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Composite Structure of Auxin Response Elements

Tim Ulmasov, Zhan-Bin Liu, Gretchen Hagen, and Tom J. Guilfoyle[1]

Department of Biochemistry, 117 Schweitzer Hall, University of Missouri, Columbia, Missouri 65211

The auxin-responsive soybean *GH3* gene promoter is composed of multiple auxin response elements (AuxREs), and each AuxRE contributes incrementally to the strong auxin inducibility of the promoter. Two independent AuxREs of 25 bp (D1) and 32 bp (D4) contain the sequence TGTCTC. Results presented here show that the TGTCTC element in D1 and D4 is required but not sufficient for auxin inducibility in carrot protoplast transient expression assays. Additional nucleotides upstream of TGTCTC are also required for auxin inducibility. These upstream sequences showed constitutive activity and no auxin inducibility when part or all of the TGTCTC element was mutated or deleted. In D1, the constitutive element overlaps the 5′ portion of TGTCTC; in D4, the constitutive element is separated from TGTCTC. An 11-bp element in D1, CCTCGTGTCTC, conferred auxin inducibility to a minimal cauliflower mosaic virus 35S promoter in transgenic tobacco seedlings as well as in carrot protoplasts (i.e., transient expression assays). Both constitutive elements bound specifically to plant nuclear proteins, and the constitutive element in D1 bound to a recombinant soybean basic leucine zipper transcription factor with G-box specificity. To demonstrate further the composite nature of AuxREs and the ability of the TGTCTC element to confer auxin inducibility, we created a novel AuxRE by placing a yeast GAL4 DNA binding site adjacent to the TGTCTC element. Expression of a GAL4-c-Rel transactivator in the presence of this novel AuxRE resulted in auxin-inducible expression. Our results indicate that at least some AuxREs have a composite structure consisting of a constitutive element adjacent to a conserved TGTCTC element that confers auxin inducibility.

INTRODUCTION

The plant hormone auxin plays an important role in many processes in plants, including cell division, cell elongation, cell differentiation, root formation, apical dominance, and tropisms. Most, if not all, of these processes are believed to involve auxin-induced changes in gene expression. A number of auxin-responsive genes that may play roles in one or more of these processes have been cloned and sequenced (reviewed in Guilfoyle et al., 1993). Recently, several different putative auxin response elements (AuxREs) have been identified within the promoters of auxin-responsive genes (Ainley et al., 1988; McClure et al., 1989; An et al., 1990; Conner et al., 1990; Korber et al., 1991; Nagao et al., 1993; Oeller et al., 1993; Li et al., 1994; Liu and Lam, 1994; Liu et al., 1994; Ulmasov et al., 1994). Identification of these putative AuxREs has been based mainly on conservation of similar sequence elements found in a variety of genes induced by auxin. Most of these putative AuxREs have not been tested for functionality, and their direct role in response to auxin remains to be demonstrated. One of the smallest functional AuxREs (~20 bp) identified to date appears to be an octopine synthase (*ocs*) or *ocs*-like element (Ellis et al., 1993; Kim et al., 1994; Liu and Lam, 1994; Zhang and Singh, 1994); however, this element does not appear to be specific for auxin but is responsive to salicylic acid, methyl jasmonate, and inactive hormone analogs (Kim et al., 1994; Qin et al., 1994; Ulmasov et al., 1994; Zhang and Singh, 1994). Auxin-responsive genes that are specifically induced by auxins do not appear to contain the *ocs* element.

We have identified and sequenced a gene, *GH3*, from soybean that is induced specifically by auxins (Hagen et al., 1984, 1991; Hagen and Guilfoyle, 1985). In addition, we have shown that the *GH3* promoter is specifically induced by auxins when fused to the β-glucuronidase (*GUS*) reporter gene and tested in transgenic tobacco and Arabidopsis plants (Hagen et al., 1991; Z.-B. Liu, G. Hagen, and T.J. Guilfoyle, unpublished results). The *GH3* gene is one of the most rapidly induced auxin-responsive genes that has been identified. Transcriptional activation of this gene is observed within 5 min after auxin application (Hagen and Guilfoyle, 1985). Understanding the mechanism of *GH3* gene activation is likely to provide general insights into auxin-regulated gene expression because of the very rapid activation of the *GH3* gene in response to auxin, the primary nature of the response (i.e., protein synthesis is not required for auxin to induce transcription of this gene), the specificity of the response to auxins, and the wide range of tissue and organ types in which the *GH3* gene is activated by auxin (Hagen and Guilfoyle, 1985; Gee et al., 1991).

Recently, we showed that the *GH3* promoter contains at least three AuxREs that can function independently of one another (Liu et al., 1994). In combination, these AuxREs incrementally increase the level of response to exogenous auxin. Two of these AuxREs are only 25 and 32 bp and are found within a larger 76-bp auxin-inducible fragment. The 25- and 32-bp elements

[1] To whom correspondence should be addressed.

are the smallest AuxREs identified to date that are specific for auxin and function as monomers when fused to a minimal cauliflower mosaic virus (CaMV) 35S promoter. Both of the small AuxREs within the 76-bp fragment contain a conserved sequence element TGTCTC. Here, we report that the 25- and 32-bp AuxREs in the *GH3* promoter have a composite structure, consisting of a constitutive element adjacent to an element, TGTCTC, that is required for auxin inducibility. In the absence of added auxin, the TGTCTC element repressed or down-regulated the adjacent constitutive element, and when auxin was added, the AuxREs were derepressed and up-regulated. We further showed that a novel AuxRE can be created by fusing a constitutive yeast promoter element adjacent to the TGTCTC element. Our results suggest that two AuxREs in the *GH3* promoter have a composite structure similar to some hormone response elements found in mammalian genes.

RESULTS

Linker Scanning and TGTCTC Mutations

Previously, we identified a 76-bp auxin-responsive fragment (−183 to −108) in the *GH3* promoter that could be separated into two independent AuxREs of 25 and 32 bp, referred to as D1 and D4, respectively (Liu et al., 1994). D1 and D4 each contain the sequence TGTCTC and were shown to function as single- or multiple-copy AuxREs when fused upstream of a minimal CaMV 35S promoter and tested by transient assays in carrot protoplasts. A diagram of this promoter fragment is shown in Figure 1. To define the AuxREs in the *GH3* promoter better, we carried out linker scanning analysis in region D4 by substituting two base pairs between −142 to −123 in the context of a 72-bp AuxRE (−181 to −110), which was fused to the minimal −46 CaMV 35S promoter (Figure 2A). These promoter constructs were fused to the *GUS* reporter gene and tested for auxin inducibility in carrot protoplasts by transient assays. The unmutated 72-bp fragment was induced greater than fivefold by the addition of 50 μM α-naphthalene acetic acid (NAA). Two base pair substitutions within the 5′ region of D4 revealed two domains important for maximal auxin inducibility (Figure 2A). Mutations M1, M2, and M4 within an 8-bp element, CACGCAAT, in the 5′ region of D4, resulted in decreased activity in the absence or presence of auxin. A second region that resulted in decreased auxin-inducible promoter activity was seen with mutation M8 within the TGTCTC element. This latter mutation did not, however, decrease the promoter activity in the absence of auxin; in fact, a small increase was observed.

Because a second AuxRE, D1, is present in the 72-bp fragment used for linker scanning, two base pair substitutions throughout D4 did not reveal complete loss of auxin inducibility. However, the linker scanning results with D4 suggested that a region 5′ to the TGTCTC element and the TGTCTC element itself are important for auxin inducibility. To gain more information on the importance of the TGTCTC element, we mutated TGTCTC elements in both D1 and D4 of the 72-bp promoter fragment to either TGgCTC (M71; mutated nucleotides are indicated in lowercase letters), TGTaTC (M72), or TGTaaa (M73). Figure 2B shows that all three of these double mutations resulted in almost complete loss of auxin inducibility, suggesting that the TGTCTC elements are required for auxin induction. In the absence of auxin treatment, each double mutation construct showed constitutive activity severalfold higher than that of the minimal CaMV 35S promoter and slightly higher than that of the unmutated promoter. The double mutation results along with results from the M1, M2, and M4 linker scanning mutations and previously reported linker scanning results (Liu et al., 1994) suggested that sequences in addition to TGTCTC within the 72-bp fragment contribute to the activity of this promoter fragment.

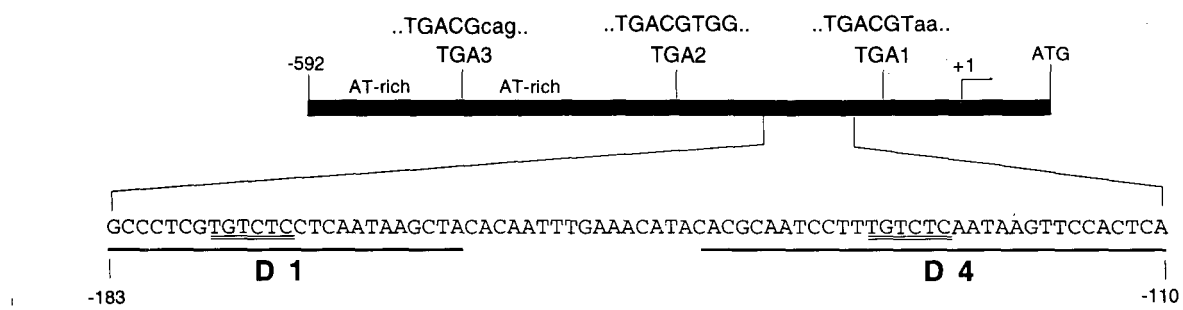

Figure 1. Diagram of the Soybean *GH3* Promoter.

The three TGA boxes and two AT-rich regions in the 592-bp promoter are shown. Residues that differ from the G-box (TGA2) are shown in lowercase letters above TGA1 and TGA3. The transcription start site is shown as +1 and the translation start site as ATG. The nucleotide sequence of a 72-bp auxin-inducible fragment (−183 to −110) of the promoter is shown. The positions of two independent AuxREs, D1 (−183 to −159) and D4 (−142 to −110), are underlined in bold, and the TGTCTC elements within each of these AuxREs are double underlined.

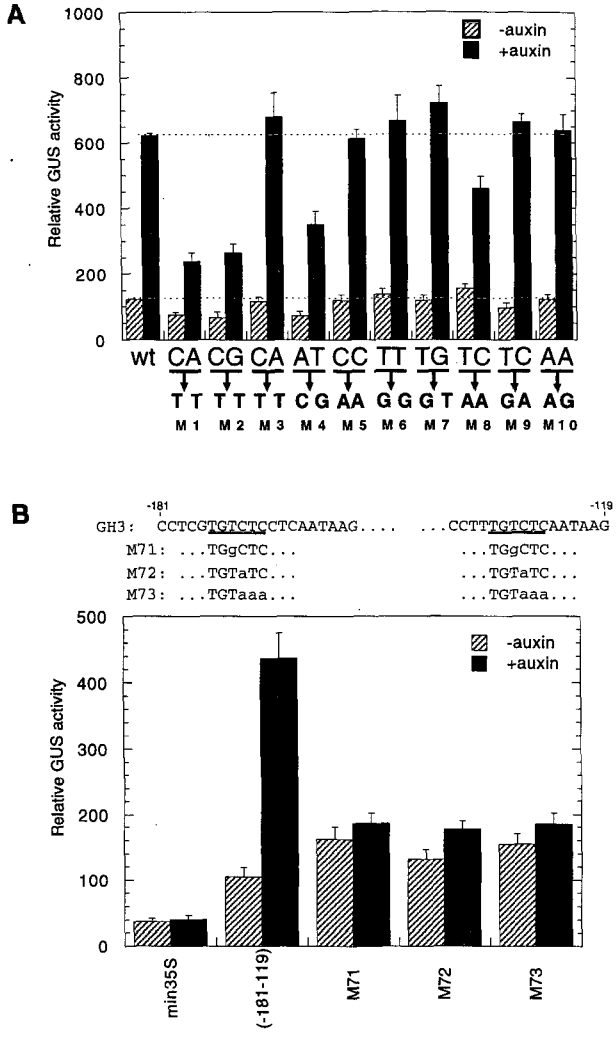

Figure 2. Quantitative Fluorometric GUS Assays for Auxin Inducibility of the Mutated 72-bp AuxRE.

(A) Assays for two base pair linker scanning mutations between −142 to −123 of D4 within the context of the 72-bp auxin-inducible fragment shown in Figure 1. Wild type (wt) and linker scanning mutations (M1 to M10) were cloned upstream of the −46 CaMV 35S promoter–*GUS* reporter gene. Mutated nucleotides are indicated under each bar graph. Carrot protoplasts were transfected and incubated for 24 hr in the absence (diagonally striped bars) or presence (black bars) of 50 μM NAA. Transfections and GUS assays were carried out as indicated in Methods, and standard errors are shown above bar graphs. GUS activity is given in relative units and was standardized using luciferase as an internal standard for each transfection.

(B) Assays for double mutations in the TGTCTC elements within the context of a 63-bp auxin-inducible fragment (−181 to −119) of the *GH3* promoter. The DNA sequences around the TGTCTC elements (double underlined) in D1 and D4 are indicated (GH3), and the sequences of the double mutations within these elements are shown below the wild-type fragment (M71, M72, and M73). Dots indicate additional unmutated nucleotides between −181 to −119 for each construct. Mutated residues in M71, M72, and M73 are indicated by lowercase letters. Wild-type and mutant fragments were cloned upstream of the −46 CaMV 35S promoter–*GUS* reporter gene and assayed as described in **(A)**. Relative GUS activity is shown for a −46 CaMV 35S promoter (min35S), an unmutated 63-bp fragment (−181 to −119), and the double mutant constructs. Protoplast incubations were carried out in the absence (diagonally striped bars) or presence (black bars) of 50 μM NAA.

A Constitutive Element and a TGTCTC Element Make Up a Composite AuxRE in D4

To define further the minimal AuxREs in the 72-bp fragment described above, we analyzed subfragments of and site-directed mutations in D1 and D4 as independent AuxREs isolated from one another. Within the 32-bp D4 fragment, three overlapping oligonucleotides, D4-1, D4-2, and D4-3, were cloned as multiple copies (two to four copies, all in correct orientation relative to the *GH3* promoter) upstream of the minimal CaMV 35S promoter (Figure 3A). These constructs were tested in the absence and presence of auxin by transient assays in carrot protoplasts. None of these constructs showed more than 1.3-fold induction by auxin, whereas a single-copy and a four-copy multimer of the D4 element (−142 to −110) showed threefold and sevenfold auxin inducibility, respectively (data not shown; see Liu et al., 1994, and construct D4-6 in Figure 3B).

The four-copy D4-1 construct was 30-fold (minus auxin) to 45-fold (plus auxin) more active than the minimal 35S promoter. The two-copy D4-3 construct and a three-copy D4-2 construct were only slightly more active than the minimal CaMV 35S promoter plus or minus auxin. The D4-2 construct contained the TGTCTC and AATAAG elements that are found in both the 25- and 32-bp D1 and D4 AuxREs in the −183 to −108 fragment of the *GH3* promoter (Liu et al., 1994). To confirm that neither the TGTCTC nor the AATAAG element was sufficient for auxin induction, we tested a seven-copy construct of TGTCTC (TGT1) and a nine-copy construct of CAATAAG (CAA1). These constructs showed no auxin inducibility and had activities approximately equal to that of the minimal CaMV 35S promoter. On the other hand, a four-copy D4-4 fragment, CACGCAAT, found upstream of the TGTCTC element in D4-1, showed activity 40- to 50-fold greater than that of the minimal 35S promoter. Linker scanning mutations (see Figure 2A) suggested that the CACGCAAT sequence contributed to the promoter activity in the D4 AuxRE. The D4-4 and D4-5 constructs, like the D4-1 construct, showed little induction in the presence of auxin.

Because the 32-bp D4 element is auxin inducible (Liu et al., 1994) but subfragments of this element, which were tested as described above, were either constitutively active (i.e., equally active in the absence or presence of auxin) or inactive (i.e., activities similar to the minimal CaMV 35S promoter), our results suggested that a constitutive element in the context of the TGTCTC or TGTCTCAATAAG element might function as a minimal AuxRE. To test this possibility further, additional 24-bp constructs (D4-6, D4-7, and D4-8) containing both the constitutive element (CACGCAAT) and the TGTCTCAATAAG

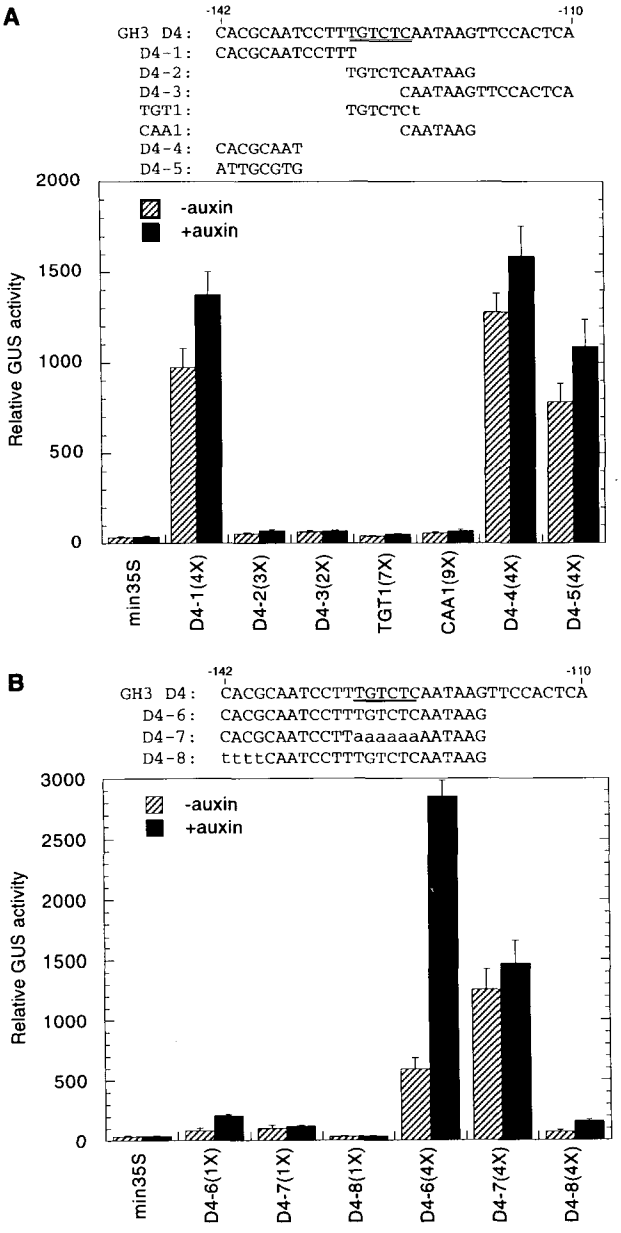

Figure 3. Quantitative Fluorometric GUS Assays for Auxin Inducibility of Subfragments and Site-Directed Mutations in the D4 AuxRE.

(A) Assays with subfragments of D4. Subfragments of the D4 AuxRE were multimerized in tandem (all in the correct orientation, with the number of repeats from 2× to 9×) and fused to a −46 CaMV 35S promoter–*GUS* reporter gene. Carrot protoplast transfections and assays were as described in Figure 2A. The sequences of the 33-bp D4 element (GH3 D4) and subfragments that were tested for auxin inducibility are shown at top.

(B) Assays with site-directed mutations in D4. A wild-type truncated D4 element (D4-6; −142 to −119), an element with a mutation in the constitutive region (D4-7), and an element with a mutation in TGTCTC (D4-8) were fused as monomers (1×) or tetramers (4×) to a −46 CaMV 35S promoter–*GUS* reporter gene and tested for auxin inducibility by transient assays in carrot protoplast.

Site-directed mutations are indicated in lowercase letters. Double underlining shows the position of the TGTCTC element. min35S is a −46 CaMV 35S promoter–*GUS* reporter gene. Protoplast incubations were carried out in the absence (diagonally striped bars) or the presence (black bars) of 50 μM NAA. Standard errors are indicated above each bar graph.

element as well as mutations in these elements were assayed in carrot protoplasts. Figure 3B shows that a single copy of the unmutated fragment (D4-6) fused to a minimal CaMV 35S promoter was induced about threefold by auxin. Mutation (D4-7) of TGTCTC to AAAAAA resulted in loss of auxin inducibility and an increased level of constitutive expression; mutation (D4-8) of the CACG in the 5′ portion of the constitutive element resulted in loss of both constitutive expression and auxin inducibility. Four-copy constructs of D4-6, D4-7, and D4-8 gave qualitatively similar results compared with those of the single-copy constructs, except that the levels of expression were increased in each case. The four-copy D4-6 construct was induced approximately fourfold by auxin, the D4-7 construct showed a high level of constitutive expression, and the D4-8 construct was induced approximately twofold by auxin. The low level of auxin inducibility seen with the D4-8 construct may have resulted from multimerization and appropriate spacing of the TGTCTC element (see Discussion). In total, the results with D4 indicated that this AuxRE consists of two separate elements, a constitutive element and an adjacent TGTCTC element. The constitutive element, when isolated from TGTCTC, was approximately equally active in the absence or presence of auxin. The constitutive nature of this element within D4 was only revealed by mutating the TGTCTC element. The results suggested that the TGTCTC element conferred auxin inducibility by repressing or down-regulating the adjacent constitutive element in cells, which had not been exposed to auxin, and that derepression and up-regulation resulted when auxin was added.

The Constitutive Element in D4 Binds Nuclear Proteins in a Sequence-Specific Manner

To determine whether the 8-bp sequence CACGCAAT, which showed constitutive activity in D4-4 (see Figure 3A), bound specifically to nuclear proteins in plant cell extracts, we tested a tetramer of this element in gel shift assays. Nuclear extracts from soybean plumules, pea plumules, cauliflower curd, and wheat germ were tested, and each extract showed specific binding to this element. Results with the pea and wheat extracts are shown in Figure 4. The pea extract showed only one major binding complex (lane 2), but the wheat extract produced two binding complexes (lane 7). The specific competitor, CACGCAAT, competed with the labeled probe for binding in both pea (lane 4) and wheat (lane 9) extracts. Nonspecific competitors,

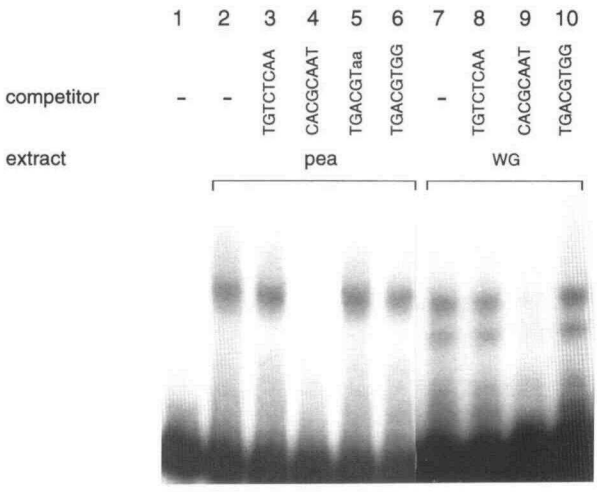

Figure 4. Gel Mobility Shift Assays with the Constitutive Element CACGCAAT in D4.

Gel mobility shift assays were performed with a ^{32}P-labeled (10^7 cpm/μg; 1 ng per lane) tetramer D4-4 probe, CACGCAAT (see Figure 3A), in the presence of nuclear extracts from pea plumules (4 μg per lane) and wheat germ (WG) (3 μg per lane). Assays were performed in the absence (lanes 1, 2, and 7) and presence (lanes 3 to 6 and 8 to 10) of competitor oligonucleotides. Competitors were added as monomers at 200 times molar excess to the probe. Lane 1 contained no nuclear extract.

including TGACGTGG (TGA2 shown in Figure 1), TGACGTAA (TGA1 shown in Figure 1), and the TGTCTC element, did not compete for binding to the probe (lanes 3, 5, 6, 8, and 10). These results indicated that a nuclear protein with DNA sequence binding specificity bound to the constitutive element upstream of TGTCTC in D4.

The AuxRE in the D1 Fragment Contains a Constitutive Element Overlapping a TGTCTC Element

The observation that the D4 construct contained a constitutive element upstream of a TGTCTC element prompted us to investigate whether the D1 fragment, which also has the sequences TGTCTC and AATAAG found in D4, contained a constitutive element separable from the TGTCTC sequence. Figure 5 shows that an 11-bp D1-4 fragment in D1 was about fivefold more active than the minimal CaMV 35S promoter in the absence of auxin treatment and was induced about sixfold after the addition of auxin when assayed as a four-copy construct in carrot protoplasts. Mutation of the TGTCTC portion of this promoter fragment to TGTaaa (D1-3 construct) resulted in a twofold increase in constitutive activity and a loss of auxin inducibility. Likewise, a mutation of CCTCGTGTCTC to CCTCGTGgaTC (D1-5 construct) resulted in constitutive expression with loss of auxin inducibility. A construct with a mutation in the 5′ half of D1-4 to aaaaaTGTCTC (D1-6 construct) had activity only slightly greater than that of the minimal 35S promoter and was not auxin inducible. These results suggested that D1, like D4, contained a constitutive element and a TGTCTC element.

To define the borders of the constitutive element, the sequence elements immediately upstream of TGTCTC were tested. The sequence upstream of TGTCTC in D1 is GCCCTCG. Examination of the sequence just 5′ to D1 (see the 5′ extension of D1 that is not underlined in GH3 D1; Figure 5 inset) revealed an imperfect direct repeat of a CG-rich 6-bp CCCT (C/G)G consensus sequence. Only part (i.e., CCCTCG) of one of these repeats is present in the D1 AuxRE previously identified by gain-of-function assays (Liu et al., 1994). The CCCT (C/G)G repeat led us to test whether one or both copies of this repeat might show constitutive activity. Therefore, we tested a four-copy D1-2 construct consisting of the 6-bp direct repeat. This construct exhibited no constitutive activity and, in fact, had no greater activity than the minimal CaMV 35S promoter.

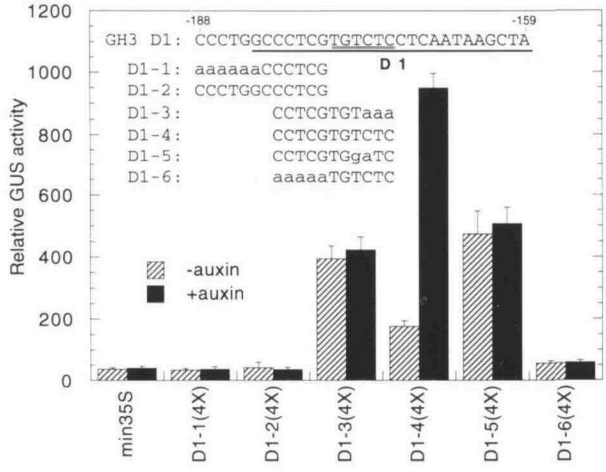

Figure 5. Quantitative Fluorometric GUS Assays for Auxin Inducibility of Site-Directed Mutations in the D1 AuxRE.

Subfragments of the D1 AuxRE (D1; −183 to −159 with bold underline, plus five additional nucleotides extending to −188, are shown in the GH3 D1 sequence) were multimerized in tandem (tetramers with all repeats in the correct orientation) and fused to a −46 CaMV 35S promoter–*GUS* reporter gene. Constructs D1-1 and D1-2 contained five additional nucleotides just upstream of the D1 AuxRE originally defined by Liu et al. (1994). A −46 CaMV 35S promoter (min35S) was also tested. Carrot protoplast transfections and assays for auxin inducibility were as described in Figure 2A. The sequences of the D1 element (bold underline in GH3 D1) and subfragments that were tested for auxin inducibility are shown in the inset. The 5′ extension of D1 is not underlined in GH3 D1. Site-directed mutations are indicated in lowercase letters. The position of the TGTCTC element is shown by a double underline. The 4× indicates that each construct was tested as a tetramer. Protoplast incubations were carried out in the absence (diagonally striped bars) or the presence (black bars) of 50 μM NAA. Standard errors are indicated above each bar graph.

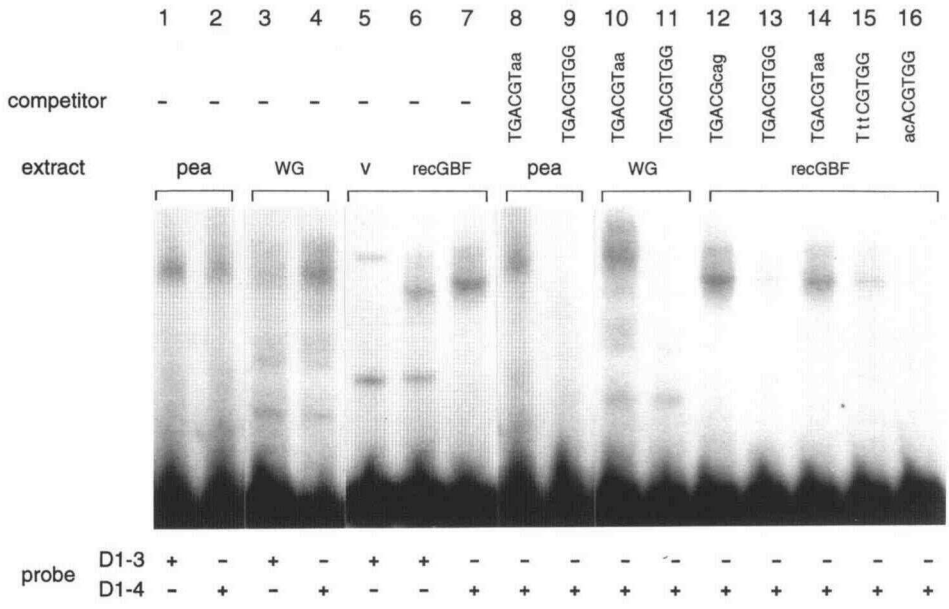

Figure 6. Gel Mobility Shift Assays with the Constitutive Element CCTCGTGT in D1.

Gel mobility shift assays were carried out with ^{32}P-labeled (10^7 cpm/μg; 1 ng per lane) tetramer D1-3 (CCTCGTGTaaa) and D1-4 (CCTCGTGTCTC) probes (see Figure 5). Probes were incubated with nuclear extracts from pea plumules (4 μg per lane), wheat germ (WG) (3 μg per lane), or recombinant soybean G-box binding factor (recGBF). The recombinant GBF used in lanes 6, 7, and 12 to 16 was 1 μg of *E. coli* crude extract expressing SBGBF. A control *E. coli* extract (v) was prepared from cells containing a vector without the SBGBF cDNA. The D1-3 or D1-4 probe used in each lane is indicated by (+) at bottom. Competitor oligonucleotides are shown at top (lanes 8 to 16). Competitors were added as monomers at 200 times molar excess to the probes. The (−) above lanes 1 to 7 indicates that no competitor oligonucleotide was added. Variations in the competitor TGA-box oligonucleotides (see Figure 1 and Liu et al., 1994) are shown in lowercase letters. *E. coli* extracts prepared from cells containing a vector without the SBGBF cDNA (v) produced two complexes with the D1-3 probe (a minor complex that migrated more slowly than the specific complex that formed with recombinant SBGBF and a more abundant complex that migrated more rapidly than the SBGBF complex).

Because only the 3′ half of the direct repeat is present in the original D1 AuxRE (Liu et al., 1994), we tested a D1-1 construct in which each nucleotide in the 5′ half of this direct repeat was mutated to an A residue. A four-copy D1-1 construct showed only minimal activity similar to the D1-2 construct. These results suggested that the sequence just upstream of the TGTCTC, when isolated from the rest of the D1 element, did not confer constitutive expression to the minimal CaMV 35S promoter. On the other hand, an element composed of CCTCG plus the TG or TGT of the adjacent TGTCTC element (D1-3 and D1-5) showed constitutive activity, indicating that the TGTCTC in D1 not only conferred auxin inducibility but made up part of a constitutive element. These results suggested that the AuxRE in D1, like that in D4, consists of a constitutive element and a TGTCTC element; however, in contrast with D4, in which these elements are separated from one another, the elements in D1 overlap.

The Constitutive Element in D1 Binds to Nuclear Proteins with G-Box Specificity

The 11-bp CCTCGTGTCTC AuxRE (D1-4) and the mutated element, CCTCGTGTaaa (D1-3), that showed constitutive activity were tested with plant cell nuclear extracts in gel mobility shift assays. Both elements bound nuclear proteins from soybean plumules, pea plumules, cauliflower curd, and wheat germ. Figure 6 shows results with pea and wheat nuclear extracts. Both D1-3 and D1-4 probes formed complexes in gel mobility shift experiments with pea and wheat extracts (lanes 1 to 4). The binding was competed with D1-3 and D1-4 (data not shown), but also with the competitor TGACGTGG, which is the TGA2 box found in the *GH3* promoter (Hagen et al., 1991; Liu et al., 1994; see Figure 1). On the other hand, competition was not observed with the competitor TGACGTAA, which is the TGA1 box in the *GH3* promoter. These results suggested that the D1-3 and D1-4 probes might bind to basic leucine zipper (bZIP) transcription factors with G-box specificity, even though the probes lacked a perfect ACGT core.

The CCTCGTGT portion of D1-3 and D1-4 in inverse orientation, ACACGAGG, has a seven out of eight nucleotide match with the abscisic acid response element (ABRE), ACACGTGG, found in the wheat *Em* promoter (Marcotte et al., 1989). This ABRE is known to bind G-box factor EmBP-1 (Guiltinan et al., 1990). To test directly whether D1-3 and D1-4 probes could specifically bind to a bZIP transcription factor with G-box specificity, we carried out gel mobility shift experiments with a recombinant G-box binding factor (SBGBF) isolated from a soybean

cDNA expression library (Z.-B. Liu, G. Hagen, and T.J. Guilfoyle, unpublished data). This factor has a proline-rich N-terminal domain and a bZIP domain. SBGBF is 38% identical to Arabidopsis GBF2 (Schindler et al., 1992) and 52% identical to parsley common plant regulatory factor CPRF-1 (Weisshaar et al., 1991). The full-length recombinant SBGBF protein bound to both D1-3 and D1-4 probes (Figure 6, lanes 6 and 7). Binding of the recombinant SBGBF to the D1-4 probe was competed for most strongly by G-box oligonucleotides TGACGTGG and ACACGTGG (lanes 13 and 16), but some competition was also observed with TttCGTGG (lane 15), which lacks a perfect ACGT core. The competitors TGACGTAA (TGA1 in the *GH3* promoter) and TGACGCAG (TGA3 in the *GH3* promoter), which lack the 3′ GG, failed to compete with the D1-4 probe. The oligonucleotide TGTCTC also failed to compete for SBGBF binding to the D1-4 probe (data not shown).

A Minimal D1 Element Is Specifically Induced by Auxins

Although it was demonstrated previously that the *GH3* promoter is specifically induced by auxins and not by other plant hormones (Hagen et al., 1984; Hagen and Guilfoyle, 1985), this promoter consists of multiple AuxREs (Liu et al., 1994), and no single AuxRE has been tested for auxin specificity in isolation from the others. At the same time, the D1-4 construct contains a sequence related to an ABRE and binds a G-box–specific transcription factor. Therefore, we tested the D1-4 element for auxin specificity. Induction of a four-copy D1-4 construct was tested in carrot protoplasts with auxin, salicylic acid, abscisic acid, cytokinin, and methyl jasmonate (Figure 7). Only auxin induced this minimal AuxRE. Thus, the 11-bp AuxRE CCTCGTGTCTC (D1-4 construct; Figure 5) represents the smallest independent AuxRE identified to date that shows strict auxin specificity. The auxin specificity observed with the D1-4 AuxRE differs strikingly from the 20-bp *ocs*-type AuxRE, which is inducible by salicylic acid and methyl jasmonate as well as auxin (see Introduction).

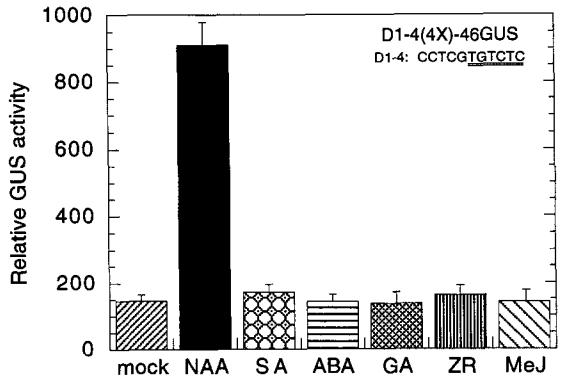

Figure 7. Auxin Specificity of the Composite AuxRE in D1.

Quantitative fluorometric GUS assays in transfected carrot protoplasts were performed as described in Figure 2A in the absence (mock) or presence of different plant hormones. A minimal D1-4 AuxRE (inset) was used as a tetramer (4×) fused to the −46 CaMV 35S promoter–*GUS* reporter gene. The position of the TGTCTC element is shown by a double underline. All hormones, with the exception of methyl jasmonate, were incubated for 24 hr in transfected carrot protoplasts at a concentration of 50 μM. Methyl jasmonate was tested at 10 μM. Standard errors are indicated above each bar graph. SA, salicylic acid; ABA, abscisic acid; GA, gibberellic acid; ZR, zeatin riboside; MeJ, methyl jasmonate.

A Tetramer of the 11-bp D1-4 Element Functions as an AuxRE in Transgenic Tobacco Plants

It is clear from the above-mentioned results that the D1-4 element is a functional AuxRE in transient assays with carrot protoplasts. To demonstrate that the D1-4 element functioned as an AuxRE in stably transformed plants, we tested a four-copy D1-4 element fused to the −46 CaMV 35S–*GUS* reporter gene for auxin inducibility in transgenic tobacco plants. Five independent D1-4 transformants showed auxin inducibility and identical tissue-specific and organ-specific patterns of expression after incubation in 50 μM NAA for 24 hr. GUS histochemical staining for one of these transformants is shown in Figure 8. In the absence of auxin treatment, staining was limited to the primary root tip, lateral root tips and meristems, and the root–shoot transition zone. This staining pattern was similar to that observed with a 592-bp *GH3* promoter–*GUS* reporter gene (Hagen et al., 1991; Liu et al., 1994); however, *GUS* expression in untreated root tips was greater than that observed with a 592-bp *GH3* promoter and promoter deletions. Auxin treatment resulted in increased GUS staining in the hypocotyl and roots (within and above the root tip) of tobacco seedlings. In contrast with the *GH3* promoter and promoter deletions, the D1-4 construct showed less activity in the cotyledons (Liu et al., 1994). Our results established that an 11-bp composite AuxRE is functional in stably transformed plants as well as transient assays with protoplasts.

TGTCTC Confers Auxin Inducibility to a Heterologous Constitutive Element

Because mutations in the 3′ half of TGTCTC in the D1 and D4 AuxREs resulted in loss of auxin inducibility and an increase in basal or constitutive expression, the TGTCTC element appeared to function by down-regulating or repressing the constitutive element in the absence of auxin and up-regulating the element in the presence of auxin. As an additional test of the inherent auxin inducibility of the TGTCTC element, we tested the effect of the TGTCTC element on a heterologous constitutive element (i.e., the *Saccharomyces cerevisiae* GAL4 DNA binding site; the DNA binding site recognized by the GAL4 transcriptional activator). Transfection experiments were carried out with four different promoter–*GUS* reporter genes. One

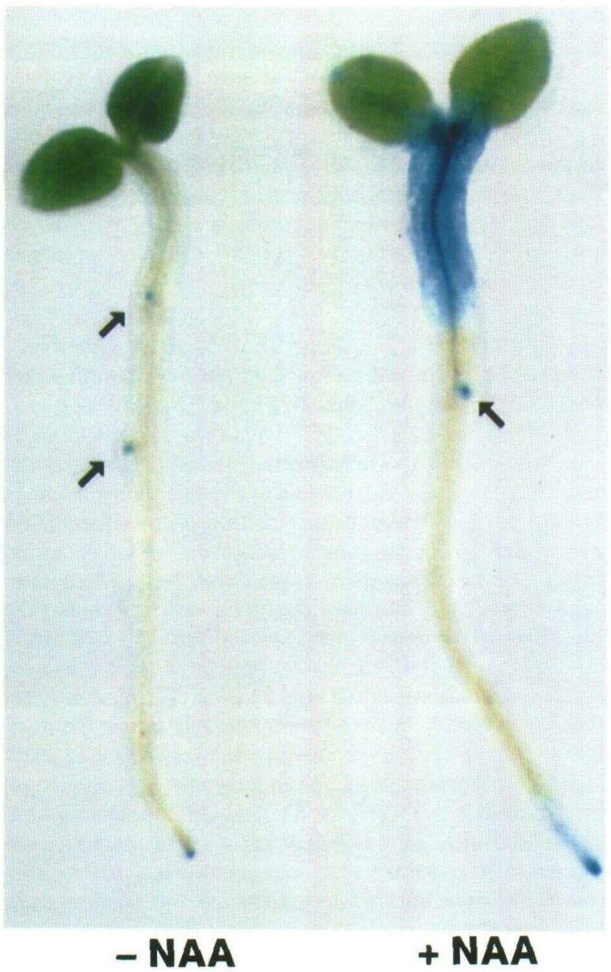

Figure 8. GUS Histochemical Staining in R_1 Transgenic Tobacco Seedlings Transformed with a D1-4 Tetramer Fused to the −46 CaMV 35S Promoter–*GUS* Reporter Gene.

Seedlings were incubated in the absence (−) or presence (+) of 50 μM NAA for 24 hr prior to histochemical staining for GUS activity. Arrows indicate the positions of developing lateral roots.

of these (minimal [min]35S) was a −46 CaMV 35S promoter containing no GAL4 DNA binding sites; a second (G4M) contained GAL4 DNA binding sites (Vashee et al., 1993) upstream of a −46 CaMV 35S promoter, a third (G4T) contained GAL4 DNA binding sites fused to TGTCTC elements upstream of the minimal 35S promoter, and a fourth (mG4T) contained mutated GAL4 DNA binding sites fused to TGTCTC elements upstream of the minimal 35S promoter. Each of these reporter genes was tested in the presence and absence of an effector gene, the double enhancer CaMV 35S promoter driving the expression of a transactivator. The transactivator (GAL4-c-Rel) is composed of the DNA binding domain (amino acids 1 to 147 of the yeast GAL4 protein) fused to the acidic activation domain of the chicken c-Rel protein (amino acids 265 to 588 of c-Rel; Morin and Gilmore, 1992).

Figure 9 shows that in the absence of the GAL4-c-Rel transactivator, all *GUS* reporter constructs had low activity in the presence or absence of auxin. In the presence of the GAL4-c-Rel transactivator, GAL4 DNA binding sites (G4M construct) conferred constitutive activity that was severalfold higher than that of the minimal CaMV 35S promoter–*GUS* reporter gene (min35S). In the presence of the GAL4-c-Rel transactivator, the activity of the G4T construct was lower than that of the G4M construct in cells not treated with auxin. This indicated that the TGTCTC element repressed the constitutive activity of GAL4 DNA binding sites in the absence of hormone addition. The addition of auxin relieved this repression and stimulated the activity of the G4T construct, resulting in a fivefold increase in reporter gene activity. The mG4T mutation, which destroyed the GAL4 DNA binding sites (Vashee et al., 1993), resulted in promoter activity approximately equal to that of the G4M or minimal CaMV 35S promoter in the presence and absence of GAL4-c-Rel activator and auxin.

DISCUSSION

Composite AuxREs Containing a Constitutive Element Juxtaposed to a TGTCTC Element

Our results showed that at least two AuxREs in the *GH3* promoter function as composite elements consisting of a constitutive element adjacent to a TGTCTC element. A schematic diagram of these AuxREs is shown in Figure 10. The TGTCTC element functioned by down-regulating or repressing the activity of nearby constitutive elements in cells deprived of exogenous auxin. The addition of auxin eliminated this repression and up-regulated the activity of these AuxREs. The constitutive elements within the AuxREs are adjacent to (D4) or are part of (D1) the conserved element TGTCTC. The constitutive elements were revealed by site-directed mutations within the TGTCTC element. These mutations resulted in loss of auxin inducibility and derepression of the AuxREs in the absence of added auxin. The constitutive elements in D1 and D4 did not appear to share sequence similarity, suggesting that TGTCTC might be able to confer auxin inducibility on a variety of constitutive elements. In fact, a heterologous constitutive element, the GAL4 DNA binding site, when placed upstream of TGTCTC, can be made responsive to auxin in the presence of a GAL4-c-Rel activator. Thus, novel AuxREs can be created by juxtaposing a heterologous constitutive element next to a TGTCTC element (see Figure 10).

The 11-bp D1-4 element, CCTCGTGTCTC, is the smallest AuxRE with strict auxin specificity shown to function in stably

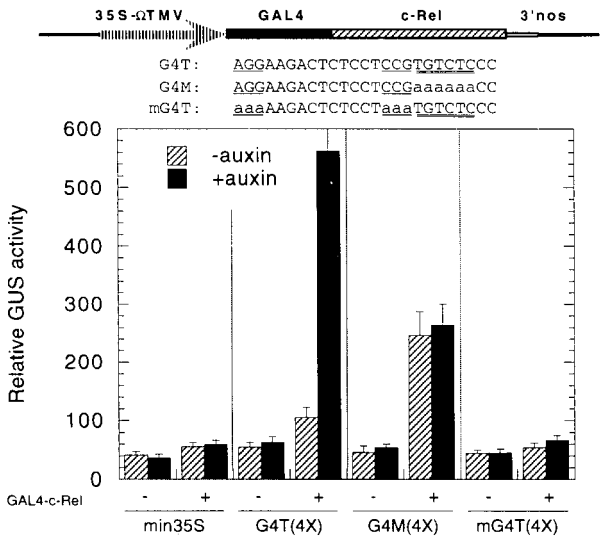

Figure 9. Quantitative Fluorometric GUS Assays for Auxin Inducibility of a Novel Composite AuxRE in the Presence and Absence of a Transactivator.

Quantitative fluorometric GUS assays with transfected carrot protoplasts were performed as described in Figure 2A. Carrot cells were either transfected with a reporter construct alone (−) or cotransfected with a reporter construct and a construct expressing the transactivator GAL4-c-Rel under the control of the CaMV 35S promoter (+). A −46 CaMV 35S promoter (min35S) was also tested. A schematic diagram of the transactivator construct is shown at top. The arrow represents a CaMV 35S promoter (35S) that was fused to a tobacco mosaic virus translational enhancer (ΩTMV); the black, diagonally striped, and open rectangles represent the GAL4 DNA binding domain (GAL4), c-Rel transactivator domain (c-Rel), and nopaline synthase (3'nos) 3' untranslated region, respectively. For details regarding the transactivator construct, see Methods and Results. The sequences of the reporter constructs G4T, G4M, and mG4T are given above the bar graphs. The position of the TGTCTC element is shown by a double underline. Single underlines indicate residues required for GAL4 transcription factor binding in G4T and G4M constructs (Vashee et al., 1993); the light underlining in the mG4T constructs shows mutations in these residues. Mutated nucleotides are given in lowercase letters. Reporter constructs were tested as tetramers (4×) fused to a −46 CaMV 35S promoter–*GUS* reporter gene in the absence or presence of a GAL4-c-Rel transactivator construct. Protoplast incubations were carried out in the absence (diagonally striped bars) or the presence (black bars) of 50 μM NAA. Standard errors are indicated above each bar graph.

transformed plants as well as in transient assays with carrot protoplasts. In transgenic tobacco, the D1-4 AuxRE showed a pattern of tissue-specific and organ-specific expression that was similar but not identical to that of the 592-bp *GH3* promoter and promoter deletions (Hagen et al., 1991; Liu et al., 1994) before and after auxin treatment. Unlike the *GH3* promoter and promoter deletions, the D1-4 element was highly active in root tips and lateral root meristems in the absence of auxin treatment and did not appear to be strongly induced by auxin in cotyledons. These results indicated that the D1-4 AuxRE displayed a slightly different pattern of organ-specific expression than did the *GH3* promoter, which contains multiple AuxREs.

Our results showed that nuclear proteins bound in a sequence-specific manner to the constitutive elements in D1 and D4. One of these binding sites (D1) has sequence similarity with a G-box binding site, and we have shown that a recombinant G-box binding factor from soybean interacts with this element in vitro in a sequence-specific manner. The nature of the transcription factor(s) that bound to the D4 constitutive element is not known, but because the DNA sequences within the constitutive elements of D1 and D4 differ, it is likely that the D4 element binds to a different transcription factor than that bound by the D1 element. Furthermore, although TGACGTGG (TGA2) competed with the D1 probe in gel mobility shift assays with nuclear extracts, it did not compete with the D4 probe. It is possible, however, that the constitutive element in D4, like that in D1, may bind to a bZIP protein, because the sequence CACG (inverse orientation in D1; see Results) is identical in both of these elements. Whether bZIP proteins interact with the D1 and D4 elements in vivo remains to be determined, and transcription factors unrelated to bZIP proteins possibly interact with these AuxREs.

With the D1 and D4 elements, we have not been able to demonstrate binding to TGTCTC that is sequence specific. Failure to observe specific binding may have resulted from weak protein interactions with this site and/or masking of specific binding by an abundant high mobility group (HMG)–like protein (i.e., identical to that reported in Yamaguchi-Shinozaki and Shinozaki, 1992). This HMG-like protein was repeatedly cloned by screening a cDNA expression library prepared from

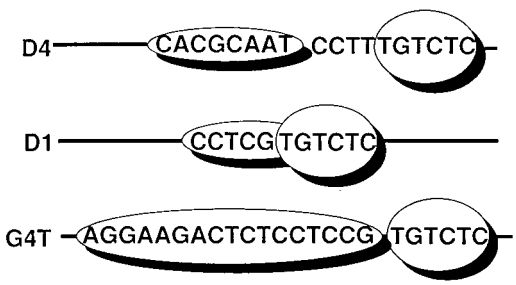

Figure 10. Diagrams of Composite AuxREs.

Diagrams are shown for the D4 element in which the constitutive element is separated from the TGTCTC element, which confers auxin inducibility; for the D1 element in which the constitutive element overlaps with the TGTCTC element; and for a G4T element in which a GAL4 DNA binding site is juxtaposed to a TGTCTC element.

Arabidopsis suspension culture cells with a multimerized TGTCTC probe (T. Ulmasov, unpublished results). The HMG-like protein has a molecular mass of 72 kD and differs from smaller HMG proteins that bind to AT-rich sequences. It is possible that HMG-like proteins that bind TG/C sequences may interact with TGTCTC and related elements to modify the architecture of AuxREs and facilitate protein–protein interactions within one AuxRE or between different AuxREs and basal transcription factors.

Repression by the TGTCTC Element

It was proposed previously that auxin activates some genes by blocking the action of a labile repressor (Theologis et al., 1985; Ballas et al., 1993). This model for auxin action is based on the observation that protein synthesis inhibitors increase the abundance of some auxin-responsive mRNAs (e.g., small auxin up RNAs [SAURs] of soybean and indole acetic acid–inducible *PS-IAA4/5* and *PS-IAA6* mRNAs of pea). According to this model, increases in these specific mRNAs in response to protein synthesis inhibitors result from destruction of a short-lived repressor of the auxin-responsive genes (i.e., continued protein synthesis is required to maintain the repressor). Direct evidence supporting this model has not been reported. It is clear, however, that the *SAUR* genes are not derepressed by protein synthesis inhibitors and that inhibition of protein synthesis causes the SAURs to increase in abundance by stabilizing these mRNAs (Franco et al., 1990). Although the TGTCTC element in the *GH3* gene appears to function by repressing the transcription of constitutive elements, the mechanism for this repression clearly differs from the model described above. Repression of *GH3* transcription is not released by the inhibition of protein synthesis (Hagen and Guilfoyle, 1985; Franco et al., 1990), indicating that short-lived repressors are not involved in regulating the expression of this gene. Thus, auxin cannot activate the *GH3* gene by destabilizing a labile repressor protein. On the other hand, it is possible that auxin stimulation may lead to a conformational change in a protein(s) that binds to the AuxREs in the *GH3* promoter, and this could lead to derepression and up-regulation.

Although the TGTCTC element appears to act by repressing or down-regulating adjacent constitutive elements, the factor(s) that interacts with the TGTCTC element may be more than a simple repressor element. We have observed very strong auxin inducibility (greater than 30-fold induction) with a multicopy construct containing TGTCTC spaced at defined intervals with appropriate surrounding nucleotides (T. Ulmasov, unpublished results). This suggests that the TGTCTC element is auxin inducible in itself and that cooperative interactions among TGTCTC binding proteins may occur when the TGTCTC element is multimerized and spaced appropriately in the context of surrounding nucleotides. The placement of a constitutive element adjacent to or within the TGTCTC element in natural auxin-inducible promoters (i.e., *GH3*) may facilitate the binding of a protein to the TGTCTC element via protein–protein interactions.

Because multimers of TGTCTC with appropriate spacing do not appear to require an adjacent constitutive element for auxin-inducible expression (T. Ulmasov, unpublished results), we favor a model in which the TGTCTC element functions by causing passive repression as opposed to active repression (reviewed in Cowell, 1994). In the case of passive repression, placement of a TGTCTC element adjacent to a constitutive element could result in repression or down-regulation by interfering with transcriptional activators that bind to constitutive elements. This interference could result from competition of transcription factors for specific DNA binding sites or from steric hindrance between transcription factors and the basal transcriptional machinery (Johnson, 1995; Roberts and Green, 1995). Auxin could cause derepression and up-regulation by relieving this interference.

Composite AuxREs and Modular Organization of AuxREs

The results with GAL4 DNA binding sites and a GAl4-c-Rel activator suggest that a variety of unrelated constitutive elements might function as parts of AuxREs if they are near or adjacent to a TGTCTC element. We predict that a variety of AuxREs in different auxin-inducible genes will consist of composite elements that include a constitutive element near or adjacent to a TGTCTC element (or an element with sequence similarity with TGTCTC). This composite structure of AuxREs might explain why some auxin-inducible genes show different patterns of tissue-specific and organ-specific gene expression compared with other auxin-inducible genes (e.g., *SAUR* versus *GH3* genes; see Gee et al., 1991). Although the TGTCTC element might function in a wide variety of cell types, the activity of different constitutive elements might depend on the concentrations of specific transcription factors in different cell types at various states of growth and development. Thus, composite AuxREs would be activated by auxin only in those cells in which the constitutive element is active (i.e., bound to a transcriptional activator).

The composite structure of AuxREs along with their modular organization in auxin-inducible promoters might explain how diverse sets of genes are regulated by auxin in a wide variety of growth and developmental processes (e.g., root initiation, tropisms, apical dominance, cell division, elongation, and differentiation). We have shown previously that some auxin-responsive genes contain a number of AuxRE modules. At least three independent AuxREs are found in the *GH3* promoter: two of these, D1 and D4, contain the TGTCTC element; a third element, E1, appears to lack an element related to TGTCTC (Liu et al., 1994). Recently, Ballas et al. (1995) also found that the *PS-IAA4/5* gene contains at least two AuxREs. We propose that a single hormone such as auxin could trigger a diversity of responses through a signaling process that ultimately targets

different types of composite AuxREs (i.e., different types of constitutive elements fused to TGTCTC-type elements) arranged as modules in auxin-responsive genes. The organization of AuxRE modules and the types of composite AuxREs that make up these modules would allow for a wide variety of developmental and spatial patterns of auxin-induced gene expression.

Relationship of the TGTCTC Element to Sequence Elements in Other Auxin-Responsive Genes

We suggested previously that D1 and D4 TGTCTC elements in the *GH3* promoter and these same elements in the *SAUR* promoters may be related to the TGTCCCAT and the TGTCAC elements found in a 164-bp *PS-IAA4/5* AuxRE of pea (Li et al., 1994; Liu et al., 1994). Based on the available evidence, the *PS-IAA4/5* promoter appears to function through a different mechanism than does the GH3 promoter, because deletion and linker scanning analyses have revealed no repressor-like element in the *PS-IAA4/5* promoter (Ballas et al., 1993, 1995). This has led Ballas et al. (1993, 1995) to propose that the labile repressor in their model for auxin-induced gene expression does not bind directly to AuxREs (Ballas et al., 1993, 1995). However, more extensive analysis (e.g., high-resolution site-directed mutagenesis) is required to define more precisely the minimal AuxREs in the *PS-IAA4/5* promoter. It remains possible that the TGTCCCAT and/or TGTCAC elements in the *PS-IAA4/5* gene could function as repressor elements similar to the TGTCTC element identified by us.

Site-directed mutagenesis of the D1 and D4 AuxREs in the *GH3* promoter revealed that the TGTCTC element functioned like a repressor element in cells deprived of auxin. Addition of auxin released this repression and up-regulated the promoter. The TGTCTC element not only represses or down-regulates the activity of nearby constitutive elements in cells not exposed to auxin but confers up-regulation or activation when cells are exposed to auxin. Evidence that the TGTCTC element confers more than repression is indicated by experiments showing that the presence of the TGTCTC element resulted in auxin-induced activation that was greater than the activity observed with the constitutive element alone (e.g., see Figure 3B, D4-6 versus D4-7; Figure 5, D1-4 versus D1-3 or D1-5; and Figure 9, G4T versus G4M). In addition, as mentioned above, the TGTCTC element itself is auxin inducible if multimerized, spaced at defined intervals, and found adjacent to appropriate surrounding nucleotides. Our results are consistent with a transcription factor (i.e., a repressor and/or activator) binding directly to the TGTCTC element in the *GH3* promoter. However, it remains possible that repression and activation are brought about by protein–protein interactions and not directly through the interaction of a repressor or activator with the TGTCTC element. It is not clear from our analysis whether the same or different transcription factors are associated with composite AuxREs before and after auxin addition.

Relationship of Composite AuxREs to Other Plant Hormone Response Elements and Composite Hormone Response Elements in Animals

The composite organization of the D1 and D4 AuxREs in the *GH3* promoter shows similarity with other types of plant hormone response elements and with composite hormone response elements found in some animal hormone–inducible promoters. At least some gibberellin and abscisic acid response elements consist of two elements, a coupling element and an element that confers gibberellin or abscisic acid inducibility (Rogers and Rogers, 1992; Shen and Ho, 1995). Both of these elements are required for hormone activation, and it has been suggested that the coupling factor confers tissue specificity to the hormone response elements (Rogers and Rogers, 1992). On the other hand, the composite structure of AuxREs with two juxtaposed or overlapping elements is similar to composite hormone response elements described in animal hormone–inducible promoters. For example, composite hormone response elements have been described that contain overlapping DNA binding sites for activator protein-1 (AP-1) and glucocorticoid receptor transcription factors (Diamond et al., 1990; reviewed in Yamamoto et al., 1992; Pfahl, 1993). Although AP-1 and the glucocorticoid receptor are transcriptional activators, repression rather than activation can be observed when their DNA binding elements overlap. In these composite elements, transcription factors appear to "cross-modulate" one another (reviewed in Herrlich and Ponta, 1994). This cross-modulation occurs by two heterodimeric or homodimeric transcription factors interacting not only with their sequence-specific DNA binding sites but also with one another via protein–protein interactions. Conformational changes caused by these protein–protein interactions can lead to inactivation of transactivation domains.

METHODS

Protoplast Transfection and GUS Assays

Carrot protoplasts were isolated and used for transfection assays as previously described (Liu et al., 1994). At least three replicate transfections were conducted with each construct tested in the presence and absence of auxin with a given protoplast preparation, and each construct was tested in at least three independent protoplast preparations. β-Glucuronidase (GUS) activities were standardized by cotransfections with a cauliflower mosaic virus (CaMV) 35S promoter–luciferase reporter gene as described by Liu et al. (1994).

Linker Scanning

Linker scanning analysis was performed using standard methods (Ausubel et al., 1995) with two base pair substitutions from −142 to −123 in the context of a 71-bp (−181 to −110) auxin-inducible *GH3* promoter fragment (Liu et al., 1994; see Figure 1). Each linker scanning

construct was sequenced using Sequenase (U.S. Biochemical) to confirm the sequences of the mutant constructs.

Construction of Chimeric −46 CaMV 35S Promoter-*GUS* Reporter Genes

Reporter plasmids for gain-of-function experiments were made by cloning one or several copies of double-stranded oligonucleotides upstream of the −46 CaMV 35S promoter (min35S; −46 truncated version of the CaMV 35S promoter) fused to the tobacco mosaic virus translational enhancer and *GUS* reporter gene (Liu et al., 1994). The expression plasmid was made by cloning the chimeric activator gene (GAL4-c-Rel) under the control of a 35S promoter with a duplicated enhancer (Skuzeski et al., 1990) and a tobacco mosaic virus translational enhancer. The GAL4-c-Rel fusion has been described (Morin and Gilmore, 1992), and the construct was provided by M. Hannink (University of Missouri, Columbia). The GAl4-c-Rel construct encodes amino acids 1 to 147 of the yeast GAL4 DNA binding domain and amino acids 265 to 588 of the chicken c-Rel activation domain. The GAL4 DNA binding site chosen for analysis in the *GUS* reporter construct was AGGAAGACTCTCCTCCG (Vashee et al., 1993), because a higher affinity binding site CGGAAGACTCTCCTCCG showed some endogenous activity in carrot cells without addition of the activator GAL4-c-Rel. The 35S–luciferase construct, pW216 (Barnes, 1990), used as a standard in each transfection assay, was provided by Dr. John Rogers (University of Missouri, Columbia).

Preparation of Nuclear Extracts and Gel Mobility Shift Analysis

The isolation of nuclei from soybean plumules, pea plumules, cauliflower curd, and wheat germ, the preparation of nuclear extracts, and the use of gel mobility shift analysis have been described previously (Liu et al., 1994). DNA fragments containing four copies of D4-4 (CACGCAAT), D1-3 (CCTCGTGTaaa; mutated residues are indicated by lowercase letters), or D1-4 (CCTCGTGTCTC) were isolated from constructs used for gain-of-function experiments. The constructs were end labeled with phosphorous-32 and gel purified. Two micrograms of poly(dI-dC) (Pharmacia Biotechnology) was used as bulk competitor DNA in all gel shift experiments. Specific and nonspecific competitor oligonucleotides were added at 200 times molar excess to the probe. The following double-stranded oligonucleotides were used for competition experiments: TGT2, CTCAATAAGTGTCTCAATAAGTGTCTCAAT; CAC1, GCAATCCTTTCACGCAATCCTTT; TGA1, CGCAACCATGACGTAATTCTG; TGA2, CACTTTTGTGACGTGGCGAC; TGA3, CGCCCTCTATTGACGCAGTTATAC; TGA2M1, CACTTTTGTttCGTGGCGAC; and TGA2M2, CACTTTTGacACGTGGCGAC (competitor elements as shown in Figures 3 and 5 are underlined).

D1-4 Expression in Transgenic Tobacco Plants

A tetramer D1-4 element was fused to a −46 CaMV 35S promoter–*GUS* reporter gene with a nopaline synthase 3′ untranslated region and used for transformation with *Agrobacterium tumefaciens* pMON505, as described by Liu et al. (1994). Tobacco leaf discs were transformed as previously described (Liu et al., 1994). R_1 seedlings were treated with 50 μM α-naphthalene acetic acid for 24 hr and stained for GUS activity as described by Hagen et al. (1991) and Liu et al. (1994).

Soybean GBF Recombinant Protein

A G-box binding factor (SBGBF) cDNA was isolated from a soybean cDNA expression library (Uni-ZAP XR vector; Stratagene) using a multimer of TGA2 as probe (see Figure 1; Z.-B. Liu, G. Hagen, and T.J. Guilfoyle, unpublished data). Crude *Escherichia coli* extracts were prepared using standard methods (Ausubel et al., 1995).

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NOTE ADDED IN PROOF

The sequence of a soybean G-box binding factor (SBGBF-2) was published (Hong et al., 1995) while this manuscript was in press. The derived amino acid sequence is identical to the sequence of the recombinant SBGBF used in experiments reported here.