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## Two auxin-responsive domains interact positively to induce expression of the early indoleacetic acid-inducible gene *PS-IAA4/5*

(auxin action/linker-scanning mutagenesis/enhancer/titration of transcription factors/cooperativity)

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**ABSTRACT** The plant growth hormone indole-3-acetic acid (IAA) transcriptionally activates expression of several genes in plants. We have previously identified a 164-bp promoter region (–318 to –154) in the *PS-IAA4/5* gene that confers IAA inducibility. Linker-scanning mutagenesis across the region has identified two positive domains: domain A (48 bp; –203 to –156) and domain B (44 bp; –299 to –256), responsible for transcriptional activation of *PS-IAA4/5* by IAA. Domain A contains the highly conserved sequence 5'-TGTCCCAT-3' found among various IAA-inducible genes and behaves as the major auxin-responsive element. Domain B functions as an enhancer element which may also contain a less efficient auxin-responsive element. The two domains act cooperatively to stimulate transcription; however, tetramerization of domain A or B compensates for the loss of A or B function. The two domains can also mediate IAA-induced transcription from the heterologous cauliflower mosaic virus 35S promoter (–73 to +1). *In vivo* competition experiments with icosamers of domain A or B show that the domains interact specifically and with different affinities to low abundance, positive transcription factor(s). A model for transcriptional activation of *PS-IAA4/5* by IAA is discussed.

The plant growth hormone auxin, typified by indole-3-acetic acid (IAA), mediates various aspects of plant development by regulating fundamental processes of plant cell growth and function (1, 2). Growth by cell elongation in response to auxin is one of the fastest hormonal responses, occurring with a lag period of 15–25 min (3, 4). Auxin specifically induces gene expression earlier than or concomitantly with the initiation of cell growth, suggesting that activation of gene expression may be responsible for initiating cell elongation.

Two auxin-inducible genes, *PS-IAA4/5* and *PS-IAA6*, have been isolated from pea and characterized as early genes (5, 6). Their encoded proteins, which are very similar to proteins encoded by auxin-inducible genes from various plants (6–10), are short lived, nuclear localized, and contain a putative  $\beta\alpha$  motif reminiscent of the  $\beta$ -sheet, DNA-binding domain of prokaryotic repressor polypeptides (10).

Although several other early auxin-inducible genes have been isolated and characterized (5–12), the mechanism responsible for their transcriptional activation by the hormone is unknown. We have previously defined a 164-bp auxin-responsive region (AuxRR) in the promoter of the early *PS-IAA4/5* gene (13). Herein, we further analyze this regulatory sequence, subdivide it into its component sequence elements (domains), and find that these elements interact with specific positive transcription factors which are present in limiting amounts in elongating pea cells.

## MATERIALS AND METHODS

**Plasmid Construction.** All plasmids were constructed in the pBluescript II KS(+) vector by using standard recombinant DNA techniques (14), and their authenticity was confirmed by DNA sequencing (15). The construction of the chimeric chloramphenicol acetyltransferase (CAT)–nopaline synthase (NOS) genes driven by the *PS-IAA4/5* (–92 to +96) or the cauliflower mosaic virus (CaMV) 35S (–73 to +1) core promoters, the chimeric CAT–NOS gene driven by CaMV 35S promoter (35S-CAT; –430), and the promoterless CAT–NOS gene (pCAT) have been described (13). The wild-type *PS-IAA4/5* promoter (–318 to +96) was synthesized by PCR as a *Cl*I–*N*co I restriction fragment and fused to the CAT gene, giving rise to the *PS-IAA4/5*-CAT plasmid.

Domain A (–203 to –156), domain B (–299 to –256), and domain C (–255 to –202) were inserted upstream of the *PS-IAA4/5* (–92 to +96) or the CaMV 35S (–73 to +1) core promoter sequence as double stranded synthetic oligonucleotides; Am is a mutant of domain A that corresponds to the linker scanning (LS) mutation LS-181 (see Fig. 1), where 6 bp of the conserved 5'-TGTCCCATgtt-3' sequence (6) have been mutated to 5'-TGAagctagcaa-3'. Domain A<sub>Aux2-11</sub> (48 bp; –219 to –172) from the *Arabidopsis AtAux2-11* gene contains the conserved sequence TGTCCAC (5'-16 bp-TGTCCAC-24 bp-3') (8). The CaMV 35S enhancer (–345 to –90; ref. 16) was synthesized by PCR. The *Sal*I restriction enzyme fragment of rice *OS-ACSI* cDNA (+1143 to +1786; ref. 17) was inserted between domain A and domain B (construct 14; see Fig. 2). The plasmids containing tetramers or icosamers (in tandem) of domain A, Am, A<sub>Aux2-11</sub>, B, or C were constructed in a head-to-tail orientation by the method of Xiao and Lis (18).

**Construction of Linker-Scanning Mutations.** Twenty LS mutations were constructed across the *PS-IAA4/5* promoter region from –318 to –144 by using the method described by Kunkel (19). In each mutation, 10 bp of the promoter sequence was substituted with the sequence 5'-AAGCTAGCAA-3' which contains an *Nhe*I site. Mutations were generated with oligonucleotides (40-mer) containing the *Nhe*I sequence flanked by 15 bp of the wild-type sequence on each side (20).

**Protoplast Transfection and CAT Assay.** Isolation of pea protoplasts from the third internodes of the 7-day-old etiolated pea seedlings and transfection of plasmid DNA using polyethylene glycol have been described (13). For the *in vivo* competition experiments, 40  $\mu$ g of the reporter plasmid (*PS-IAA4/5*-CAT or 35S-CAT) was cotransfected with increasing amounts of competitor plasmid DNA. The total amount of transfected DNA was adjusted to 70  $\mu$ g by using the promoterless pCAT plasmid (13) as carrier. The protoplasts were

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Abbreviations: IAA, indole-3-acetic acid; AuxRR, AuxRD, AuxRE, auxin-responsive region, domain, element, respectively; LS, linker scanning; CAT, chloramphenicol acetyltransferase; CaMV, cauliflower mosaic virus; NOS, nopaline synthase.

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incubated for 18 h with or without 20  $\mu$ M IAA and assayed for CAT activity (13).

## RESULTS AND DISCUSSION

**LS Mutagenesis.** To identify precisely the essential regulatory elements within the AuxRR (13) for transcriptional regulation of *PS-LAA4/5* by IAA, we analyzed the requirement for every consecutive 10 bp across the AuxRR by using LS mutagenesis (21). Fig. 1 shows the position of the LS mutants and their effect on the expression of the CAT gene in pea protoplasts in the presence or absence of IAA.

The analysis defined two domains required for IAA-induced gene expression: a 48-bp domain between -203 and -156 has been designated domain A [auxin-responsive domain A (AuxRD A)]; a 44-bp domain between -299 and -256, domain B (AuxRD B). The 54 bp between these two domains (from -255 to -202), domain C, can be mutated without any pronounced effect on promoter function (Fig. 1). The domain A mutation LS-181, which has the most severe effect, overlaps with most of the conserved 5'-TGTC<sup>3</sup>CCAT-3' motif found in many promoters of auxin-inducible genes (Fig. 1 and refs. 6 and 13), suggesting that the 5'-TGTC<sup>3</sup>CCAT-3' may be the core sequence motif, the auxin-responsive element (AuxRE), in domain A. However, this motif alone is not sufficient to confer auxin inducibility, since mutations in its flanking sequences also result in low levels of CAT expression in the presence of IAA (LS-159 and LS-201; Fig. 1). It is possible that the flanking sequences function as binding sites for auxiliary factors that stabilize the binding of nuclear factor(s) to the core motif (22). Similar observations have been reported for the

cAMP-responsive element (CRE), where the conserved core motif 5'-TGACGTCA-3' is necessary but not sufficient for cAMP inducibility (23). Mutations in domain B also result in a significant decrease in CAT gene expression (Fig. 1). Altering the 10-bp conserved palindromic motif (5'-ACATGCT-CATGT-3') found in several auxin-responsive promoters (6, 13) does not severely affect CAT gene expression (Fig. 1). Therefore, the analysis did not identify a potential core element in domain B. Interestingly, none of the LS mutations across the 144 bp (-299 to -156) derepress CAT gene expression in the absence of IAA (Fig. 1), suggesting that the putative labile repressor postulated to be responsible for the regulation of *PS-LAA4/5* gene expression by IAA does not bind directly to the AuxRR (5, 13, 24).

**Functionality of Domains A and B.** To assess the role of the individual domains in auxin inducibility, domain A and/or domain B were fused to the -92 to +96 *PS-LAA4/5* core promoter. Fig. 2 (lines 1-5) shows the activity of each domain relative to the wild-type *PS-LAA4/5* promoter (line 1). Domain A has a relatively low activity (30%) compared with that of the full-length promoter (Fig. 2, compare line 3 with line 1). The activity of domain B is negligible (Fig. 2, compare line 4 with lines 1 and 3). These results are consistent with the data obtained with the LS mutation analysis, suggesting that domain A contains the major AuxRE(s). The results may also explain the high residual activity observed with the LS mutants in domain B and the relatively low activity with the LS mutants in domain A (see Fig. 1). However, the addition of domain B upstream of domain A enhances the level of induction by the A domain (Fig. 2, compare line 5 with line 3). The activity of the A and B domains does not depend on the *PS-LAA4/5* core

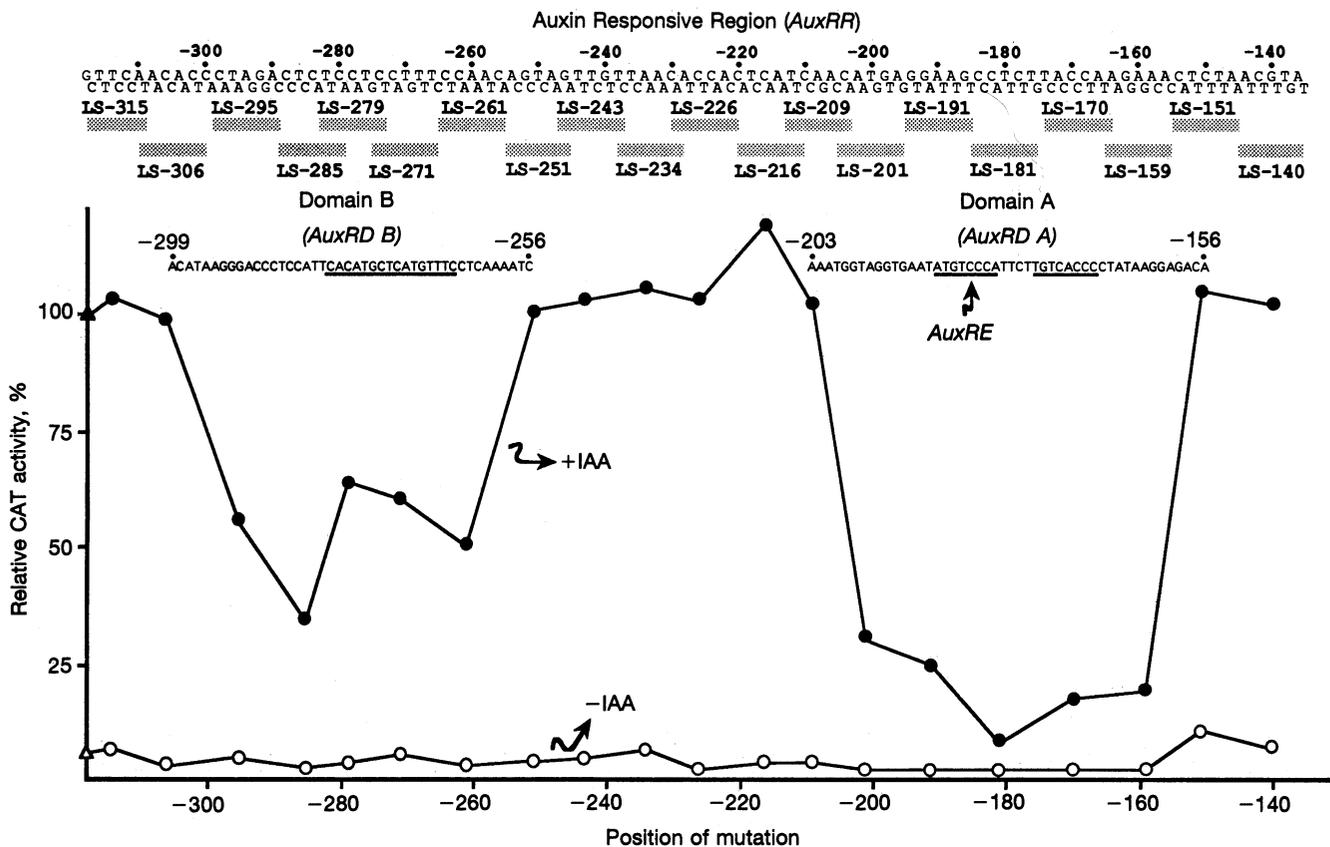


Fig. 1. LS mutagenesis of the AuxRR in the *PS-LAA4/5*-CAT gene (-318 to +96). The *PS-LAA4/5* promoter sequence from -318 to -135 (13) is shown at the top. The positions of the LS mutants are shown in shaded boxes. Each LS mutant is designated by the position of the 5th bp of the mutated sequence from the 5' end point (the T nucleotide in the *Nhe* I site). ●, +IAA; ○, -IAA. CAT activity is expressed as a percentage of the wild-type *PS-LAA4/5*-CAT promoter activity. The underlined sequences in AuxRD A and AuxRD B are conserved motifs found in auxin-regulated genes (6). The nucleotide sequence of the AuxRR is presented in two lines because of space limitation. It is read by following the sequence in the two lines (i.e., GCTTTC...).

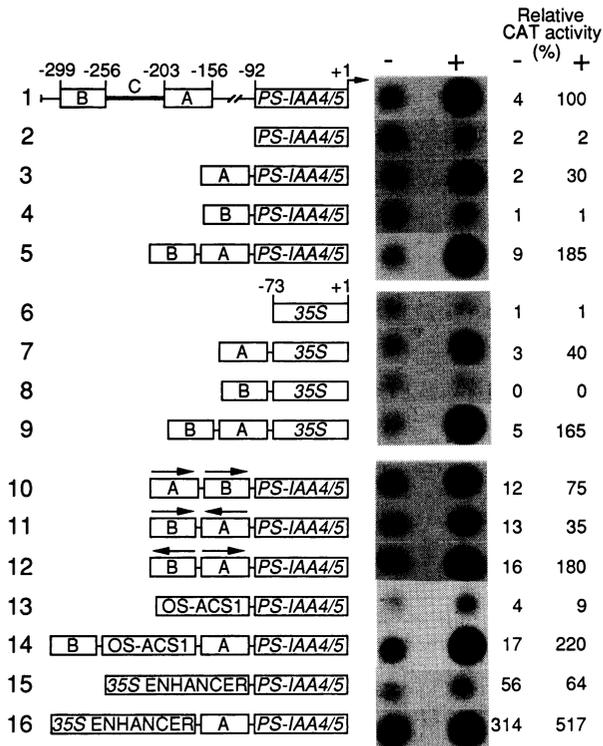


FIG. 2. Activity of the A and B domains. Line 1, the wild-type *PS-IAA4/5* promoter. Lines 2–9, activity of domain A and/or domain B with the *PS-IAA4/5* or CaMV 35S core promoters. Lines 10–14, effect of position, orientation, or distance on the activity of domains A and B. Lines 15 and 16, effect of the CaMV 35S enhancer on the activity of the *PS-IAA4/5* promoter core with or without domain A. The arrowheads show the orientation of the domains. CAT activity without (–) or with (+) IAA is expressed as a percentage of the wild-type promoter (line 1).

promoter, because similar results were obtained using the heterologous CaMV 35S core promoter (Fig. 2, compare lines 2–5 with lines 6–9).

The activity of domain A is orientation and position dependent (Fig. 2, compare lines 10–12 with line 5), whereas the activity of domain B is orientation and position independent (Fig. 2, compare line 5 with lines 10–12 and 14). The orientation-independent activity of domain B is a characteristic feature of enhancers (25). Furthermore, domain B has another feature of enhancers; it can function over a long distance (Fig. 2, compare line 14 with line 5). Insertion of a heterologous sequence between domain A and domain B does not affect the activity of domain B (Fig. 2, compare line 14 with line 5). Replacing domain B with the CaMV 35S enhancer results in increased levels of both the basal and the induced CAT gene expression (Fig. 2, line 16), suggesting that the function of domain B is distinct.

**Multimerization of Domains A and B.** To study further the functional capacity of each domain, we determined the effect of tetramerization on their activity (see Fig. 3). Tetramerization of domain A confers a higher level of IAA-inducible CAT gene activity than domain A alone or the domain B plus domain A arrangement (Fig. 3, compare line 4 with lines 3 and 2). Even higher activity is observed when the tetramer is linked to the heterologous 35S core promoter (Fig. 3, compare line 5 with lines 2–4). Surprisingly, similar results were obtained by tetramerizing domain B (Fig. 3, compare lines 8 and 7 with line 6), but the activities were lower than those observed with the tetramer of domain A (Fig. 3, compare lines 7 and 8 with lines 4 and 5). The nonfunctional domain C remains inactive even after tetramerization (Fig. 3, compare line 10 with lines 4 and 7).

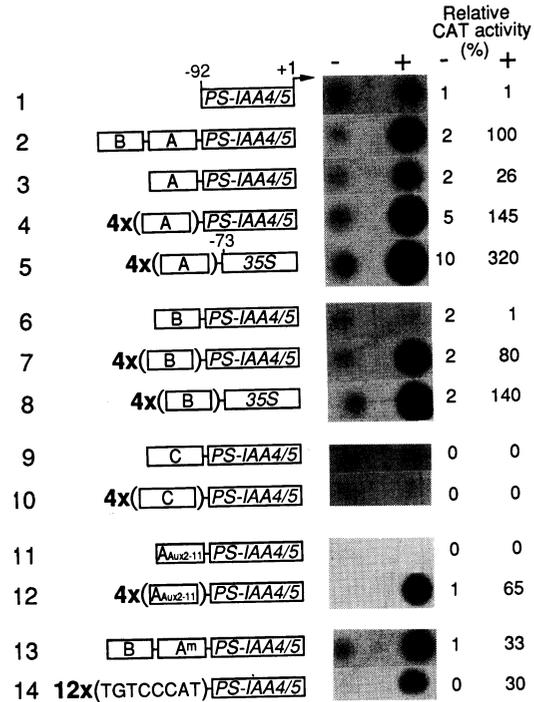


FIG. 3. Effect of multimerization of the A and B domains. Lines 1–12 are constructs similar to those shown in Fig. 1, except that in some cases domain A, B, or C is present in four tandem copies.  $A_{Aux2-11}$  is the A domain from the auxin-responsive gene *AtAux2-11* of *Arabidopsis*. Line 13, the construct B/Am/*PS-IAA4/5* contains a mutated A domain in which the 8-bp conserved sequence 5'-TGTCCTCAT-3' was mutated to the sequence 5'-AGCTAGCA-3' (*A-Nhe* I-A). Line 14 is a dodecamer of the conserved sequence 5'-TGTCCTCAT-3'. CAT activity without (–) or with (+) IAA is expressed as a percentage of the B/A/*PS-IAA4/5* activity (line 2).

Multimerization of inducible enhancer motifs can stimulate expression by enhancing the cooperative binding of transcription factors that interact with these motifs (26, 27). The unmasking of auxin-inducible activity by multimerizing domain B (Fig. 3, compare line 7 with line 6) may be due to the following: (i) the presence of a weaker AuxRE in domain B, or (ii) the interaction of domain-A-specific factor with domain-B-specific factor in the absence of domain A (protein–protein interaction).

To test whether a domain A (5'-TGTCCTCAT-3' and its flanking sequence) from other *PS-IAA4/5*-like genes, such as the *Arabidopsis*  $A_{Aux2-11}$  domain, functions as does AuxRD, we determined the activity of the  $A_{Aux2-11}$  monomer and tetramer in the pea transient assay system (Fig. 3). Tetramerization of domain  $A_{Aux2-11}$  is required for IAA-inducible CAT activity (Fig. 3, compare lines 11 and 12 with line 3). This result may be due to the lower affinity of the pea factor A for the  $A_{Aux2-11}$  domain. The fact that domain A from both pea and *Arabidopsis* is auxin responsive indicates that the conserved sequence 5'-TGTCCTCAT-3' is indeed the core motif (AuxRE) in domain A. A mutation in the conserved 5'-TGTCCTCAT-3' sequence of the pea domain A (Am) results in a 67% inhibition in its wild-type activity (Fig. 3, compare line 13 with line 2). Dodecamerization of this conserved sequence results in significant IAA-inducible CAT activity (Fig. 3, line 14), although not as high as the domain A tetramer (Fig. 3, compare line 14 with line 4). These results indicate that additional sequences in domain A are necessary for full auxin-regulated activity.

**In Vivo Titration Experiments.** To demonstrate the functional interaction of specific transcriptional factors with domains A and B, we have used an *in vivo* competition assay (28). Fig. 4 shows that increasing amounts of competitor domain A (Fig. 4A) or domain B (Fig. 4B) dramatically reduces the

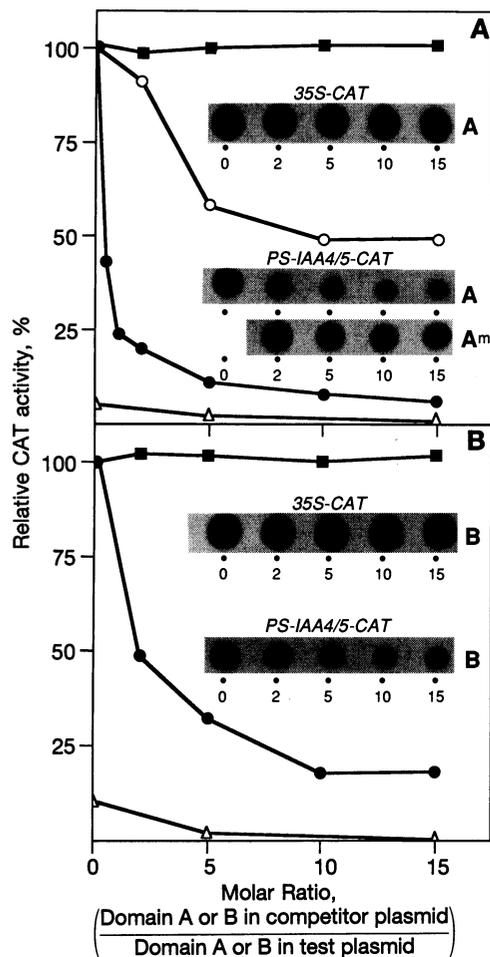


FIG. 4. Inhibition of PS-IAA4/5-CAT gene expression by *in vivo* competition with multimers (20-mer) of domain A (A) or domain B (B). The reporter plasmid PS-IAA4/5-CAT (-318 to +96) or 35S-CAT (-430 to +1) was cotransfected with increasing amounts of competitor plasmid containing 20 tandem repeats of domain A or domain B. After transfection, the protoplasts were incubated with (■, ●, ○) or without (△) 20  $\mu$ M IAA. CAT activity is expressed as percent of the PS-IAA4/5-CAT (●) or 35S-CAT (■) activities without competitor. ○, Activity of PS-IAA4/5-CAT reporter in the presence of increasing concentration of competitor mutant domain Am (A). The Am mutation is the same as in the LS-181 mutant shown in Fig. 1.

PS-IAA4/5-CAT gene expression in the presence of IAA. A partially defective A domain (Am), mutated in the conserved 5'-TGTCCT-3' (LS-181) sequence that has been proposed to be the core motif in the AuxRD A (13), is only partially active in the competition assay (Fig. 4A). The partial inhibition (50%) of CAT activity by domain Am (Fig. 4A) suggests that flanking sequences other than the conserved motif are necessary for proper interaction of the putative factor(s) A with domain A. This result is consistent with the LS analysis where LS mutations outside the core motif in domain A strongly inhibit its activity (Figs. 1 and 3, lines 2 and 13). The observed inhibition of CAT activity is unlikely to result from competition for general transcription factors, because it is not evident when the 35S-CAT is used as a reporter plasmid (Fig. 4A and B). The competition data also show that domain A is a better competitor than domain B. The nonfunctional domain C (Fig. 3) is inactive in the competition assay (Table 1). We hypothesize that the differential competition efficiency reflects differential affinity of the factor(s) to the two sites (A > B; ref. 28). Since the competitors do not derepress the PS-IAA4/5-CAT gene in the absence of IAA (Fig. 4A and B and Table 1), the promoter is not negatively regulated by a repressor that

Table 1. Inhibition of tetramer A or tetramer B activity by icosamers of domains A and B

Reporter	Competitor	Relative CAT activity, %	
		-IAA	+IAA
1. PS-IAA4/5-CAT	—	0	100
	20×A	1	12
	20×B	0	27
	20×C	1	93
2. 4×A/PS-IAA4/5 CORE-CAT	20×Am	ND	55
	—	2	100
	20×A	ND	23
	20×B	ND	100
3. 4×B/PS-IAA4/5 CORE-CAT	—	2	100
	20×A	ND	12
	20×B	ND	16
4. 4×A <sub>Aux2-11</sub> /PS-IAA4/5 CORE-CAT	—	0	100
	20×A	ND	11
	20×Am	ND	80
	20×B	ND	56

The molar ratio of the competitor plasmid to the test plasmid was 15:1 in all transfections. CAT activity is expressed as a percentage of the activity of the reporter plasmid in the absence of the competitor. Reporter plasmid 1 contains the -318 to +96 region of the PS-IAA4/5 gene (Fig. 2, lane 1). Reporter plasmids 2, 3, and 4 are lanes 4, 7, and 12 in Fig. 3. Am is the LS-181 mutant (Fig. 1). A<sub>Aux2-11</sub> is domain A of the *Arabidopsis* gene *Aux2-11* (8). The PS-IAA4/5 CORE corresponds to the core promoter sequence of PS-IAA4/5 (-92 to +96; ref. 6). ND, not determined.

directly binds to the DNA (ref. 24 and Fig. 5), consistent with the results of the LS analysis (Fig. 1).

To determine whether domain-A-specific factor interacts with domain-B-specific factor, we carried out "cross-competition" experiments using the 4×A/PS-IAA4/5 and the 4×B/PS-IAA4/5 constructs as the reporter plasmids and the 20×A and 20×B constructs as the competitor plasmids (Table 1). The 4×A activity is inhibited by the domain-A multimer but is unaffected by the domain-B multimer (Table 1, construct 2). Surprisingly, the 4×B activity is inhibited by both domain-A or -B multimers (Table 1, construct 3). These results are consistent with the idea that both domains are recognized by the same factor(s) but with different affinities (A > B). Such a transcription factor that binds two spatially-distant DNA elements may function as an architectural protein, bringing together two separate regulatory elements in the PS-IAA4/5 promoter (29). Alternatively, different factors could bind to

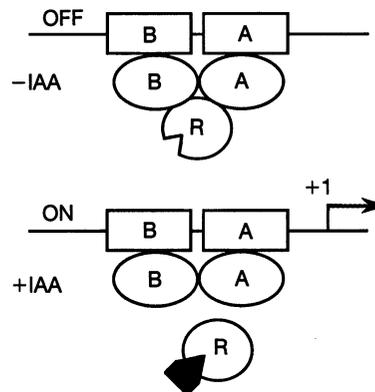


FIG. 5. A model for transcriptional activation of the PS-IAA4/5 gene by IAA. R; Repressor. A and B, transcriptional factors that bind to the A and B domains of the PS-IAA4/5 promoter, respectively; ■, represents (i) the IAA molecule (direct effect) or (ii) a biochemical modification of the repressor—e.g., phosphorylation/dephosphorylation.

domains A and B if factor A interacts with factor B by protein-protein interaction. According to this view, domain A inhibits 4×B activity because factor A, which binds to the competitor A domain, titrates factor B by protein-protein interaction. However, domain B does not inhibit the 4×A activity because factor B has higher affinity for factor A than for domain B.

The protein-DNA and protein-protein interactions proposed above may also be the basis of auxin inducibility of the heterologous A<sub>Aux2-11</sub> domain of *Arabidopsis* in pea protoplasts (Fig. 3, line 12 and Table 1, construct 4). The *Arabidopsis* 4×A<sub>Aux2-11</sub> activity is inhibited by the pea 20×A, suggesting that the pea factor A can recognize both elements. However, the pea domain-B multimer partially inhibits (44%) the *Arabidopsis* 4×A<sub>Aux2-11</sub> activity, in sharp contrast to that discussed above (Table 1, compare construct 2 activity with construct 4 activity, in the presence of 20×B). The ability of the pea domain B to inhibit the *Arabidopsis* 4×A<sub>Aux2-11</sub> and not the pea 4×A activity may indicate that the pea factor A has higher affinity for the pea factor B than for the *Arabidopsis* A<sub>Aux2-11</sub> domain.

**The AuxRR.** The experiments described above reveal the presence of two functional modules (domain A and domain B) in the inducible enhancer defined previously as AuxRR (13). The two modules interact with low-abundance positive transcription factor(s) (A and B, respectively) that enhance transcription by protein-protein interaction in the presence of IAA. Modularity is a hallmark of promoters, as well as constitutive or inducible enhancers (25, 30, 31). The modular organization of enhancers allows transcription to be regulated in a tissue-specific manner and in response to diverse intracellular signals (30). A minimum of two enhancer modules is required for the activity of the simian virus 40 enhancer, the prototype enhancer, and duplication of any one module fully compensates for loss of the other (30). The AuxRR has similar features. It contains two modules and multimerization of either of the modules compensates for the loss of the other. The intact AuxRR is not active in both orientations because module A is orientation dependent (13). Module B, however, shows many features of a typical enhancer: it can act in an orientation-independent manner and over a long distance. The tolerance of domain-B activity to LS mutagenesis (Fig. 1) is also consistent with the features of enhancers, which, due to the presence of several functional subelements, are largely forgiving of sequence alterations (31). The AuxRR can also activate heterologous promoters and functions in a tissue-specific manner (L.-M.W. and A.T.; Y. Ono and A.T.; unpublished data). It has not yet been determined whether module A, module B, or both are responsible for tissue-specific expression.

**A Model for Transcriptional Activation by IAA.** The *PS-LAA4/5* gene is induced by protein synthesis inhibitors due to both transcriptional activation and mRNA stabilization (5, 13). These results have been interpreted to suggest that the *PS-LAA4/5* gene is under the negative control of a short-lived repressor (5). Such eukaryotic repressors have been described (32). Fig. 5 shows a simplified view of how IAA may transcriptionally activate the *PS-LAA4/5* gene on the basis of results obtained thus far. We propose that in the absence of IAA the positive factors A and B interact with the A and B domains of the AuxRR, respectively. However, the factors are inactive because a labile repressor prevents the interaction of positive factors A and B with each other or with other factors in the transcription initiation complex (32). IAA may mediate transcriptional activation by altering the interaction of the repressor with the activators. This may occur (i) directly by

inactivation of the repressor by IAA, or (ii) indirectly by modification of the repressor by phosphorylation/dephosphorylation. The second view has IAA interacting with an auxin-binding protein (33) located in the plasma membrane which activates a signal transduction pathway that results in repressor inactivation. Further elucidation of the molecular details of transcriptional activation by IAA will require purification and characterization of the transcription factors that interact with the modules of the AuxRR.

We dedicate this article to Professor Kenneth V. Thimann, the father of auxin, for his 90th birthday. This work was supported by a grant from the National Institutes of Health (GM-35447) to A.T.

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