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In Vivo Footprinting Reveals Unique *cis*-Elements and Different Modes of Hypoxic Induction in Maize *Adh1* and *Adh2*

Anna-Lisa Paul and Robert J. Ferl[1]

Department of Vegetable Crops, University of Florida, Gainesville, Florida 32611

The transcriptional activation of maize *alcohol dehydrogenase-1* (*Adh1*) and *alcohol dehydrogenase-2* (*Adh2*) is accompanied by changes in the chromatin structure within the 5′-flanking region of each gene. The positions of DNA-binding factors bound to the 5′-flanking regions were determined by in vivo dimethyl sulfate footprinting of maize suspension cultures over 8 hours of hypoxic induction. In *Adh1* there are two types of DNA-binding factors associated with the promoter region. One set of factors is constitutively associated with the *cis*-regulatory anaerobic response element, whereas two additional factors bind only after *Adh1* has been induced by hypoxic stress. Returning hypoxically stressed cells to an aerobic environment restores the dimethyl sulfate footprint observed for the uninduced *Adh1* gene. In contrast, all of the factors bound to the 5′-flanking region of *Adh2* are constitutively present and unchanged by hypoxia. There is one footprint site common to both *Adh1* and *Adh2*, but it is not an anaerobic response-like element.

INTRODUCTION

Adh1 and *Adh2* encode two of the anaerobic proteins of maize (Sachs and Freeling, 1978; Ferl et al., 1979; Sachs et al., 1980). The coding regions of the two *Adh* genes are very similar, but the 5′-flanking regions are almost completely different. Both genes remain quiescent (or are transcribed at very low levels) in roots and the cultured cell line P3377 until hypoxic stress induces transcription.

Promoter mutagenesis studies have defined sections of the *Adh1* promoter that are necessary for a positive response to hypoxia (Ellis et al., 1987; Howard et al., 1987; Lee et al., 1987) and, specifically, a 40-bp region positioned between −140 and −99. This anaerobic response element (ARE) is composed of at least two sub-elements positioned between −133 and −124 (ARE I) and −113 and −99 (ARE II) (Walker et al., 1987). The *Adh2* 5′-flanking region contains a set of ARE-like sequences, but mutational analyses to determine whether they also function as regulatory elements have not been conducted with the *Adh2* promoter.

cis-Regulatory elements are often associated with *trans*-acting DNA-binding factors. In vivo dimethyl sulfate (DMS) footprinting, in conjunction with genomic sequencing (Church and Gilbert, 1984; Church et al., 1985), detects protein interactions through the modification of guanine residues introduced in vivo by DMS. A protein closely associated with a guanine will change the local chemical environment around that G such that the ability of DMS to methylate the N7 position will be either enhanced or inhibited. In this way, the positions of DNA-binding factors can be mapped to the bases involved.

Since 1984, genomic sequencing has been used in animal systems as a tool to investigate methylation (e.g., Saluz and Jost, 1986) and to target the binding sites of transcription factors (e.g., Nick and Gilbert, 1985; Becker et al., 1987). The application of genomic sequencing to plants is more recent, beginning with the comparison of the degree of methylation for *Adh1* in maize leaves (Nick et al., 1986). Plant in vivo protein interactions were first noted in the *Adh1* gene of maize (Ferl and Nick, 1987) and the *Adh* gene of *Arabidopsis* (Ferl and Laughner, 1989), and then in the light-induced chalcone synthase gene (Schulze-Lefert et al., 1989a, 1989b) and in the phenylalanine ammonia-lyase gene (Lois et al., 1989) in parsley.

The present study investigates the protein-DNA interactions of maize *Adh1* and *Adh2* in vivo during the onset, maintenance, and reversal of hypoxic conditions in cell cultures and presents a comprehensive comparison with the available promoter deletion studies.

RESULTS

The tissue culture cell line P3377 is a model cell suspension system that is very similar to maize roots with respect to

[1] To whom correspondence should be addressed.

Adh1 and *Adh2* gene activity. Using a cultured cell suspension for in vivo footprinting obviates problems arising from cell type diversity within an organ (Ferl and Nick, 1987), but it was necessary to establish that the cell cultures gave an accurate reflection of what was occurring in maize roots during hypoxia. We used three criteria to evaluate the utility of a cell culture line: (1) how closely the *Adh1* and *Adh2* mRNA induction profiles resemble those seen in seedling maize roots (e.g., Dennis et al., 1985; Hake et al., 1985), (2) that the increase of *Adh1* and *Adh2* mRNA is due to an increase in transcription, as has also been shown with transcription run-on assays for *Adh1* in nuclei from maize seedlings (Rowland and Strommer, 1986; Dennis et al., 1988b), and (3) that the in vivo footprints are prominent and consistent between experiments. Of the three cell lines we tested, only one, P3377, met all three criteria.

Messenger RNA Levels for *Adh1* in Cell Suspensions

The increase in *Adh1* and *Adh2* mRNA levels with the length of hypoxic treatment of the cells is shown in Figures 1A and 1B. The *Adh* mRNA levels declined rapidly in hypoxic cells that had been returned to air for 2 hr (A). The levels of *Adh1* and *Adh2* mRNA in aerobic (uninduced-U) cells were very low in the P3377 cell line. The *Adh1* induction profiles of two additional cell lines were also examined: P160 (kindly provided by Dr. Indra Vasil) and Black Mexican Sweet (kindly provided by Dr. Prem Chourey). The P160 and Black Mexican Sweet cell lines were not pursued further either because the footprinting data were inconsistent or because of high levels of constitutive *Adh1* mRNA levels (data not shown). In addition, because it has been shown that high concentrations of 2,4-D will induce *Adh1* in maize seedlings (Freeling, 1973), the effect of increasing the concentrations of 2,4-D in the cell cultures was investigated. Increasing the concentration of 2,4-D from the standard 2 mg/L to 4 mg/L, 8 mg/L, or 16 mg/L did not affect alcohol dehydrogenase enzyme levels in P3377 cultures (data not shown).

An increase in the rate of *Adh1* and *Adh2* transcription contributes to the higher mRNA levels seen in hypoxic cells. Figure 1C shows the results of run-on transcription assays in nuclei from hypoxic (induced) and aerobic (uninduced) cells. The center lanes illustrate the positions of the vector DNA relative to the *Adh1*, *Adh2*, and rDNA inserts on the agarose gel before transfer. Transcription of *Adh1* and *Adh2* mRNAs increased with hypoxia, whereas a lighter exposure of the autoradiograph shows that transcription of ribosomal mRNA decreased slightly.

These data illustrate that the hypoxic induction of *Adh1* and *Adh2* in the P3377 suspension cultures is qualitatively and quantitatively similar to the situation observed for maize seedlings in the accumulation of *Adh* mRNA over an induction time course (Dennis et al., 1985; Hake et al.,

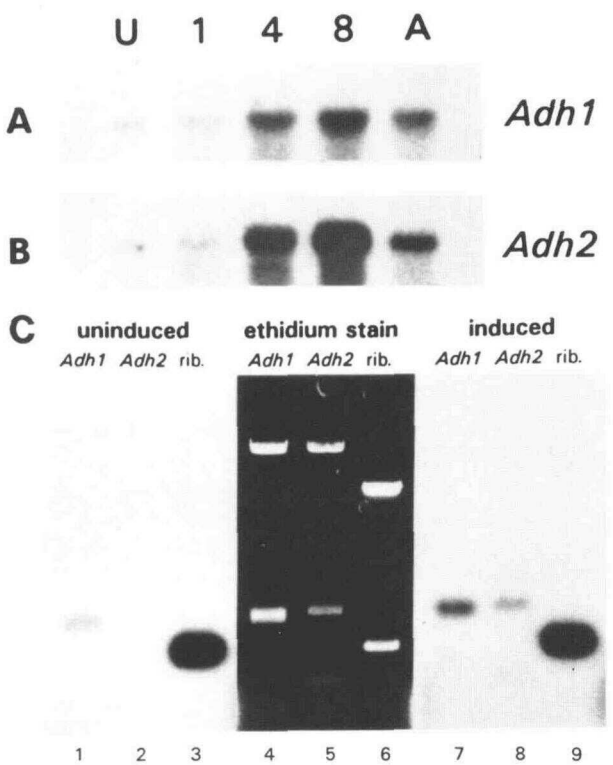

Figure 1. *Adh1* and *Adh2* mRNA Accumulation and Run-On Analyses.

(A) and **(B)** RNA gel blots of the *Adh1* and *Adh2* mRNA accumulation during hypoxia. The lanes show mRNA levels from the *Adh1* [**(A)**] and *Adh2* [**(B)**] induction time course of P3377 after 0 hr (lane U), 1 hr, 4 hr, and 8 hr of hypoxia, and after 4 hr of hypoxia followed by 2 hr of aeration (lane A). Cells were made hypoxic by bubbling argon through the cultures for the designated time.

(C) Run-on transcription assays from cell suspensions. Nuclei from aerobic (uninduced) and hypoxically stressed (induced) P3377 cells were used for the nuclear transcription run-on assays as described in Methods. Lanes 1, 4, and 7 contain an *Adh1* cDNA clone (pZmL793); lanes 2, 5, and 8 contain an *Adh2* cDNA clone (pZmL1209); lanes 3, 6, and 9 contain a plant ribosomal RNA clone (*Zamia pumila*). In all cases, the plasmids were digested to release the inserts cleanly. In the case of pZmL793, the restriction digest used to release the hybridizing fragment released a doublet, resulting in an ethidium-stained band of double the intensity of the corresponding pZmL1209 insert (where the restriction digest released only the hybridizing fragment). The center panel shows the positions of the vector (upper bands) and insert (lower bands) of each clone on the ethidium bromide-stained gel (ethidium stain) used to generate the blots for hybridization with the run-on transcripts. Lighter exposure of the autoradiographs (to put the ribosomal band within the linear range of the film) (left and right panels) shows that the level of ribosomal mRNA decreased slightly after 5 hr of hypoxic stress.

1985) resulting, at least in part, from transcriptional activation of the gene, as determined in run-on assays (Rowland and Strommer, 1986; Dennis et al., 1988b).

DMS Footprints of the 5′ Region of *Adh1* Vary with the Transcriptional State of the Gene

Contact points of DNA-binding factors are identified on genomic autoradiographs as G residues that are either darker (enhanced reaction with DMS in vivo) or lighter (protected from the DMS reaction in vivo) when compared with the control lane of naked genomic DNA reacted with DMS in vitro. In Figure 2A, autoradiographs of both the top and bottom strands show the changes in the DMS footprints that occur with the induction of *Adh1* in cell line P3377 after 1 hr, 4 hr, and 8 hr of hypoxia, and 2 hr after hypoxic cells have been returned to an aerobic environment. Densitometric scans of the autoradiographs in Figure 2A are given in Figure 2B. The densitometric scans allow a more precise comparison of relative band intensities and aid in distinguishing between (lane to lane) loading differences and true interactions.

An enhancement of the G residues in the doublet at position −178 on the top strand and a protection of the G at −182 on the bottom strand were seen in cells that had been hypoxically stressed, but were absent in cells in which *Adh1* had not been induced. Evidence of an interaction did not become prominent until after 4 hr of hypoxia. The intensity of the footprint remained constant between 4 hr and 8 hr (Figures 2A and 2B).

A DMS footprint consisting of both enhancements and protections affected several bases between −109 and −134. Protections in the top strand were seen in two positions, the G doublet at −129 and −130 and the GTGG sequence from −109 to −112. Both sets of protections could be seen even in uninduced cells, although the level of protection was reduced relative to that seen in induced cells (Figure 2B). The degree of protection intensified within the first hour of induction and then remained constant through the 8-hr time point. After *Adh1* was induced in hypoxic cells, the bottom strand of this region showed an enhancement at −131 as well.

A third footprint was centered around position −96 and was evident only in induced cells. On the bottom strand, the G residues at −92 and at least two of a triplet of G residues from −98 to −100 showed an enhancement as *Adh1* became active. The response was easily seen in the first hour and plateaued at its highest intensity by 4 hr of hypoxia (Figures 2A and 2B).

The constitutive footprints centered around −110 and −130 coincided with the AREs defined for the maize *Adh1* promoter (Walker et al., 1987). The genomic sequencing blots actually extended further 5′, beyond the region presented in Figure 2. However, no obvious indications of in vivo interactions were detected between −182 and −400.

DMS Footprinting Pattern of the Uninduced *Adh1* Gene Is Restored if the Hypoxic Stimulus Is Removed

The "A" lanes for both top and bottom strands of Figure 2 illustrate the effect on *Adh1* of removing the hypoxic stress and returning the cells to an aerobic environment. This was accomplished by bubbling cells, which had been under hypoxic stress for 4 hr, with air for 2 hr. All enhancements and protections that appeared after induction were no longer evident, and the G pattern characteristic of uninduced, aerobic cells was restored. The most illustrative example of this result can be seen in lane A, position −178 of the top strand, and lane A, positions −98 and −92 of the bottom strand in Figure 2. These enhancements clearly and completely reverted to the aerobic pattern.

DMS Footprints of the 5′-Flanking Region of *Adh2* Are Constitutive in Nature

The DMS footprinting pattern within the *Adh2* promoter did not vary with the transcriptional state of the gene. The enhancements and protections generated by in vivo DMS treatment remained constant throughout the induction time course. In Figure 3A, autoradiographs of the top and bottom strands of the *Adh2* promoter region show the positions of the enhanced and protected G residues. The densitometric scans (which have been corrected for in lane loading differences) of Figure 3B illustrate the uniformity of these in vivo footprints.

The region between −208 and −216 is composed entirely of G and C residues. This region is flanked by A residues interrupted by a single C residue at −222. Virtually all of the G residues within this region show modification on the bottom strand, with enhancement at positions −209, −210, and −211, and protection at positions −216 and −222.

The G doublet at position −160/−161 shows a dramatic enhancement over naked DNA. This enhancement is well isolated from any other footprint in the promoter but is found within the same sequence (CGGTC) as the footprint of −82/−83. The top strand enhancement of the doublet at position −82/−83 is the most TATA-proximal footprint observed for *Adh2*. The two closest G residues on the bottom strand at −84 and −87 are also footprinted, but the modification is seen as a protection rather than an enhancement.

Adh2 has two sequence elements that resemble the *Adh1* AREs. As with *Adh1*, the *Adh2* ARE-like sequence is divided into two subunits, one at position −98 to −106 and the other from −135 to −145. There was no evidence of footprinting within these sequences in *Adh2*. As with *Adh1*, the genomic sequence blots of *Adh2* extended beyond the area presented in Figure 3. There was no obvious indication of factor interactions 5′ to the footprinted region centered around −216.

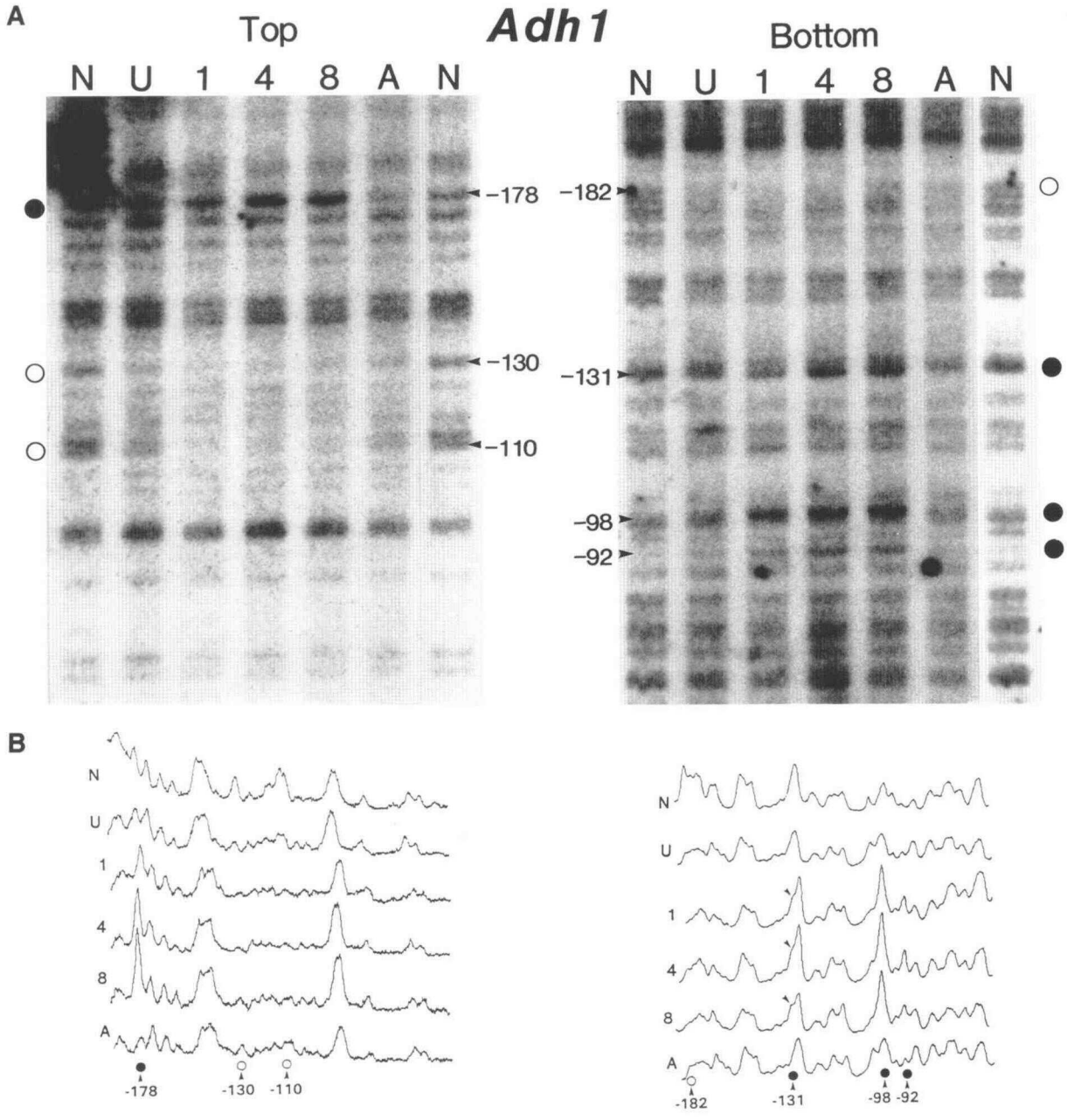

Figure 2. In Vivo DMS Footprints of the *Adh1* Promoter.

(A) Autoradiograph of a time course consisting of cells treated with DMS in vivo after 0 hr (U, uninduced), 1 hr (1), 4 hr (4), and 8 hr (8) of hypoxia, and after 4 hr of hypoxia followed by 2 hr of an aerobic environment (A, returned to air). In each panel, control lanes consisting of protein-free genomic DNA treated with DMS in vitro (N, naked) flank the in vivo treatments. Open circles (○) indicate areas of protected guanines and closed circles (●) show areas where guanine modification is enhanced. The position of each interaction relative to the start of transcription is noted on the side of the figure.

(B) Densitometric scans of the top and bottom strands of the autoradiograph shown in **(A)** illustrate the quantitative differences between the intensities of the modified G residues. The scans have been normalized to compensate for loading differences between lanes. The positions of enhanced (●) and protected (○) bands are shown at the bottom of the figure. The arrowhead indicates the position of the enhanced G at −131 of the bottom strand. The peak associated with this enhancement is slightly obscured by the adjacent peak.

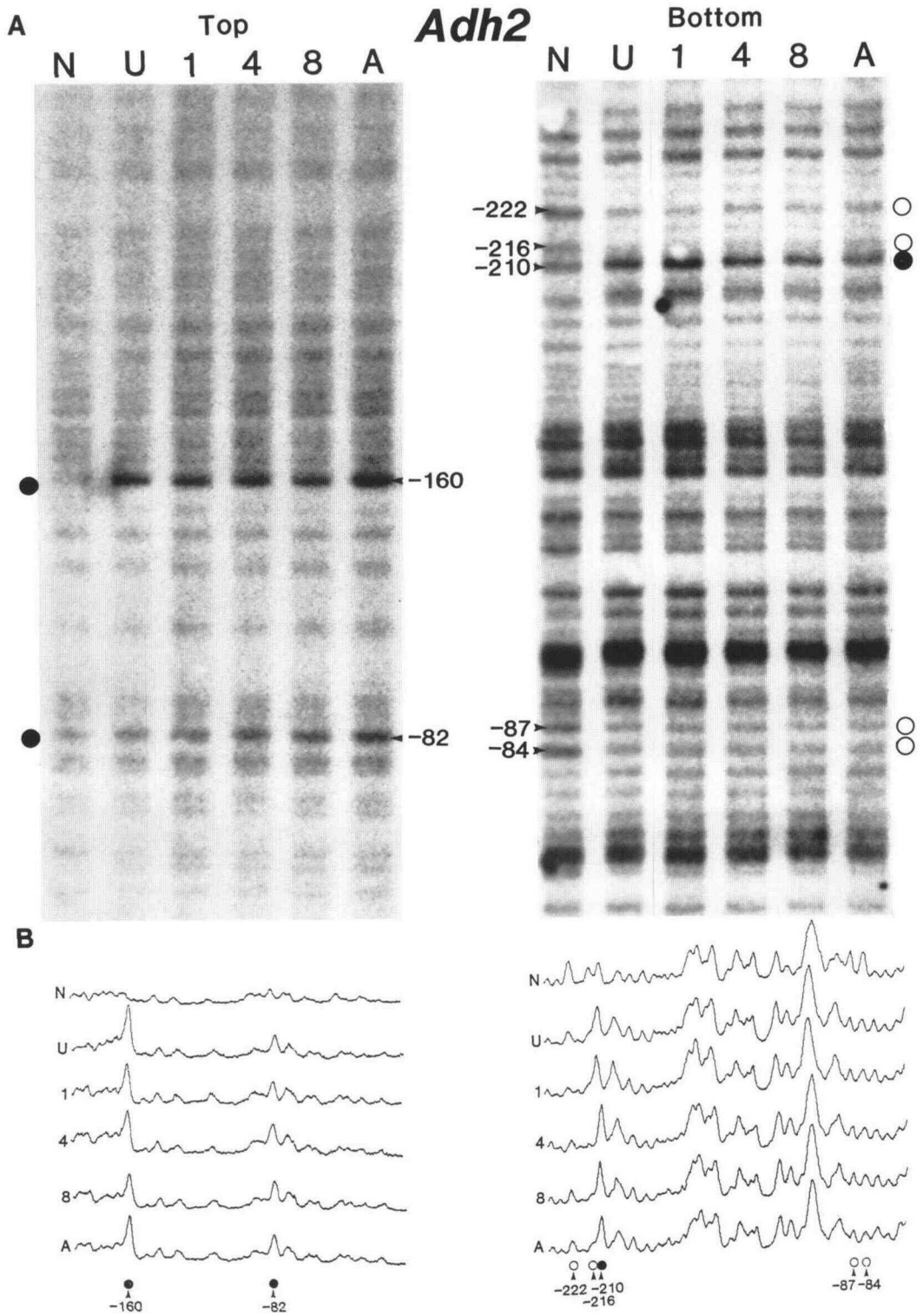

Figure 3. In Vivo DMS Footprints of the *Adh2* Promoter.

(A) Autoradiograph of a time course consisting of cells treated with DMS in vivo after 0 hr (U, uninduced), 1 hr (1), 4 hr (4), and 8 hr (8) of hypoxia and after 4 hr of hypoxia followed by 2 hr of an aerobic environment (A, returned to air). In each panel, a control lane consisting of protein-free genomic DNA treated with DMS in vitro (N, naked) is included for comparison. Open circles (○) indicate areas of protected guanines and closed circles (●) show areas where guanine modification is enhanced. The position of each interaction relative to the start of transcription is noted on the side of the figure.

(B) Densitometric scans of the top and bottom strands of the autoradiograph shown in **(A)** illustrate the quantitative differences between the intensities of the modified G residues. The scans have been normalized to compensate for loading differences between lanes. The positions of enhanced (●) and protected (○) bands are shown at the bottom of the figure.

DISCUSSION

Because *Adh1* and *Adh2* have such a similar induction response to hypoxia, our original expectation was that there would be a commonality within their mechanisms of activation. In fact, sequence inspection revealed two potentially common elements. One element consists of the two short sequence motifs that are the *cis*-acting AREs in *Adh1* (Walker et al., 1987). The other element is an 8-bp site centered around −180 in *Adh1* and −85 in *Adh2*. We expected the ARE-like sequences of *Adh2* to show evidence of interactions with DNA-binding factors in vivo as was seen for *Adh1*. This was not the case. Instead, the only common footprint was within the 8-bp element, a sequence that is not essential for anaerobic induction of *Adh1*. The other *cis*-acting elements utilized by *Adh1* and *Adh2* were unique and novel and the organization of the observed interactions was dramatically different between *Adh1* and *Adh2*.

In *Adh1*, there were two types of footprints in the promoter region, those which were constitutively present and those which appeared only after induction of the gene. In addition, an induction time course showed that the two types of factors interact with the promoter differently as induction proceeds from 1 hr through 8 hr and after hypoxic cells are returned to aerobic conditions. The *Adh1* DNA-binding factors are referred to as C (−178 to −183), B2 (−129 to −131), B1 (−109 to −112), and A (−92 to −99). [Revision of the transcription start site (Ellis et al., 1987) and promoter sequence (R.J. Ferl, unpublished data) has modified the numbering convention originally used by Ferl and Nick, 1987.]

The constitutive footprint composed of B1 and B2 contains protected G residues from −109 to −131 and corresponds to the ARE defined by Walker et al. (1987). It appears that either a single large factor (B1/B2) or two smaller factors are constitutively bound over the ARE, as shown in Figure 4. The interactions of factors B1 and B2 intensified after induction of the gene (Figure 2B). This would indicate that the factors bound to the ARE undergo a change as the gene becomes active (Ferl and Nick, 1987).

A short sequence (GGTTT) is common to the footprints of both B1 and B2 in the ARE region (Dennis et al., 1988a). A mutation of these 5 conserved bases in B2 (ARE I) reduces the induced level of *Adh1* expression by 75% in transient assays. A mutation of the same sequence in B1 (ARE II) abolishes promoter function entirely, regardless of the degree of hypoxia (Walker et al., 1987). This GGTTT sequence is also found in the ARE I-like sequence of *Adh2*, but it is not footprinted.

The inducible DNA-binding factors, A (−92 to −99) and C (−178 to −183), bound to the *Adh1* promoter only in response to hypoxia. The intensities of both footprints plateaued within 4 hr, but the mRNA levels continued to increase (Figure 1A). It is likely, therefore, that the binding of A, B1/B2, and C constitutes a fully active promoter configuration. Removing cells from hypoxic stress for 2 hr abolished the A and C footprints, thereby restoring the DMS footprint characteristic of uninduced cells and indicating that A and C require hypoxia to remain bound to the promoter.

Although neither of the footprinted sequences in A or C was required for hypoxic activation, both were necessary for full promoter function. Factor A (−92 to −99) binds to the 3′ edge of ARE II. Linker scanning mutations within this area result in a reduction of overall promoter efficiency but do not abolish the promoter's ability to respond to hypoxic stress (Walker et al., 1987). Factor C (centered around −180) is well isolated from any defined *Adh1* anaerobic regulatory sequences. However, deletion of the promoter fragment which includes C (from −410 to −138) reduces promoter function by approximately 40% (Lee et al., 1987; J. Ingersoll, R.J. Ferl, and W.B. Gurley, manuscript submitted). Thus, A and C do not appear to be essential for detecting hypoxic conditions and subsequent anaerobic induction in transient assays, but they are obviously important to quantitative aspects of promoter function.

The footprinted regions of A, B, and C are distinct, but they all share a GTGG core motif among their footprinted bases. In the case of B1 and C, the GTGG core extends to include the GGTTT motif mentioned above to become GTGGTTT. The GTGGTTTT that is found in C and B1 is similar to sequence motifs that are thought to contribute enhancer function in other systems, such as the SV40 enhancer core (GTGGTTTTG) (e.g., Benoist and Chambon, 1981). Variations on the GTGG motif are found in the promoters of a variety of light-induced and stress-induced genes, often as a symmetrical dyad (CCACGTGG) known as the "G-box" (Giuliano et al., 1988; Schulze-Lefert et al., 1989b; McKendree et al., 1990). The correlation between gene activation and DNA-binding factors associated with the G-box has been demonstrated in parsley chalcone synthase (Schulze-Lefert et al., 1989a, 1989b) and in the tomato *rbcS-3A* promoter (Ueda et al., 1989). *Arabidopsis Adh* contains a G-box that has been shown to bind a factor both in vivo (Ferl and Laughner, 1989) and in vitro (DeLisle and Ferl, 1990; McKendree et al., 1990). However, it is unclear that there is any functional relationship with either of the maize *Adh1* elements and the ideal G-box because the GTGG in A, B, and C is not present as part of a classic G-box dyad. The similarities among the separate footprints of A, B1, and C and their resemblance to known enhancer sequences suggest that the same factor, or a related set of factors, interacts with several regions of the *Adh1* promoter to influence transcriptional activity. There are no G-box dyads or GTGG sequences present in the *Adh2* promoter.

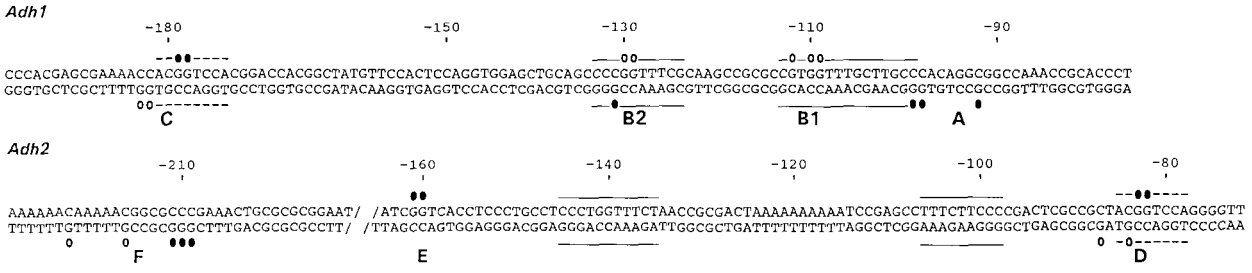

Figure 4. Summary of *cis*-Acting Functional Elements and *trans*-Acting Factors of Maize *Adh1* and *Adh2*.

The reactive G residues within these regions are marked with open (protected) circles (○) and solid (enhanced) circles (●), and the letter designation for each factor is noted below the sequence. The base pairs within the AREs and ARE-like sequences are bracketed by solid lines and the other sequence common to both promoters is bracketed by dotted lines.

The footprints found in the promoter of *Adh2* are constitutive in nature and are referred to as D (−82 to −87), E (−160/−161), and F (−209 to −222) (Figure 3). The ARE-like sequences present in the *Adh2* promoter are not discernibly footprinted in vivo and are well isolated from any footprinted sequences. In addition, *Adh2* lacks any of the GTGG motifs found (and footprinted) in the *Adh1* promoter. There are, however, 8 bp in common (ACGGTCCA) between *Adh1* footprint C and *Adh2* footprint D. This is the only footprint found in both promoters. The 8-mer is located at opposite ends of the two *Adh* promoters (from −181 to −174 in *Adh1* and from −85 to −78 in *Adh2*), but the sequence occupies a similar position relative to chromatin features of each gene. The 8-mer sequence is positioned at the 5′ boundary of the inducible DNase I hypersensitive region in *Adh1* (Paul et al., 1987) and at the 5′ boundary of the TATA-proximal DNase I hypersensitive site in *Adh2* (Ashraf et al., 1987). The 5-bp core sequence (CGGTC) from footprint D is found in *Adh2* footprint E as well.

The third factor in the *Adh2* promoter (F) is seen as a large footprint positioned over −209 to −222 on the bottom strand. Factor F is associated with an unusual sequence. The GC region from −209 to −216 is flanked by sets of adenine residues, with the 5′ set bisected by a single cytosine. This region resembles the GC-rich factor binding site in the *c*-myc (Lobanenkov et al., 1986) and the chicken adult β-globin genes (Emerson and Felsenfeld, 1984). Because no deletion data exist for *Adh2*, it is difficult to attach functional significance to the sequences and positions of the in vivo footprints.

Adh1 and *Adh2* are, then, two different genes that respond to the same environmental stimulus, yet appear to employ very different strategies in the organization and utilization of their regulatory regions. The *Adh1* gene seems to receive and process regulatory signals in at least two stages. The chromatin configuration of one portion of the promoter is constitutive, independent of the transcriptional state of the gene. Once the signal is received, however, changes in the promoter (the recruitment of new DNA-binding proteins and the appearance of new DNase I hypersensitive sites) are initiated which facilitate transcription of *Adh1*. Whether the inducible factors are recruited by the constitutive factors or whether the inducible factors recognize other aspects of the *Adh1* promoter and then effect a change in the promoter is unknown, but it is clear that the interaction of the full suite of factors with the promoter represents a terminus of the hypoxic induction pathway in *Adh1*. There are no similar stages to the activation of the *Adh2* promoter, which remains in the same constitutive conformation regardless of the transcriptional state of the gene. There is no indication that additional DNA binding factors are recruited as the gene becomes active, suggesting that the entire *Adh2* promoter is perpetually accessible to transcriptional signals. Nevertheless, *Adh1* and *Adh2* respond within the same time frame (Figure 1) and range of oxygen concentrations (Paul and Ferl, 1991) to hypoxia. Although the organization of the two *Adh* promoters is very different, there is one common factor binding site between *Adh1* and *Adh2* (C in *Adh1* and D in *Adh2*); however, in *Adh2* factor D is bound constitutively, whereas factor C in *Adh1* requires hypoxic stress to be bound. Given that this sequence is not essential for hypoxic induction in *Adh1*, it is unlikely that this common site is responsible for the coordinate induction of *Adh1* and *Adh2*.

The data reported here indicate that the few features shared in common by the *Adh1* and *Adh2* promoters are not similarly utilized. It may be that the two promoters are activated by the same signal induction pathway and factors, but these factors either interact with diverse DNA sequence elements, common but non-AREs, elements outside the scope of this analysis, or in ways we are presently unable to detect in vivo. Alternatively, *Adh1* and *Adh2* may each possess a very different transcriptional induction system. The biochemical characterization of the DNA-binding factors (Ferl, 1990) and dissection of the *cis*-acting elements of *Adh2* will address these possibilities.

METHODS

Cell Cultures

The experiments were conducted with the maize (*Zea mays*) suspension culture P3377, which originated with Dr. Jack Widholm, University of Illinois (Duncan et al., 1985). The P3377 cell line was initiated from an immature embryo of a selfed Pioneer 3377 hybrid. This cell line is distinct from the cell line used for the chromatin work of Paul et al. (1987) or by Ferl and Nick (1987). Cell suspensions were maintained in our laboratory on a commercial mixture of Murashige and Skoog salts (GIBCO Laboratories) with 2,4-D at a level of 2 mg/L.

Five time points were taken during the hypoxic induction of the *Adh* genes in P3377. Cell cultures were either analyzed for total RNA (Figure 1) or treated with DMS for footprinting analysis (Figures 2 and 3) after 0 hr (uninduced-U) 1 hr, 4 hr, and 8 hr of hypoxia. Hypoxic conditions were established by bubbling cultures with argon for the designated time. The final time point (lane A of Figures 1, 2, and 3) consisted of cells returned to aerobic conditions by bubbling with air for 2 hr after 4 hr of hypoxia. The uninduced (0 hr time point) was represented by cells taken from normal culturing conditions and processed immediately. The growth medium was not changed during the course of the experiments.

In Vivo DMS Treatment

Two to three 50-mL cultures were pooled for each treatment for a final cell mass of 4 g to 5 g. The volumes of the cell suspensions were measured, then DMS was added directly to a final concentration of 0.2%. The cultures were agitated for 2 min, filtered under vacuum onto a 10-μm screen, and then washed with 10 volumes of water to remove any residual DMS. Filtered cells were collected, weighed, frozen in liquid nitrogen, and stored at −80°C until all time points had been collected. All DMS treatments were conducted in a fume hood and the DMS filtrate was inactivated with $1/10$ volume of 5 M sodium hydroxide.

Isolation and Purification of DNA

In preparation for DNA extraction, the tissue was frozen in liquid nitrogen and powdered in a small, household coffee grinder (Sears Regal coffee and spice mill) that had been prechilled with liquid nitrogen. The frozen powder was dropped into lysis buffer (50 mM Tris, pH 8.0, 50 mM EDTA, pH 8.0, 50 mM NaCl, 0.4 mg/mL ethidium bromide, 2.0% sarcosyl) at a ratio of 1.0 mL of lysis buffer for every gram of tissue. The debris was removed by centrifugation and cesium chloride was added to the supernatant for a concentration of 1.0 g/mL. The cesium chloride/extract mixture was centrifuged (18,000 rpm for 10 min, Beckman J2-21 centrifuge, JA-20 rotor) to remove additional debris before the supernatants were prepared for ultracentrifugation (65,000 rpm, Beckman L8-M ultracentrifuge, VTi65 rotor).

DNA Preparation

Twenty micrograms of purified DNA from each time point was restricted with either HindIII (*Adh1*) or BamHI (*Adh2*) to generate a homologous end for the strand-specific, indirect end-label hybridization of genomic sequencing blots (Church and Gilbert, 1984) as described in the following section. Restriction digests were terminated with a phenol:chloroform:isoamyl (25:24:1, v/v) extraction followed by a chloroform:isoamyl (24:1, v/v) extraction. The DNA was precipitated with ammonium acetate (to 2.5 M) and ethanol (2.5 volumes). The pellet was resuspended in 50 μL of 10% piperidine and heated at 90°C for 15 min to cleave the DNA at all modified G residues. After piperidine cleavage, the reaction mixture was diluted fivefold with water and lyophilized. The resulting pellet was resuspended in 50 μL of water, lyophilized a second time, then resuspended in 5 μL of sequencing dye (13 mM EDTA in formamide plus bromphenol blue and xylene cyanol dyes). Controls consisted of naked genomic DNA restricted as above and then treated with 0.2% DMS for 2 min. Subsequent steps in preparation of the control samples were identical to the in vivo treatments.

Resolution of Modified DNA

The DNAs were resolved on a 0.75-mm, 6% acrylamide sequencing gel in TBE (0.089 M Tris, pH 8.0, 0.089 M boric acid, 0.0026 M EDTA). After electrophoresis, the gel was electroblotted onto GeneScreen (Du Pont-New England Nuclear) for 1.5 hr at 1.8 amps and then UV cross-linked to fix the DNA to the membrane. Blots were hybridized to single-stranded probes homologous to a region of the *Adh1* promoter between HaeIII (−100) and HindIII (+210) and to the *Adh2* promoter between the XbaI (−43) and BamHI (+242). The probes were generated with bacteriophage M13 clones which contain the *Adh1* HaeIII to HindIII fragment or the *Adh2* XbaI to BamHI fragment. Depending on the orientation of the fragment within the clone, either the top or bottom (coding or noncoding, respectively) strand of the promoter was hybridized in the genomic sequencing blots (Ferl and Nick, 1987). The single-stranded probes illuminate the *Adh1* promoter region from approximately −70 to −220 in Figure 2 and the *Adh2* promoter from approximately −70 to −240 in Figure 3. Hybridization conditions were as described by Church and Gilbert (1984). Blots were typically exposed to Kodak XAR film for 2 days to 3 days at −80°C with Du Pont Lightning-Plus screens.

Adh1 and *Adh2* mRNA Analyses

Total RNA was isolated from cultured cells after the method described by McCarty (1986). Total RNA was resolved on 2.2 M formaldehyde, 1.5% agarose gels, then capillary blotted to GeneScreen, UV cross-linked, and hybridized to an M13 generated probe.

Run-On Transcription Analysis

Nuclei were isolated from both aerobic and hypoxic (5 hr under argon) P3377 cells (Paul et al., 1987). The nuclear pellets were resuspended in NRB buffer (50 mM Tris, pH 8.0, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20% glycerol) and the volumes adjusted to yield an approximately equal number of nuclei per microliter. A reaction mixture consisting of 50 μL to 60 μL of nuclei in NRB, 80 units of RNasin (Promega Biotec), 100 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 0.3 μM phosphocreatin, 2.5 μg of creatin phosphokinase, and 500 μM each ATP, GTP, and CTP was incubated with 150 μCi of ^{32}P-UTP for 15 min at 30°C. The reaction mixture was

phenol extracted and the nucleic acids were precipitated with ammonium acetate and ethanol as described for DNA. Equal counts per minute of precipitated material were used to probe DNA gel blots containing 0.5 μg of plasmid DNA per lane. Plasmids containing portions of the coding regions of maize *Adh1* (pZmL 793, provided by W.J. Peacock) and *Adh2* (pZmL 1209, provided by W.J. Peacock), and *Zamia pumila* ribosomal DNA (Nairn and Ferl, 1988) were digested to release the insert. Blots were hybridized under conditions described by Paul et al. (1987).

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