The physiological and molecular bases for contrasting $\alpha$-amylase phenotypes were examined in germinating seeds of two barley (Hordeum vulgare) cultivars, Morex and Steptoe. Morex is a high-quality malting barley that develops high $\alpha$-amylase activity soon after germination. Steptoe is a feed barley that develops only low $\alpha$-amylase activity levels during this period. The expression of all high- and low-isoelectric point (pl) $\alpha$-amylase isozymes is reduced in Steptoe. The amount of $\alpha$-amylase mRNA per gram of seedling tissue is correspondingly lower in Steptoe. Southern blot analysis revealed that the cultivars have the same copy number and organization for most high- and low-pl genes. Steptoe seedlings or embryoless half-seeds produce little $\alpha$-amylase in response to exogenous applications of gibberellic acid (GA$_3$) compared with Morex. However, when isolated aleurones of both cultivars are treated with GA$_3$, they produce similar amounts of high- and low-pl $\alpha$-amylase RNAs. This suggests that a factor in the starchy endosperm is responsible for lowered $\alpha$-amylase response in Steptoe. The factor is probably not abscisic acid (ABA), since the two cultivars have similar concentrations of ABA during germination.

Molecular studies of $\alpha$-amylase gene expression in barley (Hordeum vulgare) have focused primarily on the isolated aleurone of the Himalaya cultivar. Little study has been devoted to expression in intact seedlings, especially in commercial cultivars (Chandler and Jacobsen, 1991; Karrer et al., 1991). The Morex (Rasmusson and Wilcoxson, 1979) and Steptoe (Muir and Nilan, 1973) cultivars differ greatly in $\alpha$-amylase activity during germination. Morex develops high amounts of activity soon after germination, helping to make it one of the top American six-row malting barleys. In contrast, the six-row cultivar Steptoe produces little activity and has exceptionally poor malting quality. These contrasting phenotypes provide an excellent comparative system for analyzing the control of $\alpha$-amylase genes in intact germinating seedlings.

Extensive research in the past three decades has established a central role for GA as a regulator of $\alpha$-amylase and many other hydrolytic enzymes, although the exact role played by GA is still clouded by inconsistencies (reviewed by Fincher, 1989). The regulation of $\alpha$-amylase in cereal grains has been the subject of several reviews (Ho, 1979; Enari and Sopanen, 1986; MacGregor and MacGregor, 1987; Muthukrishnan and Chandra, 1988; Fincher, 1989). Paleg (1960), Yomo (1960), and Sandergren and Beling (1959) initially discovered that GA applications increased $\alpha$-amylase activity in barley grain. The establishment of an isolated barley aleurone system, in which GA caused a dramatic induction of $\alpha$-amylase activity (Chrispeels and Varner, 1967), and the finding that GA is produced in the embryo (MacLeod and Palmer, 1967; Radley, 1967) led to the currently held view of cereal seed endosperm mobilization. In brief, GA is synthesized by the germinating embryo, diffuses to the aleurone cells, and induces a battery of hydrolytic enzyme genes, including those for $\alpha$-amylases. The GA response of isolated barley aleurones has remained an intensively studied phenomenon and forms the basis for speculation on mechanisms controlling $\alpha$-amylase genes in germinating seedlings. It remains to be seen whether this is a relevant model system or an artifactual system with a unique physiology.

It is well established that GA enhances the transcription of barley $\alpha$-amylase genes and the net accumulation of $\alpha$-amylase mRNAs in isolated aleurones (Higgins et al., 1976; Muthukrishnan et al., 1979; Mozer, 1980; Chandler et al., 1984; Huang et al., 1984; Rogers and Milliman, 1984; Deikman and Jones, 1986; Nolan et al., 1987; Khursheed and Rogers, 1988; Nolan and Ho, 1988; Chandler and Jacobsen, 1991), protoplasts (Jacobsen et al., 1985; Chandler and Jacobsen, 1991), and isolated nuclei of protoplasts (Jacobsen and Beach, 1985). Indeed, endogenous GA levels correlate with $\alpha$-amylase production in a variety of germinating commercial barleys; it has been proposed that a factor determining GA responsiveness may be active in cultivars where poor correlations are found (Kusaba et al., 1991).

The effects of ABA must be considered as a primary factor modifying the GA responsiveness of barley aleurones (Jacobsen, 1983). ABA represses the expression of $\alpha$-amylase genes and reverses the positive effect of GA on $\alpha$-amylase mRNA accumulation (Muthukrishnan et al., 1983; Chandler et al., 1984; Jacobsen and Beach, 1985; Nolan et al., 1987; Nolan and Ho, 1988). It is likely that the balance between endoge-

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nous GA and ABA concentrations strongly controls α-amylase gene expression in intact germinating seedlings, although a cultivar's sensitivity to each hormone would shift the effective (perceived) balance. ABA also induces the accumulation of the α-amylase inhibitor bifunctional α-amylase/subtilisin inhibitor (Mundy, 1984).

In addition to hormonal and other physiological controls, cultivar-specific differences in expression could be caused by gene sequence variations. However, because both the high- and low-pI α-amylases are encoded by multicity genes, variations must occur in more than one gene to cause significant phenotypic effects. The high-pI α-amylases are represented by at least seven genes or pseudogenes and the low-pI α-amylases by at least three (Knox et al., 1987; Khurseed and Rogers, 1988). Despite this, it is necessary to compare structural genes because cultivar differences may be found in a gene family member that is a dominant contributor to the transcript pool.

In the following study, the Morex and Steptoe cultivars were compared at the molecular and physiological levels. Initially, it appeared that low α-amylase activity in Steptoe could not be caused by GA-related deficiencies. Morex and Steptoe are similar in stature under field conditions. Mutants of gentamycin. Seeds were germinated in the dark at 16°C. Incubation medium was heat-treated at 70°C for 20 min on a gyratory shaker. After the first 12 h, the GA treatments were repeated. After 24 h, aleurones (or half-seeds) were blotted to remove surface moisture, weighed, and stored at −80°C prior to α-amylase enzyme analysis or RNA extraction. Incubation medium was heat-treated at 70°C for 20 min and stored at −20°C prior to analysis.

**α-Amylese Enzyme Activities**

Seedling caryopses, half-seeds, and aleurones were prepared for α-amylase assays and IEF by grinding in buffer containing 200 mM Na acetate (pH 5.5), 10 mM CaCl2, sand, and PVP-360 (Sigma) at a tissue weight-to-volume ratio of 1 g to 10 mL. Homogenates were centrifuged at 12,000 rpm in microfuge tubes for 10 min at 4°C. The supernatants were heat treated to 70°C for 15 to 20 min to inactivate β-amylase and other carbohydrases (excluding α-amylases) and centrifuged as above. α-Amylese activity was measured using a DNSA assay (Rick and Stegbauer, 1974). Assays were compared to a standard curve based upon maltose. Reactions were incubated at 30°C for 30 min and measured relative to paired samples held at 0°C prior to boiling in the DNSA chromogenic reagent. This allowed a correction for changes in background reaction levels. Units are in terms of μmol of maltose generated from the soluble starch substrate per g fresh weight of seed tissue in 30 min.

For activity gels, heat-treated homogenate was applied to the center of preformed IEF gels of ampholyte pH range 4.0 to 6.5 (Pharmacia). Gels were prerun at 1000 V for 45 min. Samples were electrophoresed at 1200 V and 10°C for 1 h. Amylese activity was detected by impregnating the gels with 1% Lintner starch (Sigma) in 200 mM ethylenediamine (pH 6.0), 10 mM CaCl2 and staining in I2/KI (Henson and Stone, 1988). After boiling and cooling the starch to 40°C, it would not sufficiently impregnate the 5% polyacrylamide gels. This was overcome by incubating the starch at 40°C with 1 μg/mL of heat-treated α-amylase (Sigma) until its A550 was reduced to 0.03. The solution was boiled again, cooled, and incubated with the gels at 40°C while shaking for 3 h. Satisfactory Polaroid photographs were obtained by employing an orange filter.
RNA Extraction

Roots, hulls, and seedling axes (roots and shoots) were removed, and caryopses were frozen in liquid N₂ and stored at −80°C until use. Up to 3 g of caryopses or isolated aleurones were ground to a fine powder in liquid N₂ and then added to 20 mL of 0°C RNA extraction buffer: 250 mM Tris-HCl (pH 9.0 at 22°C), 250 mM NaCl, 50 mM EDTA, 50 mM 2-mercaptoethanol, 1% SDS (BDH Chemicals Ltd.), 1% Na deoxycholate, and 2 mM aurintricarboxylic acid (Sigma Chemical Co.). Aurintricarboxylic acid was employed to inhibit RNase (Stern and Newton, 1984), and the main buffer components selected are known to give high-quality rice kernel RNA (O’Neill et al., 1990).

The powder was homogenized in a Polytron at high speed and centrifuged at 10,000 rpm for 10 min at 4°C in a Beckman JA-20 rotor to pellet starch. The supernatant was quickly transferred to a beaker and stirred briskly on ice with 20 mL of phenol (equilibrated with pH 8.0 Tris and then with extraction buffer). After 10 min, 10 mL of chloroform/isooctyl alcohol (24:1, v/v) was added, and stirring was continued for 5 min. The emulsion was centrifuged as above. The aqueous phase was mixed with a 10% (w/v) solution of sodium acetate, pH 5.5, and 2.5 volumes of ethanol. RNA was precipitated with K acetate. The RNA was pelleted by centrifugation at 12,000 rpm for 10 min at 4°C, pelleting β-glucans.

The RNA in the supernatant was precipitated at −20°C for 5 h in centrifuge tubes with 0.3 M (final concentration) K acetate, pH 5.5, and 2.5 volumes of ethanol. RNA was pelleted by centrifugation at 12,000 rpm for 10 min at 0°C, washed with 70% ethanol, and lyophilized briefly. The red RNA pellets were readily and completely dissolved in 4 mL of Tris-EDTA buffer (pH 8.0). Two milliliters of 6 M LiCl₂ were added, and RNA was reprecipitated for 15 h at 4°C in microcentrifuge tubes. The RNA was pelleted by centrifugation at 12,000 rpm, resuspended and washed in 3 M LiCl₂, repelleted, and dissolved in diethylpyrocarbonate-treated H₂O. The RNA was precipitated a final time with K acetate and ethanol, washed with 70% ethanol, and dissolved in H₂O.

Cloned Probes

High-pI α-amylase RNA was detected with the high-pI cDNA clone pM/C (Rogers, 1985) derived from aleurone mRNA of the Himalaya cultivar. Low-pI RNA was detected with the low-pI cDNA clone E (Rogers and Milliman, 1983) from the same source. Both were generously provided by John Rogers (Washington University). The concentrations of cloned insert DNAs used in probe synthesis and as hybridization standards were determined by spectrophotometric measurements and by Hoechst 33258 staining with fluorometric detection.

Northern Blot Analysis of RNA

Fifteen-microgram samples of RNA were electrophoresed on 1.2% Seakem (Marine Colloids) agarose-formaldehyde gels and blotted onto Nytran (Schleicher & Schuell) filters (Rave et al., 1979). After baking for 90 min in an 80°C vacuum oven, filters were prehybridized for 8 to 16 h in 50% (v/v) formamide, 2× SSPE (pH 7.4), 250 μg/mL of denatured salmon sperm DNA, 0.1% BSA, 0.1% Ficoll 400 (Pharmacia), 0.1% PVP-40 (Sigma), and 1% SDS (Thomas, 1983). Radio-labeled probe DNA was prepared from gel-purified clone inserts. Clone pM/C and clone E inserts (100 ng) were each labeled simultaneously to high specific activities (1.12 × 10⁹ cpm/μg) using a random hexamer-primed Klenow fragment reaction (Feinberg and Vogelstein, 1983) containing [α-3²P]dCTP (3000 Ci/mmol, DuPont). Probe DNA was heated to 100°C for 2 min and added to hybridization bags to give 10⁶ cpm/mL. Hybridization solution consisted of four parts of the above prehybridization buffer plus one part 50% (w/v) dextran sulfate (Pharmacia). Hybridization was conducted for 16 h at 62.5°C. Filters were washed four times in 2X SSPE and 0.1% SDS at 22°C for 15 min each and then four times in 0.2X SSPE and 0.1% SDS at 62.5°C for 15 min each.

Filters were exposed to Kodak XAR-5 film between two intensifying screens. Comparative high- and low-pI autoradiographs were made by exposing films for the same duration in the same x-ray cassette. In addition, several exposures were made to ensure that autoradiographs were within the linear response range of the film. Autoradiographs were scanned densitometrically with a soft laser scanner (Biomed).

RESULTS

Developmental Changes in Enzyme Activity

α-Amylase activity increases very slowly in Morex until d 4 (Fig. 1). Activity then increases rapidly in Morex, but the
Figure 1. Postgerminative temporal changes in α-amylase enzyme activity in the Morex and Steptoe cultivars. Plant axes and scutella were removed from seedlings at d 1 through 7 of aerobic germination, and caryopsis α-amylase activity was determined by the DNSA procedure. Units are in terms of μmol of maltose produced from potato starch during a 30-min incubation at 30°C. Morex, Solid line. Steptoe, Broken line.

Developmental increase in Steptoe does not begin until 1 to 2 d later. From d 4 to 7, the activity in Steptoe is only one-third of that in Morex.

Steptoe's relative deficiency in α-amylase activity results from lowered expression of both the high- and low-pl isozyme groups (Fig. 2A). Also, both cultivars have much more high-pl than low-pl activity. High-pl activity is evident at d 2 in both Morex and Steptoe, and it increases sharply by d 3 in both. The rapid rise seen on activity gels precedes that seen in activity assays (Fig. 1) by at least 1 d. The earlier rise seen on activity gels may result from electrophoretic removal of α-amylase inhibitors. The rise in low-pl activity occurs later than that of high-pl activity. In Steptoe, the low-pl rise lags 2 d behind that in Morex (Fig. 2B).

α-Amylase mRNA Concentrations

The relative amounts of high- and low-pl mRNAs represented on northern blots (Fig. 3) correspond to the isozymal activity levels seen on IEF activity gels (Fig. 2). Morex contains greater amounts of both the high- and low-pl mRNAs. Over the 7-d developmental period, Morex has an average of twice as much high-pl mRNA and three time as much low-pl mRNA relative to Steptoe. In both, the high-pl mRNA rises sharply between d 2 and 3, synchronous with the sharp rise in high-pl isozyme activity. High-pl mRNA reaches a plateau at d 4. The low-pl mRNA, however, continues to increase gradually throughout d 7. In Morex, there is 5.5-fold more high- than low-pl mRNA, whereas in Steptoe there is 9.3-fold more high-pl mRNA over the 7-d period. These differences between Morex and Steptoe and high- and low-pl mRNAs were highly repeatable with different seed lots and with many RNA extractions from stored seeds over a 3-year period.

The main difficulty in determining relative levels of high-versus low-pl mRNA lies in ensuring that both blots were...
hybridized with probes of the same specific activity, that the hybridization solution contained the same concentration of probe activity, and that hybridization conditions and exposure times were the same for both. These factors were rigorously monitored. Probes were synthesized simultaneously, filters were probed simultaneously (in separate bags) in the same hybridization chamber, and filters were exposed together for the same duration in the same x-ray film cassette. The concentrations of high- and low-pl cloned insert DNAs used for probe synthesis were determined to be equal, by both UV spectrophotometric readings and Hoechst 33258 staining. The inclusion of 5 ng of both DNAs as hybridization standards on each northern blot (Fig. 3, A and B), and the comparable degree of hybridization seen for each, provide evidence that the signals for both {\( \alpha \)}-amylase isozymal mRNAs portray their true abundance levels. The amount of cross-hybridization that occurs between low- and high-pl sequences is typically 1 to 5%, which would not significantly influence the interpretation of high- and low-pl mRNA levels.

Another factor that influences the amount of {\( \alpha \)}-amylase transcripts in Morex, relative to Steptoe, is the amount of RNA present per g fresh weight of tissue. Both cultivars experience the same developmental increase in total RNA levels reaching a plateau or peak between d 3 and 4 (data not presented). Before d 4, RNA concentrations are 26% higher in Steptoe, but from d 4 to 7 there is 20% more RNA/g fresh weight in Morex. Therefore, the total {\( \alpha \)}-amylase transcript availability, which is the product of {\( \alpha \)}-amylase transcripts per \( \mu \)g of total RNA (northern blot signal) and RNA concentrations per g fresh weight, is greater in Morex during the high amylolytic period of d 4 to 7. The average transcript availability in Morex, relative to Steptoe, is 2.4-fold greater for high-pl mRNA and 3.6-fold greater for low-pl mRNA over the 7-d postimbibition period.

\( \alpha \)-Amylase Gene Analysis

Because the two cultivars differ in germplasm source and in {\( \alpha \)}-amylase gene expression levels, it was possible that the cause of their phenotypic differences could be indicated by Southern blot analysis. After probing restriction enzyme-digested Steptoe and Morex genomic DNA with the high-pl clone, it was found that the high-pl gene copy number and organization of most genes are apparently identical in the two cultivars (Fig. 4A). Six high-mol wt restriction fragments were found to be identical in the two cultivars, and their signal strengths were the same between cultivars.

Similar results were found after hybridizations with the low-pl cDNA probe. Up to four high-mol wt fragments were found, and most were of the same size and signal strength in Morex and Steptoe (Fig. 4B). A polymorphism of weak intensity occurred in Xbal digests. Considering the multicity nature of the high- and low-pl genes in both cultivars, it became apparent that the lowered activity levels of both isozymes in Steptoe is due to the synthesis or response to diffusible regulatory factors, rather than to an altered complement of coding genes.
beginning of imbibition. On each day, α-amylase activity was slightly lower in Steptoe under all GA concentrations relative to untreated controls, whereas Morex responded above control levels at all GA concentrations (data not presented). Maximal response in Morex occurred at $10^{-6}$ M GA.

To remove the embryo as a possible source of inhibition of Steptoe's GA response, embryoless half-seeds were prepared and treated as above with 0, $10^{-1}$, and $10^{-3}$ M GA. Morex again responded positively to GA, with higher GA concentrations producing increased α-amylase activity (Fig. 7). A slight increase in Steptoe's α-amylase activity occurred at $10^{-6}$ M but not at $10^{-4}$ M. This suggests that either the Steptoe aleurones are unresponsive to GA, or the starchy endosperm of Steptoe contains an inhibitor or inactivator of GA.

To explore these possibilities, the GA responsiveness of Steptoe and Morex isolated aleurones was tested at the RNA and enzyme activity levels. Aleurones produced little or no α-amylase mRNA when incubated for 24 h in buffered medium without GA, but both Steptoe and Morex produced high amounts of high- and low-pI mRNA with $10^{-6}$ M GA (Fig. 8). In contrast to the large high-pI:low-pI mRNA ratios in intact seedlings (Fig. 3), isolated aleurones had similar amounts of both mRNAs. The mRNA data correlated with α-amylase isozymal activity levels (Fig. 9). Both cultivars produce similar amounts of high- and low-pI enzyme activities resulting from GA treatment. In addition, a low level of low-pI activity was produced without GA treatment, although no high-pI isozyme was produced.

**DISCUSSION**

These studies suggest that the low level of α-amylase activity found in the Steptoe cultivar could be caused by a starchy endosperm-associated inhibitor of α-amylase gene expression. Although endosperm-specific inhibitors of α-amylase enzyme activity are known, only ABA is known to inhibit α-amylase expression at the pretranslational level. ABA inhibits a wide range of genes that are positively regulated by GA3, especially α-amylase and other hydrolytic enzyme genes of germinating cereal seeds (Zeevart and Creelman, 1988). Steptoe is known to exhibit strong postharvest dormancy, or "incomplete after-ripening," and seeds of this cultivar may not germinate for 2 months after maturity (Ullrich et al., 1993). The Steptoe seeds had been stored longer than 1 year under dry conditions prior to their use in these studies. This allowed sufficient time for any dormancy
effects to have passed. It was suspected that high ABA concentrations may contribute to Steptoe's low α-amylase activity and poor response to GA. However, ABA concentrations are initially higher in Morex seeds, and by d 3 they decline to identical levels in Morex and Steptoe.

It is possible that Steptoe may be highly responsive to ABA, relative to Morex. Walker-Simmons (1987) found that a wheat cultivar subject to preharvest sprouting and a normal cultivar did not differ significantly in endogenous ABA concentrations. Instead, the preharvest sprouting cultivar was less responsive to increases in ABA. Increased ABA levels accompany late seed development and discourage premature germination in barley (Robertson et al., 1989) and in a range of monocots and dicots (King, 1982; Zeevart and Creelman, 1988). However, in two barley cultivars preharvest sprouting susceptibility was correlated with endogenous ABA concentrations (Goldbach and Michael, 1976). The question of Steptoe's responsiveness to ABA will be examined in future investigations.

Other possible mechanisms may account for Steptoe's apparent endosperm-associated inhibition. Thus, (a) the exogenous GA may not have gained access to the Steptoe aleurone, even though it did in Morex half-seeds and whole seedlings (Figs. 6 and 7); (b) the endosperm of Steptoe may bind or physically inactivate GA; (c) the endosperm of Steptoe may hydrolyze or chemically modify exogenous GA so that it is rendered ineffective; or (d) the aleurone of Steptoe may be very highly sensitive to the ABA remaining in the endosperm. There is partial merit in the first argument. When the GA treatments were repeated on half-seeds that were immersed in GA solution (exactly as in the aleurone treatments), the response of Steptoe GA- and paper-imbibed half-seeds was improved (Robertson et al., 1989) and in a range of monocots and dicots (King, 1982; Zeevart and Creelman, 1988). However, in two barley cultivars preharvest sprouting susceptibility was correlated with endogenous ABA concentrations (Goldbach and Michael, 1976). The question of Steptoe's responsiveness to ABA will be examined in future investigations.

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endosperm and diffuses to the cells of the aleurone. This possibility of chemical signaling from stored starchy endosperm compounds to the aleurone (either directly or as a result of feedback from enzymic activity released from the aleurone) has received little study.

The weaker temporal expression of α-amylase genes in Steptoe may also result from partial inactivation of endogenous GA. If this or other mechanisms effectively exclude active GA molecules from aleurone cells, particularly several days after germination, a factor other than GA must have the capacity to activate α-amylase genes up to the level seen in Steptoe. Whatever this factor is, it must have the capacity to overcome inhibitory effects that appear to arise from the endosperm. These studies suggest that endogenous factors other than ABA may be active as inhibitors of α-amylase gene expression in Steptoe. If such an inhibitor could be identified, it might have application to seed development and germination problems, such as preharvest sprouting in cereals.

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LITERATURE CITED


Muthukrishnan S, Chandra GR, Maxwell ES (1979) Hormone-induced increase in levels of functional mRNA and α-amylase mRNA in barley aleurones. Proc Natl Acad Sci USA 76: 6181-6185


acterization of cDNA clones and mRNA expression during seed germination. Mol Gen Genet 221: 239–244