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

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

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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. Intramediated 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 GA3 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 unidirectional phosphate glucose pyrophosphorylase activity and incorpo-

Abbreviations: Adh1, alcohol dehydrogenase 1; BMS, Black Mexican Sweet; BV, bromo mosaic virus; CHO, Chinese hamster ovary; DAP, days after pollination; elF, 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-A50 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-Amly-luc-A50 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 Ncol and BgIII 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 Ncol/BgIII fragment. Restriction sites for Stul and BamHI were introduced at the transcription initiation and the polyadenylation sites, respectively, of the α-amylase gene (Chandler and Huiet, 1991), and the Stul/BamHI fragment was introduced into the Stul/BamHI sites of the pT7-A50 vector. A poly(A) tract, present in pT7-Amly-luc-A50, allows the in vitro synthesis of poly(A)+ mRNA following linearization of the plasmid with Drai, which cuts 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 MgCl2, 2 mM spermidine, 100 μg/mL BSA, 0.5 mM each of ATP, CTP, and UTP, plus 160 μM GTP, 1 mM 5'GpppG, 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 so 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 CaCl2, 100 mM Glc in Gamborg's medium, pH 5.4) with 0.6 mM 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 M1035 (Genencor International, Rochester, NY), 0.25% cellulase (Worthington Biochemical, Freehold, NJ), 50 units/mL nystatin, 90 μg/mL cefotaxime, and 0.6 mM 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 mM mannitol. The protoplasts were harvested by centrifugation at 50g for 5 min and resuspended in maize MaMagMes solution (15 mM MgCl2, 0.6 mM 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 mM Ca(NO3)2, 4H2O, 0.6 mM mannitol] was added, and the solution was gently inverted until completely mixed. Following a 10-min incubation, 0.6 mL of 0.2 mM CaCl2 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 50g 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 mM 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 25g for 5 min and resuspended in endosperm protoplast electroporation buffer (4 mM CaCl2, 10 mM NaCl, 130 mM KCl, 1% BSA, 2 mM 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 130 µF and 225 V. One milliliter of endosperm incubation medium (1% BSA, 50 units/mL nystatin, 90 µg/mL cefotaxime, and 2 mM 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 CaCl2, 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 CaCl2, and 0.2 mM mannitol), and resuspended in electroporation buffer to a final concentration of 1.0 × 105 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 × 106) 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 25g were sonicated for 5 s in 100 mM Tricine, pH 7.8, 2 mM DTT, 2 mM L-diaminocyclohexane-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 (MgCO3)2Mg(OH)2, 5H2O, 2.67 mM MgSO4, 0.1 mM EDTA, 33.3 mM DTT, 270 µM CoA, and 500 µM ATP] (Promega Biotech), and the reaction was initiated with the injection of 100 µL of 0.5 µM 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 MgCl2, 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-
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) or Oh43 seed, BMS cell-suspension protoplasts isolated from 11-DAP tissue, or BMS cell-suspension protoplasts transformed using $1 \times 10^8$ to $2 \times 10^8$ 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 rate of 3' end processing and nucleocy-
Transformation of Maize Aleurone and Endosperm Protoplasts

BMS Aleurone

BMS cell suspension

Oh43 Aleurone

Oh43 Endosperm

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 Adhl 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 Adhl 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 Adhl intron substantially increased expression in aleurone and endosperm cells (Fig. 2). Although the Adhl 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 Adhl 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 synergy 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

![Image 4](https://www.plantphysiol.org)
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)$^-$ 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 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)$^-$ 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...
The presence of the \( \alpha \)-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 \( \alpha \)-amylase UTRs.

\( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-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 \( \alpha \)-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...
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 Tangoay, 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 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.

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