Plant Physiol. (1994) 106: 929-939

The Regulation of Gene Expression in Transformed Maize Aleurone and Endosperm Protoplasts[1]

Analysis of Promoter Activity, Intron Enhancement, and mRNA Untranslated Regions on Expression

Daniel R. Gallie* and Todd E. Young

Department of Biochemistry (D.R.G.) and Department of Botany and Plant Sciences (T.E.Y), University of California, Riverside, California 92521-0129

Gene expression in the aleurone and endosperm is highly regulated during both seed development and germination. Studies of α-amylase expression in the aleurone of barley (*Hordeum vulgare*) have generated the current paradigm for hormonal control of gene expression in germinating cereal grain. Gene expression studies in both the aleurone and endosperm tissues of maize (*Zea mays*) seed have been hampered because of a lack of an efficient transformation system. We report here the rapid isolation of protoplasts from maize aleurone and endosperm tissue, their transformation using polyethylene glycol or electroporation, and the regulation of gene expression in these cells. *Adh1* promoter activity was reduced relative to the 35S promoter in aleurone and endosperm protoplasts compared to Black Mexican Sweet suspension cells in which it was nearly as strong as the 35S promoter. Intron-mediated stimulation of expression was substantially higher in transformed aleurone or endosperm protoplasts than in cell-suspension culture protoplasts, and the data suggest that the effect of an intron may be affected by cell type. To examine cytoplasmic regulation, the 5′ and 3′ untranslated regions from a barley α-amylase gene were fused to the firefly luciferase-coding region, and their effect on translation and mRNA stability was examined following the delivery of in vitro synthesized mRNA to aleurone and endosperm protoplasts. The α-amylase untranslated regions regulated translational efficiency in a tissue-specific manner, increasing translation in aleurone or endosperm protoplasts but not in maize or carrot cell-suspension protoplasts, in animal cells, or in in vitro translation lysates.

The aleurone and endosperm play an essential role in storing reserves during cereal seed development as well as mobilizing those reserves during germination. The expression of several genes in the aleurone is induced following imbibition. α-Amylase synthesized and secreted from the aleurone during germination is involved in the hydrolysis of the starch reserves. The genes encoding α-amylase in barley (*Hordeum vulgare*) have been extensively characterized as an example of a gene family subject to hormonal regulation. Transcription of α-amylase is induced by GA_3 and antagonized by ABA (reviewed by Ho et al., 1987; Jacobsen and Chandler, 1987). Sequence elements within the promoter region that are responsible for the hormonal regulation have been identified by mutational analysis (Skriver et al., 1991; Gubler and Jacobsen, 1992; Lanahan et al., 1992), and much of this work was carried out using barley aleurone protoplasts (Jacobsen and Beach, 1985; Salmenkallio et al., 1990; Jacobsen and Close, 1991; Skriver et al., 1991; Gubler and Jacobsen, 1992). Isolated aleurone protoplasts are similar to the intact aleurone in both function and structure (Jacobsen et al., 1985; Hillmer et al., 1990; Gopalakrishnan et al., 1991; Jacobsen and Close, 1991) and have been used for transient transformation studies from species other than barley, including oat (*Avena sativa*; Huttly and Baulcombe, 1989) and rice (*Oryza sativa*; Sadasivam and Gallie, 1994). Similar studies in maize (*Zea mays*) have been hampered because of no procedure existed to isolate and transform aleurone protoplasts from either developing or germinating seed. Particle bombardment has been used to introduce genes involved in anthocyanin biosynthesis (Klein et al., 1989; Goff et al., 1990; Ludwig et al., 1990; Roth et al., 1991; Radicella et al., 1992); however, quantitative studies are complicated by the variability inherent in the bombardment method.

In addition to the interest in the aleurone as a system to study the hormonal or developmental control of gene expression during seed development and germination, considerable interest has focused on the control of endosperm gene expression in maize as a means to improve seed quality. Protoplasts from developing endosperm have been isolated and used for transformation studies in wheat (*Triticum aestivum*; Keeling et al., 1989; Diaz et al., 1993) and barley (Lee et al., 1991; Diaz and Carbonero, 1992). Endosperm protoplasts maintain the characteristics associated with that tissue. Endosperm from *Hevea brasiliensis* L. continue to express β-glucosidase and α-mannosidase activities (Selmar et al., 1989), and wheat endosperm protoplasts contain high levels of unidine diphosphate glucose pyrophosphorylase activity and incorpo-

[1] This work was supported by grant DF000230 from Pioneer Hi-Bred International, Inc.

* Corresponding author; fax 1-909-787-3590.

Abbreviations: *Adh1*, alcohol dehydrogenase 1; BMS, Black Mexican Sweet; BMV, brome mosaic virus; CHO, Chinese hamster ovary; DAP, days after pollination; eIF, eukaryotic initiation factor; *luc*, luciferase; ORF, open reading frame; TMV, tobacco mosaic virus; UTR, untranslated region.

rate Suc or Glc into starch at rates that are comparable to intact endosperm (Keeling et al., 1989). As with the maize aleurone, gene expression studies in endosperm tissue have been limited because of no suitable transformation system. The only protocol for the isolation of maize endosperm protoplasts (Schwall and Feix, 1988) has not been used for subsequent endosperm transformation studies. Because of the perceived difficulty in isolating protoplasts from developing maize endosperm, endosperm suspension cultures have been used as a substitute. These cell cultures synthesize anthocyanins (Racchi, 1985), recognize zein promoters (Schwall and Feix, 1988), and accumulate starch (Chu and Shannon, 1975; Saravitz and Boyer, 1987); however, these characteristics are significantly reduced in endosperm cell cultures, resulting in lower starch biosynthesis (Chu and Shannon, 1975) and zein synthesis (Shimamoto et al., 1983; Lyznik and Tsai, 1989) than in intact endosperm tissue. Consequently, these cultures are only endosperm-like, and the regulation observed does not fully reflect the regulation present in developing endosperm. Moreover, establishing endosperm cultures is laborious and often not feasible when a large number of genotypes need to be analyzed. Because of the limitations of using endosperm cell cultures, the analysis of maize endosperm gene expression has been carried out in alternative systems such as transgenic tobacco (Matzke et al., 1990), carrot, or undifferentiated maize suspension cells (Thompson et al., 1990). Although bombardment of intact endosperm overcomes the difficulties associated with endosperm cell cultures (Unger et al., 1993), it is subject to the same problem of variation as observed for aleurone tissue.

We report here the isolation and transformation of protoplasts from the aleurone of developing and germinating maize seed as well as from developing endosperm tissue. Protoplasts from these tissues were used to investigate the effect of cell type on the nuclear regulation of the *Adh1* promoter and the effect of the *Adh1* intron 1 on gene expression. Moreover, we found that the α-amylase 5′ leader and 3′ UTR conferred a tissue-specific, translational advantage to reporter mRNA in aleurone and endosperm protoplasts but not in plant cell cultures, animal cells, or in vitro translation lysates.

MATERIALS AND METHODS

mRNA Constructs and in Vitro Transcription Reaction Conditions

The pT7-*luc* and pT7-*luc*-A_{50} constructs, in which the firefly *luc*-coding region is under the control of the T7 promoter, have been previously described (Gallie et al., 1991). The *luc* constructs containing either the *Adh1* promoter, *Adh1* intron 1, or the 5′ leaders from TMV and BMV (Luehrsen et al., 1992) were a gift from V. Walbot (Stanford University, Stanford, CA). The pT7-Amy-*luc*-$_{50}$ construct was made by replacing the α-amylase ORF from a barley (*Hordeum vulgare*) cDNA (a gift from L. Huiet and P. Chandler) with the *luc* ORF. Restriction sites for *Nco*I and *Bgl*II were introduced at the initiation and termination codons, respectively, of the α-amylase ORF using site-directed mutagenesis (Kunkel et al., 1987), and the *luc* ORF was introduced as an *Nco*I/*Bgl*II fragment. Restriction sites for *Stu*I and *Bam*HI were introduced at the transcription initiation and the polyadenylation sites, respectively, of the α-amylase gene (Chandler and Huiet, 1991), and the *Stu*I/*Bam*HI fragment was introduced into the *Stu*I/*Bam*HI sites of the pT7-A_{50} vector. A poly(A)$_{50}$ tract, present in pT7-Amy-*luc*-A_{50}, allows the in vitro synthesis of poly(A)$^+$ mRNA following linearization of the plasmid with *Dra*I, which cuts at a site immediately downstream of the poly(A) tract. The concentration of the template DNAs were quantitated spectrophotometrically following linearization and brought to a concentration of 0.5 mg/mL. In vitro transcription was carried out as described by Yisraeli and Melton (1989) using 40 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 100 μg/mL BSA, 0.5 mM each of ATP, CTP, and UTP, plus 160 μM GTP, 1 mM m^7GpppG, 1 mM DTT, 0.3 unit/μL RNasin RNase inhibitor (Promega), and 0.5 unit/μL T7 RNA polymerase (New England Biolabs, Beverly, MA). Under our transcription conditions, >95% of the mRNA is capped. Each RNA construct was synthesized in triplicate in separate transcription reactions so that any variability in RNA yield would be reflected in the SD calculated as part of the expression data for each construct. The integrity and relative quantity of RNA were determined by formaldehyde-agarose gel electrophoresis as described by Melton et al. (1984).

Isolation and Transformation of Aleurone Protoplasts

Oh43 and BMS seeds were surface sterilized with 1% sodium hypochlorite solution containing 50 μL of Tween 20 for 15 min. The seeds were rinsed three times for 15 min with sterile water and germinated in sterile vermiculite at 30°C for 5 d. The aleurone layer was peeled from the seed using forceps and placed in modified Gamborg's medium (10 mM $CaCl_2$, 100 mM Glc in Gamborg's medium, pH 5.4) with 0.6 M mannitol. Following a 30-min wash to remove adhering starch, layers from six seeds were transferred to 3 mL of modified Gamborg's medium containing 1.5% cellulase, Onuzuka R-10 (Yakult Honsha Co., Tokyo, Japan), 1.5% Cytolase M103S (Genencor International, Rochester, NY), 0.25% cellulase (Worthington Biochemical, Freehold, NJ), 50 units/mL nystatin, 90 μg/mL cefotaxime, and 0.6 M mannitol and digested for 15 h. The digested layers were passed through a 50-μm nylon mesh to remove large debris. Starch grains were separated from the protoplasts by passing the solution through a 10-μm nylon mesh. The protoplasts remained on the mesh and were washed into a tube using modified Gamborg's medium containing 0.6 M mannitol. The protoplasts were harvested by centrifugation at 50*g* for 5 min and resuspended in maize MaMagMes solution (15 mM $MgCl_2$, 0.6 M mannitol, and 0.1% Mes, pH 5.4).

For transformation, 100 μg of denatured and sheared salmon sperm DNA and the test plasmid DNA or mRNA were added to 0.4 mL of protoplasts at room temperature and briefly mixed. Then 0.5 mL of PEG solution [40% PEG-8000, 0.1 M $Ca(NO_3)_2 \cdot 4H_2O$, 0.6 M mannitol] was added, and the solution was gently inverted until completely mixed. Following a 10-min incubation, 0.6 mL of 0.2 M $CaCl_2$ was added, and the tube containing the protoplasts was gently inverted until mixed. Following a 10-min incubation, the

protoplasts were harvested by centrifugation at 50*g* for 3 min, the supernatant was removed, and the cells were resuspended by gentle inversion in 1 mL of aleurone protoplast incubation medium (0.6 M mannitol, 50 units/mL nystatin, and 90 μg/mL cefotaxime in modified Gamborg's medium, pH 5.4). The protoplasts were transferred to a glass dish containing an additional 1 mL of protoplast medium and incubated at room temperature for 24 h.

Isolation and Transformation of Developing Endosperm Protoplasts

Oh43 ears were collected 11 DAP, surface sterilized with 1% sodium hypochlorite solution containing 50 μL of Tween 20 for 15 min, and rinsed three times for 15 min with sterile water. Thin transverse sections of intact seeds were removed using a razor blade, placed in protoplast medium (1.5% cellulase, Onuzuka R-10, 1.5% Cytolase M103S, 1% BSA, 1 mM DTT, 50 units/mL nystatin, 90 μg/mL cefotaxime, and 2 M glycerol in modified Gamborg's medium, pH 5.4), and digested for 4 h. The digested endosperm was gently squeezed and passed through a 105-μm nylon mesh to remove large debris. The protoplasts were harvested by centrifugation at 25*g* for 5 min and resuspended in endosperm protoplast electroporation buffer (4 mM $CaCl_2$, 10 mM NaCl, 130 mM KCl, 1% BSA, 2 M glycerol, and 10 mM Hepes, pH 7.2).

For transformation, 100 μg of denatured and sheared salmon sperm DNA and the test plasmid DNA or mRNA were added to the electroporation cuvette, followed by 0.4 mL of endosperm protoplasts. The cell-nucleic acid mixture was mixed briefly and electroporated at room temperature at 400 μF and 225 V. One milliliter of endosperm incubation medium (1% BSA, 50 units/mL nystatin, 90 μg/mL cefotaxime, and 2 M glycerol in modified Gamborg's medium, pH 5.4) was added to the cuvette, and the contents were poured into a glass dish containing an additional 1 mL of protoplast incubation medium and incubated at room temperature for 24 h.

Isolation and Transformation of BMS and Carrot Protoplasts and CHO Cells

Protoplasts were isolated from a maize (BMS) and a carrot (RCWC) cell suspension by digestion with 0.25% CELF cellulase (Worthington Biochemical), 1% Cytolase M103S (Genencor), 0.05% Pectolyase Y23 (Seishin Pharmaceutical Co.), 0.5% BSA, and 7 mM β-mercaptoethanol in isolation buffer (12 mM sodium acetate, pH 5.8, 50 mM $CaCl_2$, and 0.25 M mannitol) for 75 min. Protoplasts were washed once with isolation buffer, once with electroporation buffer (10 mM Hepes, pH 7.2, 130 mM KCl, 10 mM NaCl, 4 mM $CaCl_2$, and 0.2 M mannitol), and resuspended in electroporation buffer to a final concentration of 1.0×10^6 cells/mL. One microgram of each *luc* mRNA construct was mixed with 0.8 mL of protoplasts immediately before electroporation (500 μF and 300 V for BMS; 500 μF and 350 V for carrot) using an IBI (New Haven, CT) GeneZapper. The protoplasts were incubated overnight before assaying.

CHO cells were grown to approximately 80% confluence in Ham's F-12 medium supplemented with 10% fetal calf serum. Cells were collected from flasks by a brief incubation with 4 mM EDTA and washed twice with PBS. Cells (1×10^6) in 0.4 mL were mixed with 1 μg of test mRNA and electroporated in PBS (200 μF and 400 V). Following electroporation, the cells were incubated for 6 h in Ham's F-12 medium supplemented with 10% fetal calf serum before assaying.

In Vitro Translation

One hundred nanograms of each *luc* mRNA construct were translated in untreated rabbit reticulocyte or wheat germ lysate according to the recommendations of the supplier (Promega) with the exception that a complete mix of nonradiolabeled amino acids was used. The extent of translation was determined by assaying each aliquot for luciferase activity.

Luciferase Assay

Cells collected by centrifugation at 25*g* were sonicated for 5 s in 100 mM Tricine, pH 7.8, 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid, 10% glycerol, and 1% Triton X-100, and the cell debris was pelleted. Aliquots of the extract were added to 100 μL of luciferase assay buffer [20 mM Tricine, pH 7.8, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM CoA, and 500 μM ATP] (Promega Biotec), and the reaction was initiated with the injection of 100 μL of 0.5 mM luciferin in the *luc* assay buffer. Photons were counted using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA). Protein concentration was determined by the method described by Bradford (1976). Aliquots from in vitro translation lysates were added directly to luciferase buffer (25 mM Tricine, pH 7.5, 15 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 1 mM ATP) before assaying.

RESULTS

Isolation and Transformation of Aleurone and Endosperm Protoplasts

Protoplasts were isolated in approximately 15 h from the aleurone of 5-d germinated Oh43 and BMS inbred seed. Aleurone protoplasts were dense, uniform, and contained numerous vacuoles (Fig. 1). Although not used in this study, aleurone protoplasts were also isolated from developing 30-DAP or older seed using the same protoplast isolation protocol described for layers obtained from germinated seed. Aleurone layers could not be peeled during late seed development because the seed becomes hard.

Endosperm protoplasts were isolated in approximately 4 h from developing 11-DAP Oh43. At this developmental stage, they contained some starch grains but did not photograph well because of the lack of contrast. The 11-DAP protoplasts were uniform in appearance. Endosperm protoplasts could be isolated at an earlier stage, but any remaining nucellar tissue would have to be removed because it would also yield protoplasts. The isolation of protoplasts from older endo-

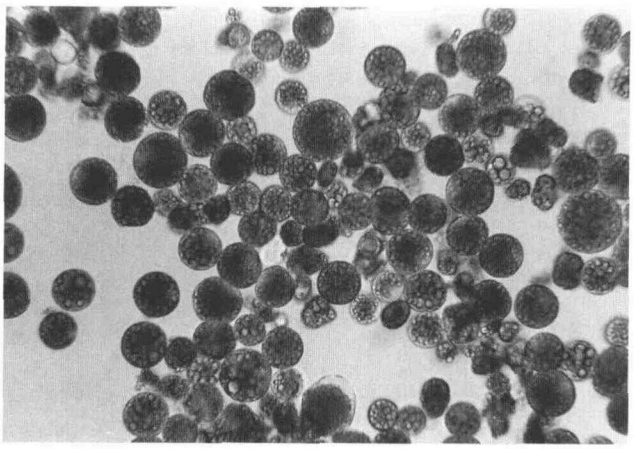

Figure 1. Protoplasts isolated from the aleurone layer of 5-d germinated Oh43 seed. Protoplasts were isolated as described in "Materials and Methods."

sperm was also possible. The protoplasts were less uniform, representing the development of the endosperm from the center to the periphery. The osmoticum and time of cell-wall digestion had to be increased with the age of the tissue, and when the endosperm cells had completely filled with starch grains, the yield of protoplasts was reduced. Because protoplasts are released from the center of the endosperm first, isolation of protoplasts from the developing aleurone and subaleurone was avoided by removing the endosperm slices before protoplasts were released from these tissues. Although Schwall and Feix (1988) described a protocol for the isolation of protoplasts from developing maize endosperm, our attempts to obtain viable protoplasts using this protocol failed. In our protocol, 1% BSA is essential to maintain protoplast viability, an observation suggesting that proteases might be present in the endosperm protoplast preparations that could compromise the integrity of the endosperm plasmalemma. Several protease activities are known to be secreted in endosperm cultures, and cells starved for nitrogen can be rescued by the addition of BSA to the medium (Miernyk and Sturch, 1991). No such BSA requirement was necessary to maintain aleurone protoplast viability.

Transformation of aleurone protoplasts was performed using PEG, whereas electroporation was used for the transformation of endosperm and cell-suspension cells. Aleurone protoplasts are very active in expressing constructs. As a result, as few as 100,000 protoplasts can be used for each transformation, although for this study approximately 500,000 protoplasts were used. Approximately 70 to 90% of the cells isolated from the aleurone were viable protoplasts depending on the inbred. Protoplast survival following PEG transformation was typically 50 to 70% 24 h following transformation. Although usually harvested 24 h following transformation, the aleurone protoplasts can survive for several more days. Neither the aleurone nor endosperm protoplasts were observed to regenerate their cell walls. Digestion of 100 seeds generates enough protoplasts for approximately 20 transformations.

Endosperm from 50 seeds (11 DAP) was sufficient for 15 transformations using 1×10^6 to 2×10^6 protoplasts for each transformation. About 90 to 100% of the cells isolated from 11-DAP endosperm were viable protoplasts. Cell survival was typically 70 to 90% 24 h following transformation; however, the percentage of cell survival is highly dependent on the electroporation conditions used. Endosperm protoplasts remained viable up to at least 8 d, the longest time tested. Electroporation of aleurone protoplasts or PEG-mediated transfection of endosperm was examined, but these methods resulted in poor cell survival. DNA constructs were transformed in duplicate, and mRNA constructs were transformed in triplicate. Each transformation was assayed in duplicate, and the resulting average is reported.

Adh1 Promoter Activity Is Influenced by Cell Type

Adh1 expression is induced in response to hypoxia, resulting in a 50-fold increase in *Adh1* mRNA within 5 h of anaerobiosis (Gerlach et al., 1982). This stress response is regulated both at the transcriptional level (Howard et al., 1987; Walker et al., 1987) and at the translational level (Sachs et al., 1980). To measure *Adh1* promoter activity in aleurone or endosperm protoplasts, the level of reporter gene expression driven by the *Adh1* promoter was compared to that from the constitutively expressed cauliflower mosaic virus 35S promoter. The firefly *luc* was used as the reporter gene, and the constructs also contained the *Adh1* intron (see Fig. 2 for construct design). Ten micrograms of each plasmid construct were introduced into aleurone protoplasts isolated from 5-d germinated BMS and Oh43 seed, Oh43 endosperm protoplasts isolated from 11-DAP tissue, or BMS cell-suspension protoplasts. The protoplasts were harvested 24 h following transformation for *luc* assays. The 35S promoter activity was significantly higher than *Adh1* promoter activity in the aleurone and endosperm cells (Fig. 2). In contrast, the *Adh1* promoter was nearly as active as the 35S promoter in BMS suspension protoplasts. The effect of cell type on relative promoter strength can be illustrated by the ratio of expression resulting from the 35S construct compared to the *Adh1* construct (Fig. 3). The observation that *Adh1* promoter activity remains low, relative to the 35S promoter-*Adh1* intron activity, in aleurone and endosperm cells but is high in cultured cells suggests that suspension cells may exist in a partially hypoxic state or may have lost the ability to repress *Adh1* promoter activity. Since all protoplasts were incubated in an identical volume of medium under identical atmospheric conditions, any hypoxic response in BMS would have to be a carryover from the cell culture before protoplast isolation. Alternatively, the loss of cellular differentiation may be the basis for the relatively high activity of the *Adh1* promoter in the suspension cells.

Intron-Mediated Enhancement of Expression Is Influenced by Cell Type

The presence of an intron within a transcript enhances both the nuclear and cytoplasmic concentration of that mRNA (Buchman and Berg, 1988). The intron effect does not increase the rate of transcription (Hamer et al., 1979) but increases the efficiency of 3′ end processing and nucleocy-

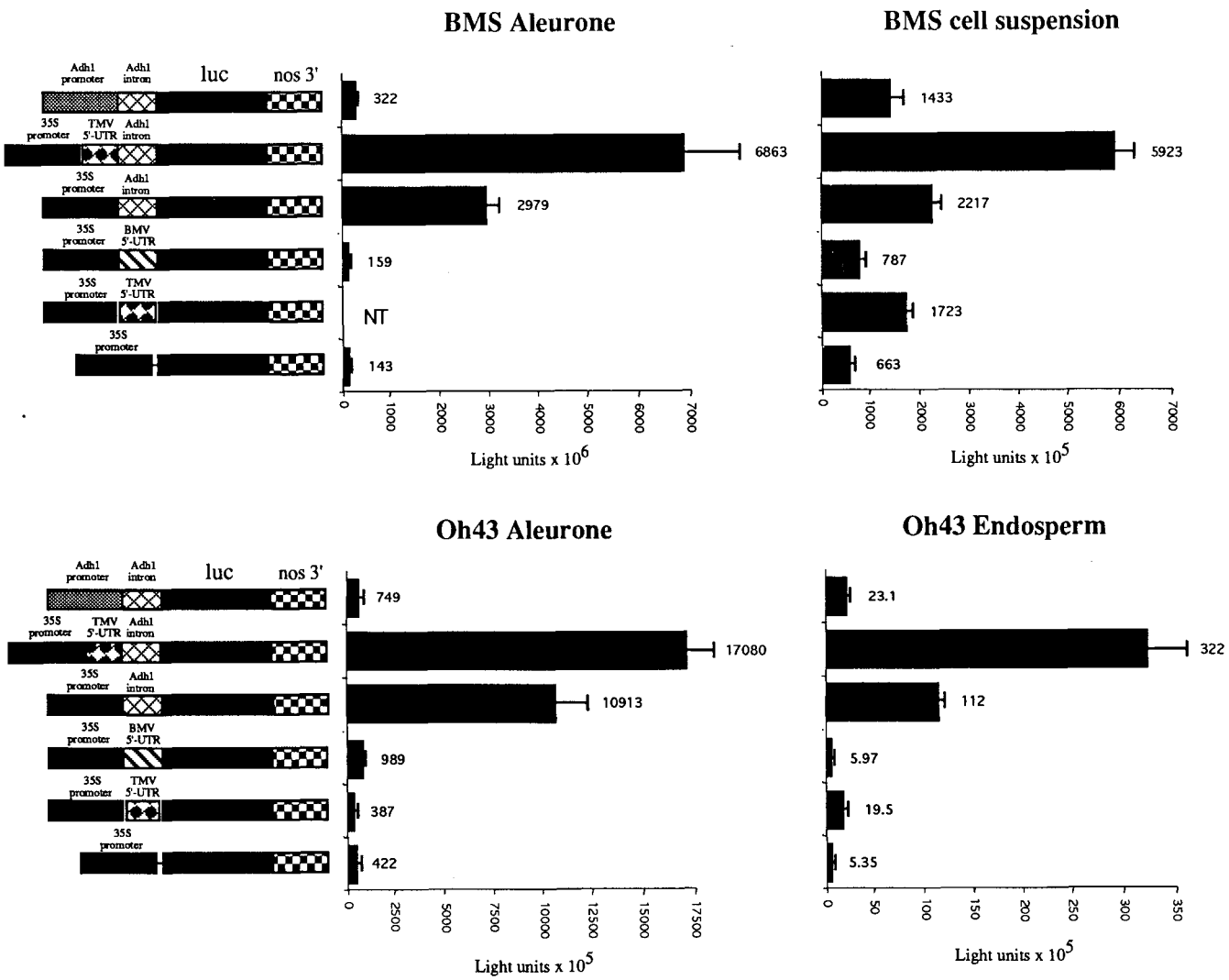

Figure 2. Comparative analysis of promoter activity, intron enhancement, and viral translational enhancer sequences on *luc* expression in maize aleurone, endosperm, and cell-suspension cells. The *luc* plasmid constructs, shown on the left, were delivered to the protoplasts as described in "Materials and Methods" and incubated for 24 h before assaying. The level of expression resulting from each construct is shown as a histogram directly to the right of the respective mRNA. The numerical value for each histogram is indicated immediately to the right of each histogram. *luc* activity is expressed as light units/mg protein except for Oh43 endosperm, for which it is expressed as light units/100,000 cells. The construct containing no intron or viral leader contains a 19-base 5′ leader, whereas the 5′ leader for the TMV and BMV constructs is 80 and 98 bases, respectively. When the *Adh1* intron is excised from the 35S construct, the resulting mRNA contains a 54-base 5′ leader. All constructs contain the nopaline synthase 3′ polyadenylation site.

toplasmic transport (Huang and Gorman, 1990). Because of the increase in the steady-state level of an mRNA, expression at the protein level also increases (Callis et al., 1987). To determine whether there was a difference in the level of the intron effect among a terminally differentiated cell type like the aleurone, a developing cell type like the 11-DAP endosperm, or the undifferentiated cells of a suspension culture, the expression from a 35S promoter-*luc* containing the first intron of *Adh1* was compared to the identical construct not containing an intron. The same set of constructs was introduced into each cell type so that any differences in the relative levels of expression could be attributed to the recipient cells.

The presence of the *Adh1* intron substantially increased expression in aleurone and endosperm cells (Fig. 2). Although the *Adh1* intron also increased expression in BMS suspension cells, the effect was considerably less than that observed in either the aleurone or endosperm cells. The effect of cell type on intron enhancement can be expressed as the ratio of expression from the intron-containing construct to the construct without the intron (Fig. 4). Since there are 12 AUG codons within the *Adh1* intron that could serve as translational initiation sites, initiation at the AUG codon of the *luc*-coding region of any unspliced mRNA that is transported to the cytoplasm would be expected to be quite low. Therefore,

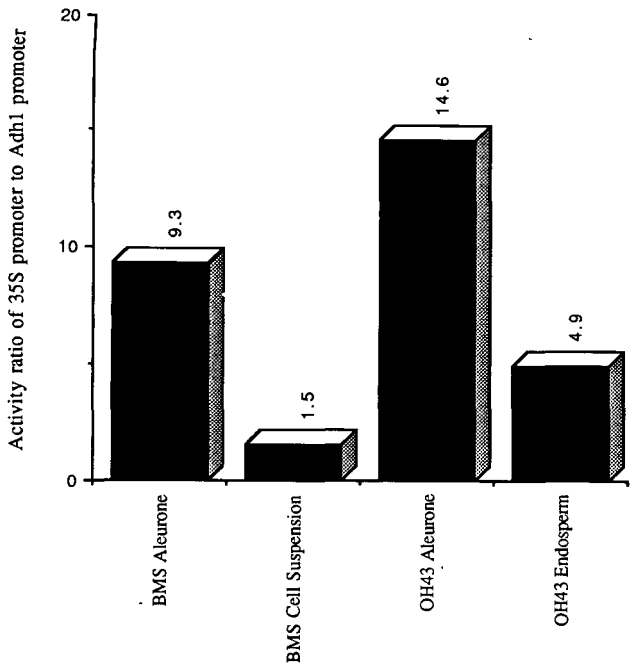

Figure 3. 35S promoter activity relative to the *Adh1* promoter in aleurone, endosperm, and suspension cells. The level of expression resulting from the *Adh1* intron-*luc* construct under the control of the 35S promoter from Figure 2 was expressed as a ratio of the analogous construct under the control of the *Adh1* promoter. Each cell type is indicated below the respective histogram.

only that mRNA in which the *Adh1* intron has been efficiently spliced out will likely be translated. These results were confirmed with a second pair of constructs that were identical with the above pair of constructs except that they contained the 5′ leader sequence from TMV. The *Adh1* intron increased expression 44.1-fold in aleurone protoplasts and 16.5-fold in endosperm protoplasts but only 3.4-fold in cell-suspension cells (Fig. 2). These data suggest that the degree to which an intron enhances gene expression may vary with the cell type or cell state.

Translational Enhancement Conferred by Viral 5′ Leaders

The 68-base 5′ leader (called Ω) of TMV confers a translational advantage to reporter mRNAs (Gallie et al., 1987, 1989). Ω increases translation in dicot species to a greater extent than in monocot species (Gallie et al., 1989). A sequence element contained within Ω is required for function (Gallie and Walbot, 1992) and is specifically recognized by an RNA-binding protein present in dicot and monocot plant extracts (Leathers et al., 1993). To determine whether cytoplasmic regulation is similar among aleurone, endosperm, and cultured cells, expression from *luc* constructs containing Ω were compared to the control *luc* construct. Ω increased luciferase expression in suspension cells to approximately the same extent (2.6-fold) whether the *luc* construct contained the *Adh1* intron or not (Fig. 2). Similar levels of enhancement were observed in transformed aleurone and endosperm protoplasts, data suggesting that Ω functions to the same extent regardless of cell type. The leader from BMV, a monocot-specific virus, was also examined. The translational enhancement conferred by this leader was not any greater than that observed by Ω and was not altered by cell type (Fig. 2).

Cap and Poly(A) Tail Function in Aleurone and Endosperm Protoplasts

Virtually all cellular mRNAs have a cap (m^7GpppN, where N represents any base) at the 5′ terminus and a poly(A) tail at the 3′ terminus that are required to promote efficient initiation of translation. As regulators of translation in higher eukaryotes, the cap and poly(A) tail are co-dependent for function because neither can function well in the absence of the other regulatory element (Gallie, 1991). Therefore, the cap and poly(A) tail are not functionally separate but work in concert, in conjunction with their associated binding proteins, to direct efficient translation.

The synergistic interaction between the cap and poly(A) tail has been observed in several plant species, including tobacco (Gallie, 1991), carrot, and maize. To determine whether the functional characteristics of the cap and poly(A) tail synergism are conserved in aleurone and endosperm protoplasts, *luc* mRNA, synthesized in vitro as uncapped and poly(A)$^-$, uncapped and poly(A)$^+$, capped and poly(A)$^-$, and capped and poly(A)$^+$ mRNA (see Fig. 5 for mRNA constructs), was delivered to aleurone and endosperm protoplasts using the same transformation protocol that was used for DNA

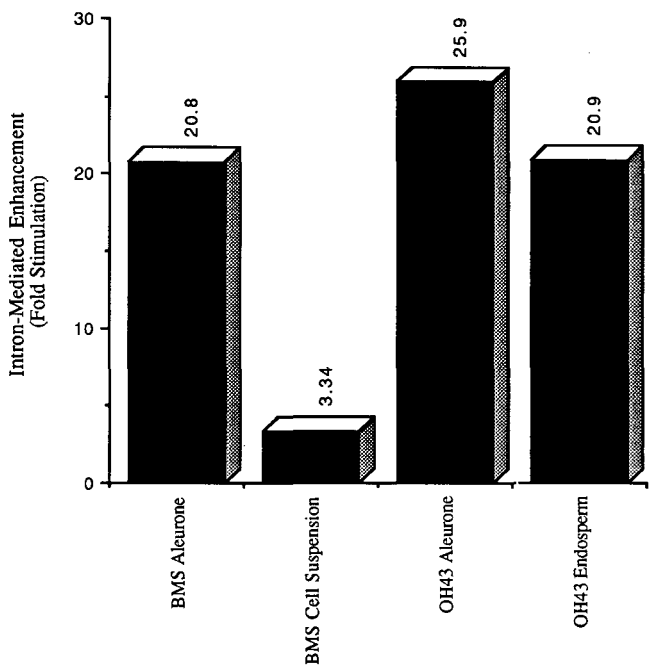

Figure 4. The effect of an intron on gene expression in aleurone, endosperm, and suspension cells. The expression levels resulting from the 35S-*luc* DNA constructs with or without the *Adh1* intron 1 from Figure 2 are shown as ratios to demonstrate the intron-mediated enhancement in each cell type. The enhancement afforded by the intron is indicated above each histogram. Each cell type is indicated below the respective histogram.

delivery for each cell type. As observed in cell-suspension protoplasts (Gallie, 1991), the level of expression from the capped, poly(A)$^+$ mRNA was considerably higher than any other form of the mRNA (Fig. 5). The effect of the poly(A) tail on the translation of a capped mRNA can be quantitated by comparing the level of expression between the poly(A)$^+$ and poly(A)$^-$ forms of capped *luc* mRNA. Similar comparisons can also be made for the poly(A)$^-$ and poly(A)$^+$ forms of uncapped *luc* mRNA to quantitate the effect of the poly(A) tail on the translation of an uncapped mRNA. The poly(A)$_{50}$ tail increased expression from capped mRNA 13.3-fold versus 1.4-fold for uncapped mRNA. This represents a 9.5-fold increase in the effect of the poly(A) tail for capped versus uncapped mRNAs and is in good agreement with previous results obtained for suspension cells (Gallie, 1991). Likewise in endosperm, the poly(A) tail stimulated expression 28.3-fold for capped mRNA but only 2.3-fold for uncapped mRNA, a 12-fold increase in the effect of the poly(A) tail for a capped message. Therefore, the efficient translation of an mRNA in aleurone and endosperm protoplasts is dependent on the presence of both a cap and a poly(A) tail.

The α-Amylase 5′ and 3′ UTRs Confer Tissue-Specific Posttranscriptional Regulation

α-Amylase is one of the most abundant proteins synthesized in the aleurone during germination. Although α-amylase mRNA represents 20% of the total mRNA present in the barley aleurone, α-amylase protein represents 60% of the protein synthesized in this tissue during germination, data suggesting the preferential translation of this mRNA (Higgins et al., 1982). α-Amylase mRNA is also long lived, exhibiting a half-life greater than 100 h (Ho, 1976). Regulatory elements controlling either the translational efficiency or stability of

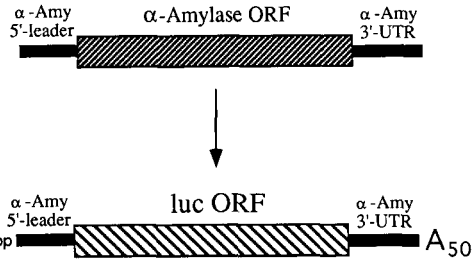

Figure 6. Illustration of the construction of the α-amylase (Amy)-*luc* chimeric mRNA.

an mRNA are often located within the 5′ or 3′ UTRs (Gallie et al., 1987; Carrington and Freed, 1990; Dickey et al., 1992; Danthinne et al., 1993; Leathers et al., 1993; Timmer et al., 1993; Zhang and Mehdy, 1994). To determine whether the α-amylase 5′ leader and 3′ UTR are required for preferential translation or message stabilization in the aleurone, the coding region from the barley α-amylase cDNA was replaced with the coding region from *luc* (Fig. 6). This chimeric α-amylase-*luc* construct was introduced into a T7 promoter-based vector that allowed the in vitro synthesis of capped mRNA that terminated in a poly(A)$_{50}$ tail.

Aleurone and endosperm protoplasts were transformed with the chimeric α-amylase-*luc* mRNA and compared to the control *luc* mRNA. The level of luciferase expression produced following incubation of the transformed protoplasts was used as a measure of the effect of the α-amylase UTRs on *luc* mRNA translation. The presence of the α-amylase UTRs increased luciferase expression 7-fold in aleurone and 3.5-fold in endosperm protoplasts (Fig. 7). The α-amylase UTRs also increased luciferase expression to the same extent

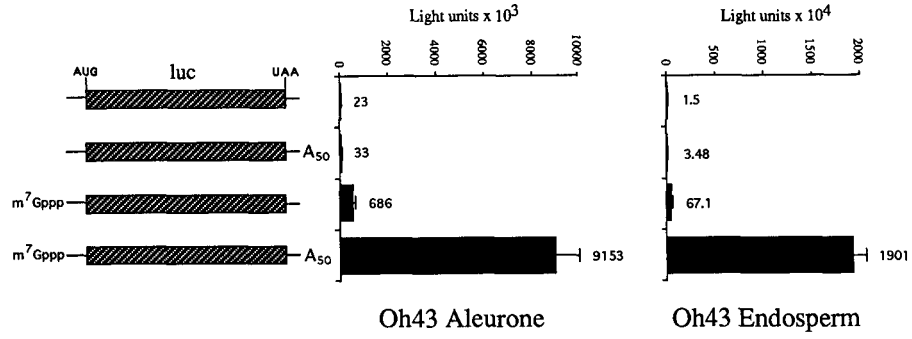

Figure 5. The effect of a cap and poly(A) tail on the translation of *luc* mRNA delivered to aleurone and endosperm protoplasts of Oh43 seed. The *luc* mRNA constructs were synthesized in vitro in the forms shown on the left. Each construct contains the same 17-base polylinker leader and a 22-base 3′ UTR. The level of *luc* expression resulting from each mRNA is shown as a histogram directly to the right of the respective mRNA. The numerical value for each histogram is indicated immediately to the right of each histogram. Luciferase activity is expressed as light units/mg protein except for Oh43 endosperm, for which it is expressed as light units/100,000 cells.

in barley aleurone protoplasts (T. Close and D. Gallie, unpublished results). In contrast, the presence of the α-amylase UTRs did not confer any translational advantage to *luc* mRNA in either maize or carrot cell culture protoplasts or in CHO cells (Fig. 7). Moreover, the α-amylase UTRs failed to enhance translation in in vitro lysates derived from wheat germ or rabbit reticulocytes. These data suggest that the α-amylase UTRs regulate translation posttranscriptionally in a tissue-specific manner.

To determine whether the α-amylase UTRs regulated translational efficiency or mRNA stability, a kinetics analysis of the translation of the chimeric α-amylase-*luc* and control mRNAs was carried out to measure directly the translational efficiency of each. Aleurone protoplasts were transformed with the in vitro synthesized *luc* mRNAs, aliquots were removed at time intervals and assayed for luciferase activity, and the level of expression was plotted as a function of time (Fig. 8). Following mRNA delivery, the rate of protein production increases as the *luc* mRNA is recruited onto polysomes. Since luciferase enzyme activity is detectable within 3 min following delivery, recruitment of the mRNA begins soon after mRNA delivery. The maximum slope of each curve represents the maximum rate of luciferase production. Between the initial loading of the mRNA onto the polysomes and its eventual degradation, there is a phase of steady-state translation in which the rate of luciferase production is both maximal and constant and is dictated by its translational efficiency. The efficiency of translation of each mRNA is measured during this steady-state phase. The mRNA is then translated for a length of time that is determined by the stability of the mRNA. The eventual degradation of the mRNA results in a decreased rate of protein accumulation. Following degradation of the mRNA, further accumulation of luciferase protein ceases, represented by the plateau of each curve at the later times.

The presence of the α-amylase UTRs increased the translational efficiency of *luc* mRNA to 411,000 light units min^{-1} mg^{-1} protein (Fig. 8). Compared to the control mRNA (94,000 light units min^{-1} mg^{-1} protein), this represents a 4.4-fold increase in the rate of translation. The length of time during which the test and control mRNAs were translationally active were approximately equivalent, with translation ceasing by 8 h following mRNA delivery. This suggests that the half-life of the *luc* mRNA was not significantly altered by the presence of the α-amylase UTRs.

α-Amylase mRNA is destabilized by heat shock in barley aleurone layers (Belanger et al., 1986; Brown and Brodl, 1988; Brodl and Ho, 1991). To determine whether the α-amylase UTRs are sufficient and necessary for the heat-mediated destabilization, barley and maize protoplasts were heat shocked at 40 and 45°C, respectively, following the delivery of either the chimeric α-amylase-*luc* mRNA or control *luc* mRNA, and the resulting level of expression was compared to that measured in non-heat-shocked (treated at 24°C) cells. Translational repression is a consequence of heat shock in plant cells with the extent of repression a function of the severity of the stress (D. Gallie, unpublished observations). However, if the α-amylase UTRs selectively destabilize the *luc* mRNA, this should result in an even lower level of *luc* expression with respect to the control construct. Heat shock reduced expression from both the test and control mRNA constructs as expected (data not shown); however, the extent of the reduction was identical for both mRNAs, data suggesting that the α-amylase UTRs are not sufficient and perhaps not necessary for the heat-mediated destabilization.

DISCUSSION

We have demonstrated the utility of maize aleurone and endosperm protoplasts in transient transformation studies

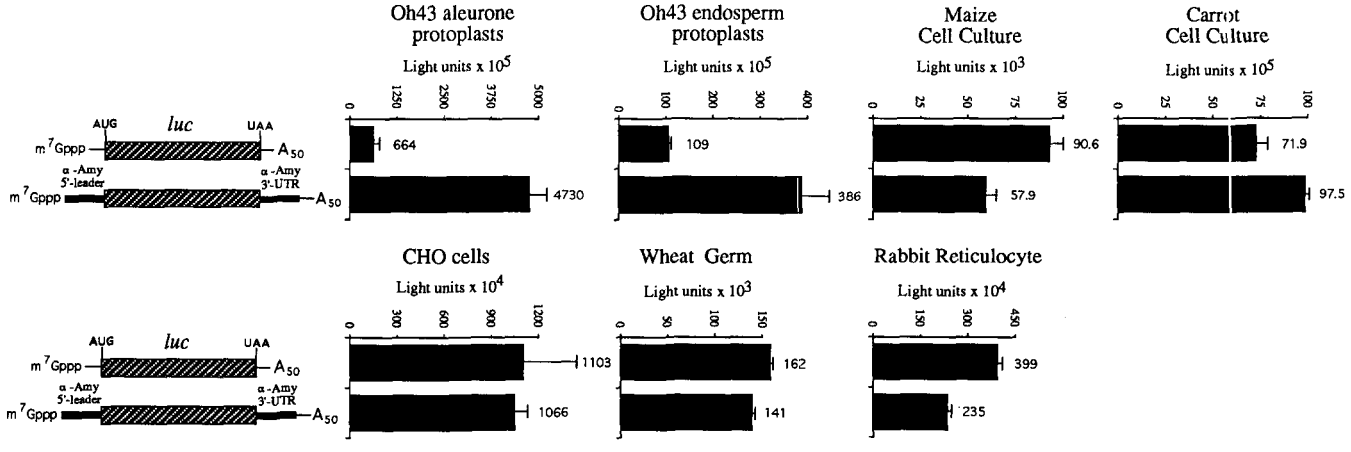

Figure 7. Regulation of *luc* translation by the α-amylase 5′ leader and 3′ UTR in Oh43 aleurone and endosperm protoplasts, maize and carrot cell cultures, CHO cells, and in vitro translation lysates. The luciferase mRNA constructs were synthesized in vitro in the forms shown on the left. The level of luciferase expression resulting from each mRNA is shown as a histogram directly to the right of the respective mRNA. The numerical value for each histogram is indicated immediately to the right of each histogram. Luciferase activity is expressed as light units/mg protein except for Oh43 endosperm, for which it is expressed as light units/100,000 cells, and for in vitro translation lysates, for which it is expressed as light units/2 μL lysate.

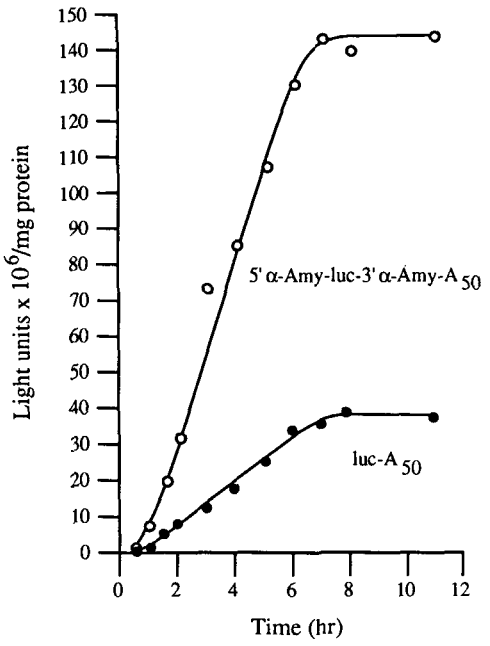

Figure 8. Kinetics analysis of the translation of the α-amylase (Amy)-*luc* chimeric and control mRNAs in transformed Oh43 aleurone protoplasts. Aliquots of protoplasts were taken at intervals following mRNA delivery, assayed for *luc* activity, and plotted as a function of time. The translational efficiency was determined from the slope of each line during the linear phase of translation.

that focus on either transcriptional or posttranscriptional regulatory events. Significant differences in regulation were observed between aleurone and endosperm cells when compared to suspension cells. *Adh1* promoter activity was low in aleurone and endosperm cells relative to the 35S promoter. No de novo synthesis of alcohol dehydrogenase was detected in scutellum tissue of maize seed germinated under aerobic conditions (Ho and Scandalios, 1975), which might also explain the low level of *Adh1* promoter activity relative to the 35S promoter in aleurone protoplasts. In contrast, *Adh1* promoter activity was high relative to the 35S promoter in BMS suspension cells. Suspension cells have been described as being in a constant state of flooding and, therefore, may be partially hypoxic (Howard et al., 1987). This may explain the high level of *Adh1* promoter activity relative to the 35S promoter in suspension cells. These results also suggest that *Adh1* promoter activity is not substantially induced in aleurone and endosperm as a result of protoplasting and subsequent incubation in medium.

The extent to which the *Adh1* intron enhanced reporter gene expression was dependent on the cell type. The intron effect was substantially greater in aleurone and endosperm cells than in suspension cells. Much of the previous work concerning the intron-mediated enhancement of gene expression was carried out in cultured cells (Callis et al., 1987; Buchman and Berg, 1988). The present data suggest that the previous studies may have underestimated the significance of the intron effect on gene expression. The observations concerning *Adh1* promoter activity and the intron effect in differentiated or developing cells versus cultured cells do not necessarily preclude the use of cultured cells. However, they do suggest that the cell state or type may influence the extent to which a general regulatory mechanism such as intron processing can affect gene expression.

In contrast to the observed differences in nuclear regulation, those cytoplasmic regulatory elements controlling translation functioned as well in aleurone and endosperm protoplasts as they had in cell-suspension protoplasts. We previously showed that in cultured plant and animal cells the basis for efficient translation is a synergistic interaction between the cap and poly(A) tail (Gallie, 1991). eIF-4F, eIF-4A, and eIF-4B, initiation factors involved in the recognition of the 5′ terminal cap structure and unwinding of any secondary structure present in the 5′ leader sequence also recognize and form a protein-RNA complex with poly(A) (Gallie and Tanguay, 1994). Initiation factor recognition may form the basis for the synergism observed between the 5′ cap structure and the poly(A) tail. If both regulatory elements are involved in translation initiation, this would explain why, in the absence of a cap, the addition of a poly(A) tail to *luc* mRNA does little to stimulate translation. It would also explain why the cap does not function well in poly(A)$^-$ mRNA.

The use of aleurone and endosperm protoplasts allowed us to investigate the tissue-specific regulation that the α-amylase UTRs exert on the posttranscriptional control of α-amylase gene expression. Because α-amylase mRNA represents 20% of the total mRNA present in the barley aleurone but α-amylase protein represents 60% of the synthesized protein, preferential translation of α-amylase mRNA has been suggested as an explanation for the difference (Higgins et al., 1982). We used the α-amylase UTRs from barley because of the reported potential for posttranscriptional regulation (Mozer, 1980; Higgins et al., 1982) and also because, until recently (Young et al., 1994), no maize α-amylase gene had been isolated. Our observation that the α-amylase UTRs increased translational efficiency in the aleurone of both barley (T. Close and D. Gallie, unpublished observations) and maize may explain the previously reported preferential translation of α-amylase in the barley aleurone. The observation that the α-amylase UTRs increase translational efficiency in maize endosperm protoplasts suggests that those factors mediating the regulation associated with the α-amylase UTRs may also be present in developing endosperm. Transient expression of α-amylase, in fact, has been observed in developing maize endosperm (Thevenot et al., 1992). Moreover, the regulation associated with the α-amylase UTRs appeared to be tissue specific because the α-amylase UTRs function in aleurone or endosperm protoplasts but not in maize or carrot cell cultures or in in vitro translation lysates. Because the α-amylase UTRs functioned not only in barley aleurone protoplasts but also in maize, the mechanism underlying the posttranscriptional regulation of α-amylase may be conserved between these two species. Comparison of the sequence of the barley (Chandler and Huiet, 1991) and maize 5′ and 3′ UTRs of α-amylase revealed a 7-base sequence present upstream of the initiation codon that was absolutely conserved in both genes. This sequence was present specifically in the high isoelectric point α-amylases of barley and maize and a rice α-amylase gene (O'Neill et al., 1990).

Whether this sequence is involved in increasing the translational efficiency of α-amylase mRNA in aleurone cells remains to be determined.

ACKNOWLEDGMENT

The authors would like to thank Dr. Virginia Walbot for the DNA plasmid constructs used in this study.

Received May 16, 1994; accepted July 25, 1994.
Copyright Clearance Center: 0032-0889/94/106/0929/11.

LITERATURE CITED

Belanger FC, Brodl MR, Ho TD (1986) Heat shock causes destabilization of specific mRNAs and destruction of endoplasmic reticulum in barley aleurone cells. Proc Natl Acad Sci USA **83:** 1354–1358

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brodl MR, Ho TD (1991) Heat shock causes selective destabilization of secretory protein mRNAs in barley aleurone cells. Plant Physiol **96:** 1048–1052

Brown PH, Brodl MR (1988) Hormonal and heat stress regulation of protein synthesis in the aleurone layers of barley seeds. Bioessays **8:** 199–202

Buchman AR, Berg P (1988) Comparison of intron-dependent and intron-independent gene expression. Mol Cell Biol **8:** 4395–4405

Callis J, Fromm M, Walbot V (1987) Introns increase gene expression in cultured maize cells. Genes Dev **1:** 1183–1200

Carrington JC, Freed DD (1990) Cap-independent enhancement of translation by a plant polyvirus 5′ nontranslated region. J Virol **64:** 1590–1597

Chandler PM, Huiet L (1991) Primer extension studies on α-amylase mRNAs in barley aleurone. I. Characterization and quantification of the transcripts. Plant Mol Biol **16:** 627–635

Chu L-JC, Shannon JC (1975) In vitro cultures of maize endosperm—a model system for studying *in vivo* starch biosynthesis. Crop Sci **15:** 814–819

Danthinne X, Seurinck J, Meulewaeter F, Van Montagu M, Cornelissen M (1993) The 3′ untranslated region of satellite tobacco necrosis virus RNA stimulates translation in vitro. Mol Cell Biol **13:** 3340–3349

Diaz I, Carbonero P (1992) Isolation of protoplasts from developing barley endosperm: a tool for transient expression studies. Plant Cell Rep **10:** 595–598

Diaz I, Royo J, Carbonero P (1993) The promoter of barley trypsin-inhibitor BTI-CMe discriminates between wheat and barley endosperm protoplasts in transient expression assays. Plant Cell Rep **12:** 698–701

Dickey LF, Gallo-Meagher M, Thompson WF (1992) Light regulatory sequences are located within the 5′ portion of the *Fed-1* message sequence. EMBO J **11:** 2311–2317

Gallie DR (1991) The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev **5:** 2108–2116

Gallie DR, Feder JN, Schimke RT, Walbot V (1991) Post-transcriptional regulation in higher eukaryotes: the role of the reporter gene in controlling expression. Mol Gen Genet **228:** 258–264

Gallie DR, Lucas WJ, Walbot V (1989) Visualizing mRNA expression in plant protoplasts: factors influencing efficient mRNA uptake and translation. Plant Cell **1:** 301–311

Gallie DR, Sleat DE, Watts JW, Turner PC, Wilson TMA (1987) The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucleic Acids Res **15:** 3257–3273

Gallie DR, Tanguay RL (1994) Poly(A) binds to initiation factors and increase cap-dependent translation *in vitro*. J Biol Chem **269:** 17166–17173

Gallie DR, Walbot V (1992) Identification of the motifs within the tobacco mosaic virus 5′-leader responsible for enhancing translation. Nucleic Acids Res **20:** 4631–4638

Gerlach WL, Pryor AJ, Dennis ES, Ferl RJ, Sachs MM, Peacock WJ (1982) cDNA cloning and induction of the alcohol dehydrogenase gene (*Adh1*) of maize. Proc Natl Acad Sci USA **79:** 2981–2985

Goff SA, Klein TM, Roth BA, Fromm ME, Cone KC, Radicella JP, Chandler VL (1990) Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulatory genes into maize tissues. EMBO J **9:** 2517–2522

Gopalakrishnan B, Sonthayanon B, Rahmatullah R, Muthukrishnan S (1991) Barley aleurone layer cell protoplasts as a transient expression system. Plant Mol Biol **16:** 463–467

Gubler F, Jacobsen JV (1992) Gibberellic-responsive elements in the promoter of a barley high-pI α-amylase gene. Plant Cell **4:** 1435–1441

Hamer DH, Smith KD, Boyer SH, Leder P (1979) SV40 recombinants carrying rabbit β-globin gene coding sequences. Cell **17:** 725–735

Higgins TJV, Jacobsen JV, Zwar JA (1982) Gibberellic acid and abscisic acid modulate protein synthesis and mRNA levels in barley aleurone layers. Plant Mol Biol **1:** 191–215

Hillmer S, Bush DS, Robinson DG, Zingen-Sell I, Jones RL (1990) Barley aleurone protoplasts are structurally and functionally similar to the walled cells of aleurone layers. Eur J Cell Biol **52:** 169–173

Ho TD (1976) On the mechanism of hormone controlled enzyme formation in barley aleurone layers. PhD thesis. Michigan State University, East Lansing, MI

Ho TD, Nolan RC, Lin L, Brodl MR, Brown PH (1987) Regulation of gene expression in barley aleurone layers. *In* JE Fox, M Jacobs, eds, Molecular Biology of Plant Growth Control. Alan R. Liss, New York, pp 35–49

Ho TD, Scandalios JG (1975) Regulation of alcohol dehydrogenases in maize scutellum during germination. Plant Physiol **56:** 56–59

Howard EA, Walker JC, Dennis ES, Peacock WJ (1987) Regulated expression of an alcohol dehydrogenase 1 chimeric gene introduced into maize protoplasts. Planta **170:** 535–540

Huang MTF, Gorman CM (1990) Intervening sequences increase efficiency of RNA 3′ processing and accumulation of cytoplasmic RNA. Nucleic Acids Res **18:** 937–947

Huttly AK, Baulcombe DC (1989) A wheat *α-Amy2* promoter is regulated by gibberellin in transformed oat aleurone protoplasts. EMBO J **8:** 1907–1913

Jacobsen JV, Beach LR (1985) Control of transcription of α-amylase and rRNA genes in barley aleurone protoplasts by gibberellin and abscisic acid. Nature **316:** 275–277

Jacobsen JV, Chandler PM (1987) Gibberellin and abscisic acid in germinating cereals. *In* RP Pharis, ed, Plant Hormones and Their Role in Plant Growth and Development. Martinus Nijhoff, Dordrecht, The Netherlands, pp 164–193

Jacobsen JV, Close TJ (1991) Control of transient expression of chimaeric genes by gibberellic acid and abscisic acid in protoplasts prepared from mature barley aleurone layers. Plant Mol Biol **16:** 713–724

Jacobsen JV, Zwar JA, Chandler PM (1985) Gibberellic-acid-responsive protoplasts from mature aleurone of Himalaya barley. Planta **163:** 430–438

Keeling PL, Baird S, Tyson RH (1989) Isolation and properties of protoplasts from endosperm of developing wheat grain. Plant Sci **65:** 55–62

Klein TM, Roth BA, Fromm ME (1989) Regulation of anthocyanin biosynthetic genes introduced into intact maize tissues by microprojectiles. Proc Natl Acad Sci USA **86:** 6681–6685

Kunkel TA, Roberts JD, Zakour RA (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol **154:** 367–382

Lanahan MB, Ho TD, Rogers SW, Rogers JC (1992) A gibberellin response complex in cereal α-amylase gene promoters. Plant Cell **4:** 203–211

Leathers VR, Tanguay RL, Kobayashi M, Gallie DR (1993) A phylogenetically conserved sequence within viral 3′ untranslated RNA pseudoknots regulates translation. Mol Cell Biol **13:** 5331–5347

Lee BT, Murdoch K, Topping J, Jones MGK, Kreis M (1991) Transient expression of foreign genes introduced into barley endosperm protoplasts by PEG-mediated transfer or into intact endosperm tissue by microprojectile bombardment. Plant Sci **78:** 237–246

Ludwig SR, Bowen B, Beach L, Wessler SR (1990) A regulatory gene as a novel visible marker for maize transformation. Science **247:** 449–450

Luehrsen KR, de Wet JR, Walbot V (1992) Transient expression analysis in plants using firefly luciferase reporter gene. Methods Enzymol **216:** 397–414

Lyznik LA, Tsai CY (1989) Protein synthesis in endosperm cell cultures of maize (*Zea mays* L.). Plant Sci **63:** 105–114

Matzke AJM, Stöger EM, Schernthaner JP, Matzke MA (1990) Deletion analysis of a zein gene promoter in transgenic tobacco plants. Plant Mol Biol **14:** 323–332

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K, Green MR (1984) Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res **12:** 7035–7056

Miernyk JA, Sturch SM (1991) Starvation for reduced nitrogen stimulates the secretion of proteases by maize endosperm cultures. J Plant Physiol **138:** 211–215

Mozer TJ (1980) Control of protein synthesis in barley aleurone layers by the plant hormones gibberellic acid and abscisic acid. Cell **20:** 479–485

O'Neill SD, Kumagai MH, Majumdar A, Huang N, Sutliff TD, Rodriguez RL (1990) The α-amylase genes in *Oryza sativa*: characterization of cDNA clones and mRNA expression during seed germination. Mol Gen Genet **221:** 235–244

Racchi ML (1985) Effect of the genes *B* and *Pl* on anthocyanin synthesis in maize endosperm culture. Plant Cell Rep **4:** 184–187

Radicella JP, Brown D, Tolar LA, Chandler VL (1992) Allelic diversity of the maize *B* regulatory gene: different leader and promoter sequences of two *B* alleles determine distinct tissue specificities of anthocyanin production. Genes Dev **6:** 2152–2164

Roth BA, Goff SA, Klein TM, Fromm ME (1991) *C1*- and *R*-dependent expression of the maize *Bz1* gene requires sequences with homology to mammalian *myb* and *myc* binding sites. Plant Cell **3:** 317–325

Sachs MM, Freeling M, Okimoto R (1980) The anaerobic proteins of maize. Cell **20:** 761–767

Sadasivam S, Gallie DR (1994) Isolation and transformation of rice aleurone protoplasts. Plant Cell Rep **13:** 394–396

Salmenkallio M, Hannus R, Teeri TH, Kauppinen V (1990) Regulation of α-amylase promoter by gibberellic acid and abscisic acid in barley protoplasts transformed by electroporation. Plant Cell Rep **9:** 352–355

Saravitz CH, Boyer CD (1987) Starch characteristics in cultures of normal and mutant maize endosperm. Theor Appl Genet **73:** 489–495

Schwall M, Feix G (1988) Zein promoter activity in transiently transformed protoplasts from maize. Plant Sci **56:** 161–166

Selmar D, Frehner M, Conn EE (1989) Purification and properties of endosperm protoplasts of *Hevea brasiliensis* L. J Plant Physiol **135:** 105–109

Shimamoto K, Ackermann M, Dierks-Ventling C (1983) Expression of zein in long term endosperm cultures of maize. Plant Physiol **73:** 915–920

Skriver K, Olsen FL, Rogers JC, Mundy J (1991) Cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid. Proc Natl Acad Sci USA **88:** 7266–7270

Thevenot C, Lauriere C, Mayer C, Simond-Cote E, Daussant J (1992) α-Amylase changes during development and germination of maize kernels. J Plant Physiol **140:** 61–65

Thompson GA, Boston RS, Lyznik LA, Hodges TK, Larkins BA (1990) Analysis of promoter activity from an α-zein gene 5′ flanking sequence in transient expression assays. Plant Mol Biol **15:** 755–764

Timmer RT, Benkowski LA, Schodin D, Lax SR, Metz AM, Ravel JM, Browning KS (1993) The 5′ and 3′ untranslated regions of satellite tobacco necrosis virus RNA affect translational efficiency and dependence on a 5′ cap structure. J Biol Chem **268:** 9504–9510

Unger E, Parsons R, Schmidt RJ, Bowen B, Roth BA (1993) Dominant negative mutants of opaque2 suppress transactivation of a 22-kD zein promoter by opaque2 in maize endosperm cells. Plant Cell **5:** 831–841

Walker JC, Howard EA, Dennis ES, Peacock WJ (1987) DNA sequences required for anaerobic expression of the maize alcohol dehydrogenase 1 gene. Proc Natl Acad Sci USA **84:** 6624–6628

Yisraeli JK, Melton DA (1989) Synthesis of long, capped transcripts *in vitro* by SP6 and T7 RNA polymerases. Methods Enzymol **180:** 42–50

Young TE, DeMason D, Close TJ (1994) Cloning of an α-amylase cDNA from aleurone tissue of germinating maize seed. Plant Physiol **105:** 759–760

Zhang S, Mehdy MC (1994) Binding of a 50-kD protein to a U-rich sequence in an mRNA encoding a proline-rich protein that is destabilized by fungal elicitor. Plant Cell **6:** 135–145