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Regulation of the Accumulation of mRNA for α-Amylase Isoenzymes in Barley Aleurone[1]

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ABSTRACT

The effect of gibberellic acid and Ca^{2+} on the accumulation of α-amylase mRNAs in aleurone layers of barley (*Hordeum vulgare* L. cv Himalaya) was studied using cDNA clones containing sequences of mRNAs for the high and low isoelectric point (pI) α-amylases. There is no significant hybridization between the two α-amylase cDNA clones under the hybridization and washing conditions employed. These clones were therefore used to monitor levels of mRNAs for high and low pI α-amylases. It is shown that although the synthesis of the high pI α-amylase proteins depends on the presence of Ca^{2+} in the incubation medium, the accumulation of mRNA for this group occurs to the same degree in the presence or the absence of Ca^{2+}. The accumulation of low pI α-amylase mRNA is also not affected by the presence or absence of Ca^{2+} in the incubation medium. These results establish gibberellic acid, not Ca^{2+}, as the principal regulator of α-amylase mRNA accumulation in barley aleurone, while Ca^{2+} controls high pI α-amylase synthesis at a later step in the biosynthetic pathway.

When aleurone layers of barley are incubated in GA_3 and Ca^{2+}, they synthesize and secrete a number of hydrolytic enzymes (reviewed in Ref. 9). *In vivo*, GA is secreted by the embryo and induces the aleurone cells to synthesize and secrete these enzymes, which allow the germinating seed to mobilize the nutrients stored in its endosperm. α-Amylase is the predominant protein synthesized by isolated aleurone layers, and it comprises more than 60% of the total protein synthesized after 24 h of incubation (8). α-Amylase is a family of isoenzymes that has been divided into two groups based on immunological properties, proteolytic fingerprints, isoelectric point (pI), and sensitivity to Ca^{2+} and pH (3, 11, 13). The isoenzymes of group A (low pI) have pIs of about 4.3 to 5.2 (3, 11), do not require Ca^{2+} in the incubation medium for their production, and are stable at low pH (13). The isoenzymes of group B (high pI) have pIs of about 5.9 to 6.6 (3, 11), require Ca^{2+} for their synthesis (6, 13, 14) and secretion (15), and are not stable at low pH (13). The sensitivity to GA_3 and the time course of appearance of the isoenzymes of the two groups are also different (3, 11). Low pI α-amylases are encoded on chromosome number 1 of barley and high pI α-amylases are encoded on chromosome number 6 (2, 18).

cDNA clones for cereal α-amylase have been isolated by several researchers (1, 4, 6, 10, 17, 19, 20). On the basis of DNA sequence analysis, the existence of two types of clones is apparent. Comparison with the amino acid sequences of the native proteins allows correlation of the two types of cDNA clones with the low and high pI groups of α-amylases (4). The two kinds of α-amylase cDNA sequences differ in about 20% of the coding region for the mature polypeptide and 45% of the signal sequence (19). Blot hybridization with cDNA clones can distinguish between the two groups of mRNAs (10, 19).

Although Ca^{2+} is required for the production of high pI α-amylases, it does not affect the accumulation of α-amylase mRNA detected with a cDNA clone isolated in our laboratory (clone 1-28; 6). From a partial DNA sequence we (J Deikman, RL Jones, unpublished data) determined that clone 1-28 is very similar to clone pHV19, which has been identified as sequences for high pI α-amylases (4). However, since no low pI α-amylase clone was available to us, we could not conclude that Ca^{2+} did not affect the accumulation of high pI α-amylase mRNA. The lack of effect of Ca^{2+} on α-amylase mRNA levels could have been due to nonspecific hybridization to α-amylase mRNAs of both groups, with the low pI increasing as the high pI decreased. We now describe the hybridization of RNA gel blots with clone 1-28 and a low pI clone, clone E, generously provided by Dr. John C. Rogers. Clone 1-28 hybridizes specifically to high pI α-amylase under the conditions employed in this and in previous work (6). The lack of effect of Ca^{2+} on the accumulation of high pI α-amylase mRNA is thereby confirmed, and the role of GA_3 as the sole exogenous regulator of α-amylase mRNA accumulation is established.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* L. cv Himalaya) caryopses were prepared and aleurone layers isolated and incubated as described previously (6).

cDNA Clones. Clone 1-28 was described by Deikman and Jones (6). It contains α-amylase cDNA sequences cloned into the Pst 1 site of pBR322. In addition, it contains approximately 0.65 kb of unidentified sequences that do not hybridize to pBR322 or to any barley mRNA (5). Clone 1-28LP is a subclone of 1-28 which contains only the 1.5 kb of α-amylase sequences (5). Clone E is a barley α-amylase clone obtained from Dr. J. C. Rogers (20). Clone pTA71 contains a complete rDNA repeat from wheat (7) and was the gift of Dr. J. Bedbrook.

DNA Gel Blot. DNA (0.5 μg) was digested with the restriction endonuclease Pst 1 as recommended by the supplier (BRL) and electrophoresed in a 1% agarose gel in 40 mM Tris (pH 8), 20 mM Na acetate, 2 mM EDTA, and 18 mM NaCl. The DNA was transferred to nitrocellulose (Schleicher and Schuell) following the procedure detailed by Maniatis *et al.* (16). Hybridization to cDNA labeled with [^{32}P]dCTP (410 Ci/mmol, Amersham) by nick translation with a Nick Translation Kit (Bethesda Research Laboratories) was in 5× SSPE (prepared from a 20× stock containing 3.6 M NaCl, 0.2 M Na-phosphate [pH 7.4], 20 mM EDTA), 0.1% SDS, 2× Denhardt's (prepared from a 100× stock

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consisting of 20 mg/ml BSA, 20 mg/ml Ficoll 400, and 20 mg/ml PVP), 50 μg/ml poly(A) (P-L Biochemicals), and 50 μg/ml denatured salmon sperm DNA (Sigma) at 68°C for about 24 h. The blot was washed at 68°C in 2× SSC (prepared from a 20× stock containing 3 M NaCl, 0.3 M sodium citrate [pH 7]) for 1.5 h and then in 1× SSC for 1.5 h.

RNA Extraction, RNA Gel Blots, and Dot Blots. Procedures were carried out as described previously (6). All blots were washed after hybridization at 68°C in 2× SSC for 1.5 h and then in 1× SSC for 1.5 h.

Autoradiogram Quantitation. Autoradiograms were quantitated by scanning with an LKB Bromma 2202 Ultrascan laser densitometer. Peak integration values were normalized within each gel to the most dense band. Each band was corrected for errors in loading the gel by dividing the integration value by the relative amount of rRNA detected by the densitometer.

RESULTS

cDNA clones 1-28 and E were digested with Pst 1, electrophoresed, and hybridized to clone 1-28LP, which contains the 1.5 kb of α-amylase sequences from clone 1-28. The cDNA inserts constitute the smaller Pst 1 bands. Figure 1 shows that clone 1-28LP hybridizes to the clone E insert (lane b, lower band) only weakly. This result is consistent with the partial sequencing data obtained for clone 1-28 (not shown) which indicates that 1-28 is very similar to pHV19, which is different from clone E in 23% of its nucleotides in the region corresponding to the mature polypeptide (4).

Clones E and 1-28 were hybridized to RNA gel blots containing

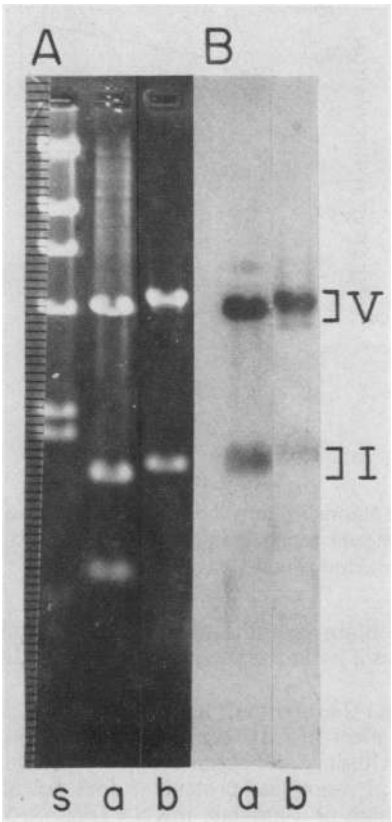

FIG. 1. Hybridization of clone 1-28LP to α-amylase cDNA clones. A, EtBr-stained 1% agarose gel of Pst 1-digested DNA. Clone 1-28 (a) and clone E (b). The sizes of the Hind III-digested λ-DNA fragments (s) are: 23.5, 9.7, 6.6, 4.3, 2.2, 2.1, and 0.6 kb. B, Autoradiogram of the blot of the agarose gel shown in A, hybridized to clone 1-28LP. V indicates the plasmid vectors and I indicates the α-amylase cDNA inserts.

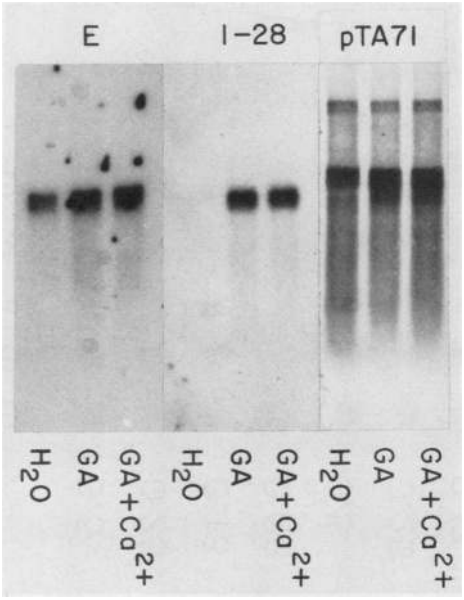

FIG. 2. RNA gel blots. Poly(A) RNA extracted from aleurone layers incubated for 12 h in H_2O, 2.5 μM GA_3, or 2.5 μM GA_3 + 5 mM $CaCl_2$. Blots were hybridized to clones E, 1-28, and pTA71.

poly(A) RNA extracted from aleurone layers incubated for 12 h in H_2O, GA_3, or GA_3 plus Ca^{2+} (Fig. 2). Figure 2 shows that while GA_3 causes an increase in the amount of mRNA homologous to clone E in isolated aleurone layers, substantial amounts of mRNA are present in H_2O-treated layers. On the other hand, almost no mRNA homologous to clone 1-28 is detectable in H_2O-treated layers while amounts equivalent to those of mRNA homologous to clone E are present in layers treated with GA_3 (Fig. 2). Ca^{2+} has no effect on the accumulation of mRNA represented by either clone 1-28 or clone E.

Clone pTA71, which contains a complete rDNA repeat (7), was hybridized to the blots to verify that equal amounts of RNA were loaded in each lane (Fig. 2). Since rRNA constitutes 97 to 98% of total aleurone RNA (12), measurement of rRNA levels by hybridization to an rDNA clone permits quantitation of RNA loading. The samples on the electrophoretogram shown in Figure 2 were passed over an oligo-dT column only once and some residual rRNA is expected to be present. Since all the samples were prepared at the same time and with the same reagents, the efficiency of poly(A) selection can be assumed to be equal for all.

To quantify the effects of GA_3 and Ca^{2+} on mRNA accumulation, RNA samples from the above experiment were spotted in a dilution series onto nitrocellulose (Fig. 3). From the dot blots one can estimate that mRNA homologous to clone E increases 2- to 3-fold and mRNA homologous to clone 1-28 increases more than 10-fold during 12 h incubation in GA_3. This blot also verifies that Ca^{2+} has no effect on the accumulation of mRNA for either α-amylase clone.

A time course of RNA accumulation in aleurone layers incubated in H_2O, GA_3, or GA_3 plus Ca^{2+} was carried out. RNA was extracted at time zero, then at 4, 8, 12, and 24 h of incubation. Total RNA was electrophoresed and blots from this experiment were hybridized to clones E, 1-28, and pTA71 (Fig. 4). The autoradiograms of the blots were scanned with a densitometer, the integration values were normalized to the 12-h GA_3 band, and errors in loading were corrected for as described in "Materials and Methods" (Fig. 5). RNA homologous to clone E increased substantially during the incubation period. As shown in Figures 2 and 3, the increase due to the presence of GA_3 in the incubation medium was about 2.5-fold at 12 h. RNA homologous to clone

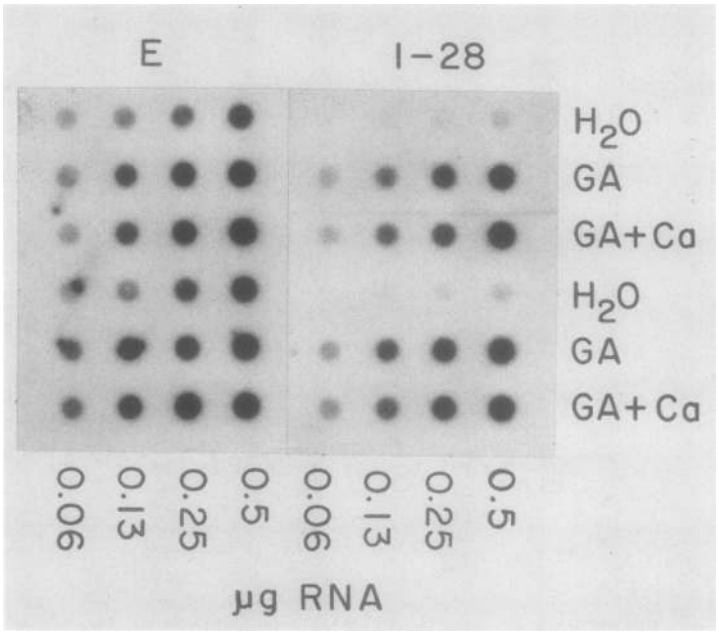

FIG. 3. Dot blot of poly(A) RNA hybridized to clones 1-28 and E. In two experiments RNA was extracted from aleurone layers incubated for 12 h in H_2O, GA_3, or GA_3 + $CaCl_2$. The RNA was spotted onto the filter in a dilution series: 0.5, 0.25, 0.13, and 0.06 µg.

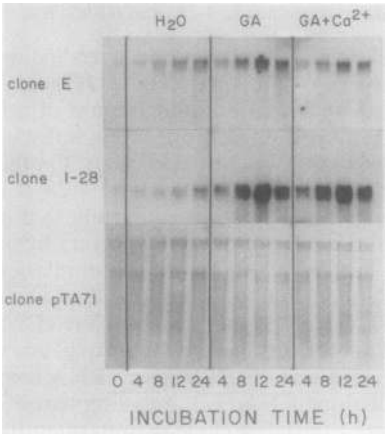

FIG. 4. Time course of RNA accumulation in layers incubated in H_2O, GA_3, or GA_3 + $CaCl_2$ for 0, 4, 8, 12, and 24 h. Total RNA (15 µg) was electrophoresed and blotted to nitrocellulose paper. The RNA gel blots were hybridized to clones 1-28, E, and pTA71.

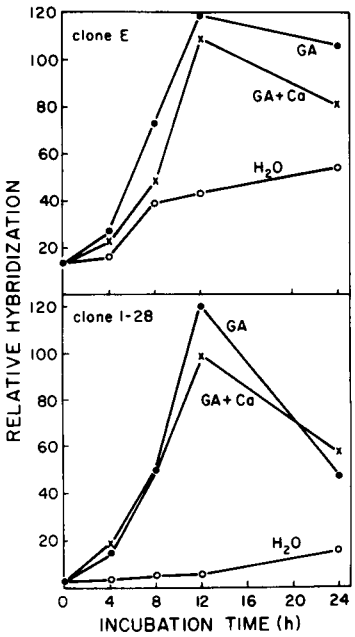

FIG. 5. The autoradiograms shown in Figure 4 were scanned with a densitometer and the results normalized to the darkest band, corrected for RNA loading, and plotted.

1-28 also increased during the incubation period, but the increase due to GA_3 was much more pronounced (19.6-fold at 12 h). The increases in RNA homologous to both clones E and 1-28 are apparent by 4 h incubation in GA_3 and peak at 12 h. The presence or absence of Ca^{2+} did not affect the pattern of mRNA accumulation for RNA homologous to either clone E or 1-28 (Figs. 4 and 5).

DISCUSSION

The patterns of hybridization of clones 1-28 and E to RNA from GA_3- and H_2O-treated layers demonstrate that clone 1-28 does not cross-hybridize to a significant degree with mRNA sequences homologous to clone E (Figs. 2–5). This result is consistent with the observation in Figure 1 that the cDNA clones do not cross-hybridize strongly with one another (Fig. 1; see also Ref. 10). The greater amount of hybridization of 1-28 sequences to the low pI α-amylase cDNA clone compared to that to low pI α-amylase mRNA may be a result of the presence of the dG-dC tails common to the cDNA clones. These results demonstrate that the isolation of cDNA fragments corresponding to the 5′ regions of the coding sequences for use as probes (19) is not required to obtain hybridization specific to only one of the mRNA groups if hybridization and washing conditions are stringent enough.

Since clone 1-28 represents high pI α-amylase, it is possible to confirm the effect of Ca^{2+} on the accumulation of α-amylase mRNA (6). Although the absence of Ca^{2+} prevents the production of high pI α-amylase protein, it does not affect the accumulation of high pI α-amylase mRNA (Figs. 2–5). In addition, the levels of low pI α-amylase mRNA detected by clone E are not affected by the presence or absence of Ca^{2+} in the incubation medium (Figs. 2–5). Therefore, Ca^{2+} must affect α-amylase synthesis at a step after mRNA accumulation and processing (6). GA_3 by itself is sufficient to cause increased α-amylase mRNA levels.

The control of α-amylase mRNA accumulation by GA_3 remains a complex problem. First, GA_3 seems to induce the two groups of α-amylase mRNAs to different degrees (Figs. 2–5; 10, 19). Second, the number of genes within each group has not been determined, and whether or not members within the group are coordinately controlled is not clear. If the control by GA_3 of mRNA accumulation parallels the control by GA_3 of protein accumulation as has been the case thus far, then one would expect, for example, that genes within the low pI group would be independently regulated by GA_3, since isoenzyme 2 is present in the absence of exogenous GA_3 and isoenzyme 1 does not appear unless GA_3 is added to the incubation medium (14). Several researchers have estimated the existence of about eight genes for α-amylase based on hybridization of cDNA clones to nuclear DNA blots (4, 17). Primer extension experiments with clone E indicated that two low pI transcripts were coordinately controlled (21). Primer extension experiments using the 5′ region of the high pI clone pM/C detected only one transcript (19). Huang *et al.* (10), however, have isolated three types of high pI cDNA clones. None of these clones included the 5′ ends of the transcripts. The primer extension experiments carried out would not distinguish between gene products with very similar 5′ ends. Perhaps examination of 3′ ends might reveal further heterogeneity within the α-amylase isoenzymes mRNA populations.

The site at which Ca^{2+} is required for the synthesis of high pI α-amylase isoenzymes remains undetermined. Possibilities include any of the steps between mRNA attachment to ribosomes and final protein processing and secretion. There is other evidence that not all α-amylase mRNA is translated with equal efficiency. For example, while α-amylase constitutes over 60% of the newly synthesized protein in barley aleurone layers incubated for 24 h, its mRNA represents only about 20% of the cell mRNA as determined by translation *in vitro* (8). One role of Ca^{2+} in the germinating seed might be to regulate the rate of translation of high pI α-amylase mRNA.

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LITERATURE CITED

1. Baulcombe DC, D Buffard 1983 Gibberellic-acid-regulated expression of α-amylase and six other genes in wheat aleurone layers. Planta 157: 493–501
2. Brown AHD, JV Jacobsen 1982 Genetic basis and natural variation of α-amylase isozymes in barley. Genet Res Camb 40: 315–324
3. Callis J, T-HD Ho 1983 Multiple molecular forms of the gibberellin-induced α-amylase from the aleurone layers of barley seeds. Arch Biochem Biophys 224: 224–234
4. Chandler PM, JA Zwar, JV Jacobsen, TJV Higgins, AS Inglis 1984 The effects of gibberellic acid and abscisic acid on α-amylase mRNA levels in barley aleurone layers studies using an α-amylase cDNA clone. Plant Mol Biol 3: 407–418
5. Deikman J 1985 The control of α-amylase synthesis in the barley aleurone: studies with a cDNA clone. PhD thesis. University of California, Berkeley
6. Deikman J, RL Jones 1985 Control of α-amylase mRNA accumulation by gibberellic acid and calcium in barley aleurone layers. Plant Physiol 78: 192–198
7. Gerlach WL, JR Bedbrook 1979 Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res 7: 1869–1885
8. Higgins TJV, JV Jacobsen, JA Zwar 1982 Gibberellic acid and abscisic acid modulate protein synthesis and mRNA levels in barley aleurone layers. Plant Mol Biol 1: 191–215
9. Ho T-HD 1979 Hormonal control of enzyme formation in barley aleurone layers. *In* I Rubenstein, RL Phillips, CE Green, BG Gengenbach, eds, Molecular Biology of Plants. Academic Press, New York, pp 217–240
10. Huang J-K, M Swegle, AM Dandekar, S Muthukrishnan 1984 Expression and regulation of α-amylase gene family in barley aleurones. J Mol Appl Genet 2: 579–588
11. Jacobsen JV, TJV Higgins 1982 Characterization of the α-amylases synthesized by aleurone layers of Himalaya barley in response to gibberellic acid. Plant Physiol 70: 1647–1653
12. Jacobsen JV, JA Zwar 1974 Gibberellic acid and RNA synthesis in barley aleurone layers: metabolism of rRNA and tRNA and of RNA containing polyadenylic acid sequences. Aust J Plant Physiol 1: 343–356
13. Jacobsen JV, JG Scandalios, JE Varner 1970 Multiple forms of amylase induced by gibberellic acid in isolated barley aleurone layers. Plant Physiol 45: 367–371
14. Jones RL, J Carbonell 1984 Regulation of the synthesis of barley aleurone α-amylase by gibberellic acid and calcium ions. Plant Physiol 76: 213–218
15. Jones RL, JV Jacobsen 1983 Calcium regulation of the secretion of α-amylase isoenzymes and other proteins from barley aleurone layers. Planta 158: 1–9
16. Maniatis T, EF Fritsch, J Sambrook 1982 Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. Muthukrishnan S, GR Chandra, ES Maxwell 1983 Hormonal control of α-amylase gene expression in barley. Studies using a cloned cDNA probe. J Biol Chem 258: 2370–2375
18. Muthukrishnan S, BS Gill, M Swegle, GR Chandra 1984 Structural genes for α-amylases are located on barley chromosomes 1 and 6. J Biol Chem 269: 13637–13639
19. Rogers JC 1985 Two barley α-amylase gene families are regulated differently in aleurone cells. J Biol Chem 260: 3731–3738
20. Rogers JC, C Milliman 1983 Isolation and sequence analysis of a barley α-amylase cDNA clone. J Biol Chem 258: 8169–8174
21. Rogers JC, C Milliman 1984 Coordinate increase in major transcripts from the high pI α-amylase multigene family in barley aleurone cells stimulated with gibberellic acid. J Biol Chem 259: 12234–12240