The Conserved B3 Domain of VIVIPAROUS1 Has a Cooperative DNA Binding Activity

Masaharu Suzuki, Chien Yuan Kao, and Donald R. McCarty

Program in Plant Molecular and Cellular Biology, Horticultural Sciences Department, University of Florida, Gainesville, Florida 32605

The biochemical activities that underlie the genetically defined activator and repressor functions of the VIVIPAROUS1 (VP1) protein have resisted in vitro analysis. Here, we show that a glutathione S-transferase (GST) fusion protein, including only the highly conserved B3 domain of VP1, has a highly cooperative, sequence-specific DNA binding activity. GST fusion proteins that include larger regions of the VP1 protein have very low activity, indicating that removal of the flanking protein sequences is necessary to elicit DNA binding in vitro. DNA competition and DNase I footprinting analyses show that B3 binds specifically to the Sph element involved in VP1 activation of the C7 gene, whereas binding to the G-box-type VP1-responsive element is of low affinity and is nonspecific. Footprint analysis of the C7 promoter revealed that sequences flanking the core TCCATGCAT motif of Sph also contribute to the recognition of the Sph element in its native context. The salient features of the in vitro GST-B3 DNA interaction are in good agreement with the protein and DNA sequence requirements defined by the functional analyses of VP1 and VP1-responsive elements in maize cells.

INTRODUCTION

In the developing maize seed, regulation of maturation and germination programs is integrated in part by the combined activator and repressor functions of the VIVIPAROUS1 (VP1) transcription factor (McCarty et al., 1989, 1991; Hattori et al., 1992; Hoecker et al., 1995). Closely related genes have been isolated from several species (Giraudat et al., 1992; Hattori et al., 1994; Bobb et al., 1995). The ABSCISIC ACID-INSSENSITIVE3 (ABI3) factor of Arabidopsis (Giraudat et al., 1992) is believed to be orthologous to VP1 and has similar functions in dicotyledonous seed development (Parcy et al., 1994). Four amino acid sequence domains that are highly conserved in VP1 and ABI3 (Giraudat et al., 1992) include the A1 domain located in the acidic N-terminal region and three basic regions here designated as B1, B2, and B3 in order from the N terminus. The 120-amino acid B3 domain in the C-terminal region comprises the largest contiguous block of amino acid identity in VP1 and ABI3.

At least two distinct types of cis-acting promoter elements can mediate gene activation by VP1 (Vasil et al., 1995; Kao et al., 1996). Transactivation of the Em maturation genes of wheat (Vasil et al., 1995) and rice (Hattori et al., 1995) is mediated principally by Em1a-like or G-box-type elements that share a core ACGT motif (Marcotte et al., 1989), whereas transactivation of the maize C7 anthocyanin regulatory gene is mediated exclusively by the Sph element (Hattori et al., 1992; Kao et al., 1996). When present in multiple tandem copies, either the G-box or the Sph element is sufficient to confer VP1 regulation to a –45 cauliflower mosaic virus 35S core promoter (Vasil et al., 1995; Kao et al., 1996). In their native promoter contexts, however, single elements are likely to function in combination with other cis-acting elements (Hattori et al., 1992; Vasil et al., 1995; Kao et al., 1996; Shen et al., 1996). In addition, VP1 functions as a repressor of the germination-specific α-amylase genes in aleurone cells (Hoecker et al., 1995). Em-β-glucuronidase (GUS) is activated by VP1 in identically treated aleurone cells, indicating that the activator and repressor functions of VP1 are determined by promoter context (Hoecker et al., 1995).

Mutations of vp1 that disrupt the highly conserved B3 domain of VP1 block expression of the Sph-coupled C7 gene but do not prevent seed maturation (McCarty et al., 1989) or eliminate the repressor function of VP1 (Hoecker et al., 1995). This has been taken as evidence that VP1 has a modular structure and that different domains may be required for activation of Sph and G-box-coupled genes (McCarty, 1995).

Although in vivo expression studies indicate that VP1 has both activator and repressor activities, the biochemical mechanisms underlying these functions remain poorly understood. The A1 domain is part of the functionally defined transcriptional activation domain (McCarty et al., 1991) and may be involved in protein–protein interactions with components of the transcription initiation complex. Previous studies with recombinant VP1 (McCarty et al., 1991; Hattori et al., 1992; Hill et al., 1996) failed to detect specific binding to either the G-box or Sph DNA sequences in vitro, leaving open the question of how VP1 is targeted to promoters of...
downstream genes (McCarty, 1995). The isolated B2 peptide nonspecifically binds DNA with low apparent affinity and can enhance DNA binding of diverse basic-domain, leucine zipper-type DNA binding proteins, including plant G-box binding factors, in vitro (Hill et al., 1996).

Here, we show that when expressed and purified as a separate peptide, the B3 domain has a highly cooperative DNA binding activity that is specific for the Sph sequence. We find that the properties of this activity are in compelling agreement with the functional analyses of VP1 and regulatory sequences in the C1 promoter. These results identify a new class of DNA binding proteins, which thus far are known only in the plant kingdom, that have critical functions in development.

RESULTS

The B3 Domain of VP1 Has a Cryptic DNA Binding Activity

The organization of conserved domains in VP1 is shown in Figure 1A. To explore the biochemical properties of the B3 domain of VP1, we expressed and purified a series of glutathione S-transferase (GST) fusion proteins that contained only the C-terminal region of the protein. Figure 1B shows that at high protein concentrations (>2 µM GST-B3 or ~2 µg of protein per 25-µL reaction), the GST-B3 fusion protein bound to a dimer of the Sph DNA sequence in gel mobility shift assays. No binding to an oligonucleotide that contained a monomer of Sph at concentrations up to 6 µM protein (data not shown) was detected. This result is consistent with our previous observation that at least a dimer of the Sph element is necessary for VP1 activation of a synthetic promoter (Kao et al., 1996).

A larger protein construct, GST-B2B3, which included both the B2 and B3 domains of VP1 (Figure 1A), had greatly reduced Sph binding activity at equivalent molar concentrations of protein. This result is consistent with our previous studies that failed to detect DNA binding activity of intact recombinant VP1 (McCarty et al., 1991; Hattori et al., 1992), suggesting that removal of the flanking protein sequences was necessary for detection of the activity in vitro. Moreover, the B2 peptide, which can enhance DNA binding of G-box binding factors in vitro (Hill et al., 1996), did not enhance DNA binding to Sph when placed either in the same molecule with B3 or in trans. A mutant derivative of the GST-B3 protein, GST-B3Δ574/600, which contained a 26-amino acid internal deletion within the B3 domain, had no activity, indicating that the intact B3 domain is essential for Sph binding.

DNA competition assays showed that the GST–B3 DNA binding activity was specific for the Sph sequence (Figure 1C). Quantitative analysis of the Sph versus self-competition experiments using a Phosphorimager and nonlinear regression curve fitting resolved a high-affinity saturable activity \( (K_{d} = 10 \pm 2 \text{nM}) \) plus a nonsaturable component that accounted for 32% of the shifted radioactivity. A dimer of Sph-128, a mutated element that blocks VP1 transactivation of C1 in vivo (Kao et al., 1996), and a dimer of the G-box DNA sequence (CCACGTGGC) failed to compete with the Sph DNA. At high concentrations of competitor DNA, the G-box and to a lesser extent Sph-128 increased the amount of Sph DNA in shifted complexes by up to twofold. The apparent stabilization of the Sph–protein complex implied that G-box DNA can interact with the Sph/GST–B3 complex but at a site that is distinct from an Sph-specific binding site. Low-affinity binding of GST–B3 to the G-box and Sph-128 sequences was confirmed by gel mobility shift assays, using these DNAs as radioactive probes (see Figure 2A).

GST–B3 DNA Binding Is Highly Cooperative

The Sph DNA binding activity of GST–B3 showed a critical dependence on protein concentration. Figure 2A shows that GST–B3 binding to DNA was highly cooperative, with a Hill constant \( (n_H) \) of 6 ± 0.3. A comparison of the apparent \( K_d \) of 10 nM for Sph binding with the half-saturating GST–B3 concentration \( (K_{app} \) of 3.4 µM protein) suggests that the effective stoichiometry of high-affinity sites is ~1 mol per 340 mol of GST–B3 at the half-saturating protein concentration. Therefore, it is likely that only a small fraction of the total protein participated in saturable binding under these conditions. This result and the sixth-order dependence on protein concentration are consistent with a model in which GST–B3 binds as a protein oligomer.

We examined the kinetics of protein binding to a series of mutant DNA sequences that functionally define the Sph element in vivo. Figure 2B shows that the Sph-128 and Sph-134 sequences that are nonfunctional in vivo (Kao et al., 1996) also had greatly reduced affinity for GST–B3. By contrast, a mutation located immediately upstream (Sph-138), which blocks abscisic acid regulation but not VP1 activation of the C1 promoter, resulted in binding that was comparable for GST–B3 and the wild-type Sph sequence. The Sph-120 mutant, which defines the 5' boundary of the minimal VP1 response element in vivo, was exceptional in that it also bound GST–B3 as effectively as did the wild-type sequence. In all cases, the protein titration results were in agreement with DNA competition experiments (Figure 1C and other data not shown). The kinetics of GST–B3 binding to the G-box dimer were intermediate between the functional and nonfunctional Sph derivatives. G-box binding was cooperative but with a low apparent affinity \( (K_{app} \) of 5.2 µM) that was similar to those of the nonfunctional Sph derivatives.

GST–B3 Footprints the Sph Motif

The identification of DNA sequences involved in the binding of the Sph dimer to the B3 domain was refined by DNase I
Figure 3A shows that the GST-B3 protein specifically footprinted the core TCCATGCAT motif of Sph. The 3' boundary of the DNase I footprint coincided with the boundary of the minimal Sph element defined by functional analysis of Sph (Kao et al., 1996). The three-base Sph-120 mutation, which affects in vivo function but not GST-B3 binding (Figure 2B), overlapped the T residue at the 5' boundary of the in vitro footprint. The fact that this mutant renders Sph nonfunctional in vivo suggested that the critical DNA contacts may extend farther 5' in the native context of the C1 promoter. Areas of enhanced DNase I sensitivity were evident in sequences adjacent to Sph as well as in distal regions of the template. To determine what role, if any, the B3 DNA binding activity might have in G-box-mediated gene activation, we footprinted a tandem G-box sequence that is known to be sufficient for VP1 transactivation in vivo (Vasil et al., 1995). As shown in Figure 3B, the core ACGT motif of the G-box element was very weakly protected by GST-B3 protein, indicating a low specificity of binding to that sequence. This result is consistent with the evidence presented in Figures 1 and 2A that the G-box sequence interacts with GST-B3 with a degree of sequence selectivity; however, the G-box and Sph sequences evidently do not compete for the same site.

Flanking Sequences Affect B3 Recognition of Sph in the C1 Promoter Context

Although dimerization of the minimal Sph motif is required for VP1 activation of a minimal promoter (Kao et al., 1996) as well as for GST–B3 binding to oligonucleotide probes, a single Sph element is able to mediate VP1 transactivation in the context of the C1 promoter. The results of DNA binding experiments shown in Figure 4 confirmed that GST–B3 bound cooperatively to the region of the C1 promoter that included the Sph motif. Compared with the Sph dimer sequence, GST–B3 binding to the native promoter element had a lower degree of cooperativity (a Hill constant of 3.4 compared with 6.0) but a somewhat higher apparent affinity ($K_{app}$ of 2.1 μM protein). Results of DNase I footprinting experiments shown...
DISCUSSION

Our results indicate that the conserved B3 domain of VP1 has a highly cooperative, sequence-specific DNA binding activity. This finding identifies a key biochemical function of the enigmatic VP1/AB13 family of regulatory proteins. The intrinsic binding affinity of the active protein species is high ($K_d$ of $10^{-8}$ M) and in the typical range of sequence-specific protein–DNA interactions. Moreover, we find that the specificity of this protein–DNA interaction is in excellent agreement
Figure 3. DNase I Footprint Analysis of the GST–B3 DNA Complex.

(A) Footprinting of a 69-bp restriction fragment containing a tandem dimer of the Sph sequence (see Figure 1B).
(B) Footprinting of a 139-bp fragment containing five tandem copies of a G-box sequence (CCACGTGGC).

The DNA templates end labeled on the bottom strand were incubated with 0, 2.5, or 5 μg of GST–B3 in binding buffer (Figure 1) and treated with 0.1 unit of DNase I for 2, 3, or 4 min, as represented by the triangles above the gels indicating increasing times. The products were resolved on a DNA sequencing gel (Sambrook et al., 1989). The G reaction products from the Maxam and Gilbert sequencing of each template (Sambrook et al., 1989) were run in adjacent lanes to provide a positional reference (only the Sph reaction is shown). The alignments of the proximal Sph (open bar) and G-box sequences (overlined at right in [B]) are shown; the distal Sph motif is indicated with a bracket. The wide solid bar indicates the three bases altered in the Sph-120 mutant, and the narrow solid bar indicates the protected bases of the DNase I footprint. Two weakly protected bases of the G-box motif (overlined at right) are indicated with asterisks.
flanking a DNA binding domain occur in mammalian Ets proteins (Jonsen et al., 1996). Conceivably, the interactions between domains of VP1 that control B3 DNA binding activity might be important regulatory mechanisms in vivo. In this respect, the regulated DNA binding activity of the mammalian steroid receptors (Godowski et al., 1987) and other regulatory proteins may be useful analogies, although other ligands of VP1 have not been identified.

B3 Is a Discrete Functional Domain of VP1

The demonstration that B3 binds DNA provides direct biochemical evidence for the proposed modular structure of VP1 (McCarty, 1995). Our finding that the B3 domain binds specifically to Sph is in full agreement with (1) the genetic

Intraprotein Interactions Inhibit VP1 DNA Binding Activity

B3 activity is cryptic in the sense that other regions of the protein must be removed to elicit DNA binding in vitro. It is possible that the N-terminal region of VP1 causes improper folding or conformational instability of the recombinant protein. We note, however, that purified GST-B2B3, which binds DNA very poorly, is no less soluble than is GST-B3. Steric interference or specific interactions within the protein might affect accessibility of the B3 domain to DNA or protein oligomerization. Simple steric interference by the additional 120 amino acids of the VP1 sequence located upstream of B3 in GST-B2B3 seems unlikely to explain the inhibitory effect because close juxtaposition of the 250-amino acid GST domain with B3 in the GST-B3 fusion protein uncovers the DNA binding activity.

The effects of the GST component on the intrinsic DNA binding, oligomerization, or other conformational properties of B3 are difficult to measure because the B3 peptide released by thrombin cleavage has very poor solubility (M. Suzuki and D.R. McCarty, unpublished results). The relatively large globular GST domain of GST-B3 evidently modifies the solubility and electrophoretic properties of the B3 peptide. In any case, it remains to be determined whether the B2 and/or the B2–B3 intervening region of VP1 has a specific role in inhibition of B3 DNA binding activity. Similar inhibitory effects of amino acid sequences immediately

with sequences identified as being functionally important for VP1 activation of the C1 gene in maize cells. Two striking features of the B3 DNA binding activity are its cryptic nature and its dependence on a critical concentration of protein.

Figure 4. GST–B3 Binds Cooperatively to the C1 Promoter.

Gel mobility shift experiments were performed and analyzed by nonlinear regression, as described for Figure 2A, using a Hindlll-Sall restriction fragment that included the region from −154 to −51 of the C1 promoter as the labeled DNA. The data for the Sph dimer are adapted from Figure 2A.

Figure 5. GST–B3 Footprints the Sph Element in the C7 Promoter.

DNase I protection experiments were performed as described in the legend to Figure 3, using the C7 promoter fragment from −154 to −51 (see Figure 4) labeled on the bottom strand. The open oval at left indicates the protected region. The solid oval indicates the core Sph motif footprinted in Figure 3. The asterisks denote bases with enhanced DNase I sensitivity. The solid rectangle specifies bases essential for VP1 transactivation, and the open rectangle indicates bases specifically required for ABA regulation of C7 in maize cells (Kao et al., 1996). The triangle above the gel indicates a gradient in protein concentrations, including 2, 4, and 6 µg of GST–B3. The G and G + A reference ladders shown in the two rightmost lanes were generated as described in Figure 3.
evidence that the B3 domain is essential for activation of the C1 gene (McCarty et al., 1989; McCarty, 1995) and (2) the fact that Sph is absolutely required for VP1 activation of C1 (Hattori et al., 1992; Kao et al., 1996).

Sph-like promoter elements, on the other hand, are not necessary for VP1 activation of G-box-coupled genes, including Em (Vasil et al., 1995). Moreover, B3 has only weak affinity for the G-box element, suggesting that the B3 activity does not directly mediate VP1 regulation of G-box-coupled genes. The quiescent embryo phenotype of vp1-McW, a truncated mutant lacking B3 (McCarty et al., 1989), confirms that Sph binding activity is not essential for induction of much of the maturation program in developing embryos. These results support a model in which functions that are sufficient for G-box-coupled gene activation map to the N-terminal region of VP1 (McCarty, 1995). This hypothesis gains additional support from the evidence that the upstream B2 region is necessary for activation of the G-box-coupled Em-GUS gene in rice cells (Hill et al., 1996). Remarkably, VP1 mutants lacking B3 also function as repressors of α-amylase genes (Hoecker et al., 1995), indicating that the Sph binding function is not needed to discriminate between activated and repressed promoter contexts in aleurone cells.

The molecular mechanisms involved in G-box–mediated activation and gene repression remain to be elucidated. It is difficult to rule out a second sequence-specific DNA binding function in the N-terminal region on the basis of negative evidence (McCarty et al., 1991; Hill et al., 1996); however, we suggest that tethering interactions mediated by other G-box binding factors bound to the DNA have a major role in directing VP1 activation and/or repression. Precedents for this type of model include the mechanism by which AP-1 defines the repressor and activator contexts of mammalian glucocorticoid receptors (Starr et al., 1996) and the protein interactions that mediate the homeodomain-independent patterning activity of FTZ in Drosophila (Copeland et al., 1996). Finally, it should be emphasized that our data do not entirely rule out a role for B3 in G-box–coupled gene activation and/or repression of signals, especially in promoters like Em that contain both G-box– and Sph-like elements. At least some DNAs, including the G-box, can cause significant stabilization of the GST–B3/Sph complex in mobility shift assays (Figure 1C). This activity and the nonsaturable component of the Sph binding activity indicate that GST–B3 may have distinct specific and nonspecific DNA binding sites.

Sequences Flanking Sph Affect B3 Cooperative Binding to DNA

The minimal functional Sph element and the core B3 binding motif TCATGCAT are defined by the mutational analysis of the sequences essential for VP1 activation of the C1 promoter in maize cells (Hattori et al., 1992; Kao et al., 1996), by the bases required for GST–B3 binding to the Sph dimer, by the DNase I footprint of the Sph dimer, and finally, by the footprint of the Sph element in its native context. Our results indicate, however, that the sequences flanking the core element contribute in an essential way to B3 recognition of a solitary Sph element embedded in the native C1 promoter. The base substitution experiments (Kao et al., 1996) indicate that the sequence requirements of the flanking region either are less constrained than in the core motif or are functionally redundant. We suggest that the principal base contacts conferring sequence specificity to the B3 interaction are located in the core Sph motif. In the native context, the DNase I footprint includes 5’ flanking bases that have been specifically implicated in ABA regulation of the C1 promoter (Hattori et al., 1992; Kao et al., 1996). It remains to be determined whether these effects are correlated in any way with subtle changes in the kinetics of B3 binding in vitro. The removal of the flanking sequence context is evidently compensated for both in vivo and in vitro by the cooperative interactions that arise from placing the core motif in a dimer configuration.

At least two modes of interaction may contribute to the critical protein concentration dependence of the B3 DNA binding activity: (1) the active protein species in solution may be an oligomer that forms only at high protein concentrations under our conditions; and (2) there may be cooperative interactions among multiple binding sites on the DNA, as suggested by the effect of Sph dimerization. Although our data do not clearly distinguish the relative contributions of these mechanisms, it is noteworthy that GST–B3 binds to the single Sph element in the C1 promoter with a Hill constant of ~3, whereas the Hill constant for binding to the Sph dimer oligonucleotide is approximately doubled to 6. This relationship suggests an effective stoichiometry of three interacting protein molecules per Sph element. Whatever the source, the potential for highly cooperative DNA binding is likely to contribute in an essential way to the function of VP1 as a developmental switch in plants.

B3 Defines a Novel Class of DNA Binding Proteins in Plants

The B3 sequence has no detectable sequence similarity to other known DNA binding proteins, and all related protein sequences identified thus far have come from the plant kingdom. Because the B3 domains of ABI3 and VP1 are 90% identical, we anticipate that ABI3 and other plant homologs of VP1 (Hattori et al., 1994) can also bind DNA. In addition to the VP1 homologs found in other species, a distinct class of more distantly related protein sequences can be identified in the plant-expressed sequence tag collections (J.M. Davis and D.R. McCarty, unpublished results), suggesting that similar proteins may function in other phases of plant development.
Preparation of Glutathione S-Transferase Fusion Proteins

We created a series of fusion proteins: glutathione S-transferase (GST)-B2B3, containing VP1 amino acids 372 to 659; GST-B2, containing VP1 amino acids 372 to 413; and GST-B3, containing VP1 amino acids 492 to 631. These fusion proteins were constructed by subcloning appropriate fragments of the VP1 cDNA into the pGEX-2T vector (Pharmacia Inc., Uppsala, Sweden). The DNA fragment for GST-B3 was prepared by polymerase chain reaction amplification from the VP1 cDNA (McCarty et al., 1991), using the following pair of primers: 5'-CTTGGATCCGACATTCACCACCGCTCCTG-3' and 5'-ACGGAATTCCTCGGAGAGCTGCGACTGTG-3', which incorporated BamHI and EcoRI sites for cloning into the pGEX-2T polylinker. The GST-B3 construct, an in-frame deletion of VP1 amino acids 574 through 600, was constructed using the same pair of primers and the 35S-Sh-VpIA574/600 plasmid as a host strain was transformed with the pGEX-2T plasmid and grown at 37°C in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside, and the cultures were sonicated in an ice bath, and the soluble protein fraction was obtained after centrifugation for 10 min at 10,000g. The GST proteins were purified by glutathione-Sepharose affinity chromatography, according to the manufacturer’s recommendations (Pharmacia Inc.), except that the proteins were eluted in GEB (20 mM reduced glutathione, 100 mM Tris-HCl, pH 8.0). The purified GST fusion proteins were analyzed for purity and integrity by SDS-PAGE. The concentration of each protein solution was determined by the method of Bradford (1976).

Construction of Sph Dimer, G-Box, and C1 Probes

The DNA probes for the DNA binding experiments were constructed from the following pairs of complementary single-stranded oligonucleotides: Sph, 5'-GATCCGGTGCGTCGTCCGATGCTGCGAGCTGATCATGCT-3' and 5'-GATCCGATGCGACGACGGATCATGCGACGCCG-3'; Sph-m134, 5'-GATCCGTCGTCGTCGACGCATGATCCGTGTCGTCGACGCATGACG-3' and 5'-GATCCCGTCGACGACGGATCATCCTCGGACGAC-3'; Sph-m128, 5'-GATCCGGTGCGTCGTCCATTACGGATCCGTCGTCGTCGACGCACG-3'; Sph-m120, 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCATGACG-3' and 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCACG-3'; Sph-m138, 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCATGACG-3' and 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCACG-3'; Sph-m134, 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCATGACG-3' and 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCACG-3'; Sph-m128, 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCACG-3'; Sph-m120, 5'-GATCCGTCGTCGTCGACGCCATGATCCGTGTCGTCGACGCACG-3'. The annealed, double-stranded oligonucleotides were subcloned into the BamHI site of pUC19. The DNA fragments were prepared by digestion of the plasmids with SstI and PstI and fragility shift assays (a 69-bp fragment) and with SstI and EcoRI for DNase I footprinting (a 77-bp fragment).

To construct the G-box dimer probe for gel mobility shift assay, G-box oligonucleotides were prepared by digestion of the chalcone synthase tetramer construct (Weisshaar et al., 1991; Vasil et al., 1995) with Sau3AI. The oligonucleotides were subcloned into the BamHI site of pUC19, and a plasmid containing two tandem G-box copies was selected. The 80-bp G-box dimer probe was prepared by digestion of that plasmid with SstI and PstI. The G-box probe for DNase I footprinting was prepared by digestion of the chalcone synthase G-box tetramer construct with HindIII and EcoRI. The DNA fragment containing the region –157 to –43 of the C1 promoter was prepared by digestion of the D29 plasmid (Hattori et al., 1992) with HindIII and SalI. The fragment was subcloned into HindIII-SalI-digested pUC19. The C1 probe was prepared by digestion of the plasmid with HindIII and Smal.

DNAase I Footprinting

DNAase I protection experiments were performed as described in Green et al. (1989). The 32P-end-labeled fragments were incubated with GST fusion proteins in 25 μL of the binding buffer used for gel mobility shift assays, and DNAase I was added to the reaction mixture and incubated for 5 min at room temperature. The digested DNA fragments were purified and separated on a 10% polyacrylamide sequencing gel. The gel and G + A ladders were prepared as described in Sambrook et al. (1989).

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