Characterization of the Arabidopsis Adh G-Box Binding Factor

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Protein-DNA interaction at an inverted repeat of the sequence 5'-GTGG-3' (G-box) has been associated with the transcription of several plant genes [Giuliano, G., et al. (1988). Proc. Natl. Acad. Sci. USA 85, 7089-7093; Ferl, R.J., and Laughner, B.H. (1989). Plant Mol. Biol. 12, 357-366; Schulze-Lefert, P., et al. (1989). EMBO J. 8, 651-656]. We characterized the binding of the Arabidopsis G-box binding factor (GBF) from whole-cell extracts and fractionated extracts to the G-box of alcohol dehydrogenase (Adh) using gel mobility shift assays. DNase I footprinting localized the region of GBF/G-box interaction to two sites, one apparent high-affinity binding site (−227 to −201) and a possible low-affinity binding site (−193 to −182). DNA-protein cross-linking demonstrated that the G-box is bound by proteins of two sizes, 31 kilodaltons and 18 kilodaltons. In addition, we found that in vitro the interaction of GBF from Arabidopsis suspension cultures or leaves with the Adh G-box is indistinguishable, and that there is evidence of multiple protein-protein interactions.

INTRODUCTION

Plants are known to respond to conditions of environmental stress by changing their pattern of gene expression (Cherry, 1989). Alcohol dehydrogenase (Adh) is an example of a plant gene that is environmentally inducible in specific organs in addition to being developmentally regulated (Sachs and Freeling, 1978). This intricate pattern of expression suggests that multiple factors are involved in controlling Adh transcription.

There are two Adh genes in corn (Adh1 and Adh2) and one in Arabidopsis, and in both plants they are regulated in similar ways (Chang and Meyerowitz, 1986). In vivo dimethyl sulfate (DMS) footprinting shows that the promoter regions of corn Adh1 and Arabidopsis Adh have similar sequences where protein-DNA interactions occur when the genes are transcriptionally active (Ferl and Nick, 1987; Ferl and Laughner, 1989). However, there are also regions of protein-DNA interactions that are not common between corn Adh1 and Arabidopsis Adh. One such region in the Arabidopsis Adh promoter is also found in the parsley chalcone synthase (CHS) promoter and shows the same pattern of in vivo DMS footprint enhancements and protections (Schulze-Lefert et al., 1989b). This common cis-DNA element, the G-box, is also present in the Antirrhinum majus CHS promoter (Staiger et al., 1989) and in several RbcS genes (Giuliano et al., 1988). Protein extracts from tobacco, Antirrhinum (Staiger et al., 1989), tomato, Arabidopsis seedlings (Giuliano et al., 1988), and suspension cultures (McKendree et al., 1990) contain specific G-box binding activity in vitro (referred to as G-box binding factor, or GBF) (Giuliano et al., 1988). Furthermore, this conserved DNA sequence is shown by deletion analyses to be important for transcription of RbcS and CHS (Ueda et al., 1989; Schulze-Lefert et al., 1989a, 1989b). However, the contribution of the G-box to Adh transcription has not been examined by deletion analyses.

Previous work demonstrates that protein extracts from Arabidopsis suspension culture cells that express Adh bind the Adh G-box in vitro (McKendree et al., 1990). Although Adh mRNA cannot be detected in Arabidopsis leaves, GBF from leaves shows specific binding to the Adh G-box in vitro.

Although Adh, RbcS, and CHS are all inducible and developmentally regulated, they are transcribed under different circumstances and in different plant organs. The fact that the G-box element is found in these different genes suggests that GBF is a general transcription factor that is involved in controlling the expression of several unrelated plant genes.

In this study, we further characterized GBF from Arabidopsis suspension cultures and leaves. Crude cell extracts from both sources were fractionated and the GBF was characterized based on its gel mobility shift, DNA-protein cross-linking, and DNase I footprinting of the Adh G-box DNA element.

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RESULTS

G-Box Binding Activity Was Present in Crude Whole-Cell Extracts from Suspension Cultures and Leaves

Figure 1 shows the DNA probes and competitors that were used in gel mobility shift assays. All sequences were derived from the Arabidopsis Adh gene (Chang and Meyerowitz, 1986) as described in Methods.

Gel mobility shift assays were used to detect GBF activity in crude whole-cell extracts from both suspension cultures and leaves. Figure 2, lanes 1 and 4, reveals that both crude extracts showed several shifted complexes of the probe. The G-box binding activities in the two extracts differed primarily in the relative mobilities of the complexes; there were slower migrating complexes in the suspension culture extract. All complexes in both extracts were specifically competed by the G-box probe but not by the 3' competitor (Figure 2, lanes 2, 3, 9, and 10). In addition, the FP oligo competed with activity in all shifted bands in the suspension culture extract, but the -190 oligo, which is homologous to the region 3' to the FP oligo in the G-box probe, did not compete to any significant extent (Figure 2, lanes 12 and 13).

In the process of scaling up the extract from suspension cultures (from 20 g to 100 g of tissue), it was noted that the fastest migrating form was predominant in the large-scale extract, but the slower migrating complexes were absent (Figure 2, lane 5). This activity showed specificity for the G-box in that it was competed by the G-box probe, the FP oligo, and not by the 3' competitor or the -190 oligo (Figure 2, lanes 5, 6, 15, and 16). As will be shown, upon further purification of either suspension cell or leaf extracts, this fast migrating form became the predominant activity that was observed on gel mobility shift assays, and it showed specific interactions on DNase I footprints. In addition, the G-box probe cross-linked to polypeptides of the same size from all extracts (see below).

The G-Box Probe Cross-Linked to Proteins of the Same Size from Suspension Cell and Leaf Extracts

Figure 3 shows crude extracts from suspension culture small-scale and large-scale preparations and from leaves cross-linked to the G-box probe and separated on a 12.5% SDS-polyacrylamide gel. All three extracts cross-linked to proteins of about 31 kD and 18 kD. In addition, a band at about 60 kD is seen in the large-scale suspension cell extract (Figure 3, lane 3) that was not reproducible between experiments. These data are additional evidence that the GBF that was in direct contact with the G-box in both suspension culture extracts and the leaf extract was the same. Furthermore, because there were so few proteins that cross-linked, the multiple complexes seen on gel mobility shift assays of crude extracts may be due to protein-protein interactions as opposed to multiple DNA-binding proteins.
G-Box Binding Activity from Suspension Culture Extracts Eluted from Heparin-Agarose between 0.3 M and 0.6 M KCl

Crude whole-cell extract from the large-scale suspension culture preparation was applied to heparin-agarose as described in Methods and resolved by stepwise elution with NEBD (20 mM Hepes-KOH, pH 7.6, 75 mM KCl, 0.1 mM EDTA, 10% glycerol, 5 mM β-mercaptoethanol) containing 0.15 M, 0.3 M, 0.6 M, and 1 M KCl. (Unless otherwise specified, all further results that are described for suspension culture extracts will be for the large-scale extract.) The fractions were assayed for G-box binding activity by gel mobility shift, as shown in Figure 4. The only fraction that contained detectable GBF activity was the one that eluted between 0.3 M and 0.6 M KCl (hereafter designated 0.6 M fraction). All other fractions showed considerable nonspecific DNA binding activity that was competed by low amounts of poly(dIdC) (0.1 μg to 0.4 μg). The gel mobility shift activity in the 0.6 M fraction was resistant to competition by high concentrations of dIdC (at least 2 μg). The 0.6 M G-box binding activity was competed by the G-box probe but not by the 3′ competitor (Figure 4, lanes 16 and 17). Therefore, the DNA binding activity that eluted from heparin agarose between 0.3 M and 0.6 M KCl was specific for the G-box sequence.

The Suspension Culture 0.6 M Heparin-Agarose Fraction and the Crude Leaf Extract Both Protected the Same Region of the G-Box Probe from DNase I Digestion

To determine whether GBF from suspension culture extracts and leaf extracts showed the same sequence-specific G-box binding, increasing amounts of the suspension culture 0.6 M fraction and the crude leaf extract were used in separate DNase I footprinting assays with both strands of the G-box probe. Figures 5A and 5B show results for the 0.6 M fraction and the leaf extract, respectively. GBF from either suspension cultures or from leaves protected the region from −225 to −201 of the top strand G-box probe from DNase I digestion. In addition, the guanosine at position −211 was enhanced in both. This footprinted...
The large-scale suspension culture extract was fractionated over heparin-agarose. The fractions were analyzed by gel mobility shift of the G-box probe in the presence of increasing amounts (µg) of poly(dldC). The fractions eluted at 0.15 M, 0.3 M, 0.6 M, and 1 M KCl are indicated across the top. Binding reactions in lanes 15, 16, and 17 all contained 4 µg of poly(dldC). Additional competitors (comp.) (0.05 µg) in lane 16 and lane 17 are described in Figure 1. Frames around different panels separate different gels.

region contains the GTGG inverted repeat as well as 7 bp upstream and 9 bp downstream of the inverted repeat. Either extract also protected the bottom strand from digestion between bases -227 and -203. As with the top strand, the protected region of the bottom strand contains the internal GTGG inverted repeat with flanking regions of 10 bp upstream and 7 bp downstream. Therefore, GFB from suspension cultures and leaves showed the same sequence-specific interactions with the G-box probe.

GBF from the FPLC Superose 6 HMW Peak Eluted from FPLC Mono Q between 0.3 M and 0.44 M KCl

To fractionate the suspension culture GFB further, the HMW FPLC Superose 6 peak was subjected to anion exchange chromatography on an FPLC Mono Q column as described in Methods. After dialysis against NEBD, the fractions were assayed for GFB by gel mobility shift of the G-box probe. Figure 7A shows a gel mobility shift analysis of only the first 10 fractions. There was no detectable GFB activity in fractions 11 to 20. One peak of GFB activity eluted from FPLC Mono Q between 0.3 M and 0.44 M KCl (fractions 5 to 7).

To determine whether the activity in these fractions protects the same DNA sequence from digestion as the 0.6 M KCl fraction from heparin-agarose, the FPLC Mono Q fractions were used in DNase I footprinting assays (Figure 7B). The FPLC Mono Q fractions that showed the most activity in gel mobility shift assays (5 to 7) also protected the same regions of the G-box probe from DNase I digestion as the 0.6 M fraction (top strand, -225 to -201; bottom strand, -227 to -203). In addition, a small footprinted region was seen on the bottom strand located downstream of the major footprint. In fractions 6 to 8 and, to a lesser extent, in fraction 5, bases from about -195 to -182 were also protected. This region contains the cryptic G-box of the -190 oligo (Figure 1). The 0.6 M
Figure 5. DNase I Footprint Analyses.
(A) Suspension culture 0.6 M KCl eluate from heparin-agarose.
(B) Crude leaf extract.
DNase I footprints of the G-box probe were done as described in Methods. Lanes are labeled with the amount of the respective extract that was used in the reaction (μL). The lanes labeled G show the Maxam and Gilbert (1980) guanine sequencing reaction of the G-box probe. Sequences that are protected from DNase I digestion are indicated. The asterisk marks the enhanced band on the top strand.

fraction and the leaf extract did not give a distinct footprint in this region. In addition, there was no obvious footprint on the other strand in the -190 region.

GBF from Leaves and from the Small-Scale Suspension Culture Extract Eluted from FPLC Mono Q at the Same Salt Concentration as GBF from the Large-Scale Suspension Culture Extract

The HMW FPLC Superose 6 peak from the leaf extract and a small-scale suspension culture 0.6 M KCl eluate from heparin-agarose were subjected to anion exchange chromatography on FPLC Mono Q. After dialysis against NEBD, the fractions were analyzed for GBF by gel mobility shift assay, as shown in Figures 8A and 8B. Each fraction from the suspension culture extract was analyzed in the presence of a 400-fold excess of dldC. A band that corresponds to the fast migrating form in the large-scale suspension culture extract eluted at the same salt concentration from the FPLC Mono Q fractionation of both the leaf and small-scale suspension culture extracts. In addition, other complexes were seen that eluted at different salt concentrations. In the suspension culture extract,
Faster migrating bands in fraction 4 were more resistant to competition by the higher amounts of dldC, but they decreased in intensity. These data are further evidence that specific G-box binding activity in all three extracts (large- and small-scale suspension culture extracts and the leaf extract) is represented in this predominant band and that slower migrating complexes are due to protein-protein interactions. In addition, these data are evidence that the GBF from suspension culture cells and leaves is the same protein.

**DISCUSSION**

We have fractionated whole cell extracts from *Arabidopsis* suspension cultures and leaves to characterize the Adh GBF. In crude extracts from both suspension cultures and leaves, there were several complexes on a gel mobility shift of the G-box probe (Figure 2). It is possible that other DNA-binding proteins recognize sequences on the probe outside of the G-box. However, all forms in gel mobility shift assays were competed by the G-box probe, as well as the oligonucleotide that is homologous to the footprinted region of the G-box probe, but not by the more 3',−190 oligonucleotide (Figures 1 and 2). Therefore, the slower migrating complexes were most likely due to protein-protein interactions between the GBF/G-box complexes and other proteins present in extracts before extensive purification of the GBF protein. The slower migrating complexes were not seen upon subsequent fractionation of the extract.

Variability was observed between different suspension culture crude extracts in the number of gel mobility shift complexes of the G-box probe. The small-scale extract had several complexes, whereas the large-scale extract had one predominant and one minor form. After fractionation of the small-scale extract, the faster migrating complex that corresponded to the predominant form in the large-scale extract persisted, whereas the other complexes disappeared, again suggesting that other proteins interacted with GBF until they were removed. Giuliano et al. (1988) observed two complexes in their gel mobility shift assays of crude nuclear extracts from *Arabidopsis* leaves that may correspond to the forms that we observed in the large-scale extract. Because Giuliano et al. (1988) observed that in light- and dark-grown plants the two forms migrated differently, it is difficult to determine how their results relate to ours. Other differences in methodologies between this study and that of Giuliano et al. (1988), such as our utilization of whole-cell extracts as opposed to nuclear extracts and the use of different probes, may account for some of the apparent differences in G-box gel mobility results.

The data presented here support the hypothesis that the GBF present in *Arabidopsis* leaves and suspension
Figure 7. Gel Mobility Shift Analysis and DNase I Footprinting of FPLC Mono Q Fractions.

The suspension culture HMW peak from FPLC Superose 6 was subjected to FPLC Mono Q chromatography as described in Methods.

cultures is the same protein. The possibility that the distribution of GBF in various plant organs is correlated with RbcS expression was raised by Giuliano et al. (1988), who reported that GBF is present in Arabidopsis leaves and stems where RbcS is expressed and present in low to undetectable amounts in roots where RbcS is not expressed. However, if GBF were required for Adh transcription, then one would expect that it would be present in roots where Adh is known to be expressed (Dolferus et al., 1985; Chang and Meyerowitz, 1986). Although it is possible that GBF shows some tissue specificity and that Adh does not require GBF for transcription in roots, it is also possible that the apparent lack of significant amounts of GBF in roots (Giuliano et al., 1988) may be due to differences in GBF stability in vivo or during extraction from roots versus leaves and stems. We found that GBF from leaf extracts was more labile in vitro than similar preparations from suspension cultures (data not shown).

Because it is not known whether or not the Arabidopsis suspension cultures express RbcS, the possibility of a correlation between RbcS expression and GBF abundance is not addressed by our data. In leaves there is no correlation between the presence of GBF and Adh expression (McKendree et al., 1990).

It seems likely that the G-box is an example of a conserved cis promoter element with a generalized function that is found in several classes of plant genes. It also seems likely that GBF works in concert with gene-specific transcription factors and promoter elements to regulate transcription. Although deletion analyses have not been done with the Arabidopsis Adh promoter, in vivo DMS footprints show that there are several regions where protein-DNA interactions occur when the gene is active, including the perfect G-box dyad at −217 to −210 and the imperfect G-box dyad at −193 to −186 (Ferl and Laughner, 1989; McKendree et al., 1990). Deletion analyses of the tomato RbcS-3A promoter demonstrate that the region from −374 to −205 is essential for promoter function (Ueda et al., 1989). This region contains a G-box and several other cis-elements that are conserved among different RbcS genes. In the parsley CHS promoter, there are a perfect G-box and an imperfect G-box and both require an adjacent cis-DNA element. Either set of elements, each set containing one G-box, can direct light-responsive transcription (Schulze-Lefert et al., 1989a). In addition to the

Figure 7. (continued).

(A) The resulting fractions were analyzed for gel mobility shift of the G-box probe. The lane labeled FT indicates the column flow-through. Subsequent numbers indicate column fractions.

(B) Fractions 5 to 8 were further analyzed for DNase I footprinting of the G-box probe. Lanes are labeled with the FPLC Mono Q fraction number, or 0 for no extract, or G for the Maxam and Gilbert (1980) guanine sequencing reaction of the G-box probe. Sequences that were protected from DNase I digestion are indicated by brackets.
genes described, all of which might be stress-inducible genes, a G-box element is present in the 5' region of the gene for the storage protein patatin, which shows yet another pattern of gene expression (Rosahl et al., 1986). Although there is no evidence at this time that the G-box is important to the transcription of patatin, it appears that the G-box and GBF may be involved in controlling transcription of a wide variety of plant genes.

The data suggest that the Adh perfect G-box dyad at −217 to −210 is a high-affinity binding site for GBF and that the imperfect G-box dyad at −193 to −186 may be a low-affinity binding site. The −190 oligo, which contains the imperfect G-box, could not compete with the G-box probe (containing both sites) in gel mobility shift assays of the crude extracts. However, the region of the G-box probe that contains the imperfect G-box was DNase I footprinted by a partially purified, concentrated extract of GBF. These data suggest that GBF may occupy both sites with preferential binding at the perfect dyad site. Binding at the imperfect dyad site may involve protein-protein cooperativity with occupancy of both sites being required for full activity. A classic example of repetitive elements with different binding affinities occurs in the Drosophila hsp 70 promoter, where the heat shock transcription factor binds the TATA proximal site with 12.5 times higher affinity than the more distal site (Topol et al., 1985). The distal site is occupied because of a cooperative interaction with heat shock transcription factor bound at the high-affinity site.

In crude extracts, the G-box probe cross-linked to polypeptides that separated on SDS-PAGE to 31 kD and 18 kD. GBF may consist of multiple polypeptides, or the 18-kD polypeptide may be a degradation product of the 31-kD polypeptide.

The dyad symmetry of the G-box 5'-CCACGTGG-3' sequences is reminiscent of DNA elements that are bound by homodimeric DNA-binding proteins. For example, the leucine zipper class of DNA-binding proteins recognizes DNA binding sites that consist of directly abutted, dyad-symmetric half sites (Vinson et al., 1989). The −190 oligo that contains sequences very similar to the G-box but with one imperfect half of the dyad was unable to compete with G-box binding in gel mobility shift assays (Figures 1 and 2). In DNase I footprints of the Arabidopsis Adh G-box (Figures 5 and 7), as well as the RbcS G-box (Giuliano et al., 1988), the inverted repeat was located in the approximate center of the protected region. In addition, in vivo DMS footprints of the Arabidopsis Adh G-box (Ferl and Laughner, 1989; McKendree et al., 1990) and the CHS G-box (Schulze-Lefert et al., 1989b) show identical, symmetric patterns of protections and enhancements centered around the inverted repeat. These results are consistent with what would be expected of DNA binding by a symmet-
rical dimeric protein. The data presented here show that after gel exclusion chromatography, GBF eluted in a high molecular weight fraction (>100 kD) (Figure 6). After SDS-PAGE, the polypeptides resolved to 31 kD and 18 kD (Figure 3). These data clearly suggest that in their native state the polypeptides are associated with other molecules. However, conclusive evidence regarding the stoichiometry of the GBF/G-box interaction will await more biochemical and physical studies of the GFB/G-box complex.

GBF appears to be distinct from any of the cloned plant DNA-binding proteins that were isolated based on their target DNA binding site. There are two reports of plant cDNA clones that were isolated using this method: HBP-1 (Tabata et al., 1989), and TGA1a and TGA1b (Katagiri et al., 1989). The clones code for proteins that are estimated to be 36.7 kD (Tabata et al., 1989) and 40.7 kD (Katagiri et al., 1989), respectively. It is possible that after processing, the proteins encoded by these cDNAs might be of molecular weights similar to that of GBF (31 kD). Both reports are for proteins that bind the Hex1 sequence (5' TGGGCCACGTCACCAATCCG-3'), which contains an imperfect dyad, such as the -190 oligo (Figure 2) and the related sequence from the corn Adh1 promoter (−185 to −170) (5'−AAACCCAGCAGTACCG-3'), which contains an imperfect 5'−GTGG-3' inverted repeat (underlined). In gel mobility shift competition assays, double-stranded oligonucleotides of imperfect dyads, such as the -190 oligo (Figure 2) and the related sequence from the corn Adh1 promoter (−185 to −170) (5'−AAACCCAGCAGTACCG-3'), (data not shown), failed to compete with the G-box probe for GBF binding. Therefore, although GBF is distinct, the similarities in binding sites suggest the possibility that GBF is related to the protein products of HBP-1, TGA1a, and TGA1b.

METHODS

Cell Cultures

Arabidopsis suspension cultures from stem explants were established and maintained as previously described (Feri and Laughner, 1989).

Plant Growth

Arabidopsis thaliana (Columbia) plants were grown in potting soil in 4-inch pots in windowsills. For protein extracts, leaves were removed from the preflowering plants and frozen in liquid nitrogen.

Fractionation of Whole-Cell Extracts

Whole-cell extracts were made from suspension cultures or leaves by a modification of Manley et al. (1980). The 20 g to 100 g of tissue were frozen in liquid nitrogen and ground in a coffee mill, followed by homogenization at 4°C with a Tissumizer (Tekmar) in 2.5 volumes of 15 mM Hepes-KOH, pH 7.6, 5 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 μM pepstatin A. All subsequent steps were carried out at 4°C unless otherwise noted. A 350 mM ammonium sulfate cut was followed by a 2.4 M ammonium sulfate precipitation. The crude pellet was resuspended in 20 mM Hepes-KOH, pH 7.6, 0.1 mM EDTA, 75 mM KCl, 10% glycerol, 5 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 0.1 μM pepstatin; dialyzed against NEBD (20 mM Hepes-KOH, pH 7.6, 75 mM KCl, 0.1 mM EDTA, 10% glycerol, and 5 mM β-mercaptoethanol); and then frozen in liquid nitrogen and stored at −75°C. The concentration of protein in the crude extracts was 1 to 3 mg/mL.

Crude suspension cell extracts were fractionated by chromatography through heparin-agarose (Bio-Rad), followed by FPLC Superose 6 (Pharmacia LKB Biotechnology Inc.) and FPLC Mono Q (Pharmacia). Crude leaf extracts were fractionated by chromatography through FPLC Superose 6, followed by FPLC Mono Q. Crude extracts and column fractions were assayed for G-box binding activity by DNA gel mobility shift (see below).

The crude suspension culture extract was applied to a 20-mL heparin-agarose column that had been equilibrated with NEBD, followed by a wash using at least 2 volumes of NEBD. Step elutions were carried out with NEBD containing 0.15 M, 0.3 M, 0.6 M, and 1 M KCl. The 0.6 M fraction, which contains the GBF activity, was diluted to 0.1 M KCl and reapplied to a 2-mL heparin-agarose column, washed with NEBD, and eluted with NEBD containing 1 M KCl. The eluate was then subjected to gel exclusion chromatography at room temperature on FPLC Superose 6 that had been equilibrated in NEBD. For analytical chromatography, an HR 10/30 column was used. Twenty-six fractions of 0.9 mL were collected. For preparative chromatography, an HR 16/50 column was used, and 33 fractions of 2 mL were collected. The high molecular weight fractions that contained G-box binding activity (see Results) were then applied to an FPLC HR 5/5 Mono Q column, at room temperature, that had been equilibrated in NEBD. The column was washed with 4 mL of NEBD and then developed with a 20-mL linear 0.075 M to 1 M KCl gradient in NEBD. Twenty fractions of 1 mL were collected.

Fractionation of the crude leaf extract on FPLC Superose 6 and FPLC Mono Q was as described above for the suspension cell extract.

DNA Probes and Competitors

The G-box probe (Figure 1) used in DNA binding and DNase I footprinting assays was constructed by filling the ends of the 58-bp Sau3A-TaqI fragment from −232 to −174 of the Arabidopsis Adh gene (Chang and Meyerowitz, 1986) with the Klenow fragment of DNA polymerase I, subcloning into the Smal site of Bluescript (Stratagene), and then isolating the insert after digestion with XbaI and EcoRI. The resulting 94-bp fragment contained 20 bp of polylinker on one end and 16 bp on the other. For gel mobility shift assays and DNase I footprints, the top strand was 3' end-labeled using Klenow fragment and α-32P-dATP, and the bottom strand was 3' end-labeled using Klenow fragment and α-32P-dCTP.

Synthetic oligonucleotide competitors were homologous to regions within the G-box probe. The region from −226 to −202 that is protected in DNase I footprint assays is referred to as the FP oligo (see Results), and the region corresponding to −196 to −182 as the −190 oligo (Figure 1).
For use as a nonspecific competitor, the 51-bp region 3' to the G-box probe (TaqI-Sall) in the Adh gene was subcloned in the same manner as the G-box probe (Figure 1). The insert was isolated using the same restriction enzymes and included the same polylinker sequences. In addition, poly(dldC) (Boehringer Mannheim) was used as a nonspecific competitor.

DNA Gel Mobility Shift Assay

In the binding reactions (10 μL to 20 μL final volume), 0.25 ng of 3' end-labeled G-box probe was incubated with extract in NEBD at room temperature for 5 min to 10 min. Variable amounts of poly(dldC) were included as nonspecific competitor (see figure legends). Reactions were separated on 5% polyacrylamide gels in 25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.3 (Singh et al., 1988) that had been prechilled to 4°C, then run at room temperature at 20 mA constant current. Gels were fixed in 10% ethanol, 5% acetic acid, dried, and autoradiographed with intensifying screens at ~80°C.

UV Cross-Linking

UV cross-linking was done by a modification of the procedure of Chodosh et al. (1986). Probe DNA was prepared for cross-linking by random primed strand synthesis using Klenow fragment in the presence of 25 μM dGTP, 5-bromo-2'-deoxyuridine triphosphate (Sigma), 50 μCi of α-32p-dCTP, and 50 μCi of α-32p-dATP (3000 Ci/mmol, Du Pont-New England Nuclear) followed by a chase that included 25 μM each dGTP, TTP, dATP, and dCTP. After binding, UV cross-linking, and nuclease digestion, the mixture was separated on an SDS-12.5% polyacrylamide gel. The gel was fixed in 10% ethanol and 5% acetic acid, dried, and autoradiographed.

DNase I Footprints

Binding reactions were performed as for gel mobility shift assays, except that they contained 1 ng to 2 ng of 3' end-labeled G-box probe in 20 μL to 200 μL. After incubation at room temperature for 5 min to 10 min, DNase I and MgCl2 were added to 5 μg/mL and 5 mM, respectively, and incubated for 1 min. Digestion was stopped by the addition of EDTA to 10 mM and transfer to 4°C, then run at room temperature for 5 min to 10 min. Variable amounts of DNase I and MgCl2 were included as nonspecific competitor (see figure legends). Reactions were separated on 5% polyacrylamide gels in 25 mM Tris, 190 mM glycine, 1 mM EDTA, pH 8.3 (Singh et al., 1988) that had been prechilled to 4°C, then run at room temperature at 20 mA constant current. Gels were fixed in 10% ethanol, 5% acetic acid, dried, and autoradiographed.

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