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Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the rbcS-3A gene

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Pea nuclear extracts were used in gel retardation assays and DNase I footprinting experiments to identify a protein factor that specifically interacts with regulatory DNA sequences upstream of the pea rbcS-3A-gene. This factor, designated GT-1, binds to two short sequences (boxes II and III) in the −150 region that are known to function as light-responsive elements (LREs) in transgenic tobacco. Binding of GT-1 to homologous sequences further upstream (boxes II* and III* in the −220 region) indicates that these boxes comprise the redundant LRE that functions *in vivo* when boxes II and III are deleted. In both box II and box II*, methylation interference experiments demonstrate that two adjacent G residues are critical for GT-binding. Single Gs present in boxes III and III* are also important. Since GT-1 is present in nuclear extracts from leaves of light-grown and dark-adapted pea plants, its regulatory role does not depend on *de novo* synthesis. Thus if GT-1 binds differentially *in vivo* it must be postranslationally modified or sterically blocked from binding by another factor in response to light.

Key words: rbcS genes/light responsive elements/*trans*-acting factor/GT-motif/DNA—protein interaction

Introduction

Light regulates the expression of many higher plant genes encoding photosynthetic enzymes (Tobin and Silverthorne, 1985). One of the best characterized enzymes is ribulose-1,5-biphosphate carboxylase, which catalyzes the primary step in photosynthetic carbon fixation. The holoenzyme is composed of eight large subunits, (rbcL), encoded in the chloroplast genome (Coen *et al.*, 1977), and eight small subunits, (rbcS), which are encoded in the nuclear genome (Berry-Lowe *et al.*, 1982; Corruzzi *et al.*, 1983; Dunsmuir *et al.*, 1983; Wimpee *et al.*, 1983). Cytoplasmic rbcS precursors are transported into the chloroplast postranslationally (Chua and Schmidt, 1978), where they are assembled into the holoenzyme (see Ellis, 1981 for a review). In the pea, rbcS is encoded by a small multigene family (Coruzzi *et al.*, 1983, 1984; Cashmore, 1983; Bedbrook *et al.*, 1980). The rbcS-3A gene is one of the most highly expressed members of this family, contributing to 40% of the total rbcS mRNA in green leaves. Expression of the rbcS-3A gene is both light inducible and organ specific, with transcripts being most abundant in leaves (Corruzzi *et al.*, 1984; Fluhr *et al.*, 1986a). In etiolated peas, light induction of the rbcS-3A gene is mediated by phytochrome, whereas both phytochrome and a blue light photoreceptor are required for full induction in mature green leaves (Fluhr and Chua, 1986).

To define the *cis*-acting DNA sequences which mediate the light response, chimeric genes have been constructed and analyzed in transgenic petunia and tobacco (Fluhr *et al.*, 1968b). The upstream region of the pea rbcS-A gene from −330 to −50 (relative to the transcription start site) is capable of enhancing the expression of a constitutive heterologous promoter in response to light and tissue type (Fluhr *et al.*, 1986b). Within the upstream enhancer region are sequence elements, between −161 and −112 (designated boxes I, II, and III), which are conserved amongst all known pea rbcS genes (for a review see Kuhlemeier *et al.*, 1987a). Kuhlemeier *et al.* (1987b) have shown that a 58-bp fragment, containing only boxes I, II, III, can act as a negative light responsive element (LRE) in transgenic tobacco. Furthermore, three copies of box II can function as a silencer in the dark, while 5′ deletion analysis suggests a similar role for box III (Kuhlemeier *et al.*, 1987b). A positive element with a 5′ boundary between −166 and −149 overlaps the negative ones, emphasizing the complex nature of the response. Deletion of all three boxes from this region reveals redundant LREs further upstream. Two boxes homologous to boxes II and III, designated boxes II* and III*, respectively, which reside around −220 have been proposed to account for this redundancy.

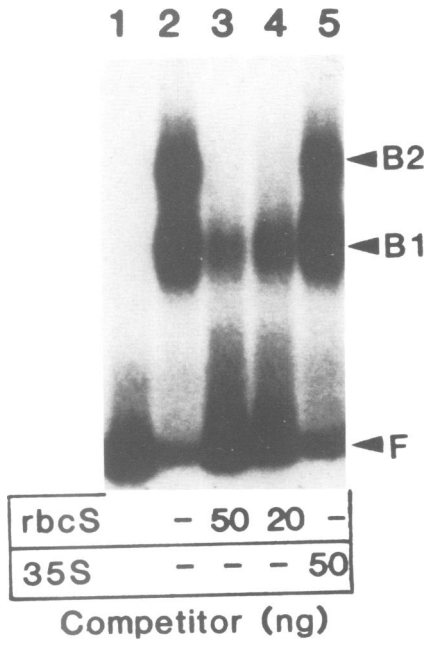

Fig. 1. Assay of rbcS-3A specific binding activity in pea nuclear extracts. The probe (rbcS-3A, −330 to −50) was incubated in the absence (**lane 1**) or the presence (**lanes 2–5**) of nuclear extracts prepared from the leaves of 10 day-old greenhouse grown pea plants. Specific competitor DNAs were included as indicated below the lanes. The unlabelled rbcS competitor was the −330 to −50 upstream fragment and the 35S competitor was an unlabelled fragment from the CaMV 35S promoter extending from −300 to +8 relative to the transcription start (Odell *et al.*, 1985). The position of the two bound species, B1 and B2 and the free probe (F) are shown by arrows. The binding activity is sensitive to heat or protease treatment (data not shown).

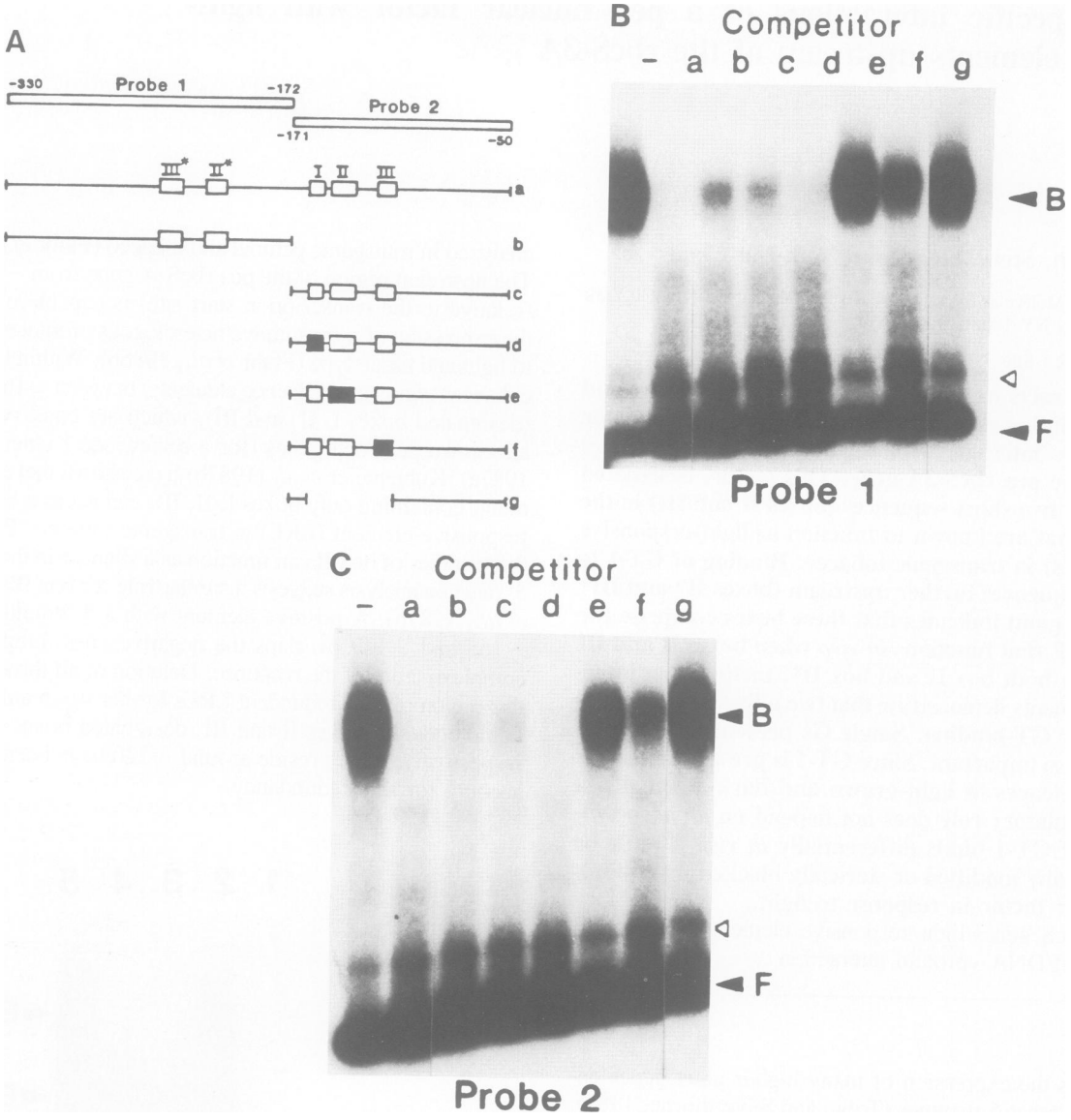

Fig. 2. Gel retardation assays with rbcS-3A probes and mutant competitor DNAs. **A**: The two probes (1 and 2) and the specific competitors (**a**–**g**) used in the gel retardation assays in Figure 2B and C are shown diagrammatically. ■ represent substitution mutations. Probes 1 and 2 were prepared by digesting singly end-labeled rbcS-3A probes at position −170. Probe 1 was labeled at −330 and probe 2 at −50. The rbcS-3A genes generated by site-directed mutagenesis and used to isolate fragments **d**–**g** have been described previously (Kuhlemeier *et al.*, 1987b). Briefly, **d** and **f** contain 8-bp substitution mutations in boxes I and III respectively; **e** has a 12-bp substitution in box II and all three boxes have been deleted from **g**. **B** and **C**: The probes (1 and 2) and specific competitors (**a**–**g**) used in the gel retardation assays are indicated below and above the gels respectively. Final competitor DNA concentration was 25 nM. Closed arrows indicate the bound (B) and free (F) species. Open arrows indicate a small amount of partial digestion product present in the probe preparations.

Well defined sequences which demonstrate *in vivo* regulatory function are likely to act as targets for the binding of protein factors (Sassone-Corsi and Borelli, 1986). For example, *Drosophila* heat shock genes have conserved sequence elements (HSEs) which bind a cognate factor, HSTF (Parker and Topol, 1984a). Similarly, the 'GC' motif of mammalian genes is recognized by the factor Spl (Dynan and Tijan, 1983), while the glucocorticoid receptor binds its target GREs (Chandler *et al.*, 1983). A ubiquitous octamer sequence which is present in immunoglobulin genes as well as others appears to be the target for many *trans* acting factors (Sive and Roeder, 1986; Staudt *et al.*, 1986). To date, only one sequence specific binding protein has been reported in plants (Maier *et al.*, 1987) although its target DNA sequence has not yet been tested for function *in vivo*.

Here, we identify a pea nuclear factor, GT-1 which specifically interacts with the rbcS-3A LREs. The binding interaction is characterized in detail at the nucleotide level, by both DNase I footprinting and methylation interference. From data obtained with site-specific mutants and cloned synthetic oligonucleotide probes, it is clear that GT-1 binds the box II and III LREs as well as boxes II* and III*. Moreover, the same binding activity is present in nuclear extracts from both dark and light grown plants, allowing us to eliminate *de novo* synthesis as a mechanism inducing GT-1 activity *in vivo*.

Results

To identify protein factors interacting with the rbcS-3A upstream regulatory region, we used a gel-retardation assay (Gardner and Revzin, 1981; Fried and Crothers, 1981) to characterize DNA–protein complexes. The rbcS-3A regulatory DNA frag-

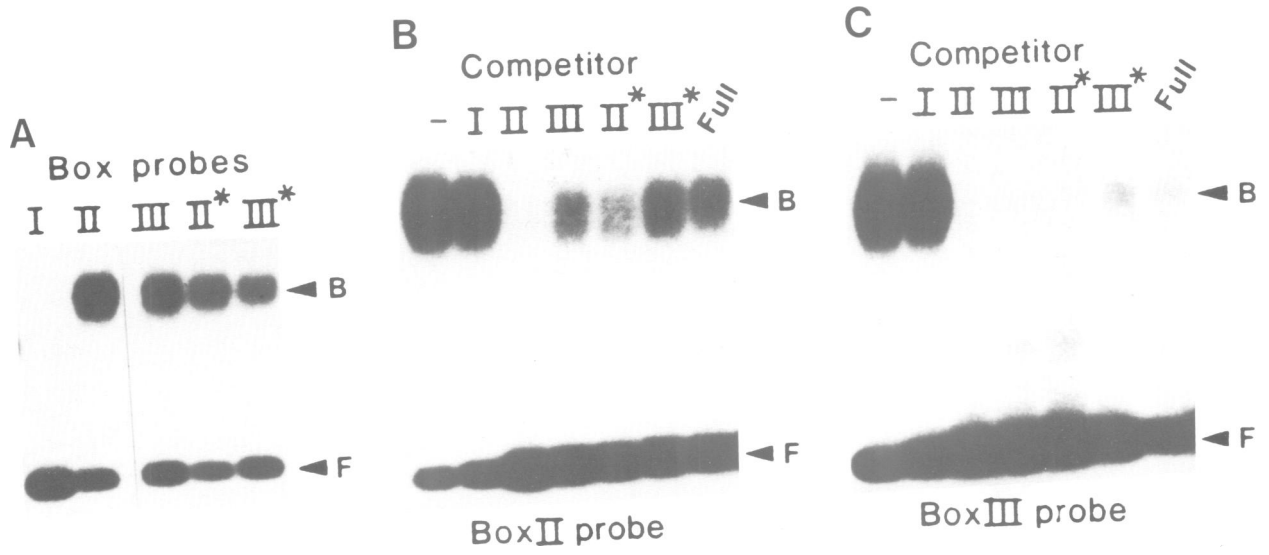

Fig. 3. Binding assays with individual box probes and competitors. **A**: 84-bp fragments containing four tandem copies of the indicated box were used as binding probes. **B**: the box II fragment was the labeled probe. The box repeated in each 84-bp fragment used as competitor (at a final concentration of 25 nM) is indicated above the corresponding lane. 'Full' represents the full-length rbcS-3A upstream fragment a (−330 to −50) used in Figure 1. **C**: As in B but the box III 84-bp fragment was used as probe. Bound (B) and free (F) species are indicated. To generate the 84-bp fragments used as probes and competitors, five sets of 84-mer oligonucleotide pairs were synthesized. Each consisted of four tandem copies (in the same orientation) of either box I (AAAATTTCAAATCTT), II (TGTGTGGTTAATATG), III (ATCATTTTCACTATC), II* (AGTGAGGTAATATCC) or III* (CACATTTACACTCTT). (The detailed sequence of these oligonucleotides is available upon request.) The pairs of oligonucleotides were cloned into bacterial plasmids and excised as double-stranded DNA fragments before use.

ment from −330 to −50 was labelled with ^{32}P and incubated with a leaf nuclear extract prepared from 10 day-old greenhouse grown peas. As shown in Figure 1, two retarded bands (B1 and B2) were observed following electrophoresis indicating the formation of DNA−protein complexes. Sequence specificity was assessed by including unlabeled specific competitor DNA fragments in the binding reactions. Binding is markedly reduced if 20 ng of the −330 to −50 rbcS fragment is present whereas 50 ng of a Cauliflower Mosaic Virus (CaMV) 35S promotor fragment (Odell *et al.*, 1985) (from −300 to +8) has little ability to compete with the rbcS-3A probe. This indicates that the factors forming B1 and B2 are specific for rbcS-3A sequences and have little or no affinity for the 35S promotor fragment. Binding reactions performed using the 35S fragment as the probe support this contention (data not shown).

In order to define more precisely the sequences involved in the interaction, shorter probes extending from −330 to −171 and −170 to −50 (probes 1 and 2 in Figure 2A, respectively) were used for binding assays. As shown in the first lanes of Figure 2 B and C, both probes form a bound complex. These bound species were sensitive to competition by the original rbcS-3A upstream fragment (fragment a in Figure 2A), and fragments corresponding to either of the short probes (fragments b and c). The ability of the probe fragments to cross-compete indicates that the same factor interacts with rbcS-3A sequences upstream and downstream of −170. Thus with the full-length probe used in Figure 1, the B2 complex may reflect binding to both regions while B1 may reflect binding to only one.

Three short sequences downstream of −170 (see boxes I−III in Figure 2A) have been identified as candidates for regulatory elements on the basis of homology with other pea rbcS genes (Fluhr *et al.*, 1986b). The box II sequence contains a GT-motif found in rbcS genes from diverse species (Nagy *et al.*, 1986; Kuhlemeier *et al.*, 1987a). To investigate the role of these conserved sequences, boxes I−III were replaced or deleted and the mutant DNAs (from −170 to −50) were used as binding competitors. Again for each of the individual competitors, similar results were obtained with either probe 1 or 2 (see Figure 2B and C). Fragment d containing an 8-bp substitution in box I competed for the binding factor as effectively as did the wild type fragment. However, the results with fragment e indicate that the 12-bp substitution of box II removed sequences that were critical for factor binding. Fragment f, a box III substitution mutant, was also impaired in its ability to compete, although to a lesser degree than the box II mutant. Consistent with the above results, little competition was observed following deletion of all three conserved boxes (see fragment g in Figure 2A−C).

The competition experiments clearly indicate that boxes II and III are involved in factor binding and that homologous sequences exist upstream of −170. Indeed homologous boxes II* and III* (see Figure 2A), identified in the −220 region are obvious candidates. To test directly which boxes are capable of interacting with the binding factor, cloned synthetic oligonucleotides, each containing four tandem copies of either box I, II, III, II*, or III* were used as probes and competitors. As shown in Figure 3A, the nuclear protein factor has little affinity for the box I probe, but has a very high affinity for the box II probe. Binding to the box III, II* and III* probes is also observed. Boxes II, III, II*, III* and the full-length −330 to −50 fragment can compete with the box II probe for binding, where box I is ineffective (see Figure 3B). Binding to the box III probe is sensitive to the same competitors (see Figure 2C). These results demonstrate that the same factor interacts with the type II and type III boxes. This factor has been designated GT-1. As Kuhlemeier *et al.* (1987b) pointed out (also see Figure 6), there is some homology between the reverse orientation of box III, and box II which contains the highly conserved GT-motif.

Based on the sequence specificity of GT-1, on the full length (−330 to −50) rbcS-3A upstream region, all four type II and III boxes should be involved in factor binding. The DNase I foot-

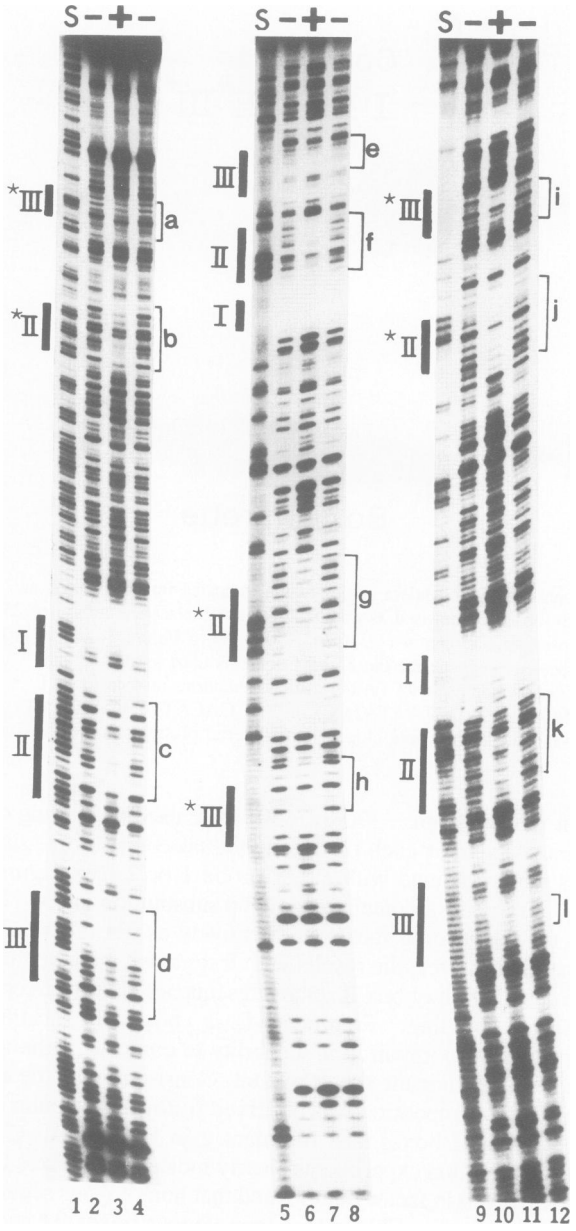

Fig. 4. Binding site determination by DNase I footprinting. The labeled ends of the DNA fragments used for binding studies were as follows: **Lanes 1–4**, 5′ bottom strand (−330 to −50); **lanes 5–8**, 5′ top strand (−330 to −50); **lanes 9–12**, 3′ top strand (−330 to −50). Top and bottom strands refer to the sequence summary in Figure 6. Standards were chemical sequencing (S) reactions: **lane 1**, A>C: **lanes 5 and 9**, G only. The (+) above **lanes 3, 7** and **11** indicates the binding reactions containing nuclear extract. Flanking (−) lanes correspond to minus extract controls with BSA. The conserved boxes are labeled to the left, and protected regions to the right of each set of lanes. Reactions performed in the absence of BSA gave the same cleavage as (−) lanes (data not shown).

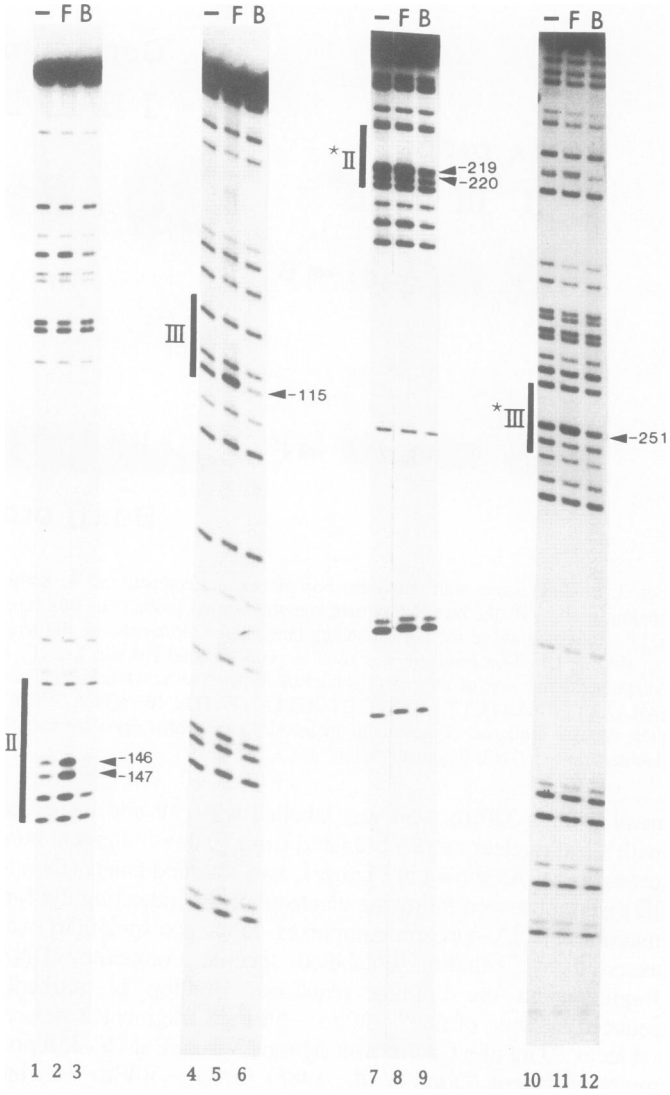

Fig. 5. Identification of residues critical for GT-1 binding by methylation interference. The labeled ends of the DNA fragments used were as follows: **lanes 1–3**, 5′ top strand (−170 to −50); **lanes 4–6**, 5′ bottom strand (−170 to −50); **lanes 7–9**, 5′ top strand (−330 to −171); **lanes 10–12**, 5′ bottom strand (−330 to −171). Top and bottom strands refer to the summary sequence in Figure 6. (−) indicates DNA eluted from minus extract controls, (F) from the free band and (B) from the bound band following gel-retardation assays with partially methylated probes and piperidine cleavage. Boxes are labeled to the left and critical Gs to the right of each set of lanes.

printing results shown in Figure 4 demonstrate that this is the case. On the bottom stand labelled at the 5′ end, boxes II, III, II* and III* are protected from DNase I digestion by the nuclear extract (compare lane 3 to minus extract controls in lanes 2 and 4). The boxes are similarly protected on the opposite strand labelled on either end (compare lanes 7 and 11 to the flanking control lanes). In contrast, the two cleavage sites located in box I on the bottom strand are not protected. No strong DNase I cleavage sites are present in this region on the top strand. The footprinting results on all probes are summarized on the rbcS-3A upstream sequence in Figure 6.

To obtain further fine structure information about the GT-1 binding sites methylation interference experiments (Hendrickson and Schleif, 1985; Weinberger *et al.*, 1986) were performed (see Figure 5). For these experiments the double stranded binding probe is first partially methylated with dimethyl sulfate (DMS) before use in gel retardation assays. Both the bound and the free probe are then eluted from the gel, deproteinized and cleaved at methylated G residues with piperidine. Following electrophoresis of the cleavage products on a sequencing gel, the methylated Gs enriched in the free fraction and depleted in the bound factions are identified. Gs displaying such a pattern are located in positions critical for factor binding because their

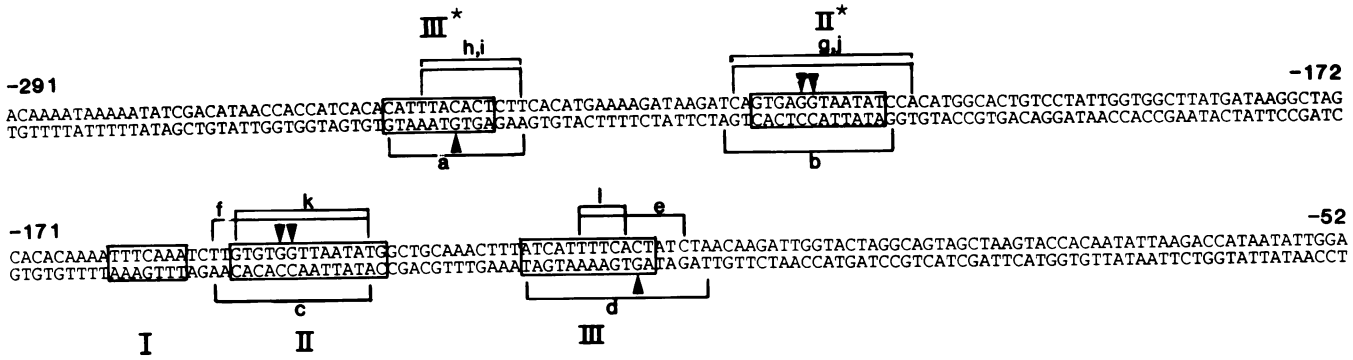

Fig. 6. Summary of DNase I protected regions and critical Gs for GT-1 binding to the rbcS-3A upstream region. The partial DNA sequence of the fragments used for binding studies in Figures 4 and 5 is shown. The conserved boxes I, II, III, II* and III* are boxed. Lettered lines correspond to the lettered regions protected in the footprinting experiments (Figure 4). Arrows indicate Gs that are critical for binding as determined by methylation interference (Figure 5). Numbering is relative to the rbcS-3A transcription start at +1.

methylation interferes with binding. Comparison of the methylation products in control, free and bound fractions (lanes 1–3 of Figure 5) reveals that the double Gs at positions −146 and −147 in Box II are most critical for GT-1 binding; methylated derivatives of these two Gs are nearly absent in the bound fraction. The G at position −149 is also depleted but to a much lesser degree. On the other DNA strand, methylation of the G at position −115 in box III interferes with GT-1 binding (compare lanes 4–6). Similar to the box II situation, the double Gs in box II* are important for binding (lanes 7–9), but binding to boxes III and III* is not entirely equivalent. Whereas methylation of the most 5′ G on the bottom strand in box III interferes with binding, it is the second most 5′ G (at position −251) that interferes in box III* (see lanes 10–12). This may be due to the different orientations of the type III boxes relative to the type IIs. As summarized in Figure 6, Gs in each of the type II and type III boxes are critical for GT-1 binding in the gel retardation assay, and each of these Gs lies in a protected region of the DNase I footprints.

The nuclear extracts used in Figures 1–5 were prepared from greenhouse-grown 10-day old pea leaves. To determine whether GT-1 is present in the dark, nuclear extracts from dark-adapted pea leaves were assayed (see Figure 7). With the −330 to −50 probe, the dark extract also forms two bound complexes with the same competition patterns seen with the light extract. This activity is most likely GT-1 because binding is detected with the box II probe and the same sites are protected in footprinting experiments with light and dark extracts (data not shown). However, it will be necessary to purify GT-1 from both light and dark extracts to determine whether the proteins are identical. We note that the binding activity from the dark extract is lower (about 2-fold normalized to extract protein) and the retarded species have slightly faster mobilities compared to those from the light extract (compare lanes 1 and 2, and 6 and 7 in Figure 7). At present, we do not know whether these characteristics (seen with two independent dark extracts) are physiologically significant or arose during preparation.

Discussion

Light regulation of rbcS-3A transcription in transgenic plants is mediated by LREs present in the 5′ flanking sequences between −330 and −50. The next step in understanding how the transcriptional apparatus responds to light is to characterize *trans*-acting protein factors that interact with these elements. GT-1, the factor identified in this report, binds to known LREs in the −150 region of rbcS-3A, namely boxes II and III, as well as

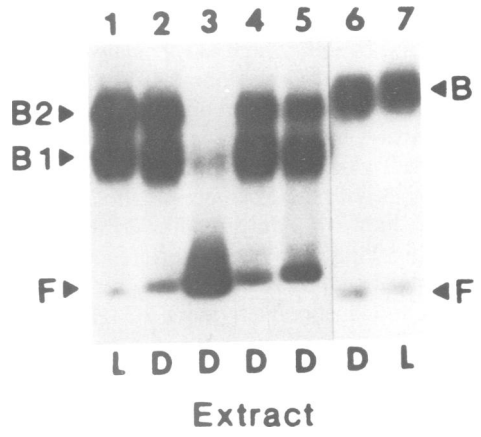

Fig. 7. Comparison of binding activity in nuclear extracts from light-grown and dark-adapted pea leaves. Reactions contained the full-length rbcS-3A −330 to −50 probe (**lanes 1–5**) or the box II probe (**lanes 6 and 7**), described in Figures 1 and 3 respectively. Binding assays were as in Figure 1 except that twice the usual amount of nuclear extract protein was used for lanes 2–4. Nuclear extracts from light-grown (L) pea leaves (**lanes 1 and 7**) were prepared as described in Materials and methods. For dark-adapted (D) nuclear extracts (lanes 2–6), peas were grown under a normal light cycle for 9 days and then in total darkness for 2 days. The leaves were manipulated in dim green safelight until after homogenization. **Lane 3** contained 25 nM unlabeled rbcS-3A competitor (the −330 to −50 fragment) and **lane 4**, 25 nM of the −300 to +8 35S promoter as competitor.

homologous boxes II* and III* located further upstream. The latter interaction substantiates our previous proposal that boxes II* and III* are the redundant LREs that function in transgenic plants when boxes II and III are deleted (Kuhlemeier *et al.*, 1987b). Our data are consistent with two or four GT-1 binding sites between −330 and −50. Judging from the relative competition levels in Figure 3, GT-1 does not bind all type II and III boxes with the same affinity. If each box constitutes a separate site then the deleterious effects that the individual box II and III substitutions (Figure 2) have on binding to the −170 to −50 probes may be due to cooperativity. Clearly GT-1 binds probes containing only one set of boxes and fragments containing either set of boxes can mediate light regulation (Kuhlemeier et al., 1987b). However, a binding interaction between both sets of boxes may occur in the intact gene. Preliminary fractionation data (J.Yamaguchi, P.J.Green and N.-H.Chua, unpublished) indicates that formation of the B2 (upper) complex in Figures 1 and 7 requires more GT-1 activity than B1. Therefore B2 probably contains more GT-1 per DNA than B1.

The GT-motifs to which GT-1 binds are similar to the SV40 enhancer core sequence GTGGA/TA/TA/TG (Weiher *et al.*, 1983). However, two pieces of evidence indicate that more than just a core sequence is required for GT-1 to bind. First, the −300 to +8 fragment from the CaMV 35S promoter fails to bind GT-1 effectively even though it contains two perfect and several imperfect cores. Second, unlike the SV40 sequence, each of the type II and type III boxes contain an AT stretch of at least 5 bp. It will be interesting to see what effect point mutations in this region have on binding. Our methylation interference experiments predict that mutation of the double Gs in the type II boxes should inactivate GT-1 binding. This should be a particularly important mutation to test in transgenic plants.

The comparison of nuclear extracts from light-grown and dark-adapted pea leaves indicates that there is little or no *de novo* synthesis of GT-1 in response to light. The constitutive presence of GT-1 leaves us with two models to consider; GT-1 could either bind to the LREs constitutively or binding may be light-modulated *in vivo*. In the first model, where GT-1 is always bound to the LREs, regulatory and binding domains could be functionally distinct as in the GCN4 protein from yeast (Hope and Struhl, 1986). Alternatively, in a differential binding model the GT-1 binding event itself would directly influence transcription. This model is consistent with the existence of another factor *in vivo* that responds to light by tightly binding to the LREs thereby blocking GT-1 binding. Such steric interference between factors is thought to bring about differential binding in the beta-interferon gene in response to induction with poly I−C (Zinn and Maniatis, 1986).

The transcriptional activity of many DNA-binding proteins in animal cells is thought to be regulated by post-translational modification. For example, the binding of a tissue specific factor to the octamer sequence of the immunoglobulin gene enhancer is activated post-translationally by phorbol esters or lipopolysaccharide (Sen and Baltimore, 1986). Phorbol esters similarly activate *c*-fos transcription (Prywes and Roeder, 1986) presumably via protein kinase C. Post-translational modification is consistent with either of the models for GT-1 binding discussed above. In the constitutive binding model, light could trigger modification of the independent regulatory domain of GT-1. Such an event could explain the slight mobility difference we observe between bound complexes with light and dark extracts. For the differential binding model, a light-dependent modification could control GT-1 binding which would directly influence transcription. However, if GT-1 is regulated in this way we would have to argue that the modification differences were not well preserved during our extract preparation since similar GT-1 binding activities were observed in light and dark samples. In this respect we note that the factor that binds a metal regulatory element (MRE) from the mouse metallothionein gene (Seguin and Hamer, 1987) requires the presence of cadmium *in vitro* to observe binding activity, (even if the extracts were prepared from cells grown in the presence of cadmium).

Due to the presence of overlapping *cis* elements, we do not yet know whether GT-1 is involved in negative or positive regulation of rbcS-3A transcription. Recently we have shown that boxes II and III, to which GT-1 binds, act to decrease transcription in dark-adapted transgenic tobacco (Kuhlemeier, *et al.*, 1987b). Thus GT-1 may be involved in dark-specific repression. However, deletion analysis of rbcS-3A has positioned the 5′ border of a positive element between −166 and −149. Since box II begins at −151, it must also contain sequences for positive regulation. Based on the results presented in this report, point mutations can be designed that eliminate binding and these can be tested for their effects on positive and negative regulation in transgenic plants. With this approach and others such as *in vivo* footprinting (Ephrussi *et al.*, 1985; Becker *et al.*, 1986) the combined potential of *in vitro* and *in vivo* analysis of plant gene expression can be most fully realized.

Materials and methods

Preparation of nuclei and nuclear extracts

Peas (*Pisium sativum L.* var. Progress no. 9) were grown under a 16 h light/8 h dark cycle for 10 days unless otherwise noted in the figure legends. Leaves (with stems and petioles) were harvested (~1 kg), immediately chilled on ice and washed sequentially with water and homogenization buffer. Tissue was disrupted in a buffer filled razor-blade homogenizer (Kannangara *et al.*, 1977) for 7−10 s. The homogenates were filtered through 1000 μ and 80 μ nylon mesh and nuclei were prepared using the buffer system of Gallagher and Ellis (1982) or Watson and Thompson (1986). For either method, the nuclei were pelleted from the homogenate at 3000 *g* for 10 min, carefully resuspended with a paint brush, and purified on percoll gradients. The nuclei were then washed twice to remove residual percoll and triton. Except for the final sedimentation, nuclei were never pelleted but were recovered from 85% w/v sucrose cushions. Typically the yield of nuclear protein was ~100 mg/kg leaf tissue. Nuclei were stored at −70°C in the presence of 20% v/v glycerol.

Nuclear extracts were prepared by a modification of the method described by Parker and Topol (1984b). Nuclei(~100 mg protein) were sedimented at 3000 *g* for 5 min. The pellet was then gently resuspended on ice in 20−40 ml of nuclear lysis buffer (110 mM KCl, 15 mM Hepes/KOH pH 7.6, 5mM $MgCl_2$, 1 mM DTT, 5 μg/ml antipain, 5 μg/ml leupeptin) and transferred to Beckman Ti 70 centrifuge tubes. One tenth vol of 4 M $(NH_4)_2SO_4$ was added and the tubes were rocked gently at 4°C for 30 min. Chromatin and particulate material were pelleted by centrifugation at 40 000 r.p.m. for 60 min. Proteins were precipitated by the addition of 0.3 g/ml freshly ground $(NH_4)_2SO_4$ with stirring for 30 min at 4°C. Following centrifugation at 10 000 *g* for 15 min, the pellet was resuspended in 1 ml of nuclear extract buffer (NEB; 25 mM Hepes/KOH pH 7.6, 40 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 5 μg/ml antipain and leupeptin) and dialyzed against NEB (without protease inhibitors and with 5 mM 2-mercaptoethanol substituted for DTT) for 4 h. Insoluble material was removed by centrifugation at 12 000 *g* for 10 min. Extracts were aliquoted, frozen in liquid nitrogen and stored at −70°C. Protein concentration generally ranged from 5−10 mg/ml. Comparable extracts were obtained from nuclei prepared with either buffer system.

Probe and competitor DNA preparation

DNA manipulations were done essentially as described by Maniatis *et al.*, (1983). Probes were 5′-end labeled with T4 polynucleotide kinase or 3′-end labeled with the Klenow fragment of DNA polymerase I. Total incorporation of radioactivity (usually 10^4-10^5 c.p.m./ng of probe) was assayed on DE81 paper and used to quantitate the purified probes. Probes and competitor DNA fragments were isolated, following electrophoresis in 5% polyacrylamide gels, by electroelution. Non-radioactive DNA was quantitated by comparison to standards on ethidium bromide stained agarose gels.

Gel retardation assays

Binding reactions (10 μl) contained 1.5 fmol end-labeled DNA probe, 3.3 μg poly(dIdC)·poly(dIdC), 45 mM KCl, 25 mM Hepes pH 7.6, 1.1 mM EDTA, 0.5 mM DTT, 5% v/v glycerol and specific competitor DNAs as indicated in the figure legends. Reactions were begun by the addition of nuclear extract protein to 0.8 μg/ml. Reactions were incubated at room temperature for 10 min and loaded on 1% agarose gels (in 10 mM Tris, 1 mM EDTA pH 7.5) under tension. Following electrophoresis (at ~10 V/cm with constant recirculation), gels were dried onto DE81 paper and autoradiographed.

DNase I footprinting experiments

DNase I footprinting was performed by a modification of the method of Galas and Schmitz (1978). Reactions (20 or 50 μl) contained 0.15 to 0.45 fmol/μl of the indicated DNA fragment, 0.2−0.3 μg/μl poly dIdC, 38 mM KCl, 24 mM Hepes pH 7.5, 0.5 mM DTT, 1 mM EDTA, and 9.5% v/v glycerol. Reactions were started by the addition of nuclear extract (or BSA in controls) to a final concentration of 3 μg/μl, and incubated for 20 min at room temperature. $MgCl_2$ (to 5 mM final concentration) and DNase I (to 3.5−12 μg/ml final concentration) were then added and incubation continued for 10 s at room temperature. Reactions were stopped with 4 vols of 6.25 mM EDTA, 0.125% SDS, 0.375 M Na acetate pH 7.5, and 62.5 μg/ml tRNA, immediately followed by phenol extraction. DNA samples were denatured and loaded on 6% gradient sequencing gels.

Methylation interference

These experiments were performed by modification of the methods of Hendrickson and Schleif (1985), and Weinberger *et al.* (1986) Methylation reactions (120 μl) containing 270 fmols end-labelled DNA fragment, 0.02% DMS, 48 μg poly dIdC, 25 mM sodium cacodylate, pH 8, 5 mM $MgCl_2$, and 0.05 mM EDTA, were incubated for 25 min at 37°C. Methylation was stopped with 30 μl of 1 M Tris−acetate, pH 7.5, 1 M 2-mercaptoethanol, 1.5 M sodium acetate, 1 mM EDTA, and 100 μg/ml poly dIdC and precipitated essentially as described by Maxam and Gilbert (1980). Gel retardation assays (75 μl) containing the methylated DNA were as described above except the probe concentration was increased 4- to 5-fold, poly dIdC by 1.4-fold and final protein concentration was 0.5−0.8 μg/μl. Wet gels were exposed overnight at 4°C. The minus extract, free and bound bands were excised, the DNA electroeluted, and deproteinized by phenol extraction. Samples were cleaved in 1.4 M piperidine for 15 min at 90°C and butanol precipitated. Equal counts were loaded in adjacent lanes.

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