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Gibberellin Treatment Stimulates Nuclear Factor Binding to the Gibberellin Response Complex in a Barley α-Amylase Promoter

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The promoters of a majority of cereal α-amylase genes contain three highly conserved sequences (gibberellin response element, box I, and pyrimidine box). Recent studies have demonstrated the functional importance of four regions that either coincide with or are immediately proximal to these three conserved elements as well as an upstream Opaque-2 binding sequence. In this study, we describe the characterization of nuclear protein factors from barley aleurone layers whose binding activity toward gibberellin response complex sequences from the barley low-pI α-amylase gene (Amy32b) promoter is stimulated by gibberellin A_3 (GA_3) treatment. Barley proteins isolated from crude nuclear extracts prepared from aleurone layers incubated with or without GA_3 were fractionated by anion exchange fast protein liquid chromatography and studied using band shift assays, sequence-specific competitions, and DNase I footprinting. A GA_3-dependent binding activity eluting at 210 mM KCl was shown to bind specifically to the gibberellin response element and the closely associated box I. DNase I footprinting with the proteins in this fraction indicated interactions with sequences in the gibberellin response element and box I. A second DNA binding activity eluting at 310 mM KCl was present constitutively in extracts prepared from tissues incubated both in the absence and in the presence of hormone. Proteins in this fraction were able to bind to many DNA sequences and, in general, were largely nonspecific. DNase I footprinting with the proteins in this fraction indicated a large area of protection with a single unoccupied region located at the 3′ end of box I. The possible function of such an activity in hormone regulation of the α-amylase genes is discussed.

INTRODUCTION

The mechanism that regulates transcription of cereal α-amylase genes integrates developmental and hormonal as well as environmental cues. In a germinating seed, α-amylase production must coincide with the growth of the embryo so that metabolic products derived from starch hydrolysis are available for uptake and utilization by the growing seedling. Gibberellin (GA), a hormone released from the growing embryo, facilitates this coordination by inducing the transcription of α-amylase genes. Expression of α-amylase is suppressed by abscisic acid (ABA) during seed development (when starch is synthesized) or during conditions unfavorable to seed germination. In addition, tissue-specific control limits the expression of α-amylase to those cells surrounding the starchy endosperm, namely, the aleurone and scutellar epithelium. Therefore, the aleurone layer provides an excellent system for studying hormone-regulated gene expression and offers the opportunity to investigate complex transcriptional regulatory mechanisms.

Huttly and Baulcombe (1989) and Jacobsen and Close (1991) have demonstrated that promoter elements located within a few hundred base pairs of the transcriptional start site of α-amylase genes confer high levels of hormone-regulated reporter gene expression. In addition, Skriver et al. (1991) showed that six copies of a 17-bp oligonucleotide, derived from an element highly conserved among GA/ABA-regulated α-amylase promoters, confer GA and ABA regulation on a heterologous minimal promoter. They refer to this sequence as the GA response element (GARE). Lanahan et al. (1992) demonstrated that within its context in the promoter of the low-pI gene Amy32b, the GARE mediates hormonal control of transcription. At least three other distinct regulatory elements were found to be necessary for high levels of hormone-regulated expression. This closely associated group of elements was designated the GA response complex (GARC) and is composed of an Opaque-2 protein binding sequence (O2S), a sequence element enriched with pyrimidine nucleotides (the pyrimidine box), the GARE, and box I (TATCCAT). Recently, Gubler and Jacobsen (1992) have shown the importance of the GARE as well as box I for high-level GA-regulated expression from the high-pI gene (Amy pHV19).

As a first step toward understanding GA signal transduction and the function of the various elements of the Amy32b promoter, we are interested in identifying and characterizing the proteins responsible for conferring aleurone-specific

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and hormone (GA/ABA)-dependent expression of the cereal α-amylase genes. We have used band shift assays and DNase I protection experiments to determine the specific sequences within the promoter fragments responsible for the protein–DNA interactions. Several sites of protein–DNA interaction were discovered that are consistent with previously identified *cis*-acting elements required for proper expression of this promoter. The three sequences recognized by these proteins have some interesting similarities to promoter elements important for the regulation of other genes. Two of these sequences, the GARE and box I, are present in virtually all α-amylase promoters characterized to date (Huang et al., 1990b). A third element (the O2S) is similar to a sequence recognized by a family of transcription factors in both plants and animals (Deutsch et al., 1988; Hartings et al., 1989; Schmidt et al., 1990, 1992; Lohmer et al., 1991; Ueda et al., 1992). Finally, we show that a factor or factors interacting with both the GARE and box I bind in a gibberellin A_3 (GA_3)-dependent manner, possibly reflecting the mechanism by which transcriptional activation of the Amy32b promoter occurs. We suggest that these proteins are involved in the transduction of the hormonal and developmental signals to changes in α-amylase gene expression.

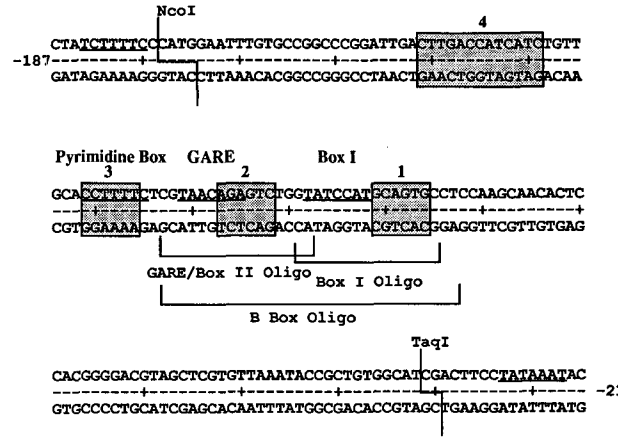

Figure 1. Barley Amy32b Promoter Sequences Essential for GA Induction.

The sequence from −187 to −23 of Amy32b is shown. Conserved sequence elements are underlined. Sequences that have been found to be functionally significant in GA induction are shown in shaded boxes. The extent of sequences found in cloned synthetic promoter fragments is indicated with brackets.

RESULTS

DNA Binding Proteins Interact with the Regulatory Regions of the Barley Amy32b Promoter

Lanahan et al. (1992) have shown through loss-of-function studies that four *cis*-acting sequences, important for high level GA_3-induced expression of the Amy32b promoter (Whittier et al., 1987), reside between positions −331 and −25. Specifically, all these elements reside downstream of position −149, as summarized in Figure 1 (shaded boxes 1 to 4). Sequences in common with or immediately proximal to three of these functionally defined sequences were identified previously as highly conserved sequence elements in α-amylase and other GA-regulated promoters (Huang et al., 1990b). Using a suggested nomenclature (Sutliff et al., 1991), these elements are designated box I, box II or the GARE as defined by Skriver et al. (1991), the pyrimidine box, and the O2S (Lanahan et al., 1992). These sequences are likely to represent the binding sites for protein factors that mediate their effects on GA-induced gene expression. Accordingly, we sought to determine if we could detect the presence of these factors in nuclear protein extracts isolated from barley deembryonated half grains incubated with GA_3.

Therefore, we developed a protein isolation procedure using crude nuclear pellets isolated from aleurone layers incubated in the presence or absence of GA_3. Band shift assays using these extracts produced a large quantity of high molecular weight–bound complex with a 305-bp probe derived from the Amy32b promoter (HindIII-BamHI fragment from pML007; data not shown). We then performed DNase I protection assays on these extracts to determine the nature of the protein–DNA interactions. As shown in Figure 2, three major regions of interactions were detected within the GARE, box I, and the O2S elements. Apparently, these protein interactions induce a conformational change in the DNA located between the GARE and box I footprints as evidenced by the generation of DNase I hypersensitive sites (Figure 2).

Nuclear Protein Fractionation and Characterization

Because these nuclear extracts contained multiple proteins capable of interacting with the Amy32b promoter, we decided to fractionate them to reduce the complexity of the extracts used in subsequent studies. We found that the DNA binding activities in which we were interested did not bind to a strong cation exchange fast protein liquid chromatography (FPLC) column (EconoPac-S; Bio-Rad) in 50 mM KCl and were contained in the flow-through (data not shown). A minor amount of purification was achieved with this column in that a small amount of protein did bind to this column and so was removed from the extracts. This column was particularly useful in the removal of unwanted debris and lipid from the sample prior to application to the Mono Q column. This flow-through was fractionated on a strong anion exchange FPLC column (Mono Q HR 5/5; Pharmacia) using a 50 to 1000 mM KCl linear gradient. As expected, the flow-through from this column lacked any relevant binding activities (data not shown). As shown in Figure 3A, when these fractions were assayed by band shift

analysis using the 152-bp probe encompassing all four important *cis*-acting elements (NcoI-TaqI fragment; Figure 1), a complicated pattern of DNA–protein complexes was observed. However, a striking difference between the extracts prepared from aleurone layers incubated in the absence or presence of GA_3 can be observed. A distinct array of complexes is present only in the 175, 210, and 243 mM KCl fractions derived from GA_3-treated aleurone layers.

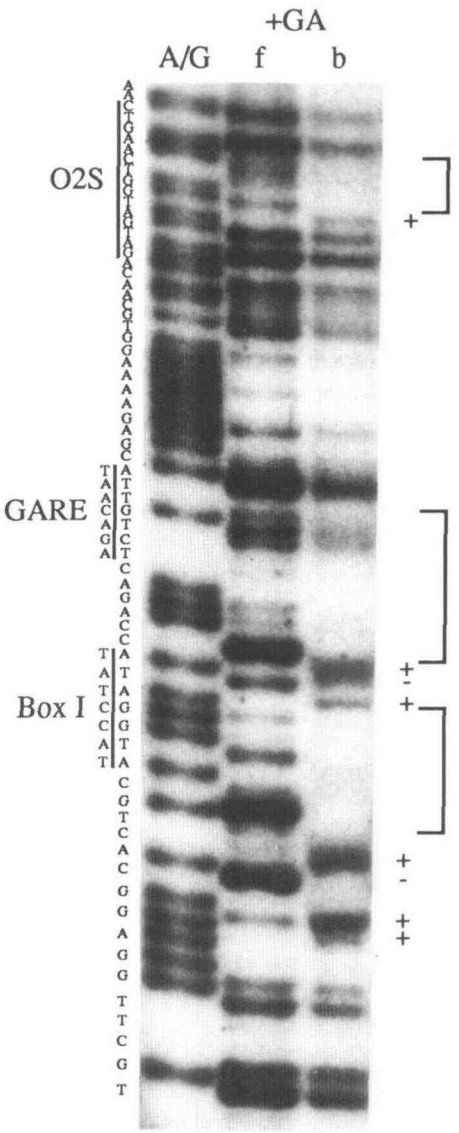

Figure 2. DNase I Footprinting Analysis with Unfractionated Barley Aleurone Nuclear Extract Demonstrating Multiple DNA–Protein Interactions.

Free and bound DNA isolated from band shift gel following DNase I treatment are designated f and b, respectively. A/G is the product of a purine-specific sequencing reaction using the Amy32b promoter fragment labeled at the 5′ end of the noncoding strand. The sequences highlighted with vertical bars are the major *cis*-acting elements. The bracketed areas on the right are the major footprints, with plus signs denoting DNase I hypersensitive sites and minus signs indicating protected sites outside the main footprints.

We have observed that fractions containing the EconoPac-S flow-through that were flash frozen in liquid nitrogen and stored at −80°C for 5 months prior to fractionation on the Mono Q column contained dramatically less (~50-fold) of this GA-dependent binding activity. The higher salt eluting binding activity (GA-independent activity), on the other hand, is unaffected by this treatment.

To delineate more precisely which sequences interact with the GA-dependent binding activity, we chose to shorten the length of the probe used for these studies. When a 36-bp B box probe containing only the GARE and box I was used as shown in Figure 3B, the GA-dependent activity was present primarily in the 210 mM KCl fraction, with a minor amount present in the 243 mM KCl fraction. Observed repeatedly on band shift gels with these two fractions was a high molecular weight probe complex that was barely able to enter the gel (Figure 3A). The GA-independent activity was most concentrated in the 310 mM KCl fraction. Furthermore, when the GA-independent binding activity is present at high concentrations (as in the 310 mM KCl fraction) and assayed with the 152-bp probe (Figure 3A), it promotes the formation of higher molecular weight shifted bands.

We further shortened the length of the probe to determine which portion of the sequences contained on the B box probe were responsible for the observed hormone-dependent differences. The smallest probe we utilized in these band shift assays was 21 bp long containing a 17-bp double-stranded region with only the GARE or box I. Figure 3C shows that both the GA-dependent and GA-independent activity are capable of binding to the GARE probe. When assayed using the box I probe, these same fractions produced only a minor amount of shifted probe (data not shown). However, as shown in Figure 4, when box I was flanked by an additional 12 bp (5′) and 51 bp (3′) of polylinker sequences (XhoI-XbaI fragment from pTS005), the same fractions were then capable of binding to this probe. Specifically, the 210 and 310 mM KCl fractions contain the highest amount of GA-dependent and GA-independent binding activities, respectively, with the shoulder fractions containing dramatically less binding activity. This pattern is similar to that observed with the GARE probe (Figure 3C). We have not observed binding of the 210 mM fraction to the XhoI-XbaI fragment from pBluescript KS+.

To better understand the nature of these DNA–protein interactions, we performed sequence-specific competitions with either the GARE or the box I competitor DNAs at 100-fold molar excess separately or in combination. As seen in Figure 5A, the GA-inducible binding to the B box probe in the 210 mM KCl fraction was competed slightly more effectively by the GARE competitor than by the box I competitor. The 310 mM KCl fraction could not be competed by either the GARE competitor or the box I competitor. Significantly, the combination of both competitors at 100-fold molar excess was only slightly

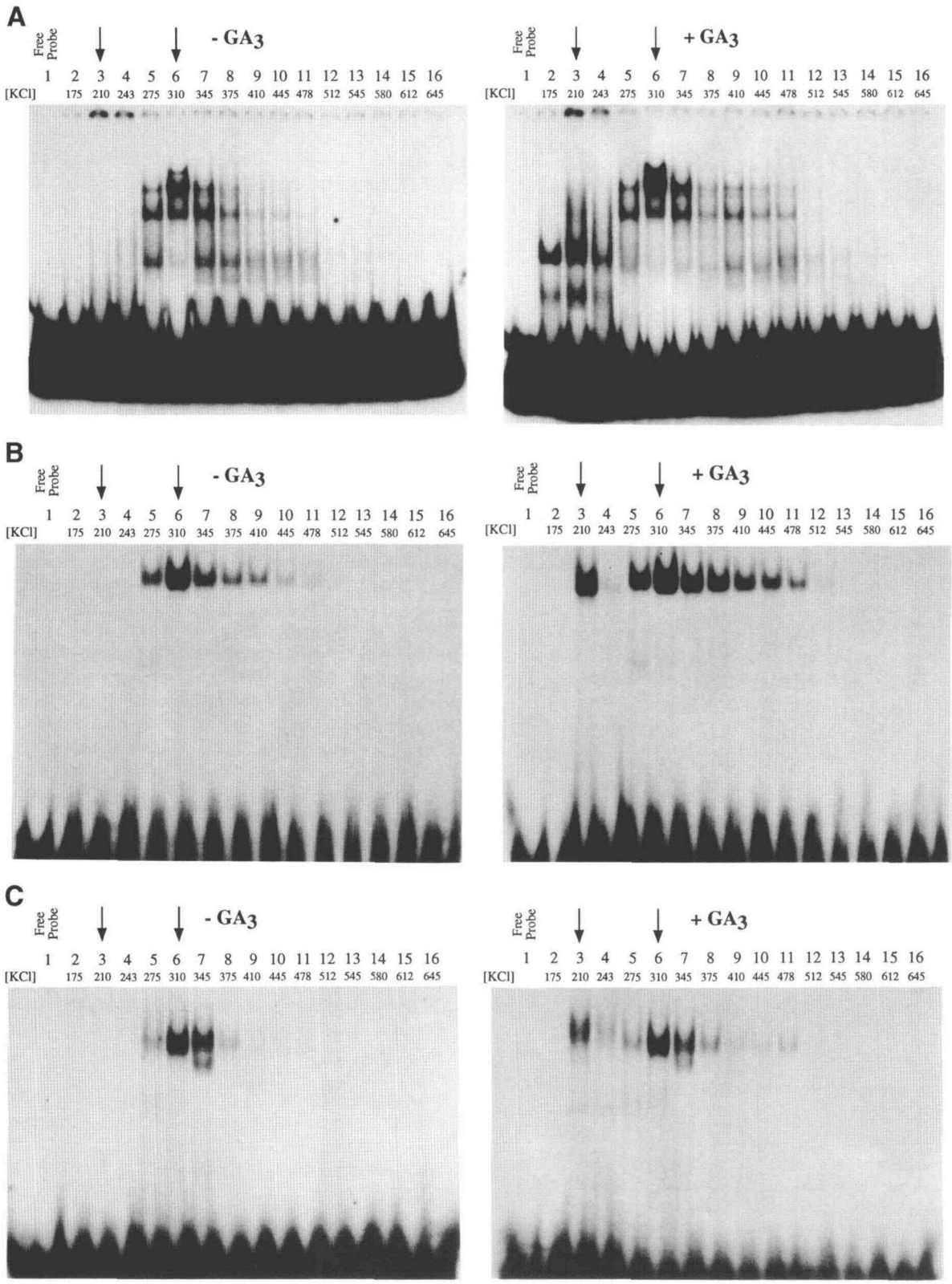

Figure 3. Band Shift Assays of FPLC Fractions Demonstrating the Presence of GA-Dependent DNA Binding Activities.

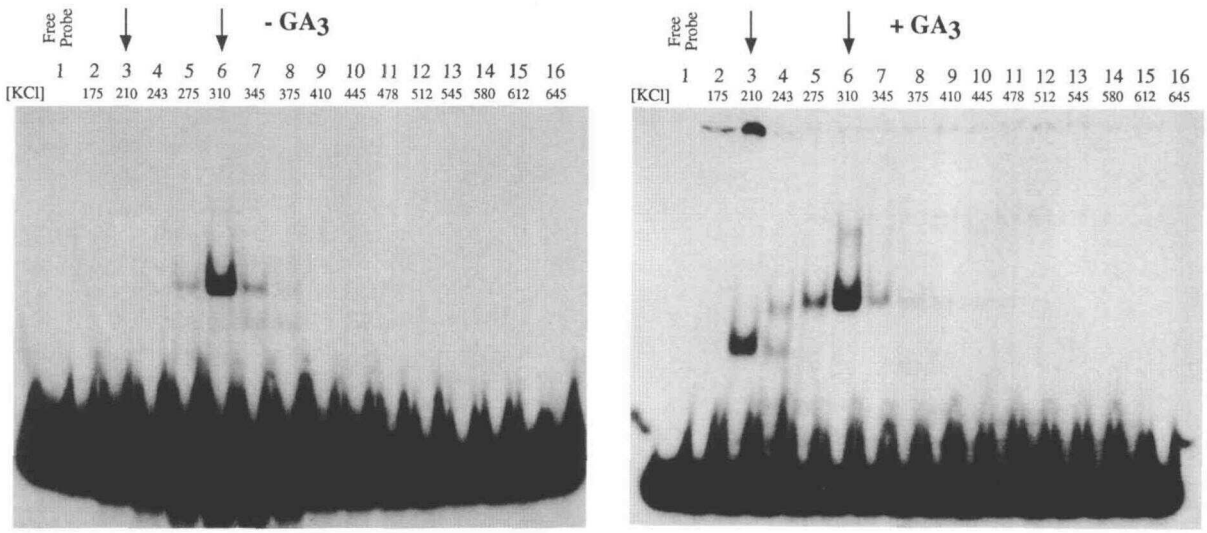

Figure 4. Effect of Using a Box I Probe in Which the Conserved Sequence Element Is Flanked by Additional Polylinker Sequence.

While unable to interact efficiently with a 21-bp BamHI fragment from pTS005, the active fractions in Figure 3 were able to bind to the XhoI-XbaI fragment from pTS005 in which box I is present in a larger DNA context. Figure details are as given in the legend to Figure 3.

inhibitory to the formation of the 310 mM KCl fraction-B box complex (Figure 5B).

We further studied the specificity of these interactions using an unrelated probe derived from the polylinker of pBluescript KS+. The XhoI-EcoRI fragment, which has a length and G+C content (48%) comparable to that of the B box (53%), was used as a probe as well as a nonspecific competitor in Figure 6. When the XhoI-EcoRI polylinker fragment was used as a probe, it was found not to bind the GA-dependent fraction (Figure 6, lane 3). The GA-independent binding activity, however, was able to bind to this probe (Figure 6, lane 6). Competition experiments performed with the GA-independent binding activity indicated that the B box could not effectively compete for this binding (Figure 6, lane 7), but that the XhoI-EcoRI polylinker fragment totally abolished this binding when used as a competitor (Figure 6, lane 8). The GA-independent binding activity is markedly different depending upon the sequences contained in the competitor DNA. For example, once bound to the XhoI-EcoRI competitor fragment, this protein(s) does not exchange at an appreciable rate and so precludes binding to the probe. However, with the B box competitor, a substantial amount was able to dissociate and bind the probe. This result implies that the GA-independent binding activity is the result of protein(s) which displays a significant amount of exchange with the B box competitor fragment similar to that observed with serum response factor (Marais et al., 1992).

The interaction of the GA-dependent binding activity with probes containing either the GARE or box I as well as with competitor fragments containing either the GARE or box I sequence elements was intriguing in that it implied interactions with both of these sequence elements. We performed DNase I footprinting studies with the 210 mM KCl fraction to determine which sequences were interacting with protein factors in this extract. Obtaining bound complex that was not contaminated with free probe was difficult with the longer 152-bp footprinting probe due to the minimal separation of the free and bound complexes. It also proved difficult to recover a sufficient amount of bound complex using the B box footprinting probe. Therefore, experiments were performed to optimize the binding reaction conditions. Binding was found to be complete after

Figure 3. (continued).

Nuclear proteins prepared from aleurone layers incubated in the presence or absence of 2×10^{-6} M GA_3 were fractionated as described in the Methods and analyzed in band shift assays.

(A) The 152-bp NcoI-HindIII restriction fragment from the Amy32b promoter subclone pML007 used as a probe.

(B) The Amy32b-derived synthetic B box BamHI restriction fragment from pML010 used as a probe.

(C) The Amy32b-derived synthetic GARE/box II BamHI restriction fragment from pTS004 used as a probe.

The signal at the bottom of the gels represents unbound probe. The KCl millimolar concentrations in FPLC fractions are given at the top of the gels. The arrows point to the peak fractions of the GA-dependent (at 210 mM KCl) and GA-independent (at 310 mM KCl) DNA binding activities.

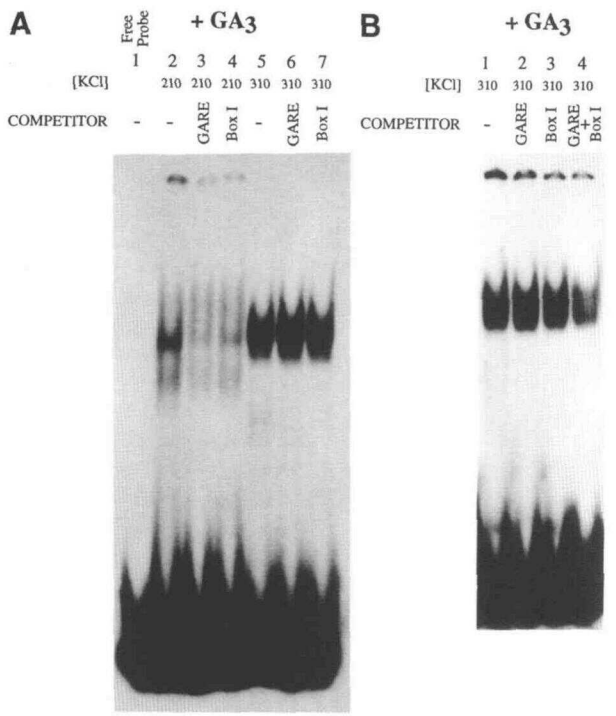

Figure 5. Sequence-Specific Competitions.

Band shift assay of the 210 and 310 mM KCl FPLC fractions using the B box BamHI restriction fragment from pML010 as probe and sequence-specific competitions.

(A) A 100-fold molar excess of either the GARE/box II competitor or the box I competitor effectively competes for the binding activity present in the 210 mM KCl FPLC fraction, whereas neither has an effect on the binding activity present in the 310 mM KCl FPLC fraction.

(B) The combination of both box I and GARE/box II competitors at a 100-fold molar excess are only slightly inhibitory to the formation of the 310 mM KCl protein–B box complex.

The signal at the bottom of the gels represents unbound probe.

a 10-min incubation at room temperature. Relative binding efficiency was found to be directly proportional to the temperature of the binding reaction at 7, 15, 22, and 30°C (data not shown). We also found that maximum bound complex formed at 100 mM KCl and 2.5 mM $MgCl_2$ (data not shown) (Robidoux et al., 1992). In addition, we used a head-to-tail trimer of the B box probe for these studies to take advantage of any cooperative interactions between proteins bound to the probe (Zhang et al., 1991).

As shown in Figure 7, this particular fraction creates an alteration of DNase I sensitivity within the GARE and box I. Whereas the central copy of the B box trimer is shown, the same pattern was present in the two other copies of the B box (data not shown). The hypersensitive site induced in the middle of the box I is particularly strong, whereas only a minor increase in DNase I sensitivity is present within the GARE. These regions are comparable to two of the three footprints generated with the unfractionated GA_3-treated, aleurone layer–derived extract (Figure 2). The generation of DNase I hypersensitive sites between these regions is analogous to those observed with the crude nuclear extract.

The induction of hypersensitive sites associated with the absence of a region of protection such as that seen here has been observed in studies with fraction AA (Carthew et al., 1985) containing USF (upstream stimulatory factor; also known as MLTF, major late transcription factor that activates transcription from the adenovirus major late promoter) by Watt and Molloy (1988). This has been observed more recently with CUP (C/EBPα undifferentiated protein) purified from HeLa cells (Vasseur-Cognet and Lane, 1993). This may be due to partial occupancy of the sites due to a low concentration of this factor in the extract and/or may indicate the importance of the other binding activities observed in the crude nuclear extract

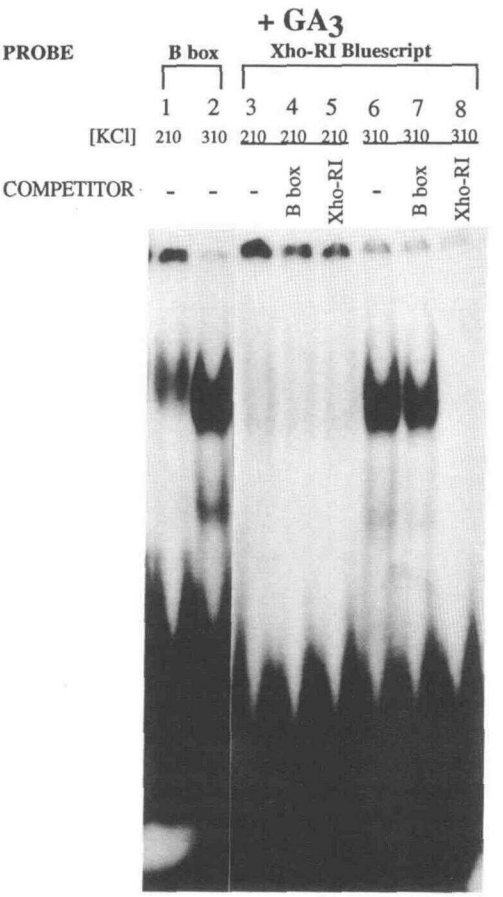

Figure 6. Use of an Unrelated Sequence to Demonstrate Differences in Specificity of Binding of the 210 and 310 mM KCl Fractions.

Band shift assay of the 210 and 310 mM KCl FPLC fractions using either the B box BamHI restriction fragment from pML010 as probe (for comparison) or the XhoI-EcoRI polylinker fragment from pBluescript KS+ as probe. Competitions using 100-fold molar excess of either the B box competitor or the XhoI-EcoRI fragment from pBluescript KS+. The signal at the bottom of the gel represents unbound probe.

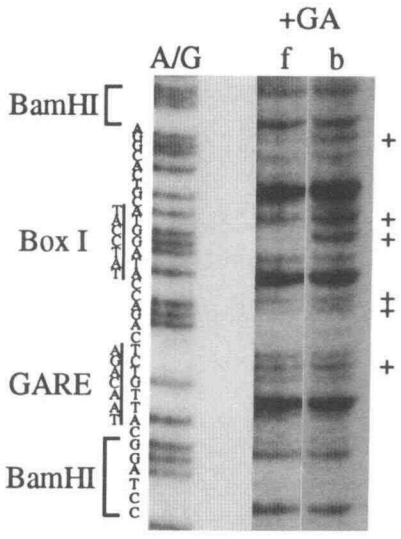

Figure 7. DNase I Footprinting Analysis Demonstrating that the GA_3-Dependent DNA Binding Activity Interacts with the GARE/Box II and Box I Region.

The binding activity in the 210 mM KCl FPLC fraction induces DNase I hypersensitive sites within the GARE and box I. The footprinting probe used in this analysis contained three head-to-tail copies of the B box oligonucleotide. The results derived from the central copy are shown (the same pattern is seen at each repeat unit). Plus signs denote DNase I hypersensitive sites. Figure details are as given in the legend to Figure 2.

for stabilizing these interactions. For example, the quality of the footprints produced by the simultaneous occupation of two adjacent sites on a DNA fragment has been observed to substantially improve the quality of the footprints compared to that of footprints generated by either factor alone (Sawadogo and Roeder, 1985; Horikoshi et al., 1988; Watt and Molloy, 1988; Roy et al., 1991; Vasseur-Cognet and Lane, 1993).

In contrast, the results of DNase I footprinting with the GA-independent binding activity (310 mM KCl fraction) were similar to that observed for UBF (upstream binding factor; HMG box containing protein that enhances polymerase I rRNA gene transcription) (Putnum and Pikaard, 1992). It occupied a large region of the probe and was most likely cooperative. As shown in Figure 8A, protein in this fraction binds to most of the probe except for a region at the 3′ end of box I. This explains why this fraction was not able to bind the shorter box I probe originally used in cases when this binding activity is apparently quite promiscuous in regard to the sequences with which it will interact. The extent of α-amylase sequence contained on this probe is indicated in Figure 8A with a bracket. As shown, the sequence contained in this probe is centered on the region to which this fraction does not bind. The nature of this binding explains the competition data in that the presence of both the GARE and box I competitors at 100-fold molar excess were able to reduce the amount of binding by only ~50% (Figure 5B). Because the proteins in this fraction can bind the B box probe at sites located between the GARE and box I (Figure 1) that are not present in the competitor DNA as a contiguous fragment, it is consistent that these proteins would still bind the probe.

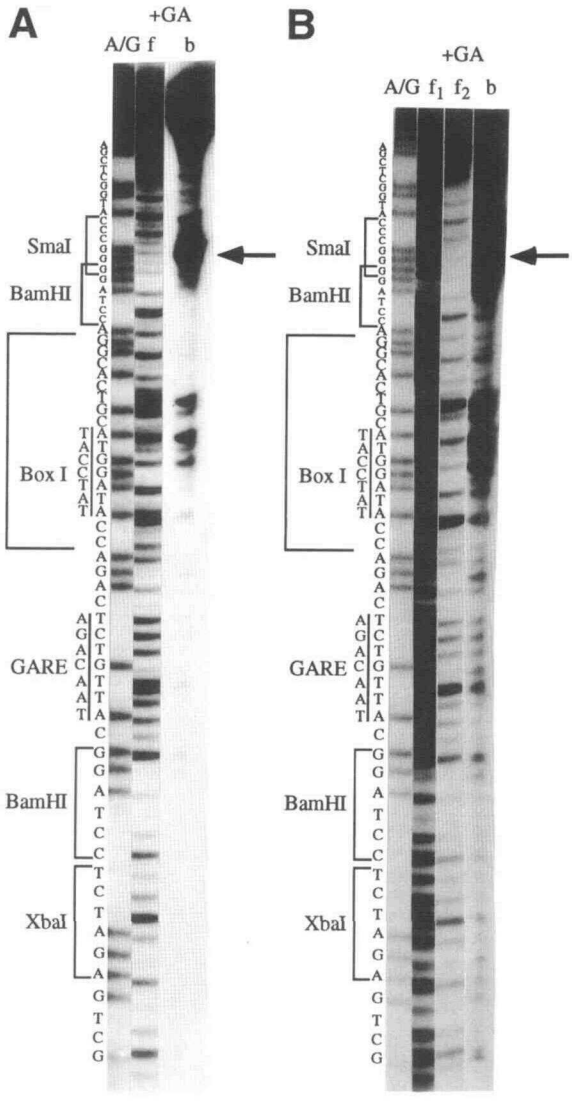

Figure 8. DNase I Footprinting Analysis Using the GA-Independent DNA Binding Activity.

(A) The binding activity in the 310 mM KCl FPLC fraction occupies a large region of the B box footprinting probe.

(B) A longer exposure of **(A)** with the f_1 free lane, same as f in **(A)**, containing 3.3 times less full-length probe than in the bound lane, in addition to the f_2 free lane containing 22 times less full-length probe than in the bound lane.

The large brackets encompassing box I in **(A)** and **(B)** denote the sequence found in the 21-bp BamHI fragment from pTS005. The arrows denote the position of a major DNase I hypersensitive site within the vector polylinker sequence. Figure details are as given in the legend to Figure 2.

Also, in a manner similar to UBF, this fraction generates a particularly strong hypersensitive site just within the vector sequence (Figure 8A; arrow). It could be argued that the bands seen in the bound lane represent hypersensitive sites instead of unoccupied sites. However, when the bound lane is exposed long enough to see additional bands (Figure 8B), an equivalent exposure of these bands in the free lane is possible only when 22-fold less free full-length probe is loaded with respect to that present in the bound lane. This indicates that the bands in the bound lane do not represent hypersensitive sites.

DISCUSSION

Our results indicate that nuclear proteins in barley aleurone layers interact with sequences in the 5′ flanking region of the Amy32b gene and that they do so in a manner consistent with what is now known about the hormone-dependent expression from this promoter. Three major protected regions are evident in footprinting assays when using an unfractionated nuclear extract derived from aleurone layers incubated in the presence of GA_3. These regions correspond to the GARE, box I, and the O2S elements. We have used anion exchange FPLC to fractionate these nuclear extracts. We have characterized a DNA binding activity directed toward the GARE and box I that is found only in tissue incubated in the presence of GA_3. In contrast, Guiltinan et al. (1990) characterized a binding activity directed toward the Em1a sequence element in the wheat ABA-regulated *Em* gene present in both untreated and ABA-treated rice suspension cells. A similar situation has been described for the transcription factor GT-1, which binds light-responsive elements and is found in both extracts from light-grown and dark-adapted pea plants (Green et al., 1987).

The GA-inducible binding activity (210 mM KCl fraction) was shown to be directed toward the GARE and box I by the use of progressively shorter probes. The GARE/box II oligonucleotide probe with only 17 bp of duplex DNA containing the underlined GARE sequence (CGTAACAGAGTCTGGT) was bound by the 210 mM KCl fraction. A similar-sized box I oligonucleotide probe containing the underlined box I sequence (GTATCCATGCAGTGC) was also bound by the 210 mM KCl fraction but with a much lower affinity. However, when a box I probe was used in which the above sequence was flanked with vector sequences, this fraction was able to bind efficiently. The binding of proteins in this fraction to box I apparently requires that this sequence be contained on a fragment with a double-stranded region larger than 17 bp. The competition data support these conclusions because both the GARE and box I competitors substantially reduce the binding of the 210 mM KCl fraction to the B box probe.

When the GA-inducible binding activity was used in a DNase I footprinting assay, the GARE and box I were associated with protein in such a way as to induce DNase I hypersensitive sites within these sequence elements as well as the generation of hypersensitive sites between these sequences. This pattern is reminiscent of bipartite binding sites that often possess intervening DNase I hypersensitive sites (Weinrich et al., 1991; Ramji et al., 1991).

Taken together, these data indicate that the 210 mM KCl fraction contains protein(s) that binds specifically to the GARE and box I. The situation may be similar to that observed for nuclear factor GT-1, which binds to three similar sequences (box II, box III, and the rice phytochrome A 3′ GT motif), albeit with different affinities (Green et al., 1987; Kay et al., 1989; Gilmartin et al., 1992). The box III site has been shown to be 20 times less effective in competing for GT-1 binding (Gilmartin and Chua, 1990). In this case, it has been determined that other proteins bind to sequences flanking this GT-1 site (3AF5 and 3AF3) and that these may contribute to stabilize the binding to a lower affinity site (Sarokin and Chua, 1992). In a similar manner, the 210 mM KCl fraction proteins may bind to the GARE-related box I sequence by means of cooperative interactions of protein factors at these sequences. Two studies support the above model: six tandem copies of the Amy6-4 GARE are required for proper hormone-regulated expression of a cauliflower mosaic virus 35S minimal promoter (Skriver et al., 1991); and duplication of GARC elements results in increased GA-induced expression (Rogers and Rogers, 1992).

It is interesting to note that the sequence element through which the transcription factor VP1 acts, known as the Sph element (TCCATGCATGCAC) (Hattori et al., 1992), is closely related to the 3′ end of box I. VP1 as a regulator of genes induced during seed development might be involved in the temporal regulation of the Amy32b gene. A VP1-like protein might be involved in effectively silencing this gene during seed development and/or high ABA concentrations. Accordingly, Rogers and Rogers (1992) have found that the Amy32b promoter responds appropriately to GA_3 treatment only in the latter half of seed development (3 weeks postanthesis). The fact that this area is not bound by the protein in the 310 mM KCl fraction may be more than a coincidence.

A possible scenario is that the binding activity present in the 310 mM KCl fraction represents a generalized repressor activity. This putative repressor, possibly in conjunction with a VP1-like protein, excludes the binding of the 210 mM KCl fraction factor to the GARE and box I until the correct developmental conditions exist for the expression of α-amylase. During germination, GA induction of the 210 mM KCl fraction binding activity in the presence of the O2S factor (Lanahan et al., 1992; Rogers and Rogers, 1992) and the loss of the VP1-like activity (McCarty et al., 1991) might cause a displacement of such repressor factors through a mechanism similar to that seen in the tissue-specific and developmentally regulated expression of α- actin in myoblasts (Lee et al., 1991) and glycophorin B in erythroid hematopoietic lineages (Rahuel et al., 1992).

The first study of a factor binding to α-amylase promoters was published by Ou-Lee et al. (1988) for a GA-inducible factor interacting with the pyrimidine box. This work was performed with the promoter region of the rice cultivar IR36 equivalent (OS*amy-a*) to the rice cultivar M202 *RAmy3C* gene (Sutliff et al., 1991). However, because no studies on the level of GA induction of this gene have been published and the fact that the promoter is atypical in regard to the region normally

containing the GARE and box I, it is unclear how this relates to the recent *cis*-acting element results. While expressed in the aleurone, the level of expression from *RAmy3C* is lower than that of *RAmy1A* (Huang et al., 1990a; Karrer et al., 1991; Sutliff et al., 1991). Accordingly, an update of this work has been published by the same group based upon the IR36 functional equivalent to the *RAmy1A* gene known as OS*amy-c* (Kim et al., 1992). This gene does contain the GARE and box I sequences at positions −154 and −130, respectively (position is the 5′ T residue in the consensus sequence). However, no significant GA-dependent differences in binding were detected. The results of methidiumpropyl-EDTA-Fe (II) footprinting using whole seed extracts were interpreted to protect three regions: the pyrimidine box and two adjacent 3′ regions located between positions −213 and −175.

The first analysis of nuclear factors isolated from wheat protoplasts incubated with or without gibberellin A_1 (GA_1) was published recently by Rushton et al. (1992). No significant GA-dependent differences in binding were described. All footprint analyses were therefore performed using GA_1-treated protoplast nuclear protein extracts. In wheat α-Amy2/*54* and α-Amy1/*18* promoters, the O2S region was protected from DNase I digestion (box 2 and box A, respectively). However, while the GARE and the immediate upstream pyrimidine box region were footprinted in the α-Amy2/*54* promoter, no footprint was evident at the GARE (TAACAAA) in the α-Amy1/*18* promoter.

It has been shown that the pyrimidine box enhances GA-dependent expression from the Amy32b promoter by 30% in its normal position and orientation (Lanahan et al., 1992; Rogers and Rogers, 1992). However, under our experimental conditions, we have not been able to detect any DNA binding activity which specifically interacts with the pyrimidine box as seen by Kim et al. (1992) and Rushton et al. (1992). A possible explanation for this discrepancy may be different protein isolation procedures as well as the fact that these extracts were prepared from three different species: namely, barley, wheat, and rice.

We have characterized a barley nuclear factor which binds to the GARE and box I sequence elements in the GARC in a hormone-dependent manner. The isolation of the cDNA equivalent of the protein responsible for the binding activity contained in the 210 mM KCl fraction will be critical for further studies of α-amylase gene regulation.

METHODS

Materials

Restriction enzymes and DNA-modifying enzymes were purchased from Promega, New England Biolabs, and Boehringer Mannheim Biochemicals. Poly(dI-dC), leupeptin hemisulfate, and soybean trypsin inhibitor were purchased from Sigma. Radioactive nucleotides were obtained from DuPont-New England Nuclear. Enzymes and reagents were used according to the instructions of the suppliers.

Nuclear Extracts

Deembryonated double-cut half grains of 1985 Himalaya barley (Washington State University, Pullman; 150 mL volume) were surface-sterilized in 3.5% Chlorox for 15 min followed by four rinses in sterile water. The embryoless half seeds were then treated with 25 mM HCl for 2 min. Following four rinses in sterile water, the half seeds were rinsed in barley buffer (20 mM sodium succinate, pH 5, 20 mM $CaCl_2$). The half seeds were imbibed at room temperature on filter paper placed over three-eighths inch of vermiculite saturated with barley buffer in glass trays. After a 4-day incubation, the half seeds were rolled between paper towels saturated in barley buffer to separate the aleurone layers from the starchy endosperm. The aleurone layers were then incubated in barley buffer supplemented with 10 μg/mL chloramphenicol with 2×10^{-6} M gibberellin A_3 (GA_3) or without hormone for 12 hr on a rotary shaker at room temperature. Following incubation, the layers were quickly blotted dry, flash frozen in liquid nitrogen, and ground to a fine powder with a mortar and pestle in liquid nitrogen.

Nuclear proteins extracts were obtained by modifying the procedure of Allen et al. (1989) as follows. Buffer A lacked EDTA but included 10 μg/mL soybean trypsin inhibitor and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). All buffers were ice cold and all subsequent procedures were performed on ice. The powdered aleurone layers were homogenized five times in 5 mL of buffer A per gram of tissue with a Brinkman polytron at level 4 for 20 sec followed by chilling on ice. The homogenate was filtered through one layer of course Nytex and two layers of 60-μm Nytex. The filtered extract was centrifuged at 5870*g* for 10 min at 4°C. This crude nuclear pellet was resuspended in an amount of buffer C (containing 15 μg/mL soybean trypsin inhibitor, 15 μg/mL leupeptin, and 0.5 mM PMSF) equal to one-sixth the volume buffer A used to homogenize the tissue and mixed for 1 hr on ice on a rotary shaker. The high-salt extracted nuclear solution was centrifuged at 10,000*g* for 30 min at 0°C. Ammonium sulfate (0.39 g/mL) was added to the supernatant in an ice/water bath at 4°C and stirred overnight. An additional amount of ammonium sulfate (equal to 0.1875 multiplied by the weight of ammonium sulfate originally added) was then added and stirred for a minimum of 6 hr in an ice/water bath at 4°C. After transfer to 50-mL Oak Ridge tubes held on ice at 4°C, the solution was mixed thoroughly and then transferred into new tubes away from precipitated ammonium sulfate.

After an overnight incubation on ice, a pellicle containing the nuclear proteins will form (Lessard et al., 1991). The solution was centrifuged at 15,000*g* for 30 min at 0°C. The supernatant was discarded and the pellicle retained. This material was resuspended in 3 mL of buffer D (25 mM Tris-HCl, pH 7.68, 50 mM KCl, 15% glycerol, 0.5 mM DTT, 0.5 mM EDTA, and 0.25 mM PMSF) supplemented with 10 μg/mL leupeptin and 10 μg/mL soybean trypsin inhibitor and centrifuged as before. The supernatant was then desalted at 4°C on a Bio-Rad P-6DG column equilibrated in buffer D supplemented with 10 μg/mL leupeptin and 10 μg/mL soybean trypsin inhibitor.

Ion Exchange Chromatography

All fast protein liquid chromatography (FPLC) was performed at 4°C. The desalted extract was loaded onto a Bio-Rad EconoPac-S FPLC column equilibrated in 50 mM KCl buffer D without leupeptin or soybean trypsin inhibitor. One milliliter fractions were collected from a 10-min 50 to 1000 mM KCl linear gradient run at 1 mL/min. The flow-through was then applied to a Mono Q HR 5/5 (Pharmacia FPLC) column equilibrated in 50 mM KCl buffer D without leupeptin or soybean trypsin inhibitor. One milliliter fractions were collected from a

30-min 50 to 1000 mM KCl linear gradient run at 1 mL/min. The resulting fractions were then flash frozen in liquid nitrogen and stored at −80°C.

DNA Probes and Competitors

Probes and competitors were derived from subclones of the Amy32b promoter (Whittier et al., 1987) or of Amy32b promoter–derived synthetic oligonucleotides (Figure 1). pML007 is a subclone of a 274-bp TaqI restriction fragment of the Amy32b promoter (−40 to −313) in the AccI site of pUC19. When digested with NcoI (site at −176) and HindIII (site in the polylinker), a 152-bp fragment is generated. pML010 contains a single copy of the 36-bp BamHI insert in pUC19 termed the B box containing both the Amy32b promoter GARE/box II (underlined) and box I (underlined). The complete sequence of this oligonucleotide as well as its orientation within the polylinker is shown below.

```
SmaI 5′-GATCCGTAACAGAGTCTGG-
     3′-     GCATTGTCTCAGACC-

             -TATCCATGCAGTGCCTG-3′
             -ATAGGTACGTCACGGACCTAG-5′
```

pTS009 contains three head-to-tail copies of the B box oligonucleotide in pBluescript KS+ with the same orientation with respect to the SmaI site as shown above.

pTS004 contains a 21-bp BamHI insert in pBluescript KS+ containing the Amy32b promoter GARE/box II.

```
SmaI 5′- GATCCGTAACAGAGTCTGGTG -3′
     3′-     GCATTGTCTCAGACCACCTAG -5′
```

pTS005 contains a 21-bp BamHI insert in pBluescript KS+ containing the Amy32b promoter box I.

```
SmaI 5′- GATCCGTATCCATGCAGTGCG -3′
     3′-     GCATAGGTACGTCACGCCTAG -5′
```

The cloned promoter fragments in pML007, pML010, pTS004, pTS005, and pTS009 have been confirmed by sequence analysis.

Labeled probes were prepared by digesting appropriate plasmids with restriction enzymes followed by treatment with calf intestinal alkaline phosphatase (Sambrook et al., 1989). Probes for band shift assays were prepared by phosphorylation of the 5′ hydroxyl termini with T4 polynucleotide kinase and γ-^{32}P-ATP (6000 Ci/mmol). Single end–labeled probes for DNase I footprint analysis were produced by extending recessed 3′ terminal hydroxyls with the Klenow fragment of DNA polymerase I with all four α-^{32}P nucleotide triphosphates (3000 Ci/mmol) followed by a secondary restriction enzyme digest. For DNA fragments in Figure 2, pML007 digested with BamHI was labeled at the 5′ end of the noncoding strand followed by digestion with HindIII. For DNA fragments in Figure 7, pML009 digested with HindIII was labeled at the 3′ end of the noncoding strand followed by digestion with XbaI. For DNA fragments in Figure 8, pML010 digested with HindIII was labeled at the 3′ end of the noncoding strand followed by digestion with EcoRI. Labeled DNA fragments were purified on 6% polyacrylamide (30:0.8) or 12% polyacrylamide (40:2) (for 21-bp probes) gels by electrophoresis in 1 × TBE (89 mM Tris-borate, 2 mM EDTA) and followed by overnight elution of the labeled DNA into TE.

Band Shift Assays

Binding reactions were performed in a volume of 40 μL containing 10 mM Tris-HCl, pH 7.68, 1 mM DTT, 1 mM EDTA, 2.5% glycerol, and either 500 ng (152-bp probe; 12.5 μg/mL; equivalent to 1811-fold weight excess) or 300 ng (36- or 21-bp probes; 7.5 μg/mL; equivalent to 3000-fold weight excess) of poly(dI-dC) and 10,000 cpm of labeled probe (Henninghausen and Lubon, 1987). Nuclear extract was then added (up to a maximum of 10 μL) such that the final KCl concentration would be 50 mM. For specific competitions, molar excesses of unlabeled restriction fragments, as indicated, were added to binding reactions containing nuclear extract and incubated for 5 min prior to the incubation with labeled probe. Following a 15-min incubation at room temperature, reactions were then electrophoresed on a 4% polyacrylamide gel (30:0.8) in 0.5 × TBE at 100 V for ~3 hr. Gels were fixed, dried, and exposed to x-ray film with an intensifying screen at −80°C.

DNase I Footprinting Assays

Binding reactions were set up as for band shift assays, except as noted below, and then incubated for 10 min at room temperature. These reactions were adjusted to 10 mM $MgCl_2$ and 2 mM $CaCl_2$ (Figure 2) or 5 mM $MgCl_2$ and 1 mM $CaCl_2$ (Figure 8). For Figure 7, a binding reaction containing twice the amount of extract (100 mM KCl) and 2.5 mM $MgCl_2$ was adjusted to 2.5 mM $CaCl_2$. The amount of DNase I and/or incubation time were optimized for specific protein extracts. The room temperature digestion was terminated by the addition of EDTA to 25 mM and immediately electrophoresed on a 4% polyacrylamide gel (30:0.8) in 0.5 × TBE at 100 V for ~3 hr. The bound and the free probes were localized in the gel by wet gel autoradiography (Carthew et al., 1985; Lessard et al., 1991) and isolated from the gel with the UEA electroeluter (International Biotechnologies, Inc., West Haven, CT). Equal amounts of each were run on a 10% sequencing gel with a purine-specific chemical sequencing reaction. Gels were fixed, dried, and exposed to x-ray film with an intensifying screen at −80°C. The amounts of full-length probe shown in Figure 8B were quantified using a 425 E PhosphorImager (Molecular Dynamics).

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