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In Vivo and in Vitro Characterization of Protein Interactions with the Dyad G-Box of the *Arabidopsis Adh* Gene

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Expression of the *alcohol dehydrogenase* (*Adh*) and ribulose-1,5-bisphosphate carboxylase small subunit (*RbcS*) genes of higher plants is cell-type-specific and environmentally inducible. However, the tissues in which these two genes are expressed, their modes of induction, and their protein functions are quite distinct. *Adh* is expressed in non-green tissue, induced by anaerobiosis, and repressed in leaves. *RbcS* is only expressed in green tissue. An 8-base pair G-box element (5′-CCACGTGG-3′) is associated with light-induced expression of *RbcS* and *chalcone synthase*. The same sequence is also present in the 5′-flanking region of *Arabidopsis thaliana Adh*, and this sequence is associated with a *trans*-acting factor in vivo. We report here that in vitro *Adh* G-box binding activity is present in crude whole cell extracts of both cell culture and leaves of *Arabidopsis*. The authenticity of in vitro *Adh* G-box binding is supported by in vivo and in vitro dimethylsulfate footprinting. A clear in vivo *Adh* G-box footprint occurs in cell cultures, but comparable in vivo binding to the *Adh* G-box does not occur in leaves. Therefore, there does not appear to be a direct correlation between the presence of the G-box factor in a tissue and its binding to the *Adh* G-box.

INTRODUCTION

The *alcohol dehydrogenase* (*Adh*) genes of higher plants are induced by hypoxic stress in specific tissue types. Maize *Adh* may be expressed in the root, but is repressed in leaves (Okimoto et al., 1980; Sachs, Freeling, and Okimoto, 1980). The hypoxic induction is due to an increase in *Adh* mRNA transcription (Ferl, Brennan, and Schwartz, 1980; Gerlach et al., 1982; Rowland and Strommer, 1986). Promoter deletion analysis of maize *Adh* shows that several regions, including the anaerobic response element, are able to modulate mRNA induction (Ellis et al., 1987; Howard et al., 1987; Lee, Fenoll, and Bennetzen, 1987; Walker et al., 1987). The *Adh* gene of *Arabidopsis* is expressed in the mature plant in a manner similar to maize, and is constitutively expressed in cultured cells in the presence of 2,4-D (Dolferus, Marbaix, and Jacobs, 1985; Chang and Meyerowitz, 1986; Ferl and Laughner, 1989). The *Arabidopsis Adh* promoter has not, however, been extensively characterized by mutational analysis in transient or stable expression systems.

Transcriptional regulation of gene expression involves the interaction of DNA binding proteins with the gene of interest (Dynan and Tjian, 1985). Localization of these sites of interaction has been facilitated by the technique of in vivo dimethyl sulfate (DMS) footprinting (Church and Gilbert, 1984; Nick and Gilbert, 1985). The in vivo DMS footprints of the maize *Adh1* and *Arabidopsis Adh* genes have revealed several sites of putative regulatory protein interaction that are similar in sequence. The footprints of both genes are associated with the 4C-box motif and a moderately conserved 5′-GTGG-3′ motif (Ferl and Nick, 1987; Ferl and Laughner, 1989). In *Arabidopsis Adh* (at position −210), the GTGG motif is present on both strands as a perfect dyad (Ferl and Laughner, 1989).

This G-box (5′-CCACGTGG-3′) has also been identified in the 5′-flanking sequence of ribulose-1,5-bisphosphate carboxylase small subunit (*RbcS*) genes of several species (Guiliano et al., 1988). Members of the *RbcS* gene family, like those of the *Adh* gene family, are environmentally inducible and expressed in a cell-type-specific manner (Tobin and Silverthorne, 1985). *RbcS* mRNA is induced in the presence of light primarily in leaf and stem tissue and is not detectable in roots (Sugita and Gruissem, 1987). Deletion analyses of chimeric constructs in transgenic plants identified specific 5′-flanking regions necessary for light-mediated regulation (Morelli et al., 1985; Nagy et al., 1985; Timko et al., 1985; Fluhr et al., 1986), and a 5′-flanking region of pea *RbcS-3A*, which contains the G-box, is correlated with cell-type-specific expression of a reporter gene (Kuhlemeier et al., 1987, 1989). The G-box of tomato *RbcS-3A* has been shown to bind specifically to protein

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from crude nuclear extracts. Gel retardation assays show that G-box elements from other *RbcS* genes, including *Arabidopsis RbcS-1A*, compete specifically for G-box protein binding (Giuliano et al., 1988).

In vivo DMS footprinting of the parsley *chalcone synthase* gene has shown that the G-box, along with two other sites of protein/DNA interaction, are bound following chalcone synthase induction by UV irradiation (Schulze-Lefert et al., 1989). Furthermore, mutational and deletion analyses of transient expression in protoplasts indicate that the G-box, along with a downstream binding domain, is necessary for significant light responsiveness (Schulze-Lefert et al., 1989).

G-box-like sequences (85% similar) are also found in non-plant genes. Near-palindromic G-box is present in the *adenovirus* major late promoter (MLP) enhancer (at position −52 to −63) and also in the rat γ-fibrinogen promoter (at position −74 to −85) (Chodosh, Buratowsky, and Sharp, 1989).

In this report we show from the in vitro binding competition assay (Fried and Crothers, 1981) and from in vitro DMS footprinting (Church and Gilbert, 1984; Treisman, 1986) that a protein component of *Arabidopsis* whole cell extracts from cultured cells and mature leaves binds the *Arabidopsis Adh* G-box in a manner comparable with that observed in cultured cells in vivo (Ferl and Laughner, 1989). However, comparison of in vivo *Adh* G-box binding in cultured cells and leaves reveals significant differences between these two tissue types. This suggests that there need be no correlation between the presence of the G-box factor in a cell and its actual interaction with a particular gene.

RESULTS

A Protein Component of *Arabidopsis* Crude Whole Cell Extracts Binds to the G-Box

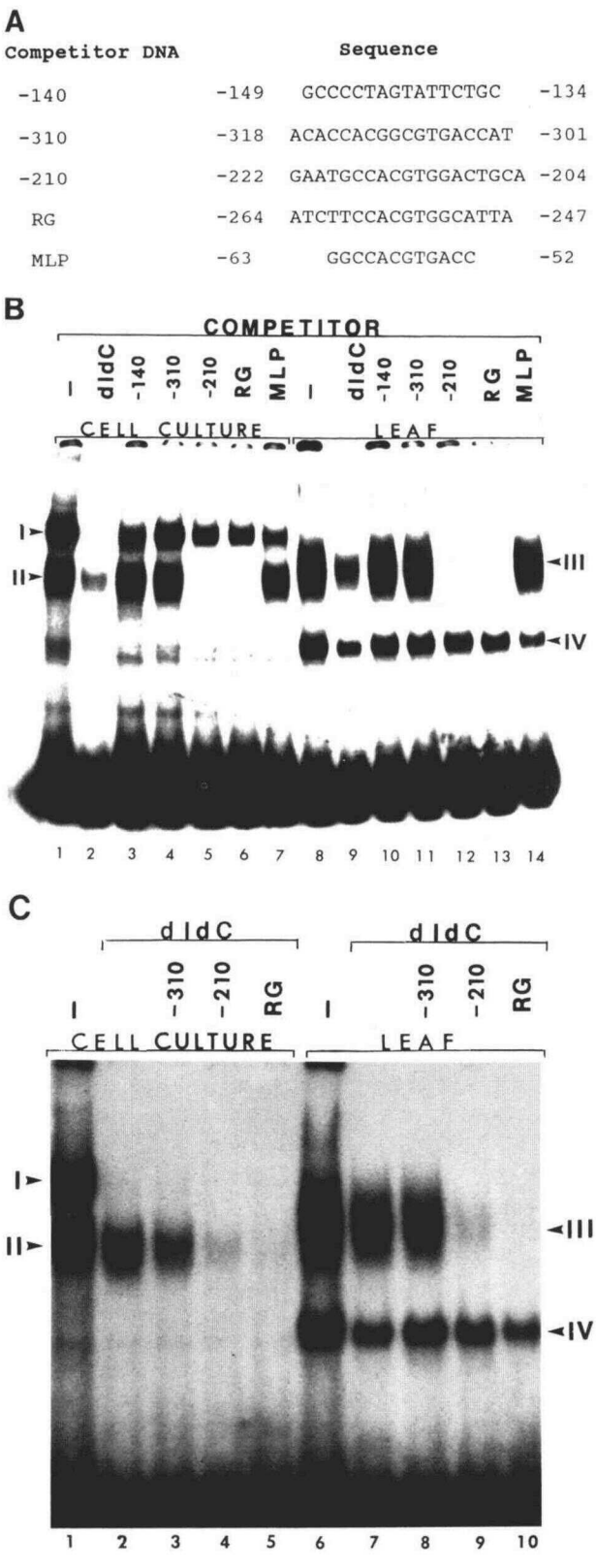
Oligonucleotides corresponding to previously determined sites of in vivo protein/DNA interactions at positions −310, −210, and −140 of the *Arabidopsis* 5′-flanking region (Ferl and Laughner, 1989) were synthesized. The −210 oligonucleotide was utilized as the probe for all in vitro gel retardation and DMS footprinting experiments. These experiments were conducted numerous times with identical results. Figure 1A shows the *Arabidopsis Adh* oligonucleotides along with those corresponding to the G-box of *Arabidopsis RbcS-1A* (RG) (Krebbers et al., 1988), and the G-box-like sequence of the adenovirus major late promoter (MLP) enhancer (Chodosh et al., 1989). The MLP oligonucleotide was synthesized identically in length and sequence to that presented by Chodosh et al. (1989) to be sufficient for specific binding to a component of yeast crude extract, and was chosen as a representative of G-box-like mammalian enhancer elements for this experiment. The specificity of G-box protein binding was determined by competition analysis using the gel retardation assay (Fried and Crothers, 1981). Crude whole cell extract (Manley et al., 1980) from cell culture exhibited two distinct types of G-box binding activity, Forms I and II (Figure 1B, lanes 1 to 7). Form I binding to the *Adh* G-box appears to be nonspecific, as it is observed in the absence of competitor DNA (lane 1), in the presence of 50 ng of both heterologous and homologous competitor oligonucleotides (lanes 3 to 7), but not in the presence of 1.0 μg of poly(dI-dC) (lane 2). Form II persists in the presence of up to 1.0 μg of poly(dI-dC) (lane 2) as well as 50 ng of heterologous competitor DNA (lanes 3, 4, and 7), yet is obliterated by 50 ng of homologous competitor *Adh* and *RbcS* G-boxes (lanes 5, and 6). Therefore, Form II appears specific for protein binding to both the *Adh* and *RbcS* G-boxes. The G-box-like MLP is not effective in competition for G-box binding.

The results of competition analysis obtained with leaf extract are similar to those from cell culture extract (Figure 1B, lanes 8 to 14) with two exceptions. Form IV persists in the presence of all competitor DNA (lanes 9 to 14), suggesting that it is nonspecific *Adh* G-box binding. The specific binding activity from leaf extract comparable with the Form II observed using cell culture extract is represented by Form III, a protein-DNA complex that was slightly reduced in migration rate relative to Form II. (This differential mobility is also seen in Figure 1C.)

Competition experiments were also conducted in the presence of 1.0 μg of poly(dI-dC) to demonstrate selective binding in excess heterologous carrier DNA. The results of this experiment are shown in Figure 1C. Binding to the G-box −210 probe in cell culture and leaf extract was conducted in the absence (lanes 1 and 6) or presence (lanes 2 to 5, and 7 to 10) of 1.0 μg of poly(dI-dC) in addition to 50 ng of the following oligonucleotide competitors: −310 (lanes 3 and 8), −210 (lanes 4 and 9), and RG (lanes 5 and 10).

Form I of the cell culture extract binding reaction is present in lane 1 and absent in lanes 2 to 5. As was seen in the previous experiment, poly(dI-dC) competitor at 1.0 μg abolishes Form I [as will 1.0 μg of the −210 G-box oligonucleotide (data not shown)]. Form II, however, persists in the presence of either 1.0 μg of poly(dI-dC) alone (lane 2) or 1.0 μg of poly(dI-dC) plus 50 ng of −310 (lane 3), yet is abolished in the presence of 1.0 μg of poly(dI-dC) plus 50 ng of −210 (lane 4) or 50 ng of RG (lane 5). We consider these results to be entirely consistent with the previous experiment (Figure 1B), and are in support of Form II being a protein-DNA complex that is specific for G-box binding.

Results obtained using leaf extract in the presence of high amounts of poly(dI-dC) (Figure 1C, lanes 6 to 10) are also consistent with the previous experiment. Bound Form III is observed in the absence of competitor DNA (lane 6),



in the presence of 1.0 μg of poly(dI-dC) (lane 7), and in the presence of 1.0 μg of poly(dI-dC) plus 50 ng of −310 (lane 8). Form III is abolished, however, when 1.0 μg of poly(dI-dC) and 50 ng of homologous competitor oligonucleotide are supplied to the binding reaction. Form IV, again, persists in the presence of all competitor DNA. Therefore, it is concluded from these results that bound Form III represents a protein/DNA interaction of a specificity equivalent to that observed for bound Form II.

In Vivo Interactions with *Adh* G-Box in Cultured Cells and Mature Leaves Correlate with mRNA Abundance

Specific DNA binding in vitro from both cell culture and leaf crude extract raises the question of whether a difference exists between the in vivo *Adh* G-box DMS footprint of these two tissue types. Figure 2A shows the in vivo DMS footprint of the *Adh* G-box for cell culture and leaves. Densitometric scans of the lanes in Figure 2B are presented in Figure 2C. To eliminate differences due to lane-to-lane variation in the amount on DNA loaded on the genomic sequencing gels, the scans are normalized so that the peaks corresponding to −229 and −232 (indicated by stars) are equal among the three samples. These bands

Figure 1. *Adh* G-Box (−210) Binding Factor Is Found in Both Cell Culture and Leaves of *Arabidopsis*.

(A) Competitor DNA oligonucleotides. The −210, −140, and −310 oligonucleotides were derived from the *Arabidopsis Adh* 5′-flanking sequence (Chang and Meyerowitz, 1986) where protein/DNA interactions are observed (Ferl and Laughner, 1989). The *RbcS* G-box oligonucleotide (RG) was synthesized from the −243 region of *Arabidopsis RbcS-1A* (Krebbers et al., 1989). The MLP oligonucleotide corresponds to the −60 region of the MLP of *Adenovirus* (Chodosh et al., 1989). Only the top strand of the sequence is shown.

(B) Crude whole cell extracts from cells in culture (lanes 1 to 7) or mature leaf tissue (lanes 8 to 14) were assayed for *Adh* G-box binding using the gel retardation assay (Fried and Crothers, 1981). The probe was 5′-end-labeled, double-stranded −210 [see **(A)**]. Binding reactions were carried out either in the absence of competitor DNA (lanes 1 and 8), or in the presence of 1.0 μg of poly(dI-dC) (lanes 2 and 9) or 50 ng of the following oligonucleotides: −310 (lanes 3 and 10), −140 (lanes 4 and 11), −210 *Adh* G-box (cold probe) (lanes 5 and 12), RG (lanes 6 and 13), or MLP (lanes 7 and 14).

(C) Crude whole cell extracts from cells in culture (lanes 1 to 5) or mature leaf tissue (lanes 6 to 10) were assayed as in **(B)**, with modification of competitive binding conditions. Binding reactions were carried out either in the absence of competitor DNA (lanes 1 and 6), or in the presence of 1.0 μg of poly(dI-dC) alone (lanes 2 and 7), or in the presence of 1.0 μg of poly(dI-dC) plus 50 ng of one of the following oligonucleotide competitor DNAs: −310 (lanes 3 and 8), −210 (lanes 4 and 9), and RG (lanes 5 and 10).

Specific competition is observed for only Forms II and III with both *Adh* and *RbcS* G-boxes.

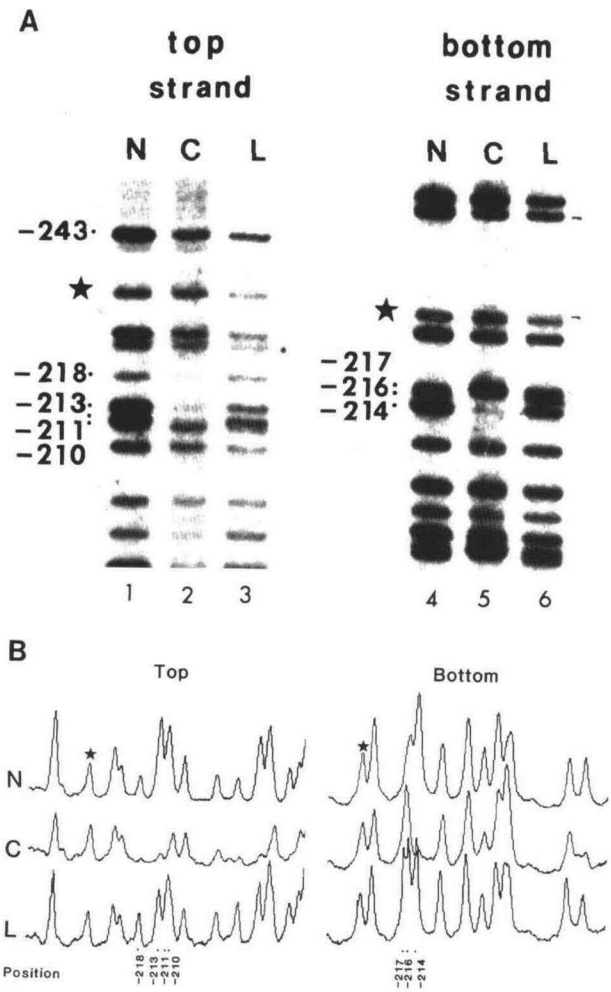


Figure 2. Genomic Sequencing Display of the in Vivo DMS Footprints of *Arabidopsis Adh* G-Box in Cell Cultures and Mature Leaves.

(A) Genomic sequencing display of top strand (left panel, lanes 1 to 3) and bottom strand (right panel, lanes 4 to 6) reactions comparing naked genomic DNA (N) with DNA isolated from cells (C) or leaves (L) treated in vivo with DMS. Duplicate filters were hybridized to strand specific probes (Ferl and Laughner, 1989) to produce the autoradiograms. The G-box core extends from approximately −210 to −220, and the specific guanine residues of the G-box are labeled and indicated by dots. Protein/DNA interactions result in the protection or enhancement (relative to naked DNA) of bands corresponding to G residues in close contact with the binding factor.

(B) Densitometric scans of the top and bottom strand of the autoradiograms presented in **(A)**. The autoradiograms of **(A)** were subjected to laser densitometry. To somewhat correct for loading differences among lanes, the output scans were adjusted so that the bands corresponding to −229 on the top strand and −232 on the bottom strand were approximately equal in peak height/area. These bands, indicated by a star, are outside the G-box (see Figure 5), and have consistently been shown to be free of interactions. The guanine residues of the G-box are indicated by dots beneath the scans, and correlate with the bands of **(A)**.

were chosen as standards because repeated experiments have shown these G residues to be outside the direct G-box footprint.

A set of striking interactions characterize the G-box footprint in the cell cultures. Guanine residues of the top strand (Figure 2A, lane 2) at positions −218, −213, and −211 are almost completely protected from methylation. The methylation at position −210 of the top strand is somewhat enhanced. Bottom strand interactions for cultured cells (lane 5) show a methylation enhancement at position −217 and strong methylation protection at positions −216 and −214. The corresponding in vivo DMS footprint of mature leaves is very different. There is some protection of −213 on the top strand (lane 3, scan L). However, there is no discernible protection of −218 or enhancement of −210. On the bottom strand (lane 6), −214 and −216 exhibit no protection, yet −217 is enhanced. Furthermore, the in vivo footprints observed in cell cultures at −310, −180, and −140 were also undetectable in leaves (data not shown).

RNA gel blot analysis of the *Arabidopsis* cell culture and mature leaves is shown in Figure 3, with *Adh* mRNA being abundant in cultured cells and not detectable in leaves.

In Vitro DMS Footprinting of the *Adh* G-Box with Cell Culture and Leaf Crude Extract

The in vitro competition analysis (Figure 1B) demonstrates the specificity of a protein factor for *Adh* G-box binding, but it does not indicate whether this factor is the same one responsible for the in vivo DMS footprint observed in cell culture. An in vitro DMS footprint of the *Adh* G-box complexes from cell culture and leaf crude extract (Forms II and III) is shown in Figure 4. Interactions that occur in vivo in cultured cells are found at identical G residues in vitro using either cell culture or leaf crude extract and are summarized in Figure 5. These results support the hypothesis that the protein component of crude extract that binds the G-box in vitro is the same protein that binds to this

Figure 3. RNA Gel Blot Analysis of *Arabidopsis* Cultured Cells and Mature Leaves.

Adh mRNA is abundant in total RNA from the cell cultures (C, lane 1), but undetectable in total RNA isolated from mature green leaves (L, lane 2).

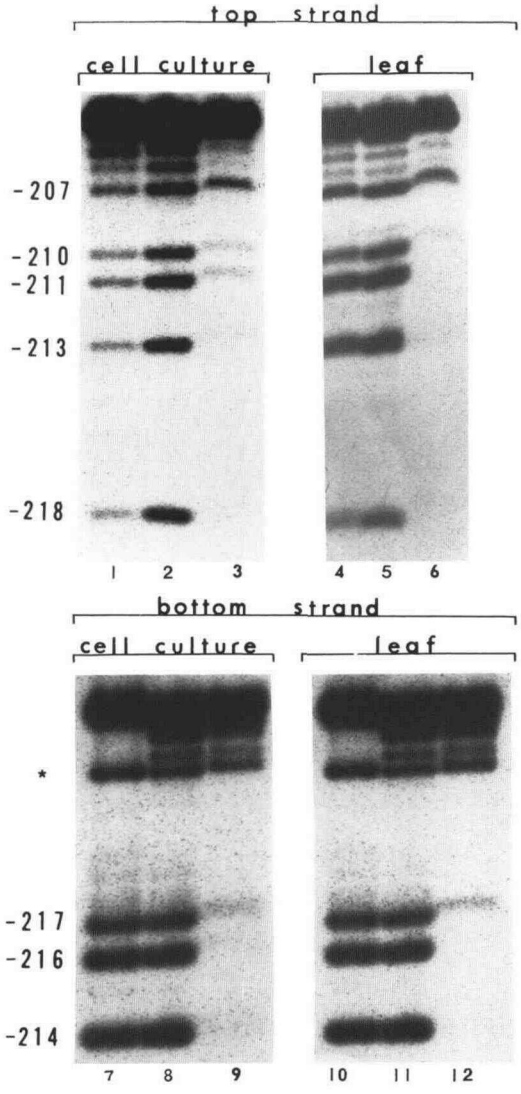


Figure 4. In Vitro DMS Footprinting of the *Arabidopsis Adh* G-Box from Cell Culture and Leaf Whole Cell Crude Extract.

A −210 *Adh* G-box (Figure 1A) double-stranded oligonucleotide containing PstI end $\begin{pmatrix} \text{GAATGCCACGTGGACTGCA} \\ \text{ACGTCTTACGGTGCACCTG} \end{pmatrix}$ was 5′-end-labeled on either the top strand only (upper panel) or lower strand only (lower panel). After binding with cell culture or leaf extract, the binding reaction was treated with DMS and subjected to preparative electrophoresis on gels similar to those in Figure 1. The bands corresponding to the free, unbound probe (lanes 2, 5, 8, and 11), bound Form II (lanes 3 and 9), and bound Form III (lanes 6 and 12) were recovered by electroelution. As an additional control, the probes were treated with DMS in binding buffer without extract (lanes, 1, 4, 7, and 10). The asterisk indicates a guanine residue outside the G-box, within the PstI end. This G-box oligonucleotide with PstI ends has identical bandshifting and competition qualities as the −210 oligonucleotide without PstI ends.

DNA sequence in vivo. In spite of the overall similarity between the in vivo and the in vitro DMS footprints, two qualitative differences are observed. An enhancement of DMS methylation is observed in vivo at position −210 of the top strand and −217 of the bottom strand, yet these G residues are protected in vitro.

DISCUSSION

The G-box sequence is highly conserved among *RbcS* genes (Giuliano et al., 1988) and some other higher plant genes (Ferl and Nick, 1987; Ferl and Laughner, 1989; Schulze-Lefert et al., 1989). In vivo DMS footprinting of an *RbcS* gene is not yet available for comparison with that of *Adh*, but in vivo studies conducted in parsley for the *chalcone synthase* 5′-flanking sequence have revealed a G-box protein footprint with DMS interactions identical to that observed in *Arabidopsis Adh* in cell culture (Schulze-Lefert et al., 1989). It is possible that the conserved G-box interaction has been selected by certain gene families to function as a transcriptional enhancer.

Although G-box sequences have been identified in several *RbcS* and other plant genes, the only *Adh* gene known to contain the conserved G-box element is that from *Arabidopsis*. Maize *Adh1* and *Adh2* do not have dyad G-box elements (Dennis et al., 1984, 1985; Ferl and Nick, 1987). Comparison of factor binding sites in *Arabidopsis* and maize by in vivo DMS footprinting (Ferl and Laughner, 1989) shows that the conserved 4C-box and a GTGG motif that is essentially half of a G-box are bound in the 5′-flanking sequence of both genes. We have found that these sequences do not compete for protein binding to the G-box in vitro (data not shown) and, therefore, suggest that these two element types are not bound by a G-box factor in vivo. The G-box factor is, therefore, separate and distinct from the factor(s) involved with the 4C-box or GTGG motifs. This indicates that a precise dyad G-box is not essential for anaerobic induction of *Adh* in maize and

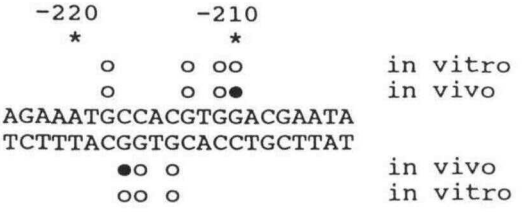


Figure 5. A Summary Comparison of in Vivo and in Vitro DMS Footprinting for the *Arabidopsis Adh* G-Box.

Protein/DNA interactions are designated by circles. Open circles denote G residues protected from DMS methylation and solid circles indicate enhanced methylation.

suggests a more general role for the G-box in transcriptional regulation.

RbcS sequences containing the G-box confer both organ-specific and photoinducible expression in transgenic plants (Fluhr et al., 1986; Kuhlemeier et al., 1987). However, a recent report (Kuhlemeier et al., 1989) has shown that a fragment containing the G-box is not essential for photoinduction of pea *RbcS-3A*, yet its presence will confer organ-specific expression in their system. These data may suggest a role for the G-box in organ-specific gene expression, but this property has not been proven to be determined solely by G-box binding. Additional experimentation of stable expression in transgenic plants of other plant genes that are cell-type-specific in expression and also contain a G-box element in the 5′-flanking sequence will be necessary to address this question properly.

G-box-like sequences of animal promoters (Chodosh et al., 1989) are known to function as enhancer elements, but our binding competition results indicate that the MLP sequence does not bind the same protein as the *Adh* G-box. Although this oligonucleotide (MLP) is 12 bp in length as opposed to the 18-bp −210 probe, the 12-bp MLP oligonucleotide has been shown to be sufficient for specific yeast protein binding (Chodosh et al., 1989). Similarly, Giuliano et al. (1988) showed that a 12-bp "core" sequence of the tomato *RbcS-3A* G-box was sufficient (to compete) for binding of the G-box factor.

It is interesting to note that the difference in electrophoretic mobility of the bound G-box between cell culture and leaf extract of *Arabidopsis* (Figures 1B and 1C) has also been observed between light-grown and dark-adapted tomato leaf extract (Giuliano et al., 1988). Extracts from dark-adapted leaf tissue, in which *RbcS* genes are not induced, exhibited a G-box-protein complex that was slightly more rapid in migration rate than that from light-grown plants. This correlates with what we observe in *Arabidopsis*, with the specific cell culture complex (Form II) migrating more rapidly than that from leaves (Form III). This observation suggests that the G-box factor we have characterized from cell culture extract (which forms the rapidly migrating complex) may not be restricted to *Adh*-inducible tissues, and that the possibility of *Adh* induction or in vivo *Adh* G-box binding in dark-adapted leaves should be examined.

The differences we have observed in DMS modification of G residues between the in vivo and in vitro DMS footprints may be a result of the artificial nature of the in vitro binding environment or a result of G-box factor binding in the absence of adjacent protein/DNA interactions (Shuey and Parker, 1986; Ferl and Laughner, 1989). The second possibility can be addressed by increasing the length of the probe used for the in vitro DMS footprint to include flanking sequences that bind (other) protein factors in vivo. This would more closely resemble the in vivo binding conditions and may assist in characterizing other putative regulatory interactions that were identified by the in vivo DMS footprint.

The presence of G-box factor(s) within a cell does not correlate with in vivo binding to the *Adh* G-box. In vitro G-box binding activity is found in both cell cultures and leaves. Although the leaf G-box binding activity forms a complex of slightly different electrophoretic mobility, it has the same guanine-specific contacts with the G-box as does the cell culture activity. However, the guanine-specific contacts of the in vivo footprints at the G-box are dramatically different between cell cultures and leaves. Although there are some potential interactions with gaunines at −217 and −213, leaves do not show a footprint that is either quantitatively or qualitatively consistent with partial or complete binding of the G-box factor as appears in vivo in cell cultures or in vitro in Form II or Form III. Because the pattern of DMS modification should be a diagnostic feature of the binding, we conclude that in leaves in vivo the *Adh* G-box is not occupied by the binding activity that we have characterized as Form III. Therefore, the presence of G-box factors is unlikely to determine tissue-specific transcription. However, G-box factor binding to any given target gene can be tissue-specific.

We suggest that the in vivo difference in *Adh* G-box binding between cell culture and leaf tissue may be due to protein/protein interactions or some cell-type variation in DNA modification or chromatin structure such that the *Adh* G-box is not available for binding to the G-box factor. The ability of *Adh* and *RbcS-1A* of *Arabidopsis* (and perhaps other genes with G-boxes such as *chalcone synthase*) to utilize the same putative regulatory protein suggests that the G-box element may participate in, but not direct, transcriptional activation of genes in diverse regulatory pathways.

METHODS

Gel Retardation Assay

Whole cell extracts were prepared by variations of Manley et al. (1980) and Wu (1984). Cell cultures or leaves were frozen in liquid nitrogen before homogenization at 4°C in 15 mM Hepes, pH 7.6, 40 mM KCl, 5.0 mM MgCl, 1.0 mM DTT, and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). One-tenth volume of 4.0 M ammonium sulfate was added and the slurry centrifuged for 30 min at 19,000 *g*, 4°C. To the supernatant, 0.3 g/mL ammonium sulfate was added and mixed on ice for 60 min. Protein was then precipitated at 15,000 *g* for 20 min, 4°C. This pellet was resuspended (1.0 mL/10 g of tissue) in 20 mM Hepes, pH 7.6, 40 mM KCl, 1.0 mM DTT, 0.5 mM PMSF, 0.1 mM EDTA, and 10% glycerol, then dialyzed 4 hr in two changes of 100 volumes of 20 mM Hepes, pH 7.6, 40 mM KCl, 0.1 mM PMSF, 0.1 mM EDTA, 10% glycerol, and 5 mM β-mercaptoethanol. After dialysis, the extract was frozen with liquid nitrogen and stored at −80°C until

use. Binding reactions were performed at room temperature in 13 μL containing 10 μL (60 μg) of crude extract. All binding reactions contained either 1 μL of competitor DNA, or extract buffer as the no-competitor control, 1 μL of end-labeled *Adh* G-box oligonucleotide (1.0 ng), and 1.0 μL of 1.0 M KCl for a final KCl concentration of 110 mM. A 5-min prebinding of competitor DNA and protein was followed by the addition of labeled oligonucleotide probe, a 5-min additional incubation, and electrophoresis on a 5% nondenaturing 89 mM Tris-Cl, 89 mM boric acid, and 2.6 mM EDTA-polyacrylamide gel at 30 mA.

In Vivo DMS Footprinting

In vivo DMS footprints of the G-box were generated for both cell cultures and mature (4-week-old) leaves using the technique of genomic sequencing as previously applied to *Arabidopsis* (Ferl and Laughner, 1989). The autoradiograms were scanned for densitometry on a Molecular Dynamics laser densitometer.

RNA Gel Blot Analysis

RNA was isolated from cell cultures and leaves by the method of Galau et al. (1981). Five micrograms of RNA were electrophoresed on a 1.5% agarose gel containing 2.2% formaldehyde (Maniatis, Fritsch, and Sambrook, 1982). The RNA was capillary blotted to nitrocellulose and probed (Finkelstein et al., 1985) with plasmid jAt3011 (Chang and Meyerowitz, 1986).

In Vitro DMS Footprinting

In vitro DMS footprinting was carried out as described (Treisman, 1986). Following electrophoretic fractionation of binding reactions or probe alone, gel slices were electroeluted, cleaved at methylated G residues with piperidine, and fractionated by electrophoresis on a 10% polyacrylamide-urea gel. Approximately equal counts were loaded per lane. The extent of methylation protection varies among G residues within a given footprint and is highly reproducible.

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