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Differential Expression and Sequence Analysis of the Maize Glyceraldehyde-3-Phosphate Dehydrogenase Gene Family

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Two cDNA clones for maize cytosolic glyceraldehyde-3-phosphate dehydrogenase are described. One is about 97% similar in coding capacity to a previously published clone [Brinkmann et al. (1987). J. Mol. Evol. 26, 320–328], while the other shows only 88% similarity. Evidence points toward the three cDNAs being the products of three genes, to be called *Gpc1*, *Gpc2*, and *Gpc3*. When the least similar clone, corresponding to *Gpc3*, was used to analyze RNA gel blots, anaerobic treatment for 6 hours induced RNA accumulation in the shoots 15.6-fold, while a 1-hour shift from 28°C to 40°C increased accumulation 5.1-fold. Roots had a higher basal level of expression, leading to a 6.0-fold anaerobic induction, and a 2.4-fold heat stress induction. RNA gel blot analysis using the clone corresponding to *Gpc2* showed decreased RNA accumulation within 6 hours of anaerobiosis, while analysis with the previously published clone, corresponding to *Gpc1*, showed a decrease within 24 hours. Neither *Gpc1* nor *Gpc2* showed heat stress induction, while some other known anaerobic genes did. Through the use of hybrid selection, in vitro translation, and immune precipitation, the relative expression of the three genes is shown. The role of the observed changes in gene expression is discussed in relation to stress physiology.

INTRODUCTION

Maize has evolved a system to ensure survival during short-term flooding (anaerobic) conditions (Lemke-Keyes and Sachs, 1989). Growth is virtually stopped during this treatment, but transcription and translation continue at a reduced level. Both of these processes are redirected toward the synthesis of anaerobic proteins, while pre-stress gene expression is inhibited (Sachs, Freeling, and Okimoto, 1980; Rowland and Strommer, 1986). All of the anaerobic proteins identified to date have been involved with glycolysis, as either core members of the pathway that also produces substrate for mitochondrial oxidation, or related gene products such as sucrose synthase, pyruvate decarboxylase, and alcohol dehydrogenase (ADH; reviewed by Sachs and Ho, 1986). Two gene families are known to be involved in the response. Both alcohol dehydrogenase genes, *Adh1* and *Adh2*, show selective RNA accumulation and preferential translation during anaerobic stress (Sachs, Freeling, and Okimoto, 1980; Dennis et al., 1985), but results are less clear with the two sucrose synthase genes (*Sh1* and *Sus1*; Springer et al., 1986; McElfresh and Chourey, 1988). Studies with *Adh1* show that transcript levels are controlled by changes in transcription and message turnover (Rowland and Strommer, 1986). Along with these changes in gene expression, there is also a simultaneous decrease in pH of the cytoplasm in excised root tips (Roberts et al., 1984) and morphological changes in the mitochondria (Aldrich et al., 1985; Vartepetian, Snkhchian, and Generozova, 1987). The mechanisms responsible for the transcriptional and translational control during anaerobic stress are not well defined.

Similar to the results in maize and other plants, mammalian cells can show increased glycolytic gene expression during reduced oxygen (hypoxic) treatment (Webster, 1987; Webster and Murphy, 1988). Additionally, heat stress is known to induce these same enzyme functions in some organisms. Heat stress in yeast promotes the selective labeling of one of the three glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins (the *TDH3* gene product) as well as phosphoglycerate kinase and enolase (reviewed in Lindquist and Craig, 1988). Work with frog embryos shows a heat shock-induced increase in GAPDH activity, and implies that two genes may exist (Nickells and Browder, 1988). Another glycolytic enzyme, pyruvate kinase, is also induced in this system (M. Marsden, R.W. Nickells, T.I. Wang, M. Kapoor, and L.W. Browder, manuscript submitted for publication). Since the induction of these genes coincides with morphological changes in the mitochondria of mammalian cells (Welch and Suhan, 1985), barley aleurone layers (Belanger, Brodl, and Ho, 1986), and soybean root tips (Chen et al., 1988),

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it has been surmised that glycolytic gene induction is necessary for survival of these organisms when mitochondrial functions are compromised, either by heat stress or reduced oxygen levels (Sachs and Ho, 1986; Webster and Murphy, 1988; M. Marsden, R.W. Nickells, T.I. Wang, M. Kapoor, and L.W. Browder, manuscript submitted for publication).

In this work, we identify two maize cytosolic GAPDH sequences by their similarity to known GAPDH clones. The two vary in degree of protein coding similarity to a previously published maize clone (Brinkman et al., 1988), but all three are completely divergent in their 3′-untranslated regions. Message accumulation during heat stress, anoxia, and light treatment of young seedlings was used to distinguish the expression of the three sequences. These sequence and expression data can be best explained by the existence of three genes for cytosolic GAPDH. We propose to name this GAPDH gene family *Gpc*, following the convention of identifying the NAD^+-dependent cytosolic enzyme used in glycolysis (EC 1.2.1.12) by the "C" designation. The previously defined gene (Brinkmann et al., 1987; Martinez, Martin, and Cerff, 1989) is then *Gpc1*, while the gene product is called GAPC1. Additionally, there is an NADP-dependent GAPDH enzyme activity localized to the plastid, which is involved in Calvin cycle reactions. It is the product of two nuclear gene families (Cerff, 1982; Cerff and Kloppstech, 1982; Shih and Goodman, 1988). These will be designated *Gpa* and *Gpb*. Genomic and cDNA clones have been identified for *Gpa1* (Brinkmann et al., 1987; Quigley, Martin, and Cerff, 1988), and its gene product will be called GAPA1. In addition to the stress-induced expression of the GAPDH gene family, similar analysis was extended to other genes known to be induced by heat stress and anoxia.

RESULTS

Sequence Analysis

A cDNA library was constructed from 6-hr anaerobically treated maize seedlings (Berkeley Fast inbred). Analysis of the library with a yeast genomic clone corresponding to *TDH3*, one of the three yeast GAPDH genes, identified a number of clones with different restriction maps. Two of the longest clones were selected for further study and are identified as pGAPC2 and pGAPC3. Sequence analysis showed that both had a high degree of protein coding similarity to the maize cytosolic GAPDH (GAPC1) cDNA sequence of Brinkmann et al. (1987). This is identified here as pZm9. The three cDNA sequences are aligned in Figure 1A, with only the differences from pGAPC2 shown in the other two sequences. It is apparent that pGAPC2 and pZm9 are more similar to each other than to pGAPC3. All sequences were even more divergent from the plastid-directed nuclear gene product GAPA1 (pZm57; Brinkmann et al., 1987), so they are assumed to code for the cytosolic enzyme used in glycolysis.

Many of the nucleotide differences between the clones are conservative, as seen in the comparison of deduced amino acid sequences shown in Figure 1B. The first amino acid shown is numbered 86 according to Harris and Waters (1976). While there is only an 80% nucleotide sequence similarity in the cloned coding region between pGAPC2 and pGAPC3, there is an 88% predicted amino acid identity. When comparing the same region of pGAPC2 to pZm9, these values are 95% and 97%, respectively. Residues defining the enzyme active site (e.g., Cys-149, the binding site of glyceraldehyde 3-phosphate; Harris and Waters, 1976) are found in all clones, implying that they code for functional enzymes.

When one looks outside the coding region to the 3′-untranslated region, all three clones show near complete divergence. The only similar region is around the AATAAA (single underline in Figure 1A), which in animals is found 15 bases to 30 bases from the poly(A) addition site, and has been shown to have a sequence-dependent (Wickens and Stephenson, 1984) and position-dependent (Fitzgerald and Shenk, 1981) function in poly(A) addition. This or a closely related sequence is similarly positioned in less than half of the plant nuclear genes (Hunt et al., 1987); its function in plants has not yet been analyzed by deletion mutations. As seen in Figure 1A, the clones of the three different GAPC transcripts have this sequence at least 100 bases from the poly(A) addition site. The two *Adh* genes in maize also have this sequence positioned far from the poly(A) addition site (Sachs et al., 1986), while in aldolase, another anaerobic gene, no similar sequence appears in the 3′-untranslated region (Kelley and Tolan, 1986; Dennis et al., 1988).

Since these three GAPC sequences show near complete divergence in their 3′-untranslated regions, it implies that there are three genes for cytosolic GAPDH. This view is also supported by the fact that pGAPC2 and pGAPC3 came from the same inbred maize cDNA library; one would not expect polymorphism in an inbred (isogenic) plant population, so the two sequences must be derived from separate genes. Furthermore, RNA analysis with gene-specific probes identifies all three sequences in the B73 inbred line, and each clone detects a different pattern of accumulation (see below). The pZm9 sequence of Brinkmann et al. (1987) was generated from a different maize library; unfortunately, none of the clones found in our library had the EcoRV restriction site (base 507) that is unique to pZm9, although the RNA analysis here implies that the sequence should be present and available for cDNA synthesis. DNA gel blot analysis by other workers (Martinez, Martin, and Cerff, 1989) also defines two to three sequences related to cytosolic GAPDH. There are, then, at least four genes in the GAPDH gene family: three

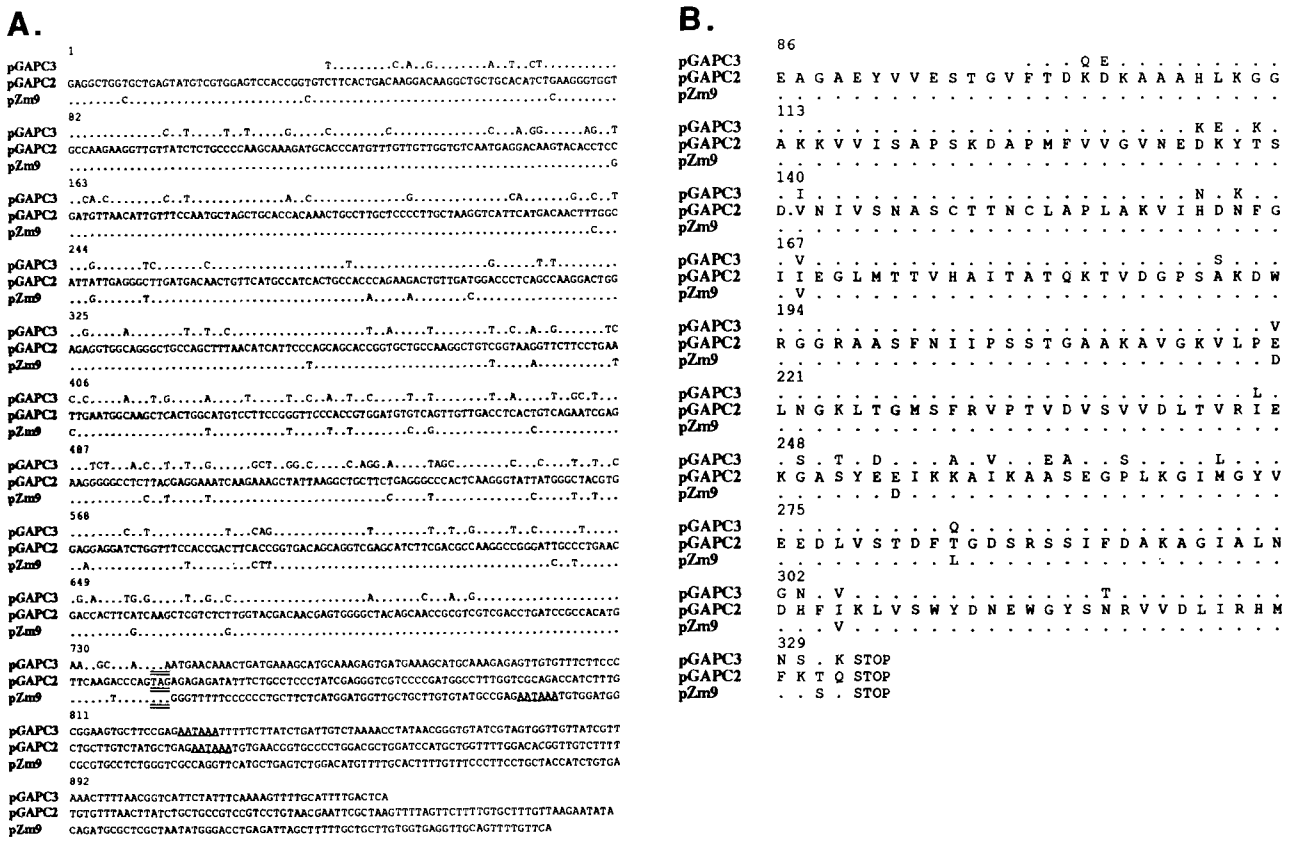

Figure 1. Aligned Sequences of the Maize GAPC cDNA Clones.

(A) The entire DNA sequence of pGAPC2 is shown, with only the differences in the coding regions of pZm9 (Brinkmann et al., 1987) and pGAPC3 indicated. The AATAAA element is underlined; the stop codon is double-underlined.
(B) A similar comparison is made between the derived amino acid sequences of the clones.

for cytosolic functions and at least one directed to the plastid.

Gpc Stress Induction

Because many genes for glycolytic enzymes show anaerobically induced RNA accumulation (Dennis et al., 1985; Springer et al., 1986), it was expected that at least one *Gpc* gene would be so induced. Because other systems show GAPDH induction at some level during heat shock (Lindquist and Craig, 1988; Nickells and Browder, 1988), there might also be induction of one of these genes by that stress. To address these points, total RNA was collected from seedlings after various times of anaerobic treatment or heat shock, as well as growth in light, and prepared for RNA gel blot analysis. To avoid potential problems of cross-hybridization, pZm9, pGAPC2, and pGAPC3 were subcloned at the conserved SalI site (base 709). These clones contained the entire 3′-untranslated regions, as well as the 11 terminal codons. Similar results, however, could be obtained with the entire clones of pGAPC2 and pGAPC3, as well as a Taq1 subclone of pGAPC2 that does not contain these 11 codons (data not shown). To correct for RNA loading errors, hybridization was normalized to the level seen with an rDNA probe. Blots from a typical experiment are shown in Figure 2. Table 1 shows the normalized hybridization signal relative to shoot control (zero hour) for each clone used. These data are the average of at least two determinations, as well as hybridizations to dilutions of the 6-hr anaerobic RNA samples.

As seen in Figure 2, RNA corresponding to HSP70 (the 70-kD heat shock protein) only shows increased accumulation during heat shock, and glycolysis-related genes (sucrose synthase 1, aldolase, ADH1) are strongly induced during anaerobic treatment. HSP70 RNA levels decrease slightly during anaerobic treatment, while the anaerobically induced genes have only a low level of

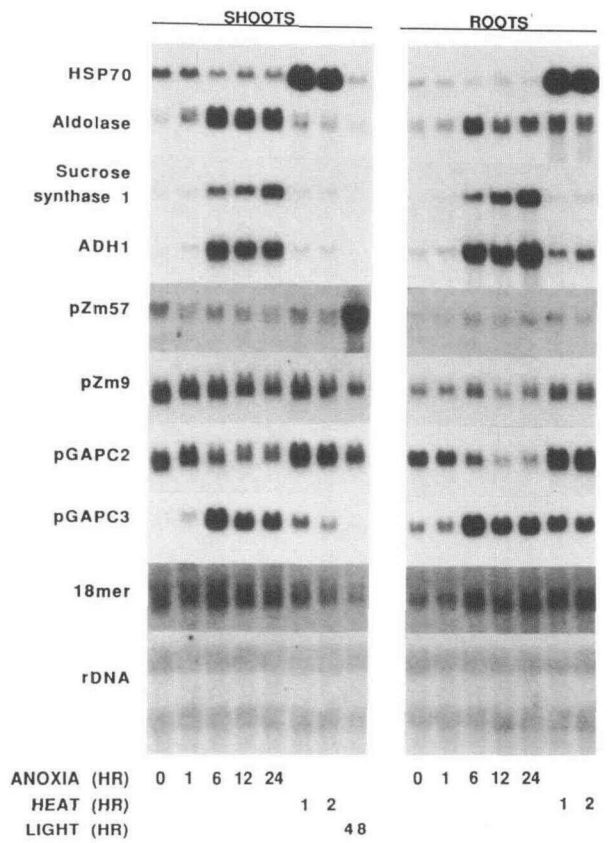

Figure 2. RNA Gel Blot Hybridization Shows the Differential Response of Various Genes to Environmental Stimuli.

Seedlings were exposed to anaerobiosis, heat stress, or light for various times as indicated at the bottom of the figure. Total RNA was collected from the roots and shoots and analyzed as described in Methods.

induction during heat stress. That level can differ in the roots and the shoots (Table 1). Organ-specific differences in the aerobic and anaerobic sucrose synthase RNA levels shown here are similar to the results of others (Springer et al., 1986). While aerobic sucrose synthase levels are lower in the root than in the shoot, most other glycolysis-related gene transcripts tested show the same or higher levels in the root. Anaerobic induction leads to similar levels of glycolysis-related RNA accumulation in both tissues.

The GAPDH sequences all show distinguishable accumulation patterns in plants under different stress conditions. The clone for the plastid-directed GAPDH, or GAPA1 (pZm57; Brinkmann et al., 1987), shows a low level of hybridization in all samples, but increases dramatically when the seedlings are in the light (Figure 2). No other family member analyzed shows a light-induced change. This corroborates an earlier study involving the immune precipitation of in vitro translated poly(A)$^+$ RNA from a variety of species (Cerff and Kloppstech, 1982). Shih and Goodman (1988) found that light induced both tobacco cytosolic and chloroplastic GAPDH, but they tested older plants under a different light/dark regime.

Table 1 shows the quantified values for RNA accumulation during anaerobic and heat stress. The clone representing the *Gpc1* transcript, pZm9, shows a decrease in RNA accumulation during anaerobic stress only after more than 6 hr of treatment, and a relatively steady level during heat shock,. GAPC2 RNA levels decrease more dramatically during anaerobic treatment, by a factor of 2 after 6 hr in roots, but again, no significant change is seen during heat shock. Of the cloned GAPDH gene family members, only GAPC3 shows anaerobic and heat shock induction. In shoots, there is a 15.6-fold increase during the first 6 hr of anaerobic stress, and a fivefold increase during heat shock. The anaerobic induction of *Gpc3* parallels that of ADH1 and aldolase (Figure 2; Gerlach et al., 1982; Rowland and Strommer, 1986). In all three cases, RNA accumulation is maximal after 6 hr of anoxia, whereas sucrose synthase 1 RNA continues to accumulate past this time point.

As shown in Table 1, GAPC3 and ADH1 transcripts have similar induction patterns during anaerobic stress since both increase from very low initial levels. However, extracts measured for enzyme activity show a fourfold to tenfold induction of ADH in the roots after 24 hr of anaerobic stress, and only a 10% to 50% increase in GAPDH specific activity (Kelley and Freeling, 1984; data not shown). Both *Adh* genes show increased RNA accumulation from low basal levels (Dennis et al., 1985), but, since the different *Gpc* genes show variable basal and induced levels, increases in GAPDH specific activity during anaerobic stress should be compared to increases in total GAPC encoding transcripts. An estimate of total GAPC-related RNA was

Table 1. RNA Levels of Various Genes Relative to the Levels in Control Shoots[a]

	Shoot			Root		
Probe	C[b]	A[c]	H[d]	C	A	H
HSP70	1	0.7	>10.0	0.5	0.4	>10.0
Sucrose synthase 1	1	6.8	1.6	0.5	6.4	1.4
Aldolase	1	5.4	1.4	1.4	5.7	3.1
ADH1	1	21.0	2.6	2.3	26.2	5.2
pZm9	1	1.0	1.3	0.5	0.5	0.6
pGAPC2	1	0.7	1.8	1.2	0.5	1.8
pGAPC3	1	15.6	5.1	4.0	24.0	9.5

[a] Data were corrected for loading error by normalizing to ribosomal hybridization. Shown are the averages of at least two determinations.
[b] Values for control (nonstressed) treatments.
[c] Values for 6-hr anaerobic treatments.
[d] Values for 1-hr heat stress treatments.

made on the RNA gel blots by hybridization with an end-labeled oligomer complementary to 18 bases in pGAPC2 and pGAPC3 starting at number 350 in the pGAPC2 sequence (Figure 1A). This hybridization is labeled "18mer" in Figure 2. Although this probe has a 1-base difference from pZm9, conditions were such that hybrids should form. Note that there are six mismatches with pZm57, and no increase in hybridization is seen in the light-induced RNA sample. This probe does show that total GAPC-related RNA increases after 6 hr of anaerobic stress. Since there are probably only three cytosolic genes, GAPC3 RNA levels after 6 hr of anaerobic stress are similar to those from the other genes at this time point; if GAPC3 levels were more significant, a greater level of induction would be seen with this probe. This relative accumulation of the GAPC transcripts is also seen when the RNA is translated in vitro (see below).

Gpc Translation Product Analysis

The relative levels of the three GAPDH transcripts can be approximated if the GAPDH products of in vitro translations could be separated. This assumes that there are similar numbers of labeled residues in each translation product, and each behaves with similar translational efficiencies in the in vitro translation system. In fact, there is 1 less methionine in the sequenced region of *Gpc3* (amino acid 271, Figure 1B). Some separation could be achieved by translating RNA from various time points in a rabbit reticulocyte system and analyzing the products on sodium dodecyl sulfate-polyacrylamide gels. As seen in Figure 3A, the total in vitro translation products from aerobic and 6-hr anaerobic root RNA samples are similar, with some new bands seen when RNA from stressed seedlings is translated (lanes 2 and 3). Similar results are seen in the shoots during anaerobic treatment, as well as heat stress: stress induces specific message accumulation, with only some decay in the pre-stress message population (lanes 4 to 6, respectively). Relatively little change is seen during light treatment (compare lanes 4 and 7). When these samples are translated and immune-precipitated with anti-GAPDH sera raised against the cytosolic mustard enzyme, an increase in immune-precipitable material is seen during anaerobic stress (compare lanes 1 to 2, 3 to 4, in Figure 3B). The linearity of the precipitation is seen in lane 8, where half the amount of incorporated counts used in lane 4 was analyzed. Additionally, the antibody recognizes two bands. During anaerobic treatment, only the faster migrating band shows an increase in labeling, roughly in parallel to the level of GAPC3 RNA seen in the RNA gel blots of Figure 2. When shoot heat shock RNA translation products are immune-precipitated, again, only the faster migrating band increases in intensity (compare lane 5 to 3). As seen with RNA gel blot analysis, light has little effect on the three cytosolic GAPDH genes. The plastid GAPDH translation product is not efficiently recognized by the anti-mustard sera (lane 6). Similar results are obtained with an antibody raised against yeast enzyme (data not shown).

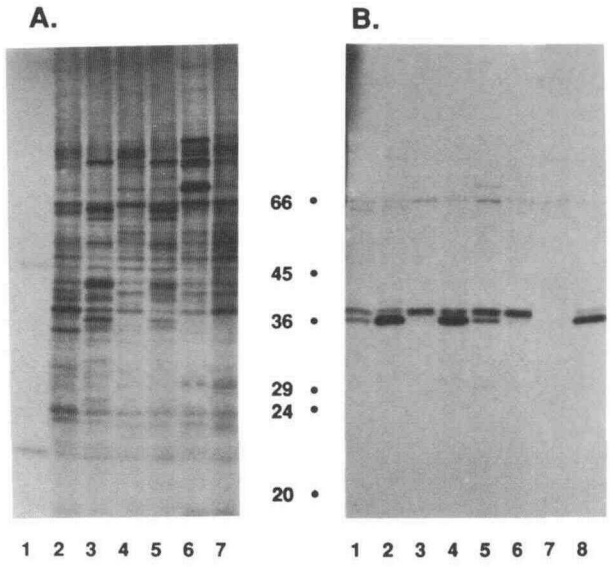

Figure 3. In Vitro Translation and Immune Precipitation Shows Changes in the Expression of *Gpc* Genes under Different Stress Conditions.

(A) Total in vitro translation products separated on an SDS-polyacrylamide gel. Equal incorporated counts per minute are analyzed from translations using RNA of aerobic roots (lane 2), 6-hr anaerobic roots (lane 3), aerobic shoots (lane 4), anaerobic shoots (lane 5), 1-hr heat-stressed shoots (lane 6), and 48-hr light-treated shoots (lane 7). Lane 1 shows the endogenous translation products.

(B) Immune precipitates of GAPC from in vitro translations. Lanes 1 to 6, 8, immune precipitations using rabbit anti-mustard GAPC sera. RNA sources were aerobic roots (lane 1), 6-hr anaerobic roots (lane 2), aerobic shoots (lane 3), 6-hr anaerobic shoots (lane 4), 1-hr heat-stressed shoots (lane 5), and light-treated shoots (lane 6). Lane 8 is as per lane 4, but precipitation was from half the amount of lysate. Lane 7 is the immune precipitation of anaerobic shoot RNA translation products with rabbit nonimmune sera. Migration of molecular weight markers is shown in kilodaltons.

Although the faster migrating band correlates in all cases with GAPC3 RNA levels as seen on the RNA gel blots, definitive proof was obtained through hybrid select translation. Seedlings were treated for 0 hr or 21 hr, and poly(A)$^+$ RNA was prepared from the pooled roots and shoots of similarly treated seedlings. The resultant translation products are shown in lanes 2 and 3 of Figure 4, with the immune precipitations shown in lanes 16 and 17. When the two RNA sets are used in hybrid selection, each immobilized maize GAPDH cDNA fragment selects a message encoding a polypeptide with a slightly different apparent mobility. As expected, the 3′-untranslated region

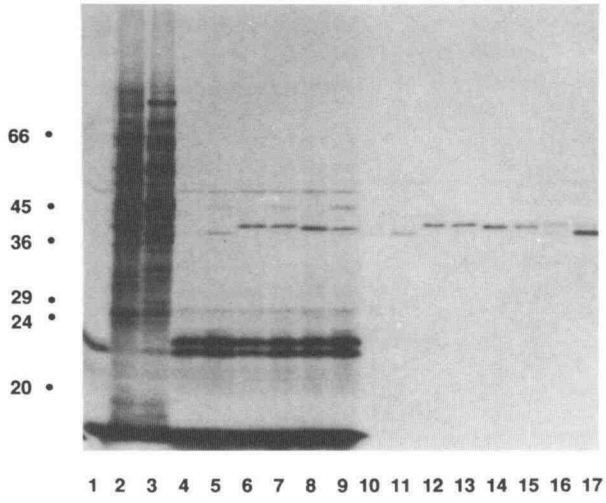

Figure 4. The Three GAPDH Sequences Encode Unique Polypeptides.

Poly(A)$^+$ RNA was prepared from aerobic and 21-hr anaerobically treated seedlings and translated in vitro. Lane 1 shows the endogenous translation products. In lanes 2 to 17, even-numbered lanes used aerobic RNA for translation, odd-numbered lanes used the anaerobic RNA. Lanes 2 and 3 represent poly(A)$^+$ translation products, which are immune-precipitated with the anti-GAPDH sera in lanes 16 and 17. Lanes 4 to 9 represent translation products of hybrid-selected RNAs using the 3′ sequences of pGAPC3 (lanes 4 and 5), pGAPC2 (lanes 6 and 7), and pZm9 (lanes 8 and 9). These samples are immune-precipitated in lanes 10 to 15, respectively. Migration of molecular weight markers is shown in kilodaltons.

sequence of pGAPC3 selects a message that is translated into the fastest migrating product (lanes 4 and 5); its level increases during anoxia, as was seen by RNA gel blot hybridization (Figure 2). By similar analysis, pGAPC2 corresponds to the slowest mobility protein product, and message levels decrease slightly during this treatment (lanes 6 and 7). pZm9 selects a message of slightly faster mobility than pGAPC2; its message level also decreases during stress (lanes 8 and 9). Most other bands present in lanes 2 to 9 are due to endogenous reticulocyte messages. The translation products seen in lanes 5, 7, and 9 with mobility and induction similar to those of ADH are not seen consistently. Only those bands in the 38-kD region of the gel are specifically precipitated by the anti-GAPDH sera (lanes 10 to 15), confirming that all three clones code for GAPDH.

DISCUSSION

Three cDNA sequences coding for maize cytosolic GAPDH are compared and are apparently encoded by three separate genes. In the case of clones pGAPC2 and pZm9, the coding regions are highly homologous, but the divergence of the 3′-untranslated regions still suggests that they are separate genes. Alternatively, there could be a single coding region spliced to alternative 3′-untranslated regions. However, introns have not been observed near or past the stop codon in *Gpc1* or other GAPDH genes (Martinez, Martin, and Cerff, 1989). We are now identifying clones pZm9, pGAPC2, pGAPC3, and pZm57 as corresponding to the transcription products of the nuclear genes *Gpc1*, *Gpc2*, *Gpc3*, and *Gpa1*, respectively. Genomic clones have been described for *Gpc1* (Martinez, Martin, and Cerff, 1989) and *Gpa1* (Quigley, Martin, and Cerff, 1988). Clones for maize *Gpb* and its transcript have not yet been identified.

All plants must have at least two GAPDH genes: one for glycolytic activity in the cytosol and a separate nuclear gene for Calvin cycle reactions in the chloroplast. By comparing various GAPDH sequences, it was reasoned that the maize plastid-directed gene product *Gpa* was derived from the ancient chloroplast progenitor during eukaryotic evolution (Brinkmann et al., 1987). The derivation of the three cytosolic GAPDH genes identified here will require more detailed analysis, such as genomic sequencing and analysis of other plant species. The divergence after the *Gpc2* and *Gpc1* stop codons is reminiscent of the *Adh* gene family. The *Adh1* and *Adh2* genes show an 82% nucleotide similarity in their coding regions, but nearly complete divergence in their introns and untranslated regions (Dennis et al., 1985). In barley, the *Adh* gene family apparently is composed of two *Adh2*-like genes and one similar to *Adh1*, implying that the duplication of the *Adh* gene occurred before the divergence of maize and barley (Trick et al., 1988).

The derivation and evolution of *Gpc3* is a potentially different story from *Gpc2* and *Gpc1* since its expression pattern is completely different from these other members of the gene family. Other plant species of variable phylogenetic distance from maize may not always have multiple cytosolic GAPDH genes and may not show anaerobic GAPDH regulation. RNA gel blot analysis of soybean RNA with the complete clones of either pGAPC2 or pGAPC3 under lower stringency conditions shows hybridization but no anaerobically induced RNA accumulation. Both the maize ADH1 and ADH2 clones do show induction under these conditions (D.A. Russell and M.M. Sachs, manuscript in preparation). Although this doesn't determine the number of soybean genes, it may imply a difference in physiology in the two highly diverged plants. In barley, a species more closely related to maize, DNA gel blot analysis identified only one gene (Chojecki, 1986). This sequence shows 92% amino acid similarity to the cloned region of *Gpc3* and an 86% similarity to the homologous region of the other two clones. Although the barley GAPDH sequence appears more related to the stress form of maize GAPC, the similarity may not be physiologically significant since many of the differences occur at positions that are normally variable in GAPDH from different species (Harris

and Waters, 1976). Recently, rice was shown to have two separable GAPDH translation products; the differential anaerobic expression of these isoforms suggests that there may be at least two genes in this monocot (Ricard, Rivoal, and Pradet, 1989).

Gpc3 is also apparently unique in that it is the only member of the gene family to show heat stress induction. Other organisms also show GAPDH heat shock induction at some level. *TDH3*, the yeast gene whose product is most highly expressed under normal conditions, is selectively synthesized over the other two gene products (Lindquist and Craig, 1988; Piper et al., 1988). Not only does *Xenopus* have increased GAPDH protein synthesis and enzyme activity during heat shock, but the extent of induction measured in vitro increases when the assays are conducted at higher temperatures; this and activity gel studies imply that there may be two different genes in this species as well (Nickells and Browder, 1988). Other glycolysis-related genes are also induced at some level by heat stress in yeast (Lindquist and Craig, 1988) and *Xenopus* (M. Marsden, R. W. Nickells, T. I. Wang, M. Kapoor, and L. W. Browder, manuscript submitted for publication). Similarly, our results show a low level of heat stress induction of some anaerobically induced genes (Table 1). The heat stress induction of anaerobic genes is less than that observed for these genes under anaerobic stress, or for HSP70 under heat stress.

The mechanism of heat stress induction of maize anaerobically inducible genes is unknown. In yeast, the gene for the glycolytic enzyme phosphoglycerate kinase has an upstream heat stress response element (Pelham, 1985) that is necessary to maintain the high expression levels of this gene during heat stress (Piper et al., 1988). When the maize anaerobic genes are examined, a perfect match to the heat stress response element can be seen in the third intron of all sequenced *Adh1* alleles, with single base differences seen in the 5′ upstream region of genes encoding ADH2 and sucrose synthase 1, as well as the second exon of the gene encoding aldolase. More stringent searches involving the periodic GAA motif of Amin, Ananthan, and Voellmy (1988) find no likely heat response elements. Heat stress-induced message accumulation of these genes instead may be due to post-transcriptional mechanisms, as seen for ADH1 in anoxic maize (Rowland and Strommer, 1986) and GAPDH in hypoxic mammalian cells (Webster, 1987; Webster and Murphy, 1988). It is also possible that heat stress induces anaerobic genes through inadvertent hypoxia, which causes a different induction pattern than anoxia (Wignarajah and Greenway, 1976). Precautions were taken to minimize hypoxia due to increased respiration, but other factors, such as reduced oxygen solubility in the warmer cytosol, were not controlled for. Additionally, effects similar to hypoxia would be created by the heat stress-induced damage to mitochondria. It has been surmised that heat stress increases glycolytic gene activity in other systems because of mitochondrial damage and/or increased ATP turnover (M. Marsden, R.W. Nickells, T.I. Wang, M. Kapoor, and L.W. Browder, manuscript submitted for publication), although low ATP levels per se do not induce chromosome puffing of heat shock loci in *Drosophila* (Leenders et al., 1974). Some of the changes seen in the mitochondria of mammalian cells during heat stress, such as more defined cristae and mitochondria outer membrane (Welch and Suhan, 1985), are also seen in barley aleurone layers (Belanger, Brodl, and Ho, 1986) and soybean root tips (Chen et al., 1988). Mitochondrial size changes and other parameters are more variable in the different organisms. In comparison, anaerobic maize root mitochondria typically show swelling, but changes in the cristae are less consistently seen (Aldrich et al., 1985; Vartapetian, Snkhchian, and Generozova, 1987).

The physiological role of GAPDH induction during anaerobic stress in maize is understandable in light of results with other glycolytic genes. To date, clones that show a pattern of anaerobically induced RNA accumulation all have been related to glycolysis. Additionally, of the messages shown to be selectively translated during anaerobic stress, all have fallen into this same class (Sachs and Ho, 1986), although the sucrose synthase gene family may be an exception (McElfresh and Chourey, 1988). The gene products also typically show an increase in terms of accumulated protein or enzyme activity; again, sucrose synthase may be an exception. Because the maize cytosolic GAPDH gene family consists of differentially regulated members, and the anaerobically inducible form has a much different protein sequence than the other two forms, it is tempting to look for a selective advantage, rather than simply differences due to neutral selection. It is intriguing that the cytoplasm of maize root tip cells is rapidly acidified during anaerobic stress (Roberts et al., 1984), and GAPDH from a variety of organisms is very pH-sensitive, with about one-third the measured activity at pH 7 versus pH 8 (Duggleby and Dennis, 1974; Harris and Waters, 1976; data not shown). However, limited tests with enzyme assays using various published buffer conditions at different pH values showed no decrease in pH optima for anaerobic versus aerobic extracts (data not shown).

Since it has been previously shown that the expression of the anaerobically induced maize genes seems to have a translational control component as well as that of message accumulation (Sachs, Freeling, and Okimoto, 1980), the relative translation of this gene family needs to be investigated. Pre-stress messages are generally not translated during anaerobic stress, but they do not immediately degrade (Sachs, Freeling, and Okimoto, 1980; Figure 3A). It is expected that GAPC1 and GAPC2 are in this class (Figure 2 and Table 1). All three gene products are shown to have different mobilities on denaturing gels (Figure 4). Because the *Gpc* gene products are separable and the family shows differential expression during anoxia, these evolutionarily related genes should be useful in defining transcriptional and translational control mechanisms in anoxic maize.

METHODS

Materials

Inbred maize line B73 was used for all expression studies, while the cDNA library was generated from the Berkeley Fast inbred line. The following materials were used in this study: pMON9501 (HSP70, a maize HSP70 genomic clone; Rochester, Winter, and Shah, 1986), pZmL793 (maize ADH1 cDNA; Dennis et al., 1984), pZmL54 (maize aldolase cDNA; Dennis et al., 1988), psh (maize sucrose synthase I genomic clone; B. Burr), pZm57 (maize glyceraldehyde-3-phosphate dehydrogenase cDNA for chloroplast functions, corresponding to GAPA1; Brinkmann et al., 1987), pZm9 (maize cytosolic GAPDH cDNA for the GAPC1 transcript; Brinkmann et al., 1987), pGmr1 (soybean genomic ribosomal clone; Zimmer, Jupe, and Walbot, 1988), pHSP35-403 (yeast genomic *TDH3* clone; S. Lindquist), rabbit anti-mustard GAPC sera (Cerff and Kloppstech, 1982), rabbit anti-yeast GAPDH sera (Nickells and Browder, 1988), and rabbit nonimmune sera (of S. Koehler). Two complementary 18-base oligomers were synthesized on an Applied Biosystems 380B DNA synthesizer for use in sequencing and RNA gel blot analysis. The sense-directed oligomer starts at base 350 in Figure 1A.

Seedling Treatment

Maize seeds were germinated 4 days to 5 days in the dark, 85% relative humidity, at 28°C, except where noted. For anaerobic stress, they were submerged for various times in 5 mM Tris-Cl, pH 8, 375 μg/mL Augmentin® (Beecham Labs) with no more than four seedlings per 30 mL of buffer. Heat stress was performed by wrapping the plants loosely with wet paper towels in a covered glass tray preincubated at 40°C. Prewarmed, water-saturated air flowed underneath the towels to prevent seedling desiccation. Light treatment was accomplished by moving the seedlings for the final 2 days of growth underneath fluorescent lamps.

cDNA Library

cDNA was synthesized from 6-hr anaerobic corn root RNA by the method of Gerlach et al. (1982) and ligated into pUC8 (Vieira and Messing, 1982). The library was probed with a nick-translated (Rigby et al., 1977) HindIII insert of pHSP35-403. Colonies that scored positive after a second screening were then further analyzed.

RNA Analysis

Total RNA was purified by a modification of MacDonald et al. (1987). Seedlings were frozen in liquid nitrogen and then pulverized in the presence of dry ice. The powder was vortexed in 5 volumes of 5 M guanidine-HCl, 10 mM Tris base, 5 mM EGTA, pH 8.5, and 0.1% lauryl sarcosine, followed by ethanol precipitation in the presence of 1/30 volume of 3 M potassium acetate, pH 6.0. The second extraction was in 2.5 volumes of 4 M guanidine-HCl, 10 mM EDTA, pH 7.0. Following precipitation and drying, the pellet was resuspended in 1/7 volume of 10 mM EDTA, pH 7.0. Extractions with chloroform/butanol (4:1) were necessary to remove green pigment from light-grown shoots. The aqueous RNA sample was spun to clear, the supernatant saved, and the pellet re-extracted three more times. The pooled supernatant was brought to 1.5 M sodium acetate, pH 6.0, and precipitated overnight at −20°C, and the precipitate was collected, resuspended in 1/14 volume of water, and precipitated by adding 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. The pellet was rinsed, dried, resuspended in water, and spun to remove insolubles. $A_{260\,nm}/A_{280\,nm}$ ratios were typically 2.0 to 2.2, with yields of 700 μg/g of shoots, fresh weight, and 300 μg/g of roots.

RNA was separated on 1.3% agarose formaldehyde gels (Ogden and Adams, 1987), but the buffer was 20 mM Mops, 5 mM sodium acetate, 1 mM EDTA, and 0.1 μg/mL ethidium bromide, and it was recirculated. Each lane contained 10 μg of total RNA. To better quantify the induction level, dilutions of the 6-hr anaerobic samples were also loaded. Capillary transfer to nylon matrix (Micron Separations Incorporated) was done in 10 × SSC. The matrix was dried, and the RNA was UV cross-linked on a transilluminator (Church and Gilbert, 1984) under optimized conditions, as determined empirically. Before use, the blots were washed for 10 min in 0.1 × SSC, 0.1% SDS.

Clones were labeled by the random hexamer method (Feinberg and Vogelstein, 1984) for use as probes. The oligomer was terminally labeled with α-[^{32}P]dCTP (Lewis et al., 1986). Hybridization and washes were patterned after Church and Gilbert (1984). Prehybridization was in 0.5 M sodium phosphate, pH 7.2, 1% BSA, 1 mM EDTA, 5% SDS, and 0.1 mg/mL sheared salmon sperm DNA at 65°C for 2 hr to 4 hr. Hybridization was in the same solution overnight. Hexamer product [1 × 10^7 cpm (Cerenkov)] or end-labeled oligomer (4 × 10^6 cpm) was used in 10 mL of solution, except for the rDNA probe, when 1 × 10^5 cpm was used. The oligomer probe was hybridized at 37°C. Blots probed with the clones were washed twice in solution 1 (40 mM sodium phosphate, pH 7.2, 0.5% BSA, 1.0 mM EDTA, 5% SDS) for 15 min and then four times in solution 2 (40 mM sodium phosphate, 1 mM EDTA, 1% SDS) for 5 min, all at 65°C. A final wash was done in solution 3 (0.1 × SSC, 0.1% SDS) for 15 min at room temperature. For the oligomer probe, the first two wash solutions were not used; 1-hr washes with solution 3 were done at sequentially higher temperatures, from 22°C to 45°C, until hybridization to the ribosomal bands was minimized. Blots were exposed to preflashed XAR-5 film (Kodak), along with an intensifying screen at −70°C. Figures were generated with exposures of 8 hr to 24 hr except for pGmr1 (40 hr to 60 hr) and the oligomer (5 days). Multiple exposures were used for densitometric scanning (Joyce-Loebl). Data were corrected for loading error with the rRNA signal. To allow reuse, blots were stripped of probe by submersion in solution 3 for 15 min at 90°C.

Sequencing

cDNAs of interest were subcloned into Bluescript plasmids (Stratagene). The Sequenase® kit (U.S. Biochemicals) was used according to manufacturer's protocols with α-[^{35}S]dATP, with both single- and double-stranded DNA as a template. The sequence was confirmed by analyzing both strands.

Protein Synthesis

Rabbit reticulocyte lysate (Promega Biotech) was used to analyze in vitro translation products according to the manufacturer's protocol, but with Trans^{35}S Label® (ICN; this product has 20% of the label in cysteine and 70% in methionine). The translations were programmed with 3 μg of total maize RNA per 10-μL reaction (a nonsaturating amount). Equal TCA-precipitable counts per minute, or a similar volume of nonprogrammed sample, were analyzed on a 10% SDS-polyacrylamide gel. Fluorography was accomplished by the method of Jen and Thach (1982). Immune precipitation was performed essentially as per Jagus (1987a) with equal TCA-precipitable counts per minute or a similar volume of nonprogrammed sample, except where noted otherwise. Before specific antisera were added, nonspecific binding products were removed by a 30-min incubation at 4°C with nonimmune sera and then cleared with protein A-Sepharose.

Hybrid Selection

Poly(A)$^+$ RNA was collected from aerobic and 21-hr anaerobic treated seedlings essentially according to Jacobson (1987) but with only one round of selection in a batch method. DNA for hybrid selection was prepared by cutting the plasmids with the appropriate restriction enzymes to liberate the SalI to 3′ untranslated region fragments, separating the fragments on agarose/Tris-acetate gels, and transferring the band of interest by continuing electrophoresis through a piece of nylon membrane (Micron Separations Incorporated) that is positioned in front of that band. Transfer was monitored by observing the ethidium bromide-stained band mobility. The membrane was then base-treated and neutralized as per Reed and Mann (1985), followed by UV cross-linking to optimize retention of the DNA and subsequent hybridization (Church and Gilbert, 1984). RNA was hybrid-selected following the method of Jagus (1987b). Three membranes, each with a cDNA fragment from a different GAPDH gene, were incubated for 3 hr at 37°C in buffer (20 mM Pipes, pH 6.4, 40% formamide, 400 mM NaCl, 2 mM EDTA, 0.1 mg/mL tRNA, 0.5% SDS). The membranes were rinsed in the same buffer once. Poly(A)$^+$ RNA from the two time points was brought to 25 μg/mL in 200 μL of the same buffer, heated 2 min at 65°C, and then incubated 8 hr with the immobilized DNA. Nonspecific RNA was removed by washing 3 × 1 min at 50°C in 1 × SSC, 2 mM EDTA, 0.5% SDS, followed by three washes in 10 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, pH 7.5. Specific RNA was eluted by two successive incubations at 90°C in 2 mM EDTA, 5 mM KCl, 10 μg/mL tRNA, followed by a quick freeze in dry ice/ethanol. Following ethanol precipitation, the RNA was resuspended in water and heated 1 min at 65°C, and the particulate matter was removed by centrifugation. RNA was translated and analyzed as above.

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