALS AND METHODS

#### *Plant material*

Barley plants, *Hordeum vulgare* L. cv. Hiproly, Sundance and Risø mutant 1508, were grown in the field. Barley seedlings were grown on sterile vermiculite at 25°C for about 8 days. Developing endosperms, young shoots and young green leaves were harvested, frozen in liquid nitrogen and stored at –80°C. An endosperm-rich milling fraction of an

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Abbreviation. SSC, standard saline citrate = 0.15 M NaCl, 0.015 M trisodium citrate.

Enzyme.  $\beta$ -Amylase (EC 3.2.1.2).

unknown two-row barley cultivar was used for the purification of the  $\beta$ -amylase [13].

*Protein purification, isolation of cyanogen bromide (CNBr) fragments and amino acid sequencing*

$\beta$ -Amylase was purified from a protein concentrate, rich in  $\beta$ -amylase, obtained during purification of barley protein Z [13]. The pure enzyme was obtained by DEAE-cellulose anion-exchange and gel filtration essentially as described by Visuri and Nummi [5]. Identity and purity were confirmed by electrophoresis, amino acid analysis and activity studies. Attempts to sequence the enzyme by Edman degradation showed that the N-terminus was blocked.

$\beta$ -Amylase was cleaved with cyanogen bromide in a 100-fold molar excess of 70% formic acid overnight in the dark. Initial separation of the resulting peptides was made on Bio-Gel P60 (1.6  $\times$  90 cm) in 30% acetic acid. The three peaks emerging first from the column were reduced and alkylated with 2-vinylpyridine as described by Friedman et al. [14] and separated by gel filtration as described above. When necessary, further purification was obtained by HPLC on a Waters Widepore C<sub>18</sub> column using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid from 10% to 60% over 45 min.

Amino acid sequencing was performed on a Beckman, model 830B sequencer as previously described [15] or on a gas-phase sequencer model 470A from Applied Biosystems according to the specifications given by the company.

*Extraction of salt-soluble proteins and analysis by SDS-PAGE*

Duplicate samples of barley flour (1 g) were stirred for 1 h at 20°C in 0.1 M phosphate buffer pH 8 with 5 mM DL-dithiothreitol to extract both 'free' and 'bound'  $\beta$ -amylase [16]. Proteins were separated by SDS-PAGE in 16% acrylamide slab gels containing 4 M urea and 4 mM dithiothreitol [17].

*Isolation, fractionation, hybrid-selection and in vitro translation of RNA*

Total polysomes, membrane-bound polysomes, polyosomal RNA and poly(A)-rich RNA from shoots, leaves and endosperms were prepared as described previously [18, 19]. Poly(A)-rich RNA derived from a membrane-bound polysome fraction was size-fractionated on 5–30% (w/v) sucrose gradients [20]. The procedure for hybrid selection was exactly as described by Kreis et al. [17]. The mRNAs were translated in a wheat-germ system containing L-[<sup>3</sup>H]leucine and L-[<sup>3</sup>H]proline. The translation products were separated by SDS-PAGE and visualized by fluorography.

*Construction of endosperm cDNA libraries*

The cDNA clones were isolated from two libraries constructed by different methods. The first method followed the procedures described previously [21, 22]. Size-fractionated poly(A)-rich RNA from Hiproly was used as a template for cDNA synthesis. Double-stranded cDNAs were given cohesive ends using *Hind*III linkers and ligated into pUC8. JM83 was transformed and selected for ampicillin resistance. The second method follows the cloning procedure described by Heidecker and Messing [23]. Size-fractionated poly(A)-rich RNA from Hiproly was used as the template, the vector was

pUC9 and *Escherichia coli* strain JM83 was transformed by the method of Hanahan [24]. 1000 transformants were recovered and plated on nylon membrane filters.

*Screening of cDNA libraries*

*Screening by 'plus-minus' (differential) hybridization.* Poly(A)-rich RNAs isolated from mutant Risø 1508 and Hiproly endosperms contain trace amounts and much increased levels, respectively, of  $\beta$ -amylase mRNAs. A 'plus-minus' hybridization technique was therefore used to screen the first endosperm-specific cDNA library for potential  $\beta$ -amylase cDNA clones by *in situ* colony hybridization [25]. Three sets of filters, each containing 400 transformants, were hybridized to three different probes. The first two sets were hybridized to ( $\gamma$ -<sup>32</sup>P)-labelled poly(A)-rich RNA (1  $\mu$ g  $\equiv$  2  $\times$  10<sup>6</sup> cpm) from Hiproly and mutant 1508 and the third set was hybridized to <sup>32</sup>P-labelled cDNAs of B and C hordein (100 ng  $\equiv$  3  $\times$  10<sup>6</sup> cpm). Hybridization was carried out at 65°C in 2  $\times$  SSC, 10  $\times$  Denhardt's solution, 0.2% SDS and 100  $\mu$ g/ml herring sperm DNA. The filters were washed in 2  $\times$  SSC, 0.1% SDS at 65°C. (Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin).

*Screening using the HindIII insert of clone pc $\beta$ H 475.* Biodyne A nylon filters containing about 1000 colonies obtained by the second cDNA cloning method were screened by *in situ* colony hybridization [25] using the <sup>32</sup>P-labelled [26] insert of pc $\beta$ H475 (see Fig. 3) as a probe. Hybridization conditions were as described above.

*Nucleotide sequencing*

The inserts of pc $\beta$ H475 and pc $\beta$ C51 were sequenced using three approaches. Firstly, restriction fragments as indicated in the legend of Fig. 4 were sub-cloned into M13 mp18 or mp19 [27]. Secondly, Bal 31 deletions [28] were generated using CsCl<sub>1</sub>-purified plasmid DNA and sub-cloned into M13. M13 sub-clones were sequenced by the dideoxy method of Sanger et al. [29]. The nucleotide sequence of the 5' end of the cDNA insert of pc $\beta$ C51 was determined by double-stranded sequencing [30].

*Primer extension analysis*

The 114-bp *Acc*I restriction fragment of pc $\beta$ C51 (see Figs 4 and 5) was used as a primer, and end-labelled using [ $\gamma$ -<sup>32</sup>P]ATP and T4 kinase. Primer (50 ng) was added to 2  $\mu$ g poly(A)-rich RNA from Hiproly, ethanol-precipitated and resuspended in 65% formamide, 0.4 M NaCl, 200 mM Pipes pH 6.4 and 2 mM EDTA in a final volume of 100  $\mu$ l. Primer and RNA were denatured at 85°C for 5 min and cooled slowly to 35°C to allow primer and RNA to anneal. The primer-RNA hybrid was recovered by ethanol precipitation and extended with reverse transcriptase (1U) in 6  $\mu$ l buffer containing 2 mM each of dATP, dGTP, dCTP, dTTP and 10 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 10 mM Tris/HCl pH 7.4. Primer extension under chain-terminating conditions was used to sequence the 5'-end of the  $\beta$ -amylase mRNA [29].

*Southern blots*

Isolation of plant DNA, digestion with restriction enzymes, gel electrophoresis, transfer of DNA to nylon membranes and hybridization were carried out as described by

Kreis et al. [31] and Williamson et al. [19]. The large *AccI* restriction fragment (789 bp) from the insert of *pcβC51* was labelled with  $^{32}\text{P}$  by nick translation ( $6 \times 10^8$  dpm/ $\mu\text{g}$ ) [26] and used as a probe. Hybridization was carried out at  $65^\circ\text{C}$  for 20 h in  $10 \times$  Denhardt's solution, 50 mM Tris/HCl pH 7.4,  $2 \times$  SSC, 1% SDS, 200  $\mu\text{g}/\text{ml}$  herring sperm DNA, and 10 ng/ml probe. Washing was in  $2 \times$  SSC, 1% SDS at  $65^\circ\text{C}$ .

#### Dot hybridization

Total polysomal RNA from leaves, shoots and endosperms of normal and mutant barley was bound to Gene Screen Plus (TM) membranes using a hybrid-dot apparatus. The large *AccI* fragment of *pcβC51* (789 bp) was nick-translated (see above) and hybridized to the RNA. Hybridization was carried out at  $42^\circ\text{C}$  for 16 h in 50% formamide,  $5 \times$  Denhardt's solution,  $5 \times$  SSPE, 0.1% SDS, 250  $\mu\text{g}/\text{ml}$  denatured herring sperm DNA, 50  $\mu\text{g}/\text{ml}$  poly U and 5 ng/ml probe. Washing of the filters was at  $50^\circ\text{C}$  in  $2 \times$  SSC in 0.1% SDS.

#### Northern blotting

Poly(A)-rich RNA from normal and mutant endosperms was denatured using glyoxal and dimethylsulphoxide [32], separated on a 1.4% agarose slab gel and transferred to Bio-dyne A membranes [19, 33]. The large *AccI* fragment was used as a probe (see above). Hybridization was at  $60^\circ\text{C}$  for 16 h in  $10 \times$  Denhardt's solution,  $4 \times$  SSPE, 1% SDS, 0.1% sodium diphosphate, 200  $\mu\text{g}/\text{ml}$  denatured herring sperm DNA and 5 ng/ml probe. Washing was in  $4 \times$  SSC, 0.5% SDS at  $60^\circ\text{C}$ .

## RESULTS

#### Synthesis of $\beta$ -amylase in vivo and in vitro in endosperms of normal and mutant barley

Salt-soluble proteins extracted from mature seeds of normal barley (cv. Sundance) and two high-lysine mutant barleys (Hipoly and Risø 1508) were separated by SDS-PAGE (Fig. 1A). The position of  $\beta$ -amylase was identified by co-migration with purified and immunoprecipitated protein (not shown). The results demonstrate that the mutant genes have opposite effects on the deposition of  $\beta$ -amylase during endosperm development. In Hipoly seeds the amount is increased by severalfold (Fig. 1A, lane 1) while in mutant 1508 a dramatic decrease is observed (lane 3) when compared to the normal cultivar Sundance (lane 2). These results are comparable to those reported previously [3, 10]. Levels of  $\beta$ -amylase similar to those present in Sundance were detected in other normal cultivars. As expected, mutant 1508 showed only low activity of 'free'  $\beta$ -amylase during endosperm development while endosperms from the normal cultivar Bomi showed 15-fold higher activity (not shown). Analysis of the *in vitro* translation products of poly(A)-rich RNA derived from a membrane-bound polysome fraction from Sundance (Fig. 1B, lane 3), Hipoly (lane 1) and mutant 1508 (lane 2) endosperms show a good correlation between the levels of *in vitro* products of  $\beta$ -amylase mRNAs, the protein deposited and the enzymatic activity during development. These results suggest that the mRNAs specifying  $\beta$ -amylase in mutant 1508 endosperms are absent or only present in very low amounts, while in Hipoly endosperms the amounts are drastically increased.

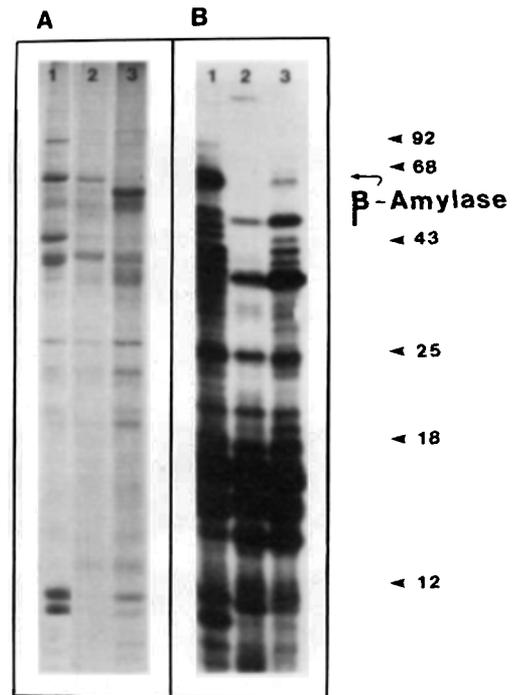


Fig. 1. Comparison of (A) *in vivo* salt-soluble proteins and (B) *in vitro* translation products of mRNA from endosperms of normal and mutant barley. (A) Salt-soluble proteins from mature grain of barley mutant Hipoly (lane 1), cultivar Sundance (lane 2) and mutant 1508 (lane 3) were separated on a 16% SDS-PAGE-urea gel and stained with Coomassie blue. (B) *In vitro* translation products of poly(A)-rich RNA prepared from 20-day-old endosperms of Hipoly (lane 1), mutant 1508 (lane 2) and Sundance (lane 3). RNA was translated in a wheat-germ system using  $^3\text{H}$ proline and  $^3\text{H}$ leucine and the labelled products were separated on a 16% polyacrylamide gel and fluorographed. The migration of  $\beta$ -amylase is known from its immunoprecipitation with  $\beta$ -amylase antibodies [7]. Numbers on the right indicate molecular mass in kDa

#### Identification of $\beta$ -amylase cDNA clones

Poly(A)-rich RNA from Hipoly endosperms was used as a template for cDNA synthesis because the RNA is highly enriched for  $\beta$ -amylase mRNAs (see above). A cDNA library of 400 transformants was constructed in pUC8 using *HindIII* linkers (see Materials and Methods). The library was screened for possible  $\beta$ -amylase cDNA clones by 'plus-minus' (differential) hybridization (Fig. 2). Three sets of nitrocellulose filters were prepared, each containing 400 recombinants, and hybridized separately to  $^{32}\text{P}$ -kinase-labelled poly(A)-rich RNA from Hipoly (Fig. 2A) and mutant 1508 (Fig. 2B), and to a nick-translated B and C hordein cDNA probe (Fig. 2C) [22]. cDNA clones containing  $\beta$ -amylase-related sequences were expected to hybridize strongly to poly(A)-rich mRNA from Hipoly but only weakly, if at all, to that from mutant 1508. However, because mutant 1508 also contains reduced levels of mRNA for B and C hordeins [31, 34, 35], the third hybridization was done to eliminate possible B and C hordein cDNA clones. Six colonies (indicated by arrows in Fig. 2) hybridized strongly to Hipoly RNA but not to mutant 1508 RNA or to the B and C hordein-specific probes. These colonies were therefore identified as possible  $\beta$ -amylase cDNA clones and were further characterized by hybrid-selection translation. Only *pcβH475* plasmid DNA selected mRNAs from the Hipoly poly(A)-rich RNA fraction which specified polypeptides with an electrophoretic mobility corresponding to *in vitro* synthesized

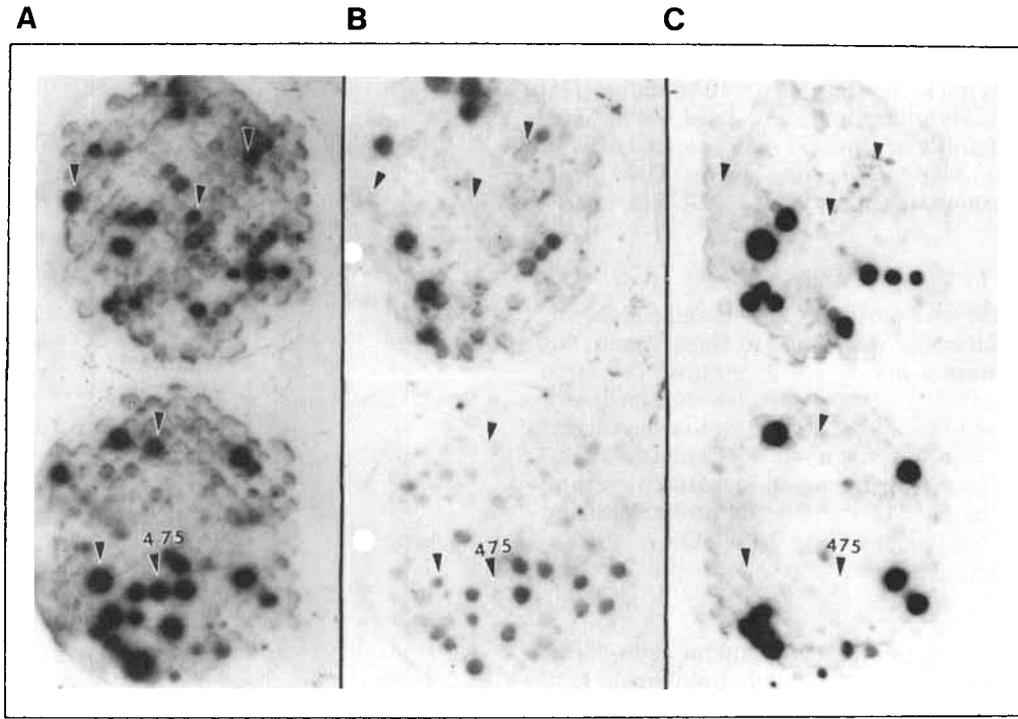


Fig. 2. Screening for  $\beta$ -amylase cDNA clones using a 'plus-minus' hybridization technique. Three sets of filters each containing 400 recombinants were hybridized separately to three probes. The first two sets of filters were hybridized to ( $\gamma$ - $^{32}$ P)-labelled poly(A)-rich RNA extracted from endosperms of Hiproly (A) and mutant 1508 (B) respectively and the last set (C) was hybridized to ( $\alpha$ - $^{32}$ P)-labelled B and C hordein cDNA probe

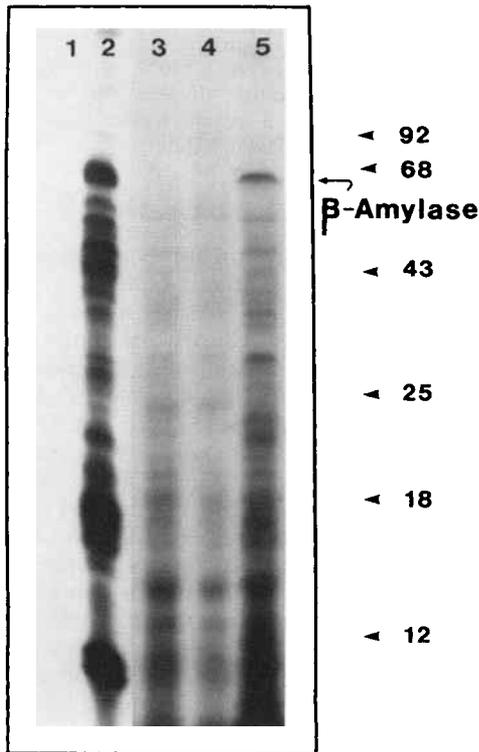


Fig. 3. Tentative identification of a  $\beta$ -amylase cDNA clone by hybrid-selection translation. mRNAs were translated in a wheat-germ system as described in Materials and Methods. No RNA was added to the *in vitro* system (lane 1); *in vitro* products of poly(A)-rich mRNA from endosperm of Hiproly (lane 2); *in vitro* products of Hiproly mRNAs which hybridized to pc $\beta$ H475 (lane 5), to pUC8 (lane 4) and with no plasmid (lane 3). The location of  $\beta$ -amylase on the gel is indicated. Numbers on the right indicate molecular mass in kDa

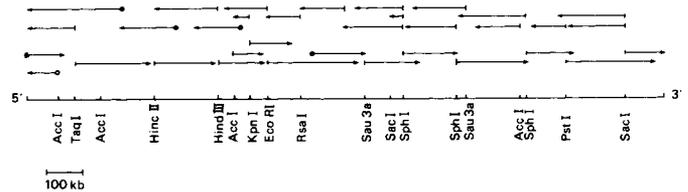


Fig. 4. Restriction map of pc $\beta$ C51 and the strategy for sequencing. Only the restriction sites used for sub-cloning are shown. Arrows indicate length and direction of DNA sequenced. Each arrow represents a separate M13 sub-clone and is marked according to the method of generation: arrows with vertical bars indicate restriction fragments sub-cloned in M13 mp18 or 19, arrows with open circles indicate double-strand sequencing and arrows with filled circles indicate Bal31 deletions which were obtained as follows. pc $\beta$ C51 was cut with *Kpn*I, digested with Bal31, then further restricted with either *Hind*III or *Bam*HI to recover deletions in both orientations. The digests were ligated into either *Hind*III/*Hinc*II-cut mp19 or *Bam*HI/*Hinc*II-cut mp18

$\beta$ -amylase (Fig. 3, lane 5). Unfortunately pc $\beta$ H475 contains a cDNA insert of only about 1000 bp and could therefore not represent a full-length copy of the  $\beta$ -amylase mRNA. Therefore a second cDNA library was constructed using a more efficient cDNA synthesis and cloning technique (see Materials and Methods). The library was screened using the insert of pc $\beta$ H475 as a probe and several further  $\beta$ -amylase cDNA clones selected. One clone (pc $\beta$ C51) had an insert of about 2 kb. This insert has the coding capacity to specify a 60-kDa protein, which was about the size of the hybrid-selected translation product.

*Sequence analysis of the  $\beta$ -amylase cDNA*

The strategy for sequencing pc $\beta$ C51 is indicated in Fig. 4. The nucleotide sequence and the deduced amino acid sequence

10 30 50  
 GATATCCAAACAAACATTGAAAGTTGAGAGCATCATCCATAGCCAGCATCCACAATGGA  
 M<sub>r</sub> E  
 F<sub>1</sub>—  
 70 90 110  
 GGTGAACGTGAAAGGCAACTATGTCCTCAAGTCTACGTCATGCTCCCTCGGACGCCGTGAG  
 V N V K G N Y V Q V Y V M L P L D A V S  
 F<sub>2</sub>—  
 130 150 170  
 CGTGAACAAACAGGTTGAGAAAGGGCGACGAGCTGAGGGCGCAATTGAGGAAGCTGGTAGA  
 V N N R R F E K G D E L R A Q L R K L V E  
 F<sub>3</sub>—  
 190 210 230  
 GGCCGGTGGAGTGGTGTATGGTAGACGCTGTGGTGGGGCTTGGTGGAGGGCAAGGGCCC  
 A G V D G V M V D V W W G L V E G K G P  
 F<sub>4</sub>—  
 250 270 290  
 CAAGCGTATGACTGGTCCGCTACAAGCAGTTGTTTGGCTGGTGCAGAAAGGCTGGGCT  
 K A Y D W S A Y K Q L F E L V Q K A G L  
 F<sub>5</sub>—  
 310 330 350  
 GAAGTCACAGCCATCATGTCTCCACAGTGTGGTGGCAAGCTCGGCGACGCCGTCAA  
 K L Q A I M S F H Q C G G N V G D A V N  
 F<sub>6</sub>—  
 370 390 410  
 CATCCCAATCCACAGTGGGTGGGGACGCTCGGCGACGGTGTATCCCGACATTTTCTACAC  
 I P I P Q W V R D V G T R D P D I F Y T  
 F<sub>7</sub>—  
 430 450 470  
 CGACGGTACGGGACTAGGAACATTGAGTACCTCCTCTTGGAGTTGATAACCGCCTCT  
 D G H G T R N I E Y L T L G V D N Q P L  
 F<sub>8</sub>—  
 490 510 530  
 CTTCCATGGAAGATCTGCCGTCAGATGTATGCCGATTACATGACAAGCTTCAGGGAGAA  
 F H G R S A V Q M Y A D Y M T S F R E N  
 F<sub>9</sub>—  
 550 570 590  
 CATGAAAGACTTCTTGGATGCTGGTGTATCGTCGACATFGAAGTGGGACTTGGCCGACG  
 M K D F L D A G V I V D I E V G L G P A  
 F<sub>10</sub>—  
 610 630 650  
 TGGAGAGATGAGGTACCCATCATCTCAGAGCCAGGATGGTCTCCAGGCGATCGG  
 G E H R Y P S Y P Q S H G W S F P G I G  
 F<sub>11</sub>—  
 670 690 710  
 AGAATTCATCTGCTATGATAAAACCTACAAGCAGACTTCAAAGCAGCAGCGCGGCT  
 E F I C Y D K Y L Q A D F K A A A A A V  
 F<sub>12</sub>—  
 730 750 770  
 TGGCCATCTGAGTGGGAATTTCTTACAGATGTCGGACAGTACAATGACACTCCCGAGAG  
 G H P E W E F P N D V G Q Y N D T P E R  
 F<sub>13</sub>—  
 790 810 830  
 AACTCAATCTTCAGAGACAACGGGACATCACTAAGTGAAGGGGAGGTTTTCCTTGC  
 T Q F F R D N G T Y L S E K G R F F L A  
 F<sub>14</sub>—  
 850 870 890  
 ATGGTACTCCAACAATCTGATCAAGCGGTGACAGGATCTGGATGAAGCAACAGGT  
 W Y S N N L I K H G D R I L D E A N K V  
 F<sub>15</sub>—  
 910 930 950  
 CTTCTGGGATACAAGGTGCAACTGGCAATCAAGATCTCTGGCATTCACTGGTGTACAA  
 F L G Y K V Q L A I K I S G I H W W Y K  
 F<sub>16</sub>—  
 970 990 1010  
 GGTTCAGCCATGACGCGAGCTCACAGCTGGTACTACAACCTTACATGATAGAGAGCG  
 V P S H A A E L T A G Y Y N L H D R D G  
 F<sub>17</sub>—  
 1030 1050 1070  
 CTACAGAACATAGCAGCGATGCTCAAAAGGCACCGTGTAGCATTAACCTCACTTGGC  
 Y R T I A R M L K R H R A S I N F T C A  
 F<sub>18</sub>—  
 1090 1110 1130  
 GGAGATGAGGGATTGGAGCAAAGCTCGCAGGCGATGAGCCACCAAGAACTAGTCCA  
 E M R D L E Q S S Q A M S A P E E L V Q  
 F<sub>19</sub>—  
 1150 1170 1190  
 ACAGGTGTGAGTGTGGATGGAGAGAGGCTTAAATGGCATGCGAAACGGCTTCC  
 Q V L S A G W R E G L N V A C E N A L P  
 F<sub>20</sub>—  
 1210 1230 1250  
 ACGATATGATCCAAGTCTTACAACCCATACTCAGGAATCGAGGCTCATGGAATCAA  
 R Y D P T A Y N T I L R N A R P H G I N  
 F<sub>21</sub>—  
 1270 1290 1310  
 CCAGAGCGGCCCTCTGAGCACAAGCTGTTGGATTACACTACCTTCGGCTGTGGAATCA  
 Q S G P P E H K L F G F T Y L R L S N Q  
 F<sub>22</sub>—  
 1330 1350 1370  
 GCTGGTGGAGGGCAAAACTATGCAATTTCAGACCTTTGTCGACAGAAATGTCGCCAA  
 L V E G Q N Y V N F K T F V D R M H A N  
 F<sub>23</sub>—  
 1390 1410 1430  
 CCTGCCCTGTGACCCATATGTTGATCCAATGGCGCCTTGCCTCAAGATCAGGGCCAGAAAT  
 L P R D P Y V D P M A P L P R S G P E I  
 F<sub>24</sub>—  
 1450 1470 1490  
 ATCGATTGAGATGATCCTACAAGCAGCAGCAAAACTGCAGCCATTCCTCCCGAGGA  
 S I E M I L Q A A Q P K L Q P F P F Q E  
 F<sub>25</sub>—  
 1510 1530 1550  
 GCACACGACCTGCCAGTAGGCCCTACTGGTGGCATGGGTGGGCGAGCTGAAGGCCCCAC  
 H T D L P V G P T G G M G G Q A E G P T  
 F<sub>26</sub>—  
 1570 1590 1610  
 CTGTGGCATGGTGGCAAGTTAAAGCCCTACTGGTGGCATGGGTGGGCGAGCTGAAGA  
 C G M C G Q V K G P T G G M G G Q A E D  
 F<sub>27</sub>—  
 1630 1650 1670  
 CCCTACTAGTGGCATAGGTGGGAGGCTCCCTGCCACCATGTAATGGAACCTTTATGATT  
 P T S G I G G E L P A T M \*  
 F<sub>28</sub>—  
 1690 1710 1730  
 ACTACCCCTTATGTTATGTTGAGTGTGACAGAGAAACCTTCTCTGCCCTTATAATAAT  
 F<sub>29</sub>—  
 1750  
 AAATAAAGCAATC

Fig. 5. Nucleotide sequence of *pcβC51* and deduced amino acid sequence of  $\beta$ -amylase. The repeats are indicated by right-angled arrows. The position of the CNBr peptides are underlined and numbered according to Table 1

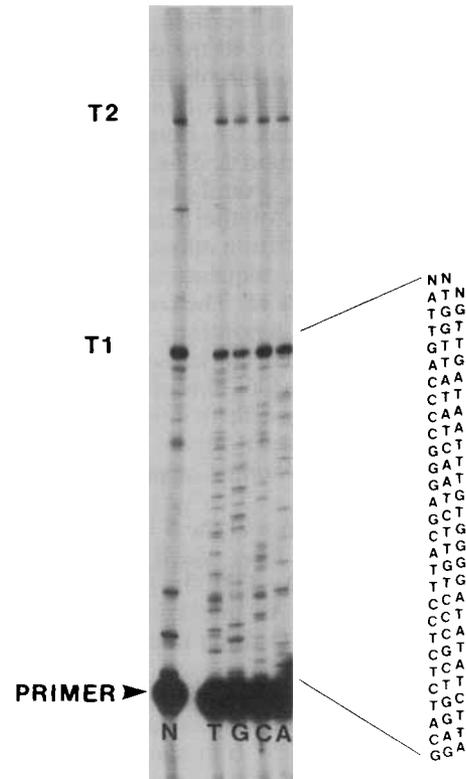


Fig. 6. Primer extension analysis of the 5'-end of the barley  $\beta$ -amylase mRNA. In lane N are indicated the 114-nucleotide primer used in the primer extension analysis, the 90–91-nucleotides extended product (T2) from the primer extension analysis. Lanes T, G, C, A show the primer extension analysis carried out under chain-termination conditions with dideoxynucleoside triphosphate to sequence the 5'-end of the  $\beta$ -amylase mRNA

are shown in Fig. 5. To confirm that *pcβC51* contains a full-length copy of a  $\beta$ -amylase mRNA, primer extension experiments were carried out on the poly(A)-rich RNA fraction of *Hipoly* (Fig. 6). The 114-bp *AclI* restriction fragment of clone *pcβC51* (see Figs 4 and 5) was used as a primer. The most prominent extended product of the 114-nucleotide primer was 90 or 91 nucleotides long. Primer extension experiments under chain-termination conditions showed that the sequence of the 5'-end of the mRNA was identical to that of the 5'-end of the *pcβC51*. The opposite strand of the 5'-end of the  $\beta$ -amylase mRNA is shown in the extension analysis (Fig. 6). This demonstrates that the *pcβC51* insert is a full-length copy of the mRNA and, because T1 is the most prominent extended product, also the main transcript of  $\beta$ -amylase. The minor extended product may result from the use of a weaker upstream initiation site on the same gene, or represent the transcript of a different gene.

The nucleotide sequence of the cDNA insert of clone *pcβC51* (Fig. 5) was 1754 nucleotides long [excluding the poly(A) tail]. The open reading frame codes for a polypeptide of 535 amino acids including the initial methionine corresponding to an  $M_r$  of 59663, a value only slightly greater than that determined previously by sedimentation equilibrium ultracentrifugation for  $\beta$ -amylase ( $M_r$ , 57200) [5]. The amino acid composition calculated from the deduced sequence agrees well with the values from amino acid analyses of purified protein [5]. The deduced amino acid sequence was compared with the amino acid sequences determined by automated

Edman degradation of ten purified  $\beta$ -amylase cyanogen bromide fragments (Table 1). All the peptides are present in the deduced amino acid sequence confirming that pc $\beta$ C51 is a  $\beta$ -amylase cDNA clone. The N-terminal sequence of the purified protein was blocked to Edman degradation and therefore no N-terminal amino acid has been determined for barley  $\beta$ -amylase. However, several observations are in agreement with our assignment of the beginning of the coding region. The presumed translation initiation codon at position 56 is the first ATG in the sequence and is preceded by a nonsense codon at position 41. The context for initiation is CCACAATGG, with an A in position -3 and a G in position +4; this fits very well with the consensus sequence for known eukaryotic initiation codons [36]. One of the peptide cyanogen bromide sequences (starting with an inferred Met) was identical to the deduced N-terminal amino acid sequence. This shows that the initial methionine is present in the mature protein.

The untranslated sequence at the 5' end of the  $\beta$ -amylase mRNA is 55 nucleotides long which is similar to many eukaryotic cellular mRNAs [37]. In the 93-nucleotide 3' untranslated region there is one consensus sequence of polyadenylation (AATAAA) seven nucleotides upstream of the poly(A) tail.

The cDNA insert of the other cDNA clone of  $\beta$ -amylase pc $\beta$ H475 is about 1000 bp and covers nucleotide 789 through to the poly(A) tail of pc $\beta$ C51 (not shown). The nucleotide sequence of this partial cDNA clone is identical to pc $\beta$ C51 except for four nucleotide substitutions at positions 983, 1139, 1270 and 1610.

It has been shown previously [7] (and Fig. 1B) that  $\beta$ -amylase is synthesized on membrane-bound polysomes. It was therefore assumed that  $\beta$ -amylase mRNA would specify a N-terminal extension with the properties of a signal peptide

Table 1. Amino acid sequence of cyanogen bromide fragments

The cyanogen bromide fragments are designated F<sub>1</sub> to F<sub>10</sub> according to the order in which they occur in  $\beta$ -amylase starting from the N-terminus

Frag-Sequence ment	
F <sub>1</sub>	E V ? V K G N Y V Q V Y
F <sub>2</sub>	L P L D A V S V N N R F E K G D E L R A Q L R K L V E A G V D G V
F <sub>3</sub>	Y A D Y
F <sub>4</sub>	T S F R E N
F <sub>5</sub>	R Y P S Y P Q S ? G
F <sub>6</sub>	L K R H R A S V N F T C A E
F <sub>7</sub>	S A P E E L V Q Q V
F <sub>8</sub>	H A N L P R D P Y D P
F <sub>9</sub>	A P L P R S G P E I S I
F <sub>10</sub>	I L Q A A Q P K L Q P F P Q E

Table 2. The short repetitive domain close to the C-terminus of  $\beta$ -amylase

G	T	A	G	G	C	C	C	T	A	C	T	G	G	T	G	G	C	A	T	G	G	G	T	G	G	G	C	A	G	G	C	T
V				G				P		T			G			G			M		G		G				Q		A			
G	A	A	G	G	C	C	C	C	A	C	C	T	G	T	G	G	C	A	T	G	G	G	T	G	G	G	C	A	A	G	T	T
E				G				P		T			C			G			M		G		G			Q		V				
A	A	A	G	G	C	C	C	T	A	C	T	G	G	T	G	G	C	A	T	G	G	G	T	G	G	G	C	A	G	G	C	T
K				G				P		T			G			G			M		G		G			Q		A				
C	A	A	G	A	C	C	C	T	A	C	T	A	G	T	G	G	C	A	T	A	G	G	T	G	G	G	A	G	C	T	C	
E				D				P		T			S			G			I		G		G			E		L				

to direct the nascent protein to the membrane. The deduced amino acid sequence, however, does not contain an N-terminal extension. We have shown in this paper that one of the peptide sequences derived from mature protein corresponds to the deduced N-terminus of  $\beta$ -amylase suggesting that it is synthesized as the mature protein.

Using a graphic matrix homology plot we have identified a short repetitive domain both at the nucleotide and amino acid level (Table 2). Four amino acid repeats, each of 11 residues, are close to the C-terminus of  $\beta$ -amylase. Five out of the 11 residues are glycine. We do not know whether these repeats have a function, if any, and what that function might be. A search of the Wisconsin protein databank using a test peptide of 11 residues (EGPTGGMGGQA) showed that similar repeats are present in other proteins including the  $\alpha$  chain of collagen [38].

#### Organization of the structural genes encoding $\beta$ -amylase in normal and mutant barleys

Genomic blots were used to study the organization and to determine the copy number of the  $\beta$ -amylase structural genes in Sundance, mutant 1508 and Hiproly. High-molecular-mass DNA was extracted from endosperms [31], digested with *Eco*RI, *Bam*HI and *Hind*III, size-fractionated on a 0.8% agarose gel and electro-blotted onto Gene-Screen Plus membranes. The filters were hybridized to the <sup>32</sup>P-labelled large *Acc*I fragment (789 bp) of the insert of pc $\beta$ C51 (Fig. 4) at a stringency of -25°C. Under these conditions the labelled insert hybridized with one *Eco*RI restriction fragment of all three genotypes (Fig. 7A). The *Bam*HI and *Hind*III digests of Sundance, 1508 and Hiproly DNA, however, contain two hybridizing fragments each, one intensively and one more weakly hybridizing (Fig. 7B and C). The more weakly hybridizing *Bam*HI fragment is larger in Hiproly than in the two other lines, which may reflect its exotic origin [8]. Comparison with a reconstruction experiment (Fig. 7C) suggests that there are at least two copies of  $\beta$ -amylase genes per haploid genome of Sundance. One copy is located on the more weakly hybridizing restriction fragment and probably two on the larger and more intensively hybridizing fragment. The results show that there are no changes in the chromosomal organization of the loci specifying  $\beta$ -amylase in Hiproly and 1508 which could alter the expression of  $\beta$ -amylase in the two genotypes.

#### Tissue-specific expression of $\beta$ -amylase in normal and mutant barleys

We have studied the expression of  $\beta$ -amylase in 20-day-old endosperms, shoots and young leaves from normal and mutant barley. Total polysomal RNA and poly(A)-rich RNA (derived from total cellular and total polysomal RNA) were extracted from these tissues of the three genotypes and

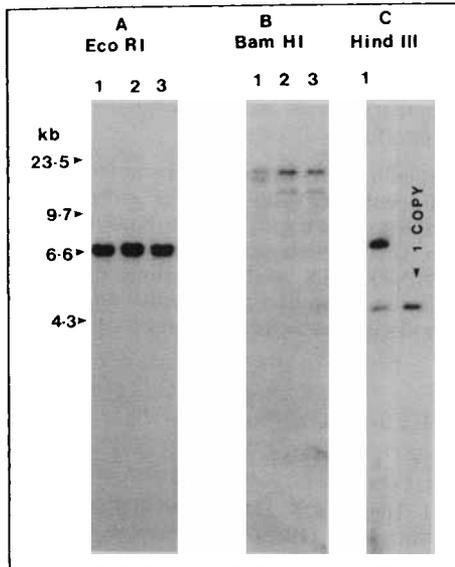


Fig. 7. Southern-blot analysis of genomic DNA from normal and mutant barley. 10  $\mu$ g of high-molecular-mass DNA from Hiproly (lane 1), mutant 1508 (lane 2) and Sundance (lane 3), were digested with *Eco*RI (A), and *Bam*HI (B). The DNA was separated on a 0.8% agarose gel and transferred to Gene Screen Plus. 10  $\mu$ g of Sundance DNA was digested with *Hind*III (lane 1) and electrophoresed alongside 7.6 pg *Bam*HI-digested pc $\beta$ 51 (=1 copy). The filter was probed with the large  $^{32}$ P-labelled *Acc*I fragment of pc $\beta$ 51. *Hind*III-digested lambda phage DNA (BRL) was included as size marker. *Bam*HI-digested pc $\beta$ 51 was used to estimate copy number; 7.6 pg plasmid is equivalent to 1 gene copy/haploid barley genome

analyzed by dot and/or northern hybridization. The large *Acc*I fragment of the insert of pc $\beta$ 51 was used as probe and hybridized to the various RNAs immobilized on Gene Screen Plus filters at low stringency ( $-28^{\circ}\text{C}$ ). A dot-blot analysis of total polysomal RNA shows that  $\beta$ -amylase transcripts are present in shoots, leaves and endosperms (Fig. 8A). The *lys*1 (Hiproly) and *lys*3a (Risø mutant 1508) genes have opposite effects on the abundance of  $\beta$ -amylase mRNAs in the endosperm from the two genotypes. Mutant 1508 and Hiproly endosperms contain respectively much reduced and increased levels of  $\beta$ -amylase. But the two genes do not affect the  $\beta$ -amylase transcripts present in shoots and leaves. Northern hybridization to poly(A)-rich RNA derived from total polysomal RNA shows similar differences in the abundance of transcripts in the endosperm (Fig. 2B). The same results were obtained using poly(A)-rich RNA derived from total cellular RNA (not shown). Comparison with glyoxylated  $\lambda$  *Hind*III fragments indicates that these transcripts are around 2 kb in length.

## DISCUSSION

Our results present the full-length nucleotide sequence for a  $\beta$ -amylase mRNA. From this the first complete amino acid sequence of  $\beta$ -amylase has been deduced, one of the most abundant salt-soluble proteins present in barley endosperm. The results suggest that the cDNA represents a full-length copy of the mRNA, which was confirmed by the primer extension results. The cDNA sequence was identified as coding for  $\beta$ -amylase on the basis of several criteria, including comigration of the polypeptide synthesized from hybrid-selected

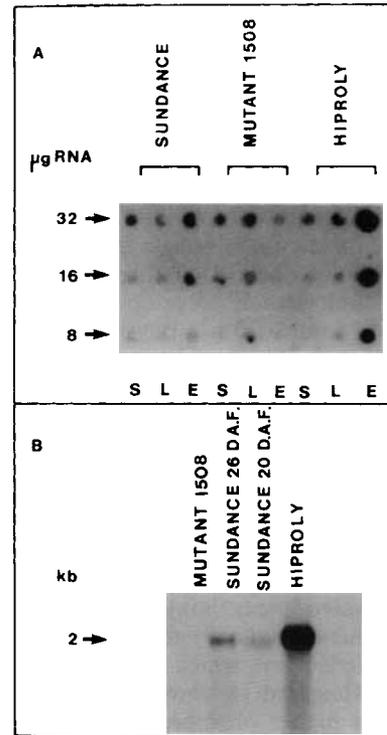


Fig. 8. Dot-blot (A) and Northern blot (B) analysis of the abundance of  $\beta$ -amylase mRNAs in shoots, leaves and endosperms from Sundance, mutant 1508 and Hiproly. (A) Glyoxal-denatured total polysomal RNA from 8-day-old shoots (S) and leaves (L), and 20-day-old endosperms (E) were dotted onto Gene Screen Plus and probed with the large  $^{32}$ P-labelled *Acc*I fragment of pc $\beta$ 51. (B) Northern analysis of  $\beta$ -amylase mRNA in poly(A)-rich RNA from 20-day-old endosperms of mutant 1508 (lane 1), Sundance (lanes 2 and 3) and Hiproly (lane 4). 3  $\mu$ g poly(A)-rich RNA from membrane-bound polysomes were denatured with glyoxal and separated by agarose gel electrophoresis, transferred to filters and probed as in A

mRNA with *in vitro* products and comparison of the deduced amino acid sequence with those determined by direct sequencing of peptides. The calculated  $M_r$  was 59663, compared with 57200 determined by sedimentation equilibrium centrifugation [5].

Comparison of the deduced N-terminal amino acid sequence with that of one of the cyanogen bromide peptides indicates that  $\beta$ -amylase is synthesized in the mature form. Therefore the initial methionine must be present and the N-terminal blocking group could be an acetyl group as shown for the proteinase A inhibitor 3 from bakers' yeast [39]. It was unexpected to find that  $\beta$ -amylase is not synthesized as a pre-form containing a signal peptide to direct the nascent protein to the membrane because it has been shown previously that it is synthesized on membrane-bound polysomes [7] (cf. Fig. 1 B). It is, however, possible that a signal sequence located internally within the coding sequence could direct the polysomes to the membrane. A hydrophobicity plot based on the deduced sequence did show the presence of hydrophobic internal regions (not shown), but there is no evidence that one of these acts as a signal. It is therefore necessary to re-examine whether  $\beta$ -amylase is really synthesized on membrane-bound polysomes and stored in protein bodies as suggested by Tronier and Ory [40]. The former result could have been due to a poor fractionation between free and bound polysomes. The latter result is highly questionable because the protein

bodies were prepared from dry barley seeds, and studies on rye [41] have shown that the protein bodies are disrupted during the later stages of seed development. Furthermore, recent results show that 'bound'  $\beta$ -amylase is not deposited together with hordein (Hejgaard, unpublished). However, it is possible that a conventional signal peptide is not required as has been shown for some microbody enzymes which are synthesized in the mature form [42, 43]. A recent study on the subcellular localization of  $\beta$ -amylase by Hara-Nishimura et al. [44] showed that the bound form was deposited at the periphery of starch granules which occurs during desiccation of the seed and results from a physicochemical rather than a biological process. Further studies on the synthesis and subcellular localization of  $\beta$ -amylase are clearly necessary.

$\beta$ -Amylase contains interesting sequence features at the carboxy-terminus. Four glycine-rich repeated sequences of 11 residues (consensus E-G-P-T-G-G-M-G-G-Q-A) were detected using a dot matrix homology plot [45]. The individual repeats differ from the consensus sequence in 1–5 residues but their role is not known. It is unlikely that they are necessary for *in vivo* activity because it has been shown [46] that limited proteolysis of the carboxy-terminal region with trypsin generated another catalytically active form of the enzyme with a higher isoelectric point, which had lost most of the repeats. Furthermore, Hejgaard [4] showed that a new form of  $\beta$ -amylase with a higher pI appeared during barley malting, which was also probably a proteolytically modified form. This form might be the product of solubilization of 'bound or latent'  $\beta$ -amylase and could correspond to that reported by Lundgard and Svensson [46]. If this is the case the repeated region could be involved in the interactions with protein Z [4] and other cellular components. The loss of these repeats may therefore be important in converting the bound form of the enzyme into the free form.

No information is available from physicochemical studies about the conformation of  $\beta$ -amylase. The elucidation of the complete amino acid sequence will facilitate such analyses and aid elucidation of its mechanism of enzymatic action.

Southern hybridization experiments suggest that barley  $\beta$ -amylase is encoded by a small gene family of at least two copies per haploid genome (Fig. 7). As expected there was no change in the chromosomal organization of these genes in 1508 or Hiproly. Therefore the differential accumulation of  $\beta$ -amylase in normal, Risø mutant 1508 and Hiproly barley must result from the action of *trans*-acting gene product(s). How do the products of the *lys3a* and *lys1* genes on chromosome 7 affect the regulation of expression of the  $\beta$ -amylase genes on chromosome 4? The effect must lie at one of the many steps from transcription to the deposition of the enzyme. The levels of  $\beta$ -amylase mRNAs in poly(A)-rich RNA and/or total polysomal RNA from shoots and leaves are similar in normal and mutant barleys indicating that the shoot/leaf gene(s) are not under the control of the *lys3a* and *lys1* genes. The mutant genes do, however, affect the abundances of  $\beta$ -amylase transcripts in the endosperm resulting in an increased level of  $\beta$ -amylase mRNAs in Hiproly and a decrease to almost zero in mutant 1508. The abundance of the  $\beta$ -amylase transcripts correlates well with the amount of  $\beta$ -amylase synthesized *in vitro* and the deposition of enzyme *in vivo* (cf. Figs 1 and 8). Therefore the deposition of  $\beta$ -amylase is mainly controlled by the abundance of its mRNA. The variation in the levels of  $\beta$ -amylase mRNA between the three genotypes could result from effects on the efficiency of transcription and/or stability of the mRNA. These results show that the  $\beta$ -amylase multigene family is under the control of a

complex regulatory mechanism(s). In addition to the strict tissue- and developmental-specific controls, the expression of the endosperm-specific genes is under the control of two mutant regulatory genes on a separate chromosome which have opposite effects.

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