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Both 5′ and 3′ Sequences of Maize *adh1* mRNA Are Required for Enhanced Translation under Low-Oxygen Conditions[1]

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Alcohol dehydrogenase-1 (ADH1) synthesis in O_2-deprived roots of maize (*Zea mays* L.) results from induced transcription and selective translation of ADH1 mRNA. The effect of ADH1 mRNA sequences on message stability and translation was studied in protoplasts of the maize cell line P3377. 5′ capped and 3′ polyadenylated mRNA constructs containing the firefly gene (*luc*) for luciferase (LUC) or the *Escherichia coli* gene (*uidA*) for β-glucuronidase (GUS) coding region were synthesized in vitro and electroporated into protoplasts that were cultured at 40 or 5% O_2. A LUC mRNA with a 17-nucleotide polylinker 5′ untranslated region (UTR) was expressed 10-fold higher under aerobic conditions than under hypoxic conditions. Expression of five chimeric ADH1-GUS mRNAs was measured relative to this LUC mRNA. An mRNA containing the 5′-UTR and the first 18 codons of *adh1* in a translational fusion with the GUS coding region and followed by the 3′-UTR of *adh1* was expressed 57-fold higher at 5% O_2. Progressive deletion of *adh1* 5′-UTR and coding sequences reduced expression of the GUS-mRNA at 5% O_2, but had little impact on expression of 40% O_2. Enhancement of expression in hypoxic protoplasts conferred by the *adh1* 5′-UTR and the first 26 codons decreased more than 3-fold when the *adh1* 3′-UTR was removed. In addition, the *adh1* 3′-UTR slightly inhibited expression in aerobic protoplasts. The physical half-lives of the GUS and LUC mRNAs were similar under both anaerobic and hypoxic conditions, indicating that expression levels were largely independent of mRNA stability. Thus, both *adh1* 5′ and 3′ mRNA sequences are required for enhanced translation in protoplasts under O_2 deprivation.

Roots of maize (*Zea mays* L.) seedlings respond to O_2 deprivation by alteration of gene expression at transcriptional and posttranscriptional levels. Genes encoding anaerobic polypeptides, including certain enzymes required for glycolysis and ethanolic fermentation, are transcribed at elevated levels and efficiently translated in O_2-deprived roots (Sachs et al., 1980; Sachs, 1994; Fennoy and Bailey-Serres, 1995). In O_2-deprived roots many genes encoding normal cellular proteins are constitutively transcribed but their mRNAs are poorly loaded with ribosomes (Fennoy and Bailey-Serres, 1995; S.L. Fennoy and J. Bailey-Serres, unpublished data). The selective translation of newly synthesized mRNAs encoding proteins that allow for ATP and NAD^+ production under anaerobiosis over translation of mRNAs encoding normal cellular proteins may provide a mechanism for survival and facilitate recovery once the stress is alleviated.

adh1, the maize gene encoding ADH1, has been extensively characterized as a model anaerobic polypeptide gene. The promoter region of *adh1* contains specific *cis*-acting sequences sufficient to confer high levels of transcription in O_2-deprived roots. A *cis*-acting sequence, the anaerobic regulatory element, was identified and characterized by promoter deletion studies in a transient expression system (Walker et al., 1987; Olive et al., 1990). Analysis of chimeric gene constructs indicated that the *adh1* 5′-UTR may also play a role in the stimulation of expression in O_2-deprived protoplasts (Walker et al., 1987). A DNA construct containing the *adh1* 5′ flanking and 5′-UTR region (−1094 to +106), fused to the bacterial chloramphenicol acetyltransferase gene-coding sequence and nopaline synthetase 3′ terminator sequence, was transcriptionally induced by O_2 deprivation in maize protoplasts (Howard et al., 1987). However, when the *adh1* 5′-UTR was replaced with that of the cauliflower mosaic virus 35S gene, chloramphenicol acetyltransferase activity was still inducible but was reduced to approximately 30% of the level detected with the construct containing both the *adh1* promoter and 5′-UTR (Walker et al., 1987). Whether the reduction in expression was due to transcriptional or posttranscriptional regulation was not tested. This observation, as well as the demonstration of efficient initiation of translation of ADH1 mRNA in response to O_2 deprivation (Fennoy and Bailey-Serres, 1995), supports the hypothesis that specific *adh1* mRNA sequences mediate translational efficiency in response to O_2 deprivation.

Regulation of plant gene expression at the level of translation has been documented (Gallie, 1993, 1996). A number of mRNA features play a role in translational control in plants. For example, a 5′-^{7}mGppp cap is required for efficient translation of native mRNAs (Callis et al., 1987; Gallie et al., 1991). The 5′-UTR of a number of plant viral mRNAs

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Abbreviations: ADH1, alcohol dehydrogenase 1; eIF, eukaryotic initiation factor; HSP, heat-shock protein; LUC, luciferase; nt, nucleotide; 6-PGD, phosphogluconate dehydrogenase; UTR, untranslated region.

contain sequences that enhance translation in host and nonhost species (Sleat and Wilson, 1992; Gallie, 1996). In most cases, translational enhancers do not affect mRNA half-life, but most likely influence the rate of initiation of translation. There is evidence for a translational enhancer in a tobacco plasma membrane ATPase (Michelet et al., 1994) and a barley α-amylase gene (Gallie and Young, 1994). The presence of a 5′ cap, the length and secondary structure of the 5′-UTR, as well as the nt sequence surrounding the start codon impact the initiation of translation (Kozak, 1994). Whether features of the coding sequence such as bias in synonymous codon usage or nt composition affect the rate of ribosome translocation in plants is unknown (Fennoy and Bailey-Serres, 1993), but the choice of a reporter gene does influence the effect of the presence or absence of a 5′ cap and/or 3′ poly(A) tail on translational efficiency in protoplasts (Gallie et al., 1991). Regulation of translation involving the 5′ cap and the 3′ poly(A) tail, most likely via protein-mRNA interactions, appears to be essential for efficient translation of plant mRNAs (Gallie, 1996). In tobacco mosaic virus mRNA these interactions can be replaced by the 5′ translational enhancer sequence ω and the highly structured, nonpolyadenylated 3′-UTR (Leathers et al., 1993). Thus, both 5′ and 3′ mRNA sequences and interactions requiring these sequences may be important in the regulation of protein synthesis in plants. Studies indicate that 5′ and 3′ mRNA interactions are also important in the regulation of translation in yeast (Jacobson, 1996).

Modulation of mRNA translation is a common feature of stress responses that limit protein synthesis. Stress may impose a requirement for a competitive advantage in the initiation of translation that is not required under nonstress conditions. For example, in globular embryos of carrots, heat shock induces selective initiation of translation of mRNAs encoding HSPs (Apuya and Zimmerman, 1992). Many non-HSP mRNAs are sequestered on ribonucleoprotein particles associated with the cytoskeleton, whereas HSP mRNAs are actively translated (Nover et al., 1989). The efficient translation of a chimeric mRNA construct in heat-shocked maize and tobacco protoplasts was conferred by the 5′-UTR of the *hsp70* gene of maize (Pitto et al., 1992). Gallie et al. (1995) presented evidence indicating that translational repression of normal cellular mRNAs in heat-shocked cells may involve the disruption of interactions between the 5′ cap and the 3′ poly(A) tail. How the leader sequence of *hsp70* mRNA allows it to escape translational repression has not been elucidated. Mutational and computer-assisted analyses of 5′-UTRs of the HSP genes in eukaryotes failed to reveal any distinct sequence or structural features (Joshi and Nguyen, 1995).

The selective translation of mRNAs under stress conditions such as heat shock or O_2 deprivation could be modulated by the translational machinery. In the cell-free translation system derived from wheat germ, differential mRNA translation results from competition between mRNAs for limited initiation factors (Browning et al., 1988, 1990; Fletcher et al., 1990; Timmer et al., 1993a, 1993b). Regulation of translation in hypoxic roots could be mediated by the rapid phosphorylation of 50% of the cellular pool of the eukaryotic initiation factor 4A (Webster et al., 1991). Modification of other initiation factors might also occur in response to stress. The modulation of ribosomal protein phosphorylation is thought to play a role in the regulation of mRNA translation in eukaryotes. A 31-kD protein, S6, is dramatically underphosphorylated in ribosomes of maize roots deprived of O_2 (Bailey-Serres and Freeling, 1990). In animal cells, mRNAs with a 5′ polypyrimidine tract appear to be regulated by the phosphorylation status of S6 (Jefferies and Thomas, 1996). It remains to be established whether phosphorylation of eukaryotic initiation factor 4A and/or the dephosphorylation of S6 is important in the selective mRNA translation in O_2-deprived roots.

To further investigate the role of mRNA sequence determinants in the posttranscriptional regulation of gene expression in O_2-deprived maize, we examined the effect of *adh1* mRNA sequences on reporter enzyme activity in a transient expression system. A reproducible response to O_2 deprivation was obtained with protoplasts from the maize cell line P3377. Synthetic mRNA constructs with a 5′ cap and 3′ poly(A) tail, 5′ and 3′ sequences of *adh1* mRNA, and the coding sequence of a reporter gene were examined in electroporated protoplasts cultured under aerobic (40 or 21% O_2) or low-O_2 (5% O_2) conditions.

MATERIALS AND METHODS

The maize (*Zea mays* L.) suspension-cell culture line P3377 was obtained from A.-L. Paul and R. Ferl (University of Florida, Gainesville). This culture was initiated from immature embryos of the hybrid line P3377 (Pioneer Hi-Bred, Johnston, IA) by J. Widholm (University of Illinois, Urbana-Champaign) (Duncan et al., 1985). The cell culture was maintained in Murashige and Skoog minimal organic medium (Sigma), which also contained 30 g/L Suc, 1 mg/L *myo*-inositol, 1.3 mg/L L-Asn, 0.13 mg/L niacin, 2.5 μg/L pyridoxine, 2.5 μg/L D-pantothenic acid, 2 mg/L 2,4-D, pH 5.8. Fifty-milliliter cell cultures were grown in 250-mL flasks shaken at 180 rpm at 28 to 30°C. Every 3.5 d, 20 mL of cell suspension was subcultured into 30 mL of fresh medium. Two weeks prior to protoplasting the cells were sieved through a 450-μm wire mesh screen to remove large clumps. Cells were subcultured 48 h prior to protoplasting.

Enzymes

Enzymes for DNA and RNA manipulations were purchased from Promega-Fisher, United States Biochemical-Amersham, or GIBCO-BRL. Enzymes for protoplasting were Pectolyase Y23 (Seishin Pharmaceutical, Tokyo, Japan) and cellulysin (Calbiochem).

Plasmid Construction

DNA manipulations were performed using standard techniques (Sambrook et al., 1989). The 5′ and 3′ sequences of the mRNA constructs were confirmed by DNA sequencing (Sequenase Kit, United States Biochemical) (Fig. 1).

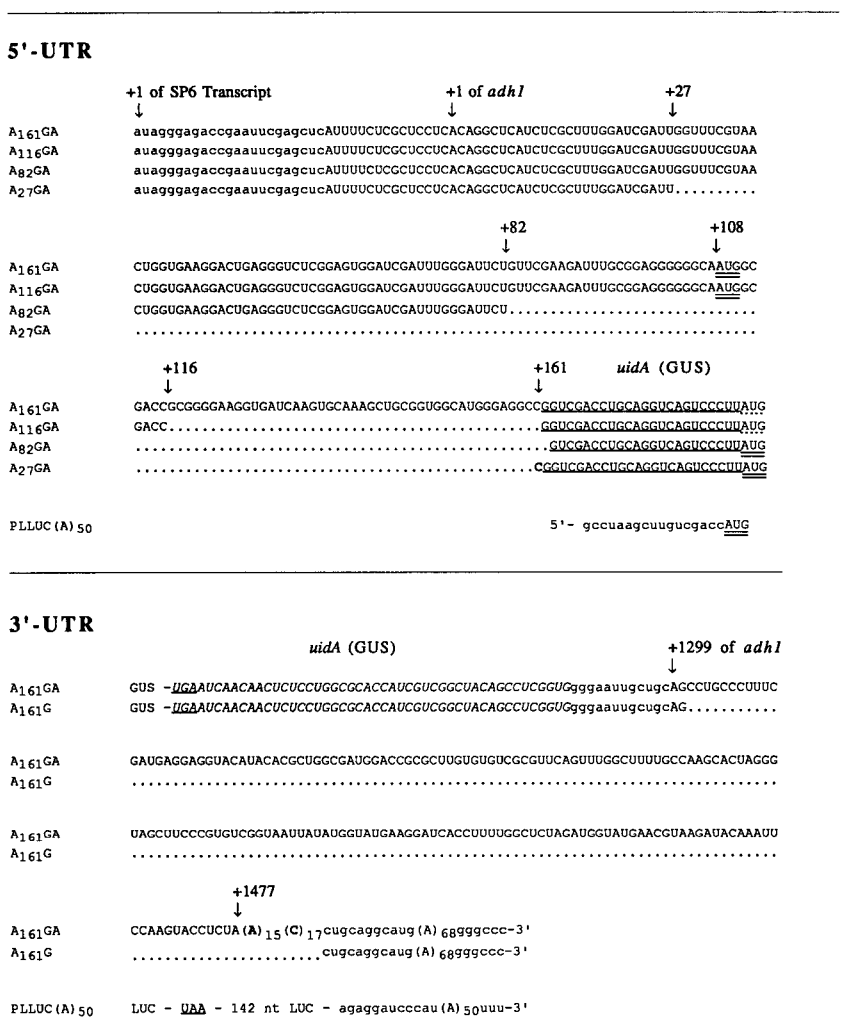

Figure 1. Sequence of 5'- and 3'-UTRs of mRNA constructs. ADH1 mRNA constructs differ in 5' sequences but have the same 3'-UTR, except for $A_{161}G$, which does not contain *adh1* 3'-UTR sequences. ADH1 mRNA sequences are in uppercase letters; the initiator Met codon (AUG) is double-underlined; the polylinker sequence 5' to the GUS coding region is single-underlined; the GUS mRNA sequence is italicized; deletions are represented by dots; sequence variations due to cloning are in boldface; +1 is the first nt of *adh1* mRNA in roots (Kloeckener-Gruissem et al., 1992); and the AUG of *adh1* is at position +108 bp. *adh1* 3'-UTR numbering is based on the cDNA clone encoding *Adh1+CroF* (GenBank accession no. X02913). PLLUC$(A)_{50}$ was described elsewhere (Gallie et al., 1991).

$pA_{161}GA$

Adh1+CroF cDNA pZmL793 (Dennis et al., 1984) was subcloned by partial digestion with *Pst*I into the *Pst*I site of the vector pGEM3 (Promega) to obtain pKD2. This cDNA begins at +31 bp, relative to the transcription start site of *adh1*. To compensate for the missing portion of the 5'-UTR, a *Pst*I-*Sac*II fragment (−140 to +114 bp) of an *Adh1+S* genomic clone (pB428, see Bennetzen et al., 1984) was exchanged with the *Pst*I-*Sac*II fragment of pKD2 from +31 to +114 bp. All but 15 bp of the 5' nontranscribed region of *adh1* was removed by excision of a *Sac*I (polylinker)-*Ban*II (−15 bp of *adh1*) fragment to obtain pKD5. Terminal deoxynucleotidyl transferase was used to add adenylate residues to the 3' end of *adh1* (beginning at the *Sph*I site in the polylinker) and to add thymidylate residues to the complementary strand. A resulting plasmid (pKD14) was partially digested with *Sal*I and *Nhe*I to remove a portion of the coding region of *adh1* (+782 to +1304 bp), which was replaced by a *Sal*I-*Bgl*II fragment of the plasmid pAM15 (obtained from M. Fromm, Monsanto, St. Louis, MO) containing the *uidA* gene of *Escherichia coli*. This plasmid (pKD16) was partially digested with *Nae*I and *Sal*I to excise +161 to +782 bp of *adh1*, which was then religated by the addition of a *Sal*I linker at the *Nae*I terminus to place the GUS coding region in frame with the first 18 codons of *adh1* and 8 codons from the polylinker sequence. A poly(A/T) tract of 68 nt was confirmed by DNA sequencing.

$pA_{116}GA$

To place the GUS coding region in-frame with the first three amino acids of *adh1*, $A_{161}GA$ was digested with *Sac*II (+116 bp of *adh1*), the termini were end-filled with Klenow polymerase, *Sal*I linkers were added, and the new *Sal*I site was ligated to the *Sal*I site that immediately precedes the *uidA* coding sequence.

$pA_{82}GA$

The internal sequences of pKD16 were removed by digestion with *Asu*II (+82 bp of *adh1*) and *Bgl*II (just proximal to the 3'-UTR of *adh1*) and exchanged with the *uidA* gene purified from pAM15 (by cleaving with *Acc*I and *Bgl*II).

$pA_{27}GA$

To construct a deletion within the 5′-UTR and coding region of *adh1*, $pA_{161}GA$ was digested with *Sac*II (+116 bp of *adh1*), treated with *Bal*31 nuclease, end-filled using Klenow polymerase and deoxyribonucleotide triphosphates, and recircularized by ligation. A clone with a deletion from +29 to +161 bp of *adh1* was identified by DNA sequence analysis.

$pA_{161}G$

To remove all of the *adh1* 3′-UTR sequence, $pA_{161}GA$ was partially digested with *Pst*I to release the 178-bp fragment of *adh1* origin (position +1299 to +1477 of *Adh1+CroF*). $pA_{161}G$ was formed by religation.

Control Plasmid

pPLLUC(A)$_{50}$ (Gallie et al., 1991) was kindly provided by D. Gallie (University of California, Riverside).

In Vitro Transcription

Plasmid DNA was purified twice over cesium chloride/ethidium bromide gradients. DNAs were linearized immediately downstream of the poly(A) tract with the appropriate restriction enzyme [*Bam*HI for ADH1-GUS constructs; *Dra*I for PLLUC(A)$_{50}$], phenol-extracted, ethanol-precipitated, and resuspended to a DNA concentration of 0.5 μg/mL. In vitro transcription was carried out in 100 μL using 2 μg of linearized template, 1× transcription buffer (Promega), 10 mM DTT, 0.5 mM each of ATP, CTP, and UTP, 0.05 mM GTP, 0.5 mM $m^7G(5')pppG(3')$ (Pharmacia), 80 units of RNasin (Promega), and 40 units of SP6 or T7 RNA polymerase (Promega) at 37°C for 1 h. An additional 20 units of RNA polymerase and 5 μL of 1 mM GTP were added and the reaction continued at 37°C for 1 h and terminated by the addition of 2.5 μL of 0.5 M EDTA and 100 μL of TE8 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). RNA was purified by phenol extraction and ammonium acetate/isopropanol precipitation, resuspended in distilled water, quantified spectrophotometrically, and frozen in 40-μg aliquots. The quality of the RNA was checked by electrophoresis in a 7% (v/v) formaldehyde-1.3% (w/v) agarose gel and staining with 0.2% (w/v) methylene blue. Typically, 1 μg of template yielded 10 μg of mRNA. More than 80% of the mRNA was capped, as determined by radioactively labeling the mRNA and measuring the percentage of mRNA with a 1-nt increase in size on polyacrylamide gels.

Protoplast Isolation and Electroporation

Protoplasts were prepared as described by Fromm et al. (1987) with the following modifications: Protoplast isolation enzyme solution contained 0.75 g/100 mL cellulase, 0.2 g/100 mL pectinase, 0.5 g/100 mL BSA, and 50 μL/100 mL β-mercaptoethanol. Digestion of cells was for 5 h. Protoplasts were separated from cell clumps by filtration through a 150-μm wire mesh screen, recovered by centrifugation, washed, and resuspended in ice-cold electroporation buffer (10 mM Hepes, pH 7.2, 130 mM KCl, 10 mM NaCl, 0.2 M mannitol, and 4 mM $CaCl_2$) at a density of 2×10^6 live protoplasts/mL as described previously (Fromm et al., 1987). Protoplasts were >80% viable as determined by staining with 0.12% (w/v) Evan's blue and examination with an inverted light microscope. Protoplasts were placed in 1-mL aliquots into a 24-well dish on ice. Five micrograms each of a GUS and LUC mRNA construct were added and cells were immediately electroporated at 275 V, 490 μF, for 10 ms (Progenetor 2 electroporation system, Hoefer Scientific, Richmond, CA). Five micrograms of a 2.5-kb transcript is approximately 3.8×10^{12} molecules of mRNA. Each electroporation was performed in triplicate. Mock (no input RNA) electroporations were performed and used to calculate background fluorescence, which was subtracted from the experimental samples. Post-electroporation incubation and the growth medium were as described previously (Fromm et al., 1987), except that cells were cultured in 60-mm Petri dishes placed into sealed chambers and aerated with air, 5% O_2/95% N_2, or 40% O_2/60% N_2 at room temperature for 18 h in the dark and under different conditions. Following the incubation protoplasts were collected by centrifugation as described previously (Fromm et al., 1987).

Determination of Dissolved O_2 Concentration

The dissolved O_2 concentration in the protoplast growth medium of a mock (no nucleic acid) protoplast electroporation was measured with a Clark electrode (model 97–08–00, Orion, Cambridge, MA) and meter (model 611, Orion). Concentrations were calculated using the standard of 272 μM O_2 in water sparged with air at 22°C. Additional standards included growth medium sparged with Ar/0.001% O_2, 5% O_2/95% N_2, or 40% O_2/60% N_2.

Assay of Enzyme Activity and Determination of Protein Concentration

GUS Assay

One-half of the cells from each co-electroporation were sonicated (Microson model XL2005 sonicator, Heat Systems, Farmingdale, NY) on ice for 5 s in 0.4 mL of buffer (200 mM Trizma-HCl, pH 7.0, or 50 mM $NaPO_4$, pH 7, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.01% [w/v] SDS, and 0.1% [v/v] Triton X-100). Cell debris was removed by centrifugation for 3 min at 16,000*g* at 4°C. The supernatant was transferred to a clean tube and kept on ice for up to 30 min before the enzyme assay. To a 400-μL aliquot of supernatant, 40 μL of 1 mM 4-methylumbelliferyl-β-D-glucuronide (Sigma) was added as substrate. An aliquot of the reaction was diluted in 0.2 M Na_2CO_3 at time 0 and every 15 min for 60 min at 37°C, and UV fluorescence was measured at a 365-nm excitation and a 455-nm emission (TKO Mini Fluorometer, Hoefer) (Jefferson, 1987). GUS specific activity is expressed as nmol methylumbelliferone produced min^{-1} mg^{-1} protein. Methylumbelliferone in 0.2 M Na_2CO_3 was used as the fluorescence standard. Protein concentration in a 50-μL aliquot of the cell extract was

determined using a protein concentration reagent (United States Biochemical-Amersham) with BSA as the standard. Nearly equal amounts of protein were present in each enzyme assay.

LUC Assays

One-half of the cells from each co-electroporation were sonicated on ice for 5 s in 0.4 mL of luciferase assay buffer (200 mM Tricine-NaOH, pH 7.8, or 50 mM $NaPO_4$, pH 7.8, 1.07 mM $[MgCO_3]_4Mg[OH]_2$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, and 33.3 mM DTT) and centrifuged as described for assay of GUS activity. A 100-μL aliquot of extract was transferred to a cuvette, 100 μL of reagent mix (270 μM enzyme CoA [Sigma], 470 μM luciferin [Analytical Luminescence Laboratories, San Diego, CA], 530 μM ATP [Phamacia]) was added and luminescence was immediately measured using a luminometer (Lumat LB 9501, Berthold Systems, Pittsburgh, PA). LUC specific activity is expressed as relative light units/mg protein. Protein concentration was determined as for GUS assays.

Calculation of Relative Expression

mRNA electroporations were performed in triplicate. GUS and LUC activities for each experimental sample were calculated after subtraction of background fluorescence or luminescence, measured in mock (no RNA) electroporation samples. To calculate the expression of the test GUS construct relative to the expression of the control LUC construct for each sample, GUS activity was divided by LUC activity. For each construct the average relative expression and SD values was calculated from the three samples. To facilitate the comparison of test construct expression, relative expression values were normalized to the relative expression of construct A_{161}GA at 40% O_2, which was given a value of 1.

ADH and PGD Assays

Two million protoplasts were lysed in 500 μL of extraction buffer (100 mM Tris-HCl, pH 8.0, 4 mM DTT) and centrifuged as described for the assay of GUS activity. 6-PGD was assayed in 250 μL of reaction mix containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, and 4 mM $NADP^+$, as described previously (Bailey-Serres and Nguyen, 1992). ADH was assayed in a 250-μL reaction mixture containing 150 mM Tris-HCl, pH 8.0, 300 μM NAD^+, and 0.66 mM ethanol. Assays were initiated by the addition of 40 μL of extract. Specific activity was determined from reactions that were linear for 2 min. One unit of enzyme activity was defined as 1 μmol of reduced NAD^+ or $NADP^+$/min. ADH and 6-PGD specific activities are expressed as units/mg protein.

mRNA Half-Life Determination

Total cellular RNA was purified from seedling roots by the guanidinium thiocyanate method (Chomczynski and Sacchi, 1987). RNA was fractionated on 13% formaldehyde-1.3% agarose gels, transferred to nylon membrane (Magna-NT, Micron Separations, Westborough, VA), immobilized by UV cross-linking (Stratalinker, Stratagene), and hybridized to ^{32}P-labeled DNA fragments. DNA fragments used as hybridization probes were as follows: GUS, 1.9-kb *Pst*I fragment of pAIGN (Klein et al., 1988); LUC, 1.8-kb *Dra*I fragment of pPLLUC$(A)_{50}$; maize ADH1 coding sequence, 893-bp *Bal*I fragment of pZmL793 (Dennis et al., 1984); and tomato 18S rRNA, 0.21-kb *Not*I-*Sfi*I fragment (DB292, D. Bird, personal communication). Hybridization was at high stringency in 7% (w/v) SDS, 50 mM $NaPO_4$, pH 7.0, at 65°C for 18 h. Filters were washed twice for 15 min at 65°C in both 2× SSC/0.1% (w/v) SDS and 0.2× SSC/0.1% (w/v) SDS. Radioactive signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Filters were stripped by two 20-min washes at 95°C in 0.1× SSC/0.5% (w/v) SDS prior to probing with a second ^{32}P-labeled fragment. Physical mRNA half-life was determined by plotting the $\log_{10}$ values against time (min) and determining the slope (m) of the best-fit line. The equation $t_{1/2} = 0.693/k$ was used, where $k = 2.303m$ (Pitto et al., 1992).

Polyribosome Analysis

One million protoplasts were sonicated in 500 μL of polyribosome resuspension buffer (0.2 M KCl, 40 mM Tris, pH 8.4, 5 mM EGTA, 30 mM $MgCl_2$, 5 mM DTT, 50 μg/mL cycloheximide, and 50 μg/mL chloramphenicol). Triton X-100, Brij-35, Tween-40, and NP-40 were added to a final concentration of 1% (v/v). The extract was clarified by centrifugation in a microcentrifuge at 16,000g at 4°C for 10 min. Two-hundred fifty microliters of extract was layered over a 5-mL, 20 to 60% (w/v) Suc density gradient, centrifuged (115,000g for 1.25 h; L8-M centrifuge, SW-55 rotor, Beckman), and analyzed as described elsewhere (Fennoy and Bailey-Serres, 1995).

In Vivo Labeling of Protein

Protoplasts were incubated with 550 μCi [^{35}S]Met (1200 mCi/mmol, New England Nuclear) in 5 mL of recovery medium under the appropriate O_2 conditions for the final 3 h of the 18-h culture period. Protoplasts were pelleted by centrifugation, the supernatant was removed, and the pellet was frozen in liquid N_2 and stored at −70°C until use. Soluble protein was recovered by homogenization of protoplasts in 400 μL of extraction buffer (0.1 M Tris-HCl, pH 7.5, 15% [v/v] glycerol, and 10 mM DTT) with a small amount of alumina and a pellet pestle (Kontes, Vineland, NJ) in a microcentrifuge tube. The extract was clarified by centrifugation at 16,000g for 5 min at room temperature. Uptake of [^{35}S]Met was calculated from the value (cpm/μL) of the sample supernatant. Incorporation of [^{35}S]Met into soluble protein (cpm/μg) was determined by precipitation with boiling 5% TCA. Incorporation of [^{35}S]Met into soluble protein was determined as a percentage of uptake of label into protoplasts. Protein concentration was determined as described for enzyme assays.

RESULTS

Effect of O_2 Deprivation on Maize Protoplasts

The suspension-cell culture line P3377, derived from immature embryos of maize (Duncan et al., 1985), is responsive

to O_2 deprivation, as demonstrated by its increased accumulation of ADH1 and ADH2 mRNAs when cultured under hypoxia (Paul and Ferl, 1991). We examined whether protoplasts of P3377 cells respond to hypoxia by induction of ADH specific activity and reduction of total protein synthesis, two hallmarks of the anaerobic stress response in roots of maize seedlings (Sachs and Freeling, 1978; Sachs et al., 1980).

The specific activity of ADH in P3377 protoplasts was measured following a mock electroporation and culture at 5, 21, or 40% O_2 for 18 h (Table I). A 2-fold higher level of ADH activity was repeatedly detected in protoplasts cultured at 5% O_2 compared with those cultured at 21 or 40% O_2 ($P \leq 0.01$). The specific activity of an oxidative pentose-phosphate pathway enzyme, 6-PGD, was measured as a control for cell viability because 6-PGD activity in roots is unaffected by O_2 deprivation (J. Bailey-Serres, unpublished data). The variation in specific activity of 6-PGD in protoplasts cultured at the three O_2 levels was not significant. ADH specific activity was even higher (2.39 units mg^{-1} protein ± 0.62) in protoplasts cultured under argon (0.001% O_2) for 24 h; however, protoplast viability was severely compromised as determined by a significant decrease in 6-PGD specific activity (0.246 unit mg^{-1} protein ± 0.007).

O_2 deprivation in roots of maize seedlings results in a significant decrease in protein synthesis (Sachs et al., 1980) and a reduction in the initiation of translation (Fennoy and Bailey-Serres, 1995). To determine if protein synthesis was impaired in P3377 protoplasts cultured at 5% O_2, we examined the uptake and incorporation of [^{35}S]Met into soluble protein in the final 3 h of an 18-h culture period. Even though the uptake of [^{35}S]Met into protoplasts was similar at all three O_2 levels, the incorporation of [^{35}S]Met into TCA-insoluble protein was reduced by 5-fold at 21% O_2 and by 20-fold at 5% O_2, compared with 40% O_2 (Table I). To determine the effect of hypoxia on the initiation of translation, polyribosomes associated with mRNA were analyzed by fractionation of crude cell lysates on Suc density gradients (Fig. 2). In hypoxic protoplasts the levels of 40S and 60S ribosomal subunits and 80S monoribosomes were slightly higher than in protoplasts cultured at 21% O_2, whereas polyribosome levels were clearly reduced in response to hypoxia. Similar polyribosome levels were observed in protoplasts cultured at 21 and 40% O_2 (data not shown). These results indicate that initiation of translation is impaired by culturing protoplasts at 5% O_2, as observed in seedling roots deprived of O_2 (Fennoy and Bailey-Serres, 1995).

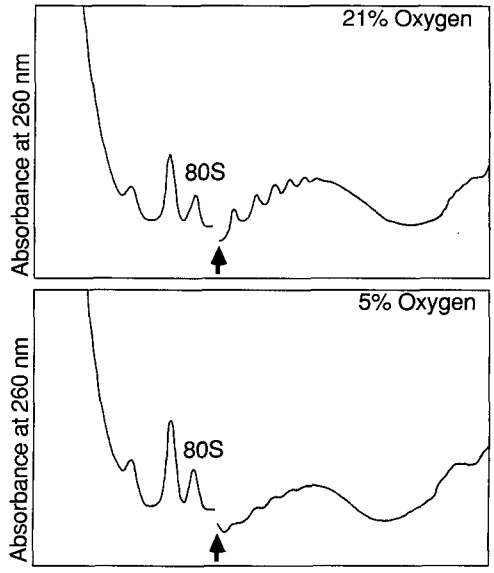

Figure 2. Sedimentation profiles of extracts from protoplasts cultured at 21 and 5% O_2. Protoplasts were collected after 18 h of culture and lysed by sonication in buffer, and the crude supernatant was layered onto 20 to 60% (v/w) Suc density gradients containing 0.2 M KCl and centrifuged. Sedimentation profiles were analyzed with a UV absorbance detector (ISCO, Lincoln, NE) at 1.0 sensitivity until the position marked by the vertical arrow, at which time the detector was switched to 0.2 sensitivity. The location of the 80S monoribosome peak is indicated. The 40S and 60S ribosomal subunit peaks are to the left of the 80S peak; polyribosome peaks are to the right of the 80S peak.

We chose 5% O_2 for deprivation treatments, since this level of O_2 causes a clear reduction in protein synthesis and increase in ADH activity, but does not compromise cell viability after 24 h of culture. Forty percent O_2 was chosen for aerobic treatments instead of 21% O_2, because ADH activity was the lowest and protein synthesis levels were the highest at this concentration, suggesting that protoplasts cultured at 21% O_2 may be O_2-deprived.

Effect of Quantity of Input mRNA and Length of Culture after Electroporation on Reporter Enzyme Activity

Pilot electroporation experiments were performed to determine the parameters required to obtain optimal and

Table I. *Dissolved O_2 concentration, ADH and 6-PGD specific activities, and [^{35}S]Met incorporation into protein in protoplasts*

Data are from one representative experiment; assays were performed in triplicate. Specific activity values followed by a different letter are significantly different ($P \leq 0.01$), as determined by the Student's *t* test.

Percent O_2 in Growth Chamber	[O_2] in Culture Medium at 22°C	Specific Activity		Percent cpm Incorporated per μg Soluble Protein (relative)
		ADH	6-PGD	
	μM	*units mg^{-1} protein ± SD*		
5	60	1.03 ± 0.01a	2.67 ± 0.20a	0.03 (5)
21	272	0.74 ± 0.11b	2.65 ± 0.26a	0.16 (27)
40	355	0.55 ± 0.05c	2.79 ± 0.15a	0.60 (100)

reliable expression of in vitro-synthesized, 5′ ^{7}mGpppG-capped, and 3′ polyadenylated mRNAs in P3377 protoplasts. To determine an appropriate quantity of input mRNA to use per electroporation, 0.5 to 5 μg of an mRNA construct containing the *uidA* gene encoding GUS from *E. coli* (A_{161}GA; Fig. 1) or the *luc* gene encoding LUC from firefly (PLLUC[A]$_{50}$; Fig. 1) were co-electroporated into two million protoplasts. GUS and LUC activities were determined after 18 h of culture at 40% O_2. A linear increase in reporter enzyme activity was observed for both constructs over the range of 1 to 5 μg of mRNA (Fig. 3A).

The kinetics of accumulation of reporter enzyme activity was examined to choose a standard length of time for cell culture after electroporation. Five micrograms of A_{161}GA and PLLUC(A)$_{50}$ mRNAs was co-electroporated into protoplasts and cells were harvested over a 24-h time course of culture at 40% or 5% O_2. Reporter enzyme activity was determined and plotted as a function of time (Fig. 3B). In aerobic protoplasts, GUS activity from the ADH1-GUS construct plateaued after 12 h of culture and remained stable. Since the half-life of GUS is greater than 24 h in plant cells (Jefferson et al., 1987), the plateau indicates that translation of A_{161}GA mRNA did not continue beyond the 12-h time point in aerobic protoplasts. GUS activity was low in hypoxic protoplasts and did not increase until after 6 h of culture. The lower level of mRNA translation is indicative of the reduction in protein synthesis (Table I) and polyribosome levels (Fig. 2) in response to O_2 deprivation. In contrast to aerobic protoplasts, a steady increase in GUS activity was observed from 6 to 24 h, indicating that the ADH1-GUS mRNA was stable and translated more efficiently after a 6-h lag-time in hypoxic protoplasts. In both aerobic and hypoxic protoplasts LUC activity increased linearly over 18 h of culture and then decreased between 18 and 24 h. A significantly lower level of LUC activity was detected in hypoxic protoplasts, indicative of poor translation of PLLUC(A)$_{50}$ mRNA in cells deprived of O_2. These data indicate that PLLUC(A)$_{50}$ mRNA translation continued for up to 18 h under both culture conditions. The decrease in LUC activity after 18 h is indicative of the short half-life of this enzyme in plant cells (Luehrsen et al., 1992). Based on these results, in subsequent experiments 5 μg each of a GUS and a LUC mRNA construct were co-electroporated into two million protoplasts, which were harvested after 18 h of culture.

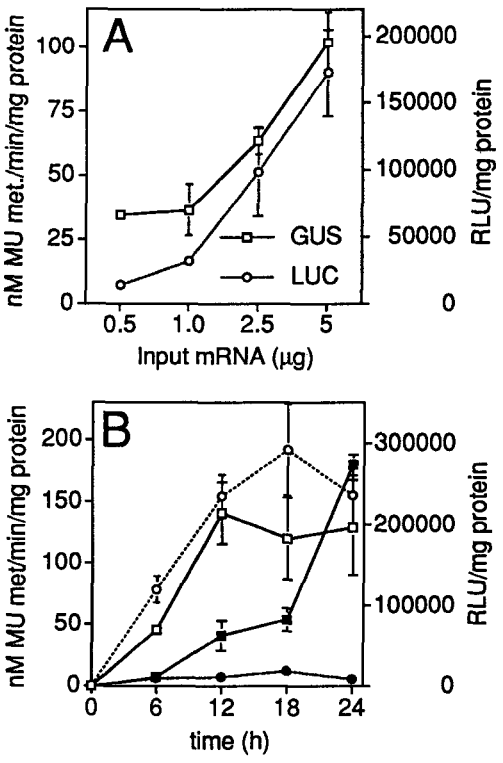

Figure 3. Effect of quantity of input mRNA and time in culture on reporter enzyme activity. A_{161}GA and PLLUC(A)$_{50}$ mRNAs (Fig. 1) were co-electroporated into two million protoplasts in triplicate; protoplasts were harvested after 18 h (A) or at the times shown (B); and GUS and LUC specific activities were determined for cells cultured at 40% O_2 (open symbols) or 5% O_2 (filled symbols). Input mRNA amounts were 0.5 to 5 μg as indicated (A) or 5 μg (B).

Effect of *adh1* Sequences on Reporter mRNA Expression in Protoplasts

Five synthetic ADH1-GUS mRNA constructs were used to test the effect of specific sequences on translational efficiency in protoplasts cultured at 5 or 40% O_2. The LUC construct [PLLUC(A)$_{50}$; Fig. 1], which contains no *adh1* sequence, was used as an internal standard in all electroporations. This construct was expressed 10.5- ± 3.1-fold more efficiently in cells cultured at 40% O_2 (Fig. 4). In each experiment a chimeric ADH1-GUS test construct (Fig. 1) was co-electroporated with the control construct PLLUC(A)$_{50}$; electroporations were performed in triplicate, GUS and LUC activities were determined, and the relative expression of GUS to LUC in each sample was calculated (see "Materials and Methods"). For ease of comparison, the relative expression data were normalized to the expression of the construct A_{161}GA at 40% O_2 (Fig. 4).

The expression of four chimeric mRNAs containing various portions of the 5′-UTR, coding region, and 3′-UTR of *adh1* were tested. An ADH1-GUS translational fusion containing the 5′-UTR and the first 18 amino acids of ADH1 and 8 amino acids from the polylinker squence (26 amino acids) (A_{161}GA) was expressed 57-fold more efficiently in hypoxic protoplasts than in aerobic protoplasts relative to the expression of PLLUC(A)$_{50}$ mRNA. Since the expression of PLLUC(A)$_{50}$ mRNA was inhibited 10.5-fold in hypoxic protoplasts, the actual enhancement of translation of A_{161}GA mRNA in hypoxic protoplasts was between 5- and 6-fold. The relative expression of the GUS reporter mRNA in hypoxic protoplasts progressively decreased as *adh1* 5′ sequences were removed in a 3′ to 5′ direction. For example, construct A_{116}GA contains the 5′-UTR and first 3 codons of *adh1* and 8 codons from the polylinker sequence (11 amino acids) in a translational fusion with *uidA*, but was expressed at a significantly lower level than A_{161}GA in hypoxic protoplasts ($P < 0.05$, Student's *t* test). A_{161}GA and A_{116}GA have identical 146-nt 5′-UTRs ending at the *adh1* initiation codon. The deletion construct A_{82}GA contains

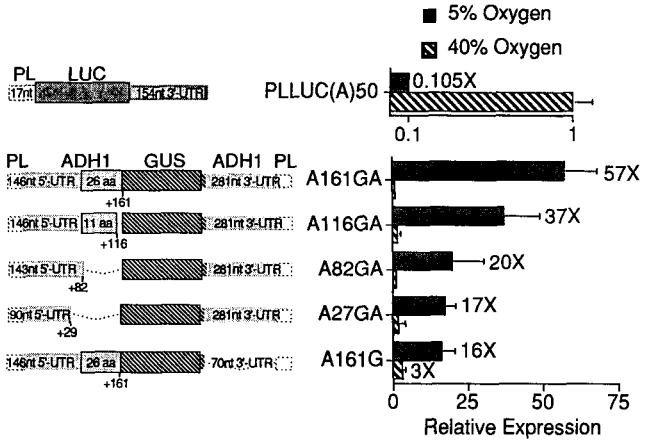

Figure 4. Relative expression of mRNA constructs in protoplasts cultured at 5 or 40% O_2. A schematic of mRNA constructs is shown on the left (see Fig. 1 for nt sequence details). Five micrograms of ADH1-GUS and LUC mRNAs was co-electroporated into two million protoplasts that were cultured for 18 h at 5 or 40% O_2. Expression data for PLLUC$(A)_{50}$ are the mean ± SD from four electroporation experiments (n = 39 samples for each O_2 level). Expression of PLLUC$(A)_{50}$ at 5% O_2 was normalized to that observed at 40% O_2 (given a value of 1). Relative expression of ADH1-GUS constructs was normalized to the expression of A_{161}GA at 40% O_2 (given a value of 1). The relative expression data were determined as follows: for each electroporation sample the specific activity of GUS (nm methylumbelliferone metabolized mg^{-1} protein min^{-1}) was divided by that of LUC (relative light units/mg protein) to obtain a relative expression value. For each experiment the mean value from the replicate samples for each construct under each O_2 concentration was determined and normalized to the expression of A_{161}GA at 40% O_2 (given a value of 1). Histogram data represent the mean ± SD of normalized expression data from multiple experiments. Number of electroporation experiments per construct: A_{161}GA (n = 4), A_{116}GA (n = 1), A_{82}GA (n = 4), A_{27}GA (n = 3), A_{161}G (n = 3).

most of the *adh1* 5′-UTR in a 143-nt leader sequence that ends at the *uidA* initiation codon. This construct was expressed in hypoxic protoplasts, but at a significantly lower level than A_{161}GA ($P < 0.01$). Construct A_{27}GA has a 90-nt leader that contains the first 27 nt of the *adh1* 5′-UTR. A_{27}GA was expressed at a significantly lower level than A_{161}GA ($P < 0.01$) and A_{116}GA ($P < 0.05$) in hypoxic protoplasts. In aerobic protoplasts, the relative expression of these ADH1-GUS constructs was not significantly affected by changes in the 5′-UTR and coding sequence.

To determine whether the *adh1* 3′-UTR sequences were necessary for enhanced expression of A_{161}GA in O_2-deprived protoplasts, a construct (A_{161}G) containing the *adh1* 5′-UTR sequences but lacking *adh1* 3′-UTR sequences was tested. The removal of the *adh1* 3′-UTR resulted in a 3.5-fold reduction in relative expression in protoplasts cultured at 5% O_2; however, this construct was expressed at a significantly higher (3-fold; $P < 0.05$) level than A_{161}GA in protoplasts cultured at 40% O_2. As mentioned, the expression of the ADH1-GUS constructs was measured relative to that of the control construct PLLUC$(A)_{50}$. If the 10.5-fold difference in expression of the *luc* reporter mRNA in aerobic and hypoxic protoplasts is considered, the expression of constructs A_{27}GA and A_{161}G in hypoxic protoplasts was only slightly higher than that in aerobic protoplasts. In summary, the presence of both 5′ and 3′ sequences of *adh1* mRNA enhanced expression of a GUS coding sequence in O_2-deprived protoplasts by greater than 5-fold.

Effect of *adh1* Sequences on Physical mRNA Half-Life in Protoplasts

The level of expression of mRNAs electroporated into protoplasts is affected by the half-life and efficiency of translation of an mRNA. Physical mRNA half-life was determined for each construct in protoplasts cultured under aerobic and hypoxic conditions. mRNAs were electroporated into protoplasts, aliquots were removed over a time course, and total RNA was extracted and fractionated by electrophoresis under partially denaturing conditions in formaldehyde-agarose gels. Full-length transcripts were detected by hybridization of ^{32}P-labeled GUS or LUC probes to RNA blots and quantitated using a PhosphorImager (Molecular Dynamics). A rRNA probe was used to confirm equal loading of total mRNA. As shown in Figure 5A, the physical half-life of A_{161}GA and PLLUC$(A)_{50}$ mRNA ranged from 91 to 125 min in aerobic and hypoxic protoplasts. For ease of comparison, the physical half-life of the control LUC and experimental ADH1-GUS constructs was determined relative to the half-life of A_{161}GA mRNA in aerobic protoplasts over the first 2.5 h in culture (Fig. 5B). There was less than 50% variation in the mRNA half-life between constructs, and less than 40% difference in mRNA half-life of a single construct under the two treatment conditions. RNA blot analysis demonstrated that full-length

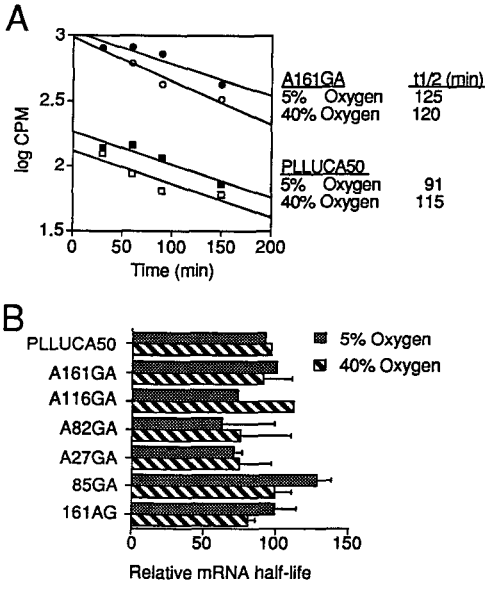

Figure 5. Analysis of physical mRNA half-life in electroporated protoplasts. A, mRNA levels were determined by RNA blot hybridization and full-length transcripts were quantified by PhosphorImager analysis as described in "Materials and Methods." Closed symbols, 5% O_2; open symbols, 40% O_2; A_{161}GA, circles; PLLUC$(A)_{50}$, boxes. B, mRNA half-life normalized to the half-life of A_{161}GA mRNA in protoplasts cultured at 40% O_2 (given a value of 1). Data are the mean ± SD from multiple experiments, except for A_{116}GA, which is from one experiment.

electroporated mRNAs were still present in aerobic and hypoxic protoplasts up to 24 h after electroporation (data not shown). Hence, the differences in mRNA half-life observed between protoplasts cultured under the two O_2 conditions was not a major determinant of the variations in reporter enzyme activity.

DISCUSSION

adh1 mRNA is efficiently translated in O_2-deprived roots of maize, whereas many normal cellular mRNAs are poorly translated (Fennoy and Bailey-Serres, 1995; S.L. Fennoy and J. Bailey-Serres, unpublished data). An RNA electroporation and transient expression system was used to examine the effect of portions of *adh1* mRNA on gene expression in aerobic and hypoxic protoplasts. The maize cell-suspension culture line P3377 is capable of inducing the accumulation of *adh1* mRNA in response to O_2 deprivation (Paul and Ferl, 1991). We showed that P3377 protoplasts cultured at 5% O_2 have a 2-fold higher level of ADH specific activity, a lower level of total protein synthesis, and fewer polyribosomes than protoplasts cultured under higher O_2 concentrations (21 or 40% O_2).

An initial observation was that 5′ capped and 3′ polyadenylated control mRNA from the construct pPLLUC$(A)_{50}$ was expressed at a 10.5-fold higher level in aerobic (40% O_2) than in hypoxic (5% O_2) protoplasts. In protoplasts cultured at 21% O_2 this construct was expressed at a 5-fold higher level compared with 5% O_2 protoplasts (data not shown), further indicating that protoplasts cultured at 21% O_2 are O_2-deprived. The half-life of PLLUC$(A)_{50}$ mRNA was about 100 min in protoplasts cultured at 5 or 40% O_2. LUC activity increased linearly in both aerobic and hypoxic protoplasts over 18 h of culture, indicating that the half-life of LUC protein was not differentially affected by the two treatments. Thus, the 10-fold lower level of expression of PLLUC$(A)_{50}$ mRNA in protoplasts cultured at 5% O_2 is most likely due to translational repression and not mRNA stability. In maize roots, translation of many normal cellular mRNAs is repressed in response to O_2 deprivation (Sachs et al., 1980; Fennoy and Bailey-Serres, 1995). Our demonstration that PLLUC$(A)_{50}$ mRNA is stable but poorly translated in hypoxic protoplasts may reflect the posttranscriptional regulation of the adenine nt translocator protein mRNA, which is maintained in hypoxic roots but poorly loaded onto polyribosomes. Translational repression of PLLUC$(A)_{50}$ was also observed in response to heat shock in carrot protoplasts; however, in contrast to what we observed with hypoxia, heat shock significantly increased the half-life of PLLUC$(A)_{50}$ (Gallie et al., 1995).

A series of chimeric mRNAs containing the *adh1* 5′- and 3′-UTR sequences and the *uidA* (GUS) reporter gene were made using restriction endonuclease cleavage sites. The expression of these 5′ capped and 3′ polyadenylated mRNAs relative to that of PLLUC$(A)_{50}$ mRNA was examined in protoplasts cultured under aerobic or hypoxic conditions. The most highly expressed construct in hypoxic protoplasts was one that included the 5′-UTR and the first 18 codons of *adh1* fused to GUS, and 186 nt of the *adh1* 3′-UTR followed by a 68-nt poly(A) tract (A_{161}GA). A time-course experiment demonstrated that the translational activity of this construct was prolonged in O_2-deprived protoplasts. Deletion of *adh1* 5′ sequences in a 3′ to 5′ direction resulted in a steady decrease in the level of reporter mRNA expression in hypoxic protoplasts. In marked contrast, the presence of the *adh1* 5′ sequences did not significantly affect the expression of these constructs in aerobic protoplasts. RNA blot hybridization analyses demonstrated that the physical half-lives of the mRNAs tested were not markedly different under the two O_2 conditions. These observations suggest that the differences in expression of the ADH1-GUS mRNA constructs in response to the O_2 concentration in the culture medium are due mainly to variations in translational efficiency (i.e. initiation or elongation of translation) or some other posttranslational effect (i.e. GUS specific activity).

Three forms of GUS, each with distinct NH_2 termini, were encoded by the ADH1-GUS constructs used in this analysis. Constructs A_{161}GA and A_{161}G contained the 5′-UTR and 26 codons (the first 18 codons of *adh1* and 8 codons from polylinker sequence) in a translational fusion with the GUS coding sequence. Construct A_{116}GA contained the 5′-UTR and 11 codons (the first 3 codons of *adh1* and 8 codons from polylinker sequence) in a translational fusion with GUS. The other ADH1-GUS constructs (A_{82}GA and A_{27}GA) encoded the native bacterial GUS. GUS is known to tolerate N-terminal translational fusions (Jefferson, 1987). The K_m values of GUS synthesized from A_{161}GA, A_{116}GA, and A_{82}GA mRNAs in a wheat germ translation system were similar (data not shown), as was the specific activity of GUS from these constructs in aerobic protoplasts. Thus, it is most likely that the differences in GUS specific activity from the *adh1* 5′ region (5′-UTR and the first 18 codons) deletion constructs in hypoxic protoplasts were due to the mRNA sequence differences and not to posttranslational effects.

Two mRNAs encoding the ADH1-GUS translational fusions A_{161}GA and A_{116}GA were efficiently expressed in hypoxic protoplasts. These constructs have the same 5′-UTR that ends at the *adh1* initiation codon, but differ in the length of *adh1* coding sequences fused to the GUS open reading frame. It is not clear why the sequence encoding codons 4 to 18 of *adh1* might enhance translation of A_{161}GA in hypoxic protoplasts. In only a few cases have sequences downstream of the initiation codon been shown to be important in the posttranscriptional regulation of gene expression in plants. Sequences within the first one-third of the coding region of the pea Fd gene are required for light-mediated regulation of mRNA abundance in transgenic tobacco (Dickey et al., 1994). This posttranscriptional regulation is abrogated if a nonsense codon is present in the first portion of the coding region (Dickey et al., 1994). Modification of the insect-control protein gene *cryIA* of *Bacillus thuringiensis* to the synonymous codon usage of the host plant increased steady-state mRNA levels and, even more significantly, increased translation of the mRNA (Perlack et al., 1991). The presence of the first 18 codons of *adh1* in A_{161}GA did not affect mRNA stability, but appeared to specifically increase mRNA translation in hypoxic proto-

plasts. Perhaps sequences within the first portion of the *adh1* open reading frame aid in the efficient recruitment of ribosomes under conditions of limited protein synthesis.

The construct $A_{82}GA$ contains a 143-bp 5′-UTR that lacks an *adh1* coding region. A significantly lower level of expression of $A_{82}GA$ was observed in hypoxic protoplasts, compared with $A_{161}GA$ ($P < 0.01$). The construct $A_{27}GA$ is a further deletion of *adh1* 5′ message sequence and was expressed at roughly the same level as the $A_{82}GA$ construct (Fig. 4). The actual level of expression of these constructs, which is based on GUS specific activity, was similar in aerobic and hypoxic protoplasts. Both $A_{82}GA$ and $A_{27}GA$ differ from $A_{161}GA$ with respect to the context of the first AUG. Assuming that translation commences at the first AUG, translation of $A_{161}GA$ begins at the AUG of *adh1* (GCAAUGG), and translation of $A_{82}GA$ and $A_{27}GA$ begins at the AUG of *uidA* (CUUAUGA). The initiation codon of bacterial *uidA* is a poor match to the initiation codon consensus for maize genes, which is (G/A)(C/A)(C/G)AUGG (Luerhsen and Walbot, 1994). Although there is evidence that an initiation codon in a "good" context will stimulate expression of a reporter gene in protoplasts, the effect is small and dependent on other features of the 5′-UTR (Gallie et al., 1987; Luerhsen and Walbot, 1994). We did not observe a significant difference between constructs with an *uidA* initiation codon or an *adh1* codon under aerobic conditions (Fig. 4; $P > 0.1$). Only under hypoxia was a difference observed (Fig. 4), indicating that the sequence between +82 to +161 of *adh1* mRNA specifically enhances translation under hypoxia. The comparison between $A_{27}GA$ and $A_{82}GA$ suggests that there are no additional sequences within the region between +27 and +82 that enhance translation under hypoxia and, further, that the length of the 5′ untranslated leader did not affect translational efficiency in this system.

Our data also suggest that the 3′-UTR has an effect on translation under hypoxia. Comparison of expression of $A_{161}GA$ and $A_{161}G$ revealed that the presence of the 5′-UTR, the first 18 codons, and the 3′-UTR of *adh1* were necessary for enhanced expression in O_2-deprived protoplasts. The 3′-UTR of $A_{161}GA$ is composed of 57 nt of the *uidA* 3′-UTR, followed by an *adh1* sequence from a *Nhe*I site (+1299 bp) located 60 nt downstream of the stop codon, to position +1477 bp, located 7 nt 3′ of a native polyadenylation site (+1470 bp) (Sachs, 1986) (Fig. 1). Although removal of the 180 nt of *adh1* 3′-UTR in $A_{161}G$ resulted in a 3.5-fold lower level of expression in hypoxic protoplasts, a 3-fold higher level of expression was measured in aerobic protoplasts. Since $A_{161}GA$ and $A_{161}G$ mRNAs have similar physical half-lives in hypoxic protoplasts and encode the same ADH1-GUS translational fusion, the significant difference in relative expression is due to the effect of the *adh1* 3′-UTR sequence on translation. It is well documented that the 3′-UTR can play a critical role in the control of translation during development in animals (reviewed by Wickens et al., 1996); in general, 3′-UTR elements reduce mRNA stability. Although the 3′-UTR of *adh1* reduced expression in aerobic cells, both 5′ sequences (5′-UTR and the first 18 codons) and the 3′-UTR of *adh1* were required to stimulate translation in response to low O_2.

Recent studies have demonstrated functional interactions between the 5′ and 3′ portions of mRNA in plants (Gallie, 1996) and other eukaryotes (Jacobson, 1996). The 5′ and 3′ ends of an mRNA may interact via protein-RNA contacts between the 5′ cap or 5′-UTR and the 3′-UTR or 3′ poly(A) tail. Proteins involved in the interaction could be translation factors, poly(A)-binding proteins, or specific mRNA-binding proteins. Interactions between the 5′ and 3′ portions of the mRNA may promote initiation or reinitiation of translation; such interactions may not be absolutely required for translation but may improve translational efficiency. Our observation that both 5′ and 3′ regions of *adh1* mRNA are required to maintain and enhance translation in hypoxic protoplasts shows some similarity to the dependency between 5′ and 3′ mRNA sequences of viral mRNAs. Enhancement of translation of tobacco mosaic virus mRNA involves a synergistic interaction between the omega sequence of the 5′ leader and a pseudoknot region of the nonpolyadenylated tail (Leathers et al., 1993). Translation of satellite tobacco necrosis virus RNA, a noncapped and nonpolyadenylated transcript in wheat germ extract, is dependent on the presence of specific 5′-UTR sequences and a higher-order structure in the 3′-UTR (Timmer et al., 1993a). Further studies are required to determine whether the 5′ and 3′ regions of *adh1* mRNA function cooperatively or independently to maintain efficient translation in O_2-deprived cells.

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