DNA sequences required for anaerobic expression of the maize alcohol dehydrogenase 1 gene

(gene regulation/transient expression/electroporation/Zea mays)

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ABSTRACT

Expression of the maize alcohol dehydrogenase 1 (Adhl) gene is transcriptionally regulated under conditions of anaerobic stress. DNA sequences required for the expression of Adhl have been identified by a functional analysis of in vitro constructed hybrid genes consisting of the Adhl promoter fused to the chloramphenicol acetyltransferase coding region. A series of 5' deletions, 3' deletions, hybrid promoters, and linker scanning mutants of the Adhl-CAT hybrid gene were introduced into maize protoplasts by electroporation and assayed for chloramphenicol acetyltransferase activity after incubation of the protoplasts under different oxygen tensions. The results indicate that a 40-base-pair DNA sequence within the Adhl promoter is required for anaerobically regulated expression of the hybrid gene. Clustered point mutations in this sequence show that it is composed of two essential regions, each ~15 base pairs, separated by a 10-base-pair DNA sequence that does not appear to be important for anaerobic expression. Attachment of this 40-base-pair element to an unrelated promoter shows that this DNA sequence is both necessary and sufficient for induction of gene expression by low oxygen stress.

Oxygen deficiency has a dramatic effect on the pattern of gene expression in maize seedlings. During the first 2 hr of anaerobic stress, normal (aerobic) protein synthesis is repressed, and a class of ~20 polypeptides is synthesized (1). The synthesis of these anaerobic polypeptides accounts for >70% of total protein synthesis after 5 hr of anaerobiosis and is the result of an increase in the steady-state levels of the mRNAs (2, 3).

The maize alcohol dehydrogenase 1 enzyme is one of the polypeptides induced in response to anaerobic conditions (1), such as flooding, and serves as the terminal dehydrogenase for continued energy production by ethanol fermentation. Adhl gene mRNA levels in aerobically grown roots are ~2% the level found in aerobic roots; mRNA levels increase in concentration during anaerobic treatment, reaching a maximum within 5 hr (3, 4). This increase in the level of Adhl gene mRNA can be attributed, for the most part, to an increase in the transcription rate of the Adhl gene (4, 5).

Howard et al. (6) have examined the expression of the endogenous Adhl gene and a chimeric Adhl gene in maize protoplasts. Over a 24-hr period there was an incremental increase in the expression of the endogenous Adhl gene with decreasing oxygen concentrations: at 10% oxygen/90% N₂, there was a 2- to 4-fold increase in the level of Adhl gene mRNA and at 5% oxygen/95% N₂, a 5- to 10-fold increase. However, at 1% oxygen/99% N₂, no increase in Adhl expression was detected. This latter observation was probably the result of reduced cell viability under these conditions. The fold increase in Adhl expression was lower than has been reported in maize seedlings (3, 4); this is due to the higher level of "aerobic" Adhl expression in the tissue culture cells, which is not surprising given the cells are grown in solution and are probably hypoxic. The Adhl chimeric gene Adh-CAT consists of the Adhl promoter linked to the chloramphenicol acetyltransferase (CAT) coding sequences and nopaline synthase (nos) 3' signal. Adh-CAT, introduced into maize protoplasts by electroporation, was expressed ~4-fold higher in low oxygen concentrations than under control conditions. By all criteria examined, expression of Adh-CAT paralleled the expression of the endogenous Adhl gene in maize protoplasts and the anaerobic response in cell culture was qualitatively similar to the response in maize seedlings.

In this report we identify the sequence elements necessary for anaerobic induction of Adh-CAT based on the expression of a series of in vitro-manipulated Adh-CAT chimeric genes. Our analyses show that there is an anaerobic regulatory element (ARE) between positions -140 and -99 of the maize Adhl promoter. The ARE is composed of at least two sequence elements, positions -133 to -124 and positions -113 to -99, both of which are necessary, and together are sufficient for low oxygen expression of Adh-CAT gene activity.

MATERIALS AND METHODS

Construction of Plasmids. All plasmids were constructed by standard recombinant DNA techniques (7) using subcloned DNA fragments of the maize Adhl-1S gene (8, 9) or the cauliflower mosaic virus (CaMV) genome (a gift from D. Merlo, Agrigenetics, Madison, WI). We have numbered the sequence (Fig. 1) with the cap site designated as +1 according to the correction published by Ellis et al. (10). The plasmid pAdhCAT, described (6), contains a BAL-31-generated promoter fragment, from positions -1094 to +106 of the Adhl-1S gene, attached to the reporter gene cassette pCN100 (CAT–Nos) (6) by a BamHI linker. A truncated Adhl promoter gene, pAdhCAT-140, was constructed by subcloning the PstI fragment of pAdhCAT, utilizing the only PstI site at position -140 in the Adhl-1S promoter and the PstI site in the pUC19 polylinker. Progressive 5' deletions of pAdhCAT-140 were obtained by BAL-31 digestion. Generation of 3' deletions was as follows: the Adhl-1S promoter fragment from position -1094 to position +106 in pUC19 was digested at the only SmaI site in the polylinker at the 3' end of the promoter, followed by BAL-31 digestion; this fragment was ligated to SalI linkers, and digested with SalI and with BamHI (at position -1094 of the Adhl-1S promoter); and the relevant fragments were isolated and cloned into pUC19. All

Abbreviations: ARE, anaerobic regulatory element; CAT, chloramphenicol acetyltransferase; CaMV, cauliflower mosaic virus; LS, linker scanning.

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deletion endpoints were characterized by restriction endonuclease mapping and dideoxy sequencing (11). Our data confirm the published sequence of the promoter region (8), except from position -123 to position -121 where we find a CC dinucleotide rather than a CAC trinucleotide.

The linker scanning (LS) mutations were constructed by joining the Sal I linkers of the appropriately matched 3' and 5' deletions. In the case of pLS-113/-99, the Sal I termini were filled in with the Klenow fragment of Escherichia coli DNA polymerase I before ligation.

The plasmid p355CN (CaMV 35S promoter -CAT-Nos) was constructed as follows: a HincII-Hph I fragment of the CaMV 35S promoter (viral nucleotides 7015-7450) (12) was ligated with BamHI linkers and inserted into the BamHI site at the 3' end of pCN100. p355SCN was constructed by BAL-31 deletion from the EcoRV site (nucleotide 7665) within the 35S promoter. p355SCN was determined to have an endpoint 45 base pairs upstream from the cap site of the 35S promoter (13) by dideoxy sequencing (11). Neither p355CN nor p355SCN contain any of the transcribed sequences of the CaMV 35S transcript.

Hybrid promoters were constructed by ligation of the Sal I site of p355SCN with the Sal I site of various 3' deletions of the Adh/-35S promoter.

Plant Material and Protoplast Isolation. A Zea mays c.v. Black Mexican Sweet XII-II suspension cell line (14) was generously provided by P. Chourely (Florida State University). The maize suspension cells were grown in a modified MS liquid medium (13) at 26°C. Protoplasts were isolated (16) and prepared for electroporation as described (6).

**Electroporation and CAT Assays.** Expression analysis of pAdhCAT mutant constructions was performed as described (6). For convenience and by analogy to the low-oxygen response in maize seedlings, which is qualitatively similar to the response in protoplasts (6), we have designated the expression of Adh-CAT activity in maize protoplasts in 20% O2/80% N2 as “aerobic” and in 5% O2/95% N2 as “anaerobic” expression.

Each construction was assayed in at least two and in most cases three to five separate electroporation experiments using different protoplast preparations. Because of variation between experiments, results given have been normalized within each experiment by assignment of a value of 100% for the anaerobic (5% O2/95% N2) expression of Adh-CAT after subtraction of the nonspecific background products.

**RESULTS**

**Analysis of 5' Deletions.** Low oxygen (5% O2/95% N2) increases expression of the hybrid gene Adh-CAT to -4-fold above the level observed under "aerobic" conditions (20% O2/80% N2) (Fig. 2). Deletion of 956 base pairs of 5' DNA sequence from pAdhCAT to the Pst I site at position -140 (pAdhCAT-140) does not alter the level of induction of the Adh-CAT chimeric gene, although the level of expression under both conditions drops ~25% (Fig. 2). Thus, ~250 base pairs (positions -140 to +106) of DNA, on the 5' side of the coding region of AdhI, is sufficient to promote expression of CAT enzyme activity under conditions of low oxygen in maize protoplasts. This Adh-CAT-140 chimeric gene is also sufficient to confer anaerobic expression of the CAT activity in transgenic tobacco when an enhancer-like element is attached adjacent to and on the 5' side of the maize AdhI sequences (10).

A series of 5' deletions of pAdhCAT-140 were constructed to delineate further the sequences necessary for anaerobic expression. Fig. 2 shows that deletion to either position -124 or position -112 dramatically reduces expression (~3-fold); however, these deletions appear to retain a slight degree of inducibility (~1.5-fold). Deletions to position -99 (Fig. 2) and below (data not shown) reduce anaerobic and aerobic levels of CAT activity to background levels. These results delimit the 5' boundary of the ARE to between positions -140 and -124. The difference in the level of expression of the position -124 and position -112 deletions compared to the position -99 deletion indicates there may be two com-

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**FIG. 1.** Structure of the plasmid pAdhCAT. pAdhCAT (5,700 base pairs) contains ~1200 base pairs of Adh-1S 5'-flanking DNA (solid bar) from position +106 to -1094 joined by a BamHI linker to the CAT coding region (hatched bar) and 3' sequences from the nos gene (open bar). Plasmid sequences from pUC19 are shown by the single line. Restriction endonuclease sites are indicated as E, EcoRI; B, BamHI; P, Pst I; H, HindIII.

**FIG. 2.** CAT activity of 5' deletions. Maize protoplasts (3x10^6 protoplasts per ml) were electroporated in the presence of pAdhCAT at 100 μg/ml (lanes 1 and 2), pAdhCAT-140 at 100 μg/ml (lanes 3 and 4) or 5' deletions of pAdhCAT-140 at 100 μg/ml with deletion endpoints at positions -124 (lanes 5 and 6), -112 (lanes 7 and 8), or -99 (lanes 9 and 10). After a 20-hr incubation in either 5% O2/95% N2 or 20% O2/80% N2, the protoplasts were harvested and assayed for CAT enzyme activity as described (6). Positions of the [3H]chloramphenicol (CM) and its acetylated derivatives 3-acetylchloramphenicol (3 AcCM) and 1-acetylchloramphenicol (1 AcCM) are indicated. The substance running between the 1 AcCM and 3 AcCM (asterisk) is a compound of unknown identity and is found in the [3H]chloramphenicol. (Lower) Relative CAT activities expressed as a percentage of CAT activity from pAdhCAT at 5% O2/95% N2, after subtraction of nonspecific background activity. The values given represent the average of at least four independent experiments with the standard error of the mean indicated. Open bars, 5% O2/95% N2; Solid bars, 20% O2/80% N2.
ponents to the ARE. A deletion to position -124 or -112 reduces, but does not abolish expression; however, further deletion to position -99 eliminates all expression above background.

**Analysis of Hybrid Promoter Fusions.** We have shown that expression of a hybrid gene containing the CaMV 35S promoter 35SCN is not regulated by anaerobic stress but shows a reduction in activity under hypoxic conditions (Fig. 3). To determine the 3' endpoint of the ARE, we have linked a series of 3' deletions of the Adhl gene upstream of the "TATA box" to a deleted CaMV 35S promoter. This defective 35SCN gene, designated Δ35SCN, is deleted to position -45 of the promoter and retains its TATA box, but upstream sequences required for expression of 35SCN have been deleted (Fig. 3) (13). Fusion of sequences from position -35 to position -1094 or position -81 to position -1094 of the Adhl gene confers anaerobically regulated expression on Δ35SCN (Fig. 3). When Adhl promoter sequences were deleted on the 3' side of position -99, expression of the Δ35SCN gene was abolished. These results show that sequences on the 5' side of position -81 are necessary and sufficient for anaerobically regulated expression of a gene in maize protoplasts.

The levels of expression of the Adh-35S hybrid promoter constructions are not equivalent to those of the Adh-CAT constructions under any oxygen concentrations. Specific sequence elements around the TATA box, at the mRNA cap site, or in the mRNA leader that may be important for the efficient expression of the Adhl gene are absent in these constructs. It may also be that the spacing of the ARE relative to the TATA box/mRNA cap site is important for the level of expression. The ARE is 20 base pairs further away from the TATA box/mRNA cap site in the Adh-35S construction containing Adhl sequences from position -35 to position -1094 than in Adh-CAT, while in the Adh-35S construction with position -81 to position -1094 of the Adhl promoter, the ARE is 36 base pairs closer to the TATA box/mRNA cap site. Although the difference in expression between these two functional hybrid promoters is only slight, indicating some flexibility in spacing requirements, neither construction places the ARE in the same position relative to the TATA box/mRNA cap site as it occurs in Adh-CAT.

**Analysis of Linker-Scanning Mutations.** We constructed a series of LS mutations in the region between positions -140 and -72 to define more precisely the DNA sequences essential for anaerobic regulation. These LS constructions result in clustered point mutations and alter the spacing between nonmutated sequences by no more than ±2 base pairs. The structure of the LS mutants and results of CAT enzyme activity assays are summarized in Fig. 4. One mutant, LS-133/-124, reduces anaerobic expression to the level observed under aerobic conditions. The importance of the region from position -133 to position -124 is consistent with the results from the 5' deletion to position -124; one difference is that LS-133/-124 does not appear to be inducible. A second region is defined by LS-133/-99; this mutant does not express CAT enzyme activity above background levels. A LS mutation between these two regions, LS-125/-117 does not affect anaerobic induction of CAT expression relative to Adh-CAT. In addition, LS mutants LS-99/-92, LS-89/-81, and LS-80/-72 have no dramatic effect on the anaerobically inducible expression of CAT enzyme activity.

From these results, the 5' boundary of the ARE lies between positions -140 and -133, and the 3' boundary lies between positions -99 and -113. The ARE appears to contain two important regions: region I is defined by LS-133/-124 and region II by LS-113/-99. The DNA sequence between these two regions can be mutated without any discernible effect on anaerobically regulated expression.

**The ARE Contains DNA Sequences Homologous to Adh-2N.** Because the Adhl gene is only one of several anaerobically expressed genes in maize (1), it might be expected that other similarly regulated genes would have ARE-related sequences. The Adh2-N gene of maize (17) is also regulated by anaerobiosis (1) and contains homology to the Adhl ARE (Fig. 5). The homology in region I of the ARE is 13 out of 16 bases, while the homology in region II is 11 out of 16 bases. There is only limited homology, in sequence and spacing in the DNA segments located between or surrounding the two regulatory elements. Whether this region of homology is important for the anaerobic control of Adh2 expression remains to be tested. The 5'-flanking regions of the Adh genes from Arabidopsis (18) and pea (19) are not >60% homologous to the maize Adhl ARE over a 10-base-pair region.

**DISCUSSION**

Our results show that the region between positions -140 and -99 of the maize Adh1 promoter is essential and sufficient for low oxygen stimulation of Adh-CAT expression in maize.
protoplasts. Since this response is analogous to the low oxygen induction of Adh in maize seedlings or the anaerobic response (1), we have designated this region of DNA as the ARE. Deletion of DNA sequences on the 3' side of Adh–CAT indicates the 3' boundary of the ARE lies between positions -140 and -124. However, sequences upstream of position -140 modestly increase the expression of the Adh–CAT constructions. The fusion of a defective 35S–CAT gene (Δ35S CN) to 3' deletions of the Adhl promoter shows that the 3' boundary of the ARE lies between positions -81 and -99. The hybrid promoter fusions are also significant because they demonstrate that sequences upstream of position -81 are not only necessary but also sufficient to confer anaerobic expression on an unrelated promoter. LS mutants were constructed to further delineate the sequences within the boundaries defined by deletion analyses. Expression analysis of these LS mutants shows the ARE to be composed of two essential sequence regions, both of which are required for expression. Mutation of region I, defined by LS-133/-124, results in a low, constitutive level of expression. Mutation of region II, LS-113/-99, results in no observable expression above background. Although the phenotypes of the two LS mutants are different, it is not clear if the elements they disrupt are independent. Mutation of the DNA segment between these two regions does not significantly alter the level of induction. This suggests that the DNA between the two essential regions does not contain important sequence information; however, it may play a role in maintaining the spatial arrangement of the two regulatory regions.

The functional analysis of the maize Adhl promoter defining the ARE as an essential regulatory element is further supported by two other observations. There is substantial sequence conservation (70–80%) in the region of the ARE between Adh1 and another anaerobically regulated gene of maize, Adh2-N. There are further regions of sequence homology between the promoter region of Adh1 and Adh2 located at other potentially important control regions, i.e., around the TATA box, the mRNA cap site, and a short sequence in the mRNA leader (20). The absence of other homologies of substantial length (>10 base pairs) between the promoters of these two genes supports the suggestion that the conserved sequences may have a functional role. The ARE also corresponds to one of the nuclease-sensitive regions of Adh1 in the chromatin of nuclei isolated from anaerobic roots (5, 21). Increased nuclease sensitivity is often associated with genes in a transcriptionally active state (22) and would suggest that the ARE may be part of a transcriptionally active region in the chromatin of anaerobic maize roots.

Our results show that the Adhl promoter has a number of regulatory sequences. We have defined two regions within the promoter that are essential for anaerobic regulation of the maize Adhl gene. Sequences upstream of the ARE are important for the level of expression. In addition, the observation that the hybrid promoter constructions are not expressed as efficiently as the Adh–CAT construction indicates that additional sequences, surrounding the TATA box, surrounding the mRNA cap site, or in the mRNA leader, may play a role in the level of gene expression.

It would be naive to suggest that the regulatory elements we have identified comprise all of the sequence elements involved in the expression of the Adh1 gene in maize plants. Our analysis has utilized a transient expression assay with cultured cells, and we have concentrated on the analysis of 5' DNA sequences. Additional components in the 5' region or sequences in the introns, coding regions, or 3'-flanking regions may also be important to the expression of the Adh1 gene. For example, Dudler and Travars (23) have demonstrated that for the Drosophila Hsp70 promoter, upstream elements are necessary for maximal expression in transformed flies. However, these upstream elements are not required for induction and maximal expression of the Hsp70 promoter in Xenopus oocytes and COS cells. The Adhl gene also is expressed at specific developmental stages (24) and additional elements are probably required for this control as
has been observed for other genes that respond to both environmental and developmental stimuli (25, 26).

Our analyses suggest that positive control factors interact with sequences of the ARE to increase gene expression during anaerobiosis. The results demonstrating that fusion of Adhl promoter sequences to a deleted 35S promoter confers not only expression, but regulated expression to the crippled gene, are strongly indicative of positive control factor(s) interacting with the Adhl sequences during anaerobiosis to activate transcription. Further, the observation that the 5' deletions and linker scanning mutations in the ARE either have no effect on the phenotype or reduce expression to the levels observed under aerobic conditions also supports a model based on positive control. However, we cannot eliminate the possibility of a negative regulatory factor acting in conjunction with the apparently positive mode of gene expression. Further elucidation of the molecular mechanisms of anaerobic regulation of gene expression will be facilitated by analysis of putative trans-acting regulatory factors and a more detailed analysis of the properties of the cis-acting DNA signals involved in the anaerobic response, for both Adhl and the other anaerobically regulated genes of maize.

Note. While this manuscript was under review a paper by Springer et al. (27) identified a sequence in the first intron (positions 431–443) of the maize anaerobically inducible sucrose synthase gene that is identical to region II (positions −113 to −101) of the ARE. The functional significance of this sequence in sucrose synthase has not been examined.

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