The Plant Cell, Vol. 1, 1069–1077, November 1989

Binding of a Pea Nuclear Protein to Promoters of Certain Photoregulated Genes Is Modulated by Phosphorylation

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There have been numerous recent reports documenting phosphorylation of DNA-binding proteins [Montminy and Bilezikjian (1987); Sorger, Lewis, and Pelham (1987); Hoeffler, Kovelman, and Roeder (1988); Jones et al. (1988); Prywes et al. (1988); Sorger and Pelham (1988); Yamamoto et al. (1988)], and the transcriptional regulatory activity of at least one of these proteins appears to be modulated by this modification [Montminy and Bilezikjian (1987); Yamamoto et al. (1988)]. We report here on a plant nuclear protein, the DNA-binding activity of which is strongly affected by phosphorylation. This protein, AT-1, binds to specific AT-rich elements (the AT-1 box) within promoters of certain nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase and the polypeptide components of the light-harvesting chlorophyll *a*/*b* protein complex. A consensus sequence of AATATTTTTATT was derived for the AT-1 box. We demonstrate that the DNA-binding ability of AT-1, from nuclear extracts of pea, can be reversibly modulated by phosphorylation. AT-1 is active in the nonphosphorylated form and loses all DNA-binding ability as a result of phosphorylation. The kinase that phosphorylates AT-1 uses both Mg-ATP and Mg-GTP as a substrate and is inhibited by heparin and spermine, indicative of an NII-type casein kinase.

INTRODUCTION

Genes encoding the chloroplast proteins ribulose-1,5-bisphosphate carboxylase small subunit (RBCS) and the chlorophyll a/b binding proteins (CAB) reside within the nucleus. These genes display complex expression characteristics including developmentally regulated, photoregulated, and tissue-specific expression (Kuhlemeier, Green, and Chua, 1987). Numerous laboratories have contributed in efforts to characterize the DNA sequences that mediate these expression characteristics. Similarly, protein factors that bind to these regulatory elements have been described. A factor, GT-1, that binds to multiple elements within a pea RBCS gene has been described (Green, Kay, and Chua, 1987). An apparently unrelated factor, the G-box binding factor (GBF), binds to a DNA sequence (the G-box) conserved in many different RBCS genes (Giuliano et al., 1988) and in other plant genes (Schulze-Lefert et al., 1989). The precise role of either of these factors is not understood, although it has been demonstrated for a truncated promoter that mutation of the binding sites for GT-1 results in loss of expression (Kuhlemeier et al., 1988). Similarly, mutation of the G-box in the *Arabidopsis thaliana rbcS-1A* promoter results in complete loss of expression in transgenic plants (R.G.K. Donald and A.R. Cashmore, manuscript submitted), and in a transient-expression assay for the chalcone synthase promoter, mutation of the G-box results in loss of light responsiveness (Schulze-Lefert et al., 1989).

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In this report we describe the identification and characterization of a new protein factor, AT-1, that binds to an AT-rich element in light-regulated genes. We also report on the regulation of the DNA-binding activity of this factor by phosphorylation.

RESULTS

AT-1 Binds Specifically to an AT-Rich Element within the Pea *rbcS-3.6* Promoter

In studies aimed at extending our knowledge of nuclear factors that influence the expression of photoregulated genes, we tested nuclear extracts from 1-week-old pea seedlings for DNA-binding activity using the promoters of the light-regulated genes RBCS and CAB. A chromatin bound factor, AT-1, showed specific binding to the −574 to −433 region of the pea *rbcS-3.6* promoter (Herrera-Estrella et al., 1984; Figure 1a). Figure 1 shows competitive binding studies using the gel retardation assay. The pea promoter fragment often showed two additional bands, with slower electrophoretic mobility, in the absence of extract (Figure 1a, lane 1). In the presence of extract, two protein-DNA complexes were observed (Figure 1a, lane 2). Both retarded bands could be competed out by excess unlabeled plasmid containing the promoter fragments

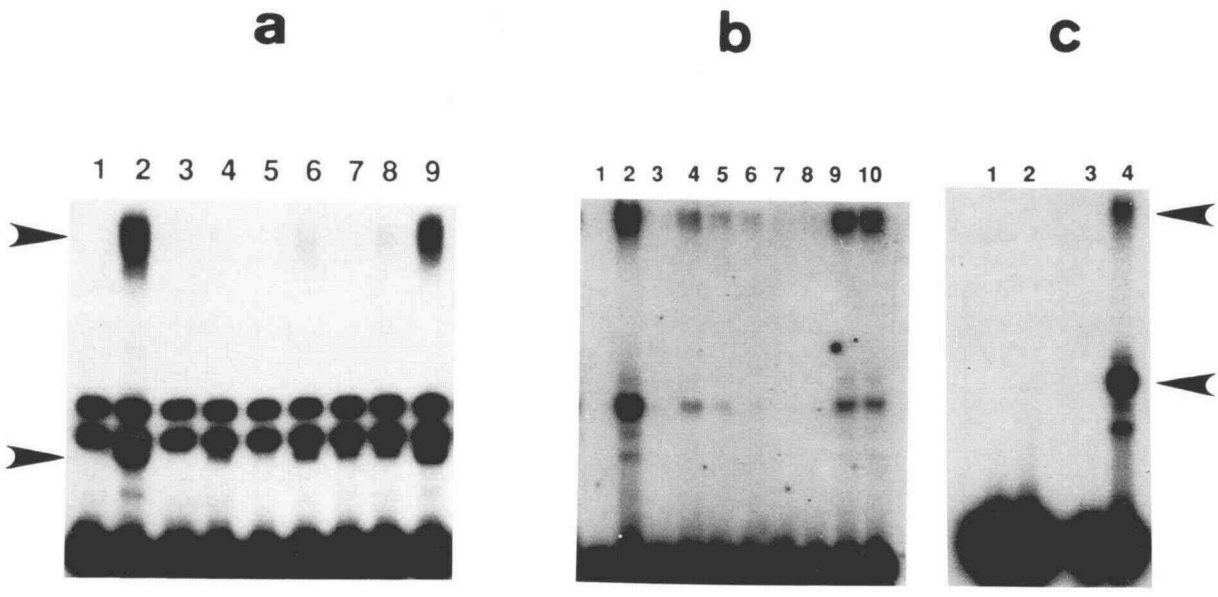

Figure 1. Competitive Protein-DNA Binding Studies.

(a) Gel retardation and competition studies with the −574 to −433 fragment of the pea *rbcS-3.6* promoter. Extracts used for these experiments were a mixture of P1 and P2 ammonium sulfate fractions, as described in Methods. Lane 1 is the probe alone. Lanes 2 to 9 are plus extract. The arrows show the two protein-DNA complexes. Lanes 3 to 9 have 400 ng of competitor DNA, in the following order: plasmids containing the fragments −574 to −298 and −574 to −433 of pea *rbcS-3.6* (lanes 3 and 4), −1182 to −970 of tobacco *cab E promoter (lane 5), −1099 to −984 of tomato rbcS-3A* promoter (lane 6), cloned oligonucleotide corresponding to −566 to −533 of the *rbcS-3.6* promoter (lane 7), cloned oligonucleotide corresponding to the AT-1 consensus sequence (lane 8), and pUC18 (lane 9).
(b) Resembles **(a)** except that cloned AT-1 box oligonucleotide was used as the probe and 20 ng of competitor DNA was used. The additional lane 10 is competition with 20 ng of Bluescript alone.
(c) Gel retardation of the cloned AT-1 box oligonucleotide (lanes 3 and 4) compared with gel retardation done under the same conditions, with an equally sized Bluescript polylinker fragment lacking the AT-1 box (lanes 1 and 2). Lanes 1 and 3 are probe alone and lanes 2 and 4 are plus extract.

−574 to −298 and −574 to −433 of pea *rbcS-3.6* (lanes 3 and 4, respectively), but not by the plasmid alone (lane 9). It is the DNA binding factor associated with the slower-moving complex that we call AT-1. We believe these complexes to be protein-DNA complexes, as they were sensitive to heat and protease treatment (data not shown).

We used the Bal31 delineation method (Jensen et al., 1988) to define further the nature of the DNA sequences mediating this protein-DNA interaction. These studies defined, for both the faster- and slower-moving complexes, a 33-bp sequence as the smallest fragment to form a protein-DNA complex, as illustrated in Figure 2. This AT-rich fragment runs from −566 to −533 in the pea *rbcS-3.6* promoter fragment (Figure 2). We prepared a synthetic oligonucleotide corresponding to this 33-bp AT-rich sequence and cloned it into the Bluescript plasmid. This cloned oligonucleotide also gave rise to two competable protein-DNA complexes (results not shown), similar to the −574 to −433 pea *rbcS-3.6* fragment, and competed for binding with this fragment (Figure 1a, lane 7).

We note that, for the binding experiments with the cloned AT-1 box oligonucleotide shown in Figures 1b and 1c, only 20 ng per assay of salmon sperm DNA was used as nonspecific competitor. In contrast, for the Bal31 experiments shown in Figure 2, 10 μg of salmon sperm DNA was used. The AT-1 box did not show significant binding in the presence of this larger amount of salmon sperm DNA (results not shown), thus explaining why the larger 33-bp fragment was defined as the minimum-size binding fragment in the Bal31 experiment. This larger 33-bp fragment clearly bound more tightly to the nuclear factor, presumably reflecting the presence of two AT-1 boxes within the fragment (see Figure 2).

Other RBCS and CAB Genes Contain AT-1 Binding Sites

We searched for sequences similar to the AT-rich binding sequence in two previously studied promoters from the *Lycopersicon esculentum* (tomato) *rbcS-3A* gene (Giuliano et al., 1988; Ueda et al., 1989) and the *Nicotiana plumba-*

ginifolia (tobacco) *cab-E* gene (Castresana et al., 1988). Related sequences were found within both of these promoters, as shown in Figure 3, and binding studies done with fragments from these *rbcS-3A* and *cab-E* promoters, containing these sequences, gave results similar to those obtained with the −574 to −433 pea *rbcS-3.6* fragment (results not shown). More significantly, these restriction fragments from the *cab-E* and *rbcS-3A* promoters competed for binding with the −574 to −433 pea *rbcS-3.6* promoter fragment (Figure 1a, lanes 5 and 6). Furthermore, it was found that, if the AT-1 binding site in the *rbcS-3A* promoter fragment was disrupted, there was a complete loss of the binding activity (see Figure 4). This was also observed in the case of the *cab-E* promoter fragment (data not shown). Based on these binding studies, a consensus sequence AATATTTTTATT (the AT-1 box) was derived (Figure 3). An oligonucleotide corresponding to this AT-1 box consensus sequence was prepared and cloned into Bluescript, and gel retardation assays were performed. This cloned AT-1 box oligonucleotide probe formed two protein-DNA complexes, similar to what had been observed for the pea promoter fragment (Figure 1b, lane 2, and Figure 1c, lane 4). When a gel retardation assay was performed with a fragment from the Bluescript polylinker equal in size to the fragment containing the AT-1 box, no band shift was observed (Figure 1c, lane 2). Specificity of

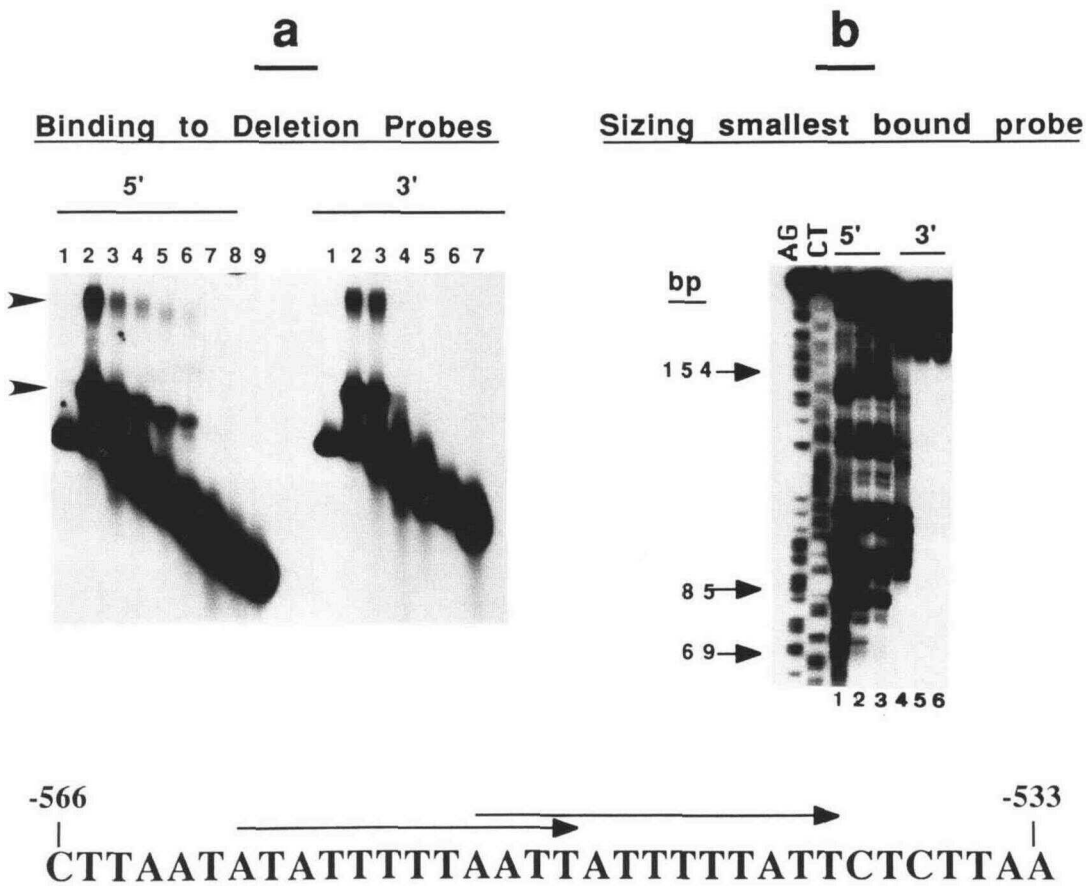

Figure 2. Bal31 Delineation of the AT-1 Binding Site in the −574 to −433 Fragment of the *rbcS-3.6* Pea Promoter, Using the 0.4 M NaCl Extract.

(a) Gel retardation assay carried out with Bal31 generated deletion probes. 5′ and 3′ denote which end of the fragment was labeled.
(b) Fractionation of the retarded and free bands on a 6% sequencing gel carried out to size the smallest probe that gave a gel retardation. AG and CT are Maxam and Gilbert sequencing reactions of the −574 to −433 fragment, *labeled at the EcoRI site. These were used as* markers and the numbers on the left are sizes (in base pairs). Lanes 1 to 3 are all the free bands (lane 1), the lower shifted bands (lane 2), and the higher shifted bands (lane 3) obtained with the 5′ deletion probes that were excised and run on this sequencing gel. Similarly, lanes 4 to 6 are the free bands (lane 4), the lower shifted bands (lane 5), and the higher shifted bands (lane 6) obtained with the 3′ deletion probes.

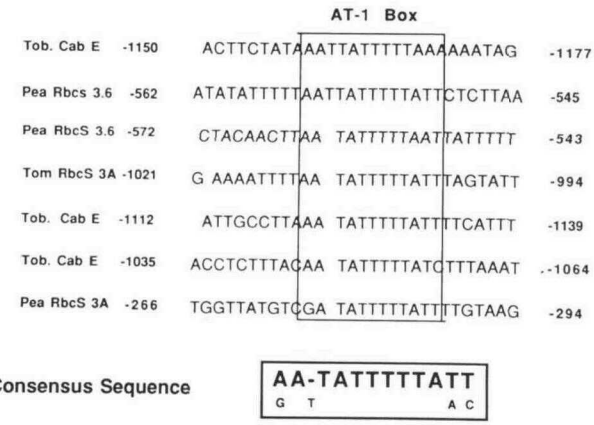

Figure 3. Sequence Homology of the AT-1 Boxes in Promoters from Pea *rbcS-3.6* (Herrera-Estrella et al., 1984), Tomato *rbcS-3A* (Ueda et al., 1989), Tobacco *cab-E* (Castresana et al., 1988), and pea *rbcS-3A* (Green et al., 1987).

Binding studies were done on all restriction fragments containing these sequences except the *rbcS-3A* fragment from pea. They all showed binding and competition characteristics similar to the pea *rbcS-3.6* fragment (data not shown). Significantly, these same fragments compete for binding to AT-1 with the pea *rbcS-3.6* −574 to −433 promoter fragment (Figure 1a). The consensus sequence derived from those studies is as shown.

binding was also demonstrated by competition (Figure 1b) with the pea *rbcS-3.6* (lanes 3 and 4), tobacco *cab-E* (lane 5), and tomato *rbcS-3A* (lane 6) promoter fragments and the two synthetic oligonucleotides cloned into Bluescript (lanes 7 and 8). Plasmid DNA alone, lacking the AT-1 box, did not give comparable competition (Figure 1b, lanes 9 and 10). The cloned AT-1 box oligonucleotide competed for binding of AT-1 to the −574 to −433 pea *rbcS-3.6* promoter fragment (Figure 1a, lane 8).

Deletion of an Upstream Promoter Fragment from the Tomato *rbcS-3A* Gene Results in an Absence of AT-1 Binding and Is Correlated with Loss in Expression

The data in Figure 3 show that an AT-1 binding site resides at approximately 1009 bp 5′ from the transcription start site for the tomato *rbcS-3A* gene. This AT-1 binding site contains within it an SspI restriction site that was used to generate two restriction fragments designated b and c in Figure 4. Neither of these two fragments, in contrast to the noncleaved restriction fragment a, bound AT-1 (Figure 4).

We have previously reported that deletion 5′ of a terminal 90-bp fragment from the tomato *rbcS-3A* promoter results in almost total loss in expression of a chimeric gene in transgenic tobacco plants (Ueda et al., 1989). This deletion involved cleavage of the SspI site described in Figure 4, and thus resulted in destruction of the AT-1 binding site. One interpretation of these data is that it is the loss of the AT-1 binding site that results in the absence of expression. Consistent with this interpretation is the observation that fusion of fragment c (Figure 4) in the inverse orientation to the −1009 *rbcS-3A* truncated promoter did not result in recovery of expression (T. Ueda and A.R. Cashmore, unpublished results). Clearly, other interpretations can be offered for these observations and more experiments are necessary to define the relationship between AT-1 and expression of the *rbcS-3A* gene. It is also of interest to note that the pea *rbcS-3.6* promoter fragment −722 to −357, containing the AT-1 boxes, acts as a positive regulatory element in transgenic tobacco cells (Timko et al., 1985). In contrast, in the tobacco *cab-E* gene (Castresana et al., 1988), the AT-1 boxes reside within the AT-rich negative regulatory element. What role the AT-1

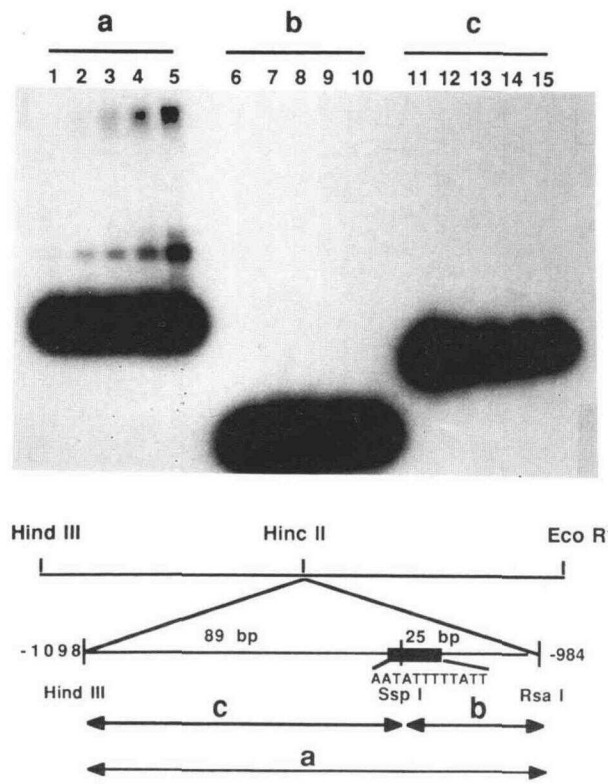

Figure 4. Binding of AT-1 to the *rbcS-3A* Promoter Fragments.

Probe a is from −1098 to −984, probe b is from −1009 to −984, and probe c is from −1098 to −1009. Lanes 1, 6, and 11 are the probes a, b, and c alone. Lanes 2 to 5, 7 to 10, and 12 to 15 are plus extract with decreasing concentrations (2 μg, 1 μg, 0.5 μg, and 0.1 μg) of salmon sperm DNA as the nonspecific competitors. Extracts used for these experiments were a mixture of P1 and P2 ammonium sulfate fractions, as described in Methods.

sequences play in either this positive or negative regulation is not known at this stage.

Binding of AT-1 Is Modulated by Phosphorylation

In our studies on AT-1 we often observed dramatic increases in the level of AT-1 activity during storage of extracts that had initially shown low activity immediately after extraction. As these changes in AT-1 activity were not likely due to proteolytic degradation, we considered post-translational modification. Because phosphorylation is a well documented form of protein modification (Cohen, 1982), we checked to determine whether phosphorylation affected AT-1 activity. From previous studies we knew that the pea nuclear 0.4 M NaCl extracts contained both protein kinase (Datta, Schell, and Roux, 1987) and protein phosphatase activity (N. Datta, unpublished results). We partially inhibited the protein phosphatase activity by adding NaF to the extract and then added either Mg-ATP or Mg-GTP as substrates for protein kinases. Incubation of the extract for 3 min with either of these substrates resulted in almost complete loss in AT-1 activity (Figure 5a, lanes 3 and 11). We interpret these results to mean that AT-1 is a substrate for phosphorylation by a protein kinase present in our extracts and that the active form of AT-1 is the nonphosphorylated form. In keeping with this model, we demonstrated that the phosphorylation of AT-1 was reversible by first incubating the extract for 2 min with Mg-GTP (sufficient to lose essentially all AT-1 activity, as seen in lane 10) and then subsequently incubating for 3 min with EDTA. The EDTA was added to inhibit any further kinase activity and, therefore, allow the residual phosphatase activity to show effect. This experiment clearly demonstrated recovery of AT-1 activity (Figure 5a, lane 14). The result is consistent with our model for phosphorylation, and eliminates the possibility that loss in AT-1 activity was due to proteolysis.

Further experiments were carried out to test the model for phosphorylation of AT-1. In the experiment illustrated in Figure 5b, we repeated the experiments of Figure 5a with the exception that NaF was omitted from the reaction mix. We anticipated that this would result in increased phosphatase activity in the extract which, in turn, would lead to increased AT-1 activity. In keeping with this expectation, high levels of AT-1 activity were observed. We note that, for the experiment depicted in Figure 5b, AT-1 activity is slightly increased by incubation with either Mg-ATP or Mg-GTP. A possible explanation for this might be that the inactive phosphorylated form of AT-1 may be more stable than the nonphosphorylated form; if this were the case, then in the absence of NaF the equilibrium between the two forms resulting from incubation in ATP/GTP could result in an overall increase in AT-1 activity. In another test for the model, we attempted to recover AT-1 activity from extracts that had been incubated with Mg-ATP by subsequent incubation with alkaline phosphatase. In Figure 5c (lane 10) it is seen that a 3-min treatment with alkaline phosphatase, after a 2-min Mg-GTP treatment, resulted in some recovery of AT-1 activity. Although the recovery was certainly not complete, this result was strongly influenced by the instability of AT-1 at the 37°C incubation temperature for this experiment (see Figure 5c, lane 11).

It was of interest to determine which of the nuclear kinases was responsible for the phosphorylation of AT-1. The use of Mg-GTP as a substrate is a characteristic of nuclear NII kinases (Thornberg et al., 1979; Erdmann, Bocher, and Wagner, 1982). These kinases are also characterized by inhibition by heparin and in certain cases by spermine (Erdmann et al., 1982; Datta et al., 1987). We preincubated the extract containing AT-1 with either heparin or spermine before initiating phosphorylation by the addition of Mg-GTP (Figure 5c). In both cases (lanes 4 and 7), the loss in AT-1 activity by phosphorylation was strongly inhibited, thus suggesting that, indeed, the phosphorylation of AT-1 was mediated by an NII kinase. The observation that the controls (spermine, lane 6, and heparin, lanes 1 and 2, added to nonphosphorylated extracts) showed slightly higher AT-1 activity could have been due to residual nucleotides resulting in some basal endogenous phosphorylation. The observation that 1 μg/mL heparin is more effective than 5 μg/mL heparin (lanes 4 and 3, respectively) might be due to an interaction of heparin with AT-1. We know that AT-1, like most DNA-binding proteins, binds to heparin columns. If the interaction were stimulated by Mg, a reasonable possibility, then increasing heparin concentration could result in an Mg-dependent decrease in AT-1 activity.

DISCUSSION

In the experiments presented, we have described the binding of a nuclear factor AT-1 to AT-rich elements within promoters derived from certain photoregulated genes. These binding experiments invariably showed two retarded bands in gel retardation experiments. The Bal31 experiment (Figure 2), the binding to the cloned AT-box oligonucleotide (Figures 1b and 1c), and the competitive binding studies all indicated that the two bands represent binding to identical, or closely related, sequences. We note, however, that the factors that give rise to these complexes can be separated by ammonium sulfate fractionation and fast protein liquid ion-exchange chromatography (results not shown), indicating some differences in the polypeptides associated with these complexes. These differences could reflect a variety of things, including proteolytic degradation products, monomers compared with dimers, polypeptides that differ by phosphorylation or some other modification; or we could be dealing with quite distinct polypeptides. In the phosphorylation studies

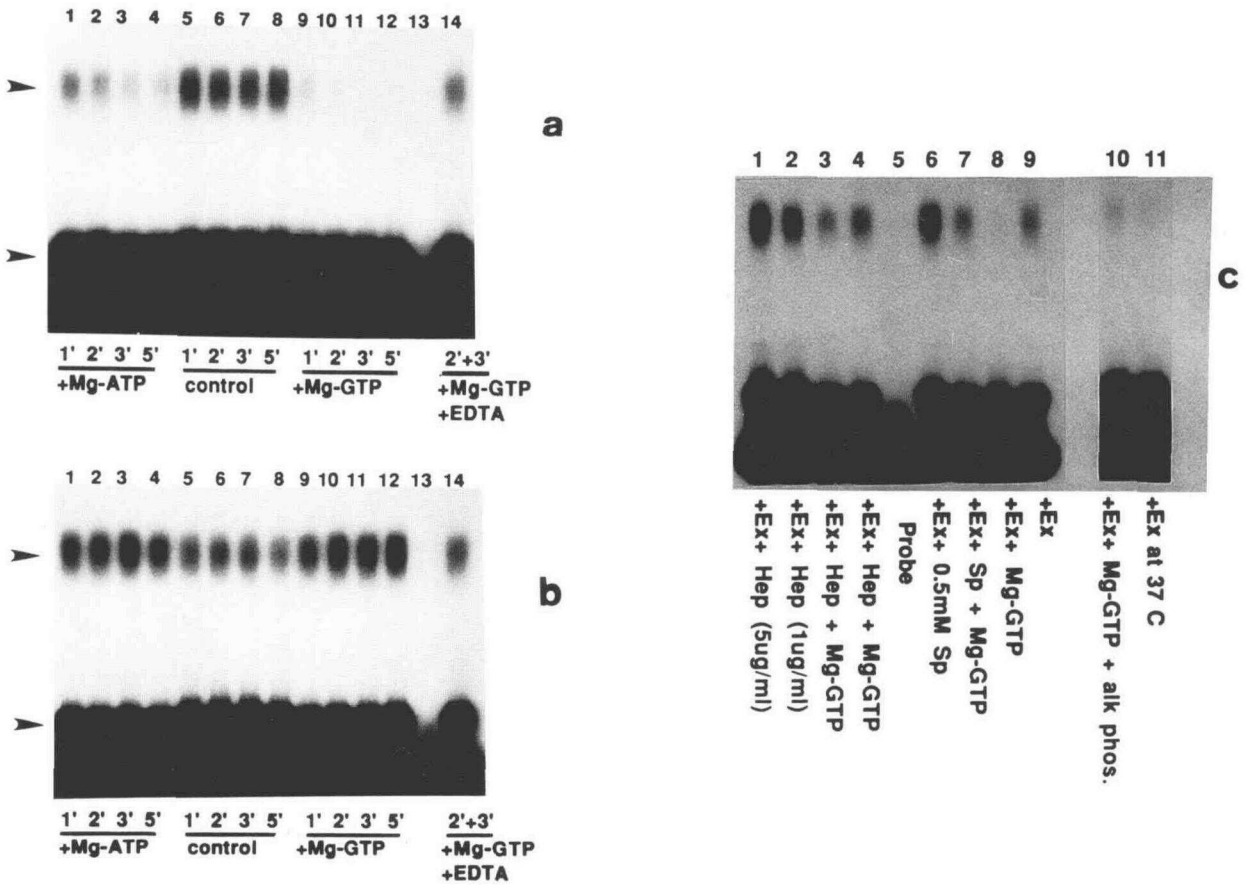

Figure 5. Inactivation of AT-1-Binding Activity by Phosphorylation.

(a) Extracts (0.4 M NaCl extract) were phosphorylated in the presence of sodium fluoride.
(b) Extracts (0.4 M NaCl extract) were phosphorylated in the absence of sodium fluoride. In lanes 1 to 4 and lanes 9 to 12, the extracts were incubated with Mg-ATP and Mg-GTP, respectively, for 1 min, 2 min, 3 min, and 5 min before starting the gel retardation assay. Lane 13 is the probe alone and lane 14 is incubation of the extract with Mg-GTP for 2 min followed by 3 min of incubation with 50 mM EDTA. Lanes 5 to 8 are control lanes, where the extract was incubated with Mg-Na for 1 min, 2 min, 3 min, and 5 min.
(c) Composite figure showing the effect of NII kinase inhibitors and alkaline phosphatase on AT-1-binding activity. Lane 5 is the free probe and lane 9 is plus extract (control). Lanes 1 and 2 show the effects of 5 μg/mL and 1 μg/mL heparin for 15 min on the gel retardation assay. In lanes 3 and 4, the extract was preincubated with 5 μg/mL and 1 μg/mL heparin for 15 min on ice before adding Mg-GTP. Lane 6 shows the effect of spermine on the gel retardation assay. In lane 7, the extract was preincubated with spermine for 15 min on ice before adding Mg-GTP. In lane 8, the extract was incubated with Mg-GTP for 2 min. Lane 10 is extract incubated with Mg-GTP for 2 min and subsequently incubated for 3 min at 37°C with 5 units of alkaline phosphatase. Lane 11 is the extract alone incubated at 37°C. [Lanes 10 and 11 are from another gel that had all the internal controls (as seen in lanes 5, 8, and 9). These are not shown here to avoid repetition.]

shown in Figure 5, the autoradiographs were heavily exposed to illustrate the changes in the slower-moving complex (AT-1). We note that the faster-moving complex was also affected by phosphorylation, but, in this case, the effect was not so pronounced.

Recently, there have been numerous reports invoking phosphorylation as a means of altering the activity of transcriptional regulators. Stimulation of transcription by RNA polymerase III in HeLa cells is apparently mediated through phosphorylation of the transcription factor TFIIIC (Hoeffler, Kovelman, and Roeder, 1988). Similarly, the DNA binding activity of the factor (SRF) that binds the serum response element responsible for the induction of the *c-Fos* protooncogene is modulated by phosphorylation (Prywes et al., 1988). In yeast, the heat shock factor (HSF) may also be phosphorylated, although its binding ability

does not appear to be affected. In this case, phosphorylation causes a change in mobility of both HSF and an HSF-DNA complex on SDS and native acrylamide gels, respectively. These changes are reversed by treating HSF with acid phosphatase (Sorger, Lewis, and Pelham, 1987; Sorger and Pelham, 1988). Another factor from yeast (*Saccharomyces pombe*) similar to the AP1 factor also shows a change in mobility of the factor-DNA complex upon treatment with alkaline phosphatase (Jones et al., 1988). The factor (CREB) that binds the cAMP-responsive element of the rat somatostatin gene shows dual regulation by phosphorylation. When it is phosphorylated by protein kinase A, CREB is involved in regulation of transcription. When phosphorylated by protein kinase C, CREB undergoes dimerization (Montminy and Bilezikjian, 1987; Yamamoto et al., 1988).

From the numerous examples we have cited, it has become increasingly clear that phosphorylation of transcription factors may play a major role in regulating gene expression. It would come as no surprise if such a mechanism played an equally important role in modulating gene expression in plants. In the experiments reported in this article, we have demonstrated an effect of phosphorylation on binding of AT-1 to promoters of certain plant photoregulated genes. The function of AT-1 and whether it acts as a transcriptional regulator is currently being studied. Also of interest is the relationship between AT-1 and factors binding to AT-rich sequences described for other plant genes (Jofuku, Okamuro, and Goldberg, 1987; Jensen et al., 1988; Bustos et al., 1989).

METHODS

Nuclear Isolation

Nuclei were isolated from plumules of 8-day-old etiolated seedlings of *Pisum sativum* var. Progress 9, using a slight modification of the method described in Datta, Chen, and Roux (1985). The seedlings were given 4 hr of white light just prior to harvesting. One hundred grams of plumules was homogenized in 1 L of buffer A [10 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 100 KIU (Kallikrein inactivating units)/0.11 trypsin inactivating units of aprotinin, and 10 mM 2-mercaptoethanol], using a polytron. The homogenate was filtered through three layers of cheesecloth, three layers of Miracloth, and two layers each of 88-μm and 30-μm nylon mesh. The filtrate was centrifuged at 4000 rpm for 10 min using a Beckman JA-10 rotor. The pellet was resuspended in 10 mL to 15 mL of buffer A using a Dounce homogenizer and then centrifuged (7000 rpm for 30 min in a Beckman JS-13 rotor) over a discontinuous Percoll gradient containing 12 mL each of 50% and 25% Percoll in buffer A. Nuclei were collected from the interface of the two layers and washed twice with buffer A to remove the residual Percoll. The purified nuclei were either used directly or stored in 20 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 50% glycerol, 1 mM PMSF, 1 mM benzamidine, 100 KIU of aprotinin, and 1 mM DTT.

Nuclear Extract

Purified nuclei from 100 g of pea plumules were lysed, and an extract containing AT-1 was prepared essentially as described for NII kinase in Datta et al. (1987). The nuclei were pelleted by centrifugation at 20,000 rpm for 5 min. (This and all subsequent centrifugation steps were done in a Beckman TL-100 tabletop ultracentrifuge.) The nuclei were resuspended in 2.5 mL of lysis buffer (50 mM Hepes, pH 8.0, 1 mM EDTA, 0.3% Triton X-100, 1.14 mM ascorbic acid, 0.2 mM ammonium molybdate, 1 mM PMSF, 1 mM benzamidine, 200 KIU/mL aprotinin, and 10 mM DTT) using a Pasteur pipette. This resuspension was allowed to sit on ice for 10 min and then centrifuged at 80,000 rpm for 15 min. The supernatant from this step was called the "soluble fraction." To the pellet, the chromatin fraction, was added 0.4 mL of high salt buffer, which consisted of 50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 0.4 M NaCl, 1.14 mM ascorbic acid, 0.2 mM ammonium molybdate, 1 mM PMSF, 1 mM benzamidine, 200 KIU/mL aprotinin, and 10 mM DTT. After stirring for 30 min, the mixture was centrifuged at 80,000 rpm for 15 min. The supernatant was called the 0.4 M NaCl extract. This extract was either used as such or was ammonium sulfate fractionated. 0% to 30% (P1) and 30% to 50% (P2) ammonium sulfate fractions gave the slower and faster migrating complexes, respectively. For the binding competition studies, the two fractions were mixed in the ratio of 10:1 to give equal amounts of the two retarded complexes.

DNA Probes for Gel Retardation Assays

The −574 to −433 fragment of pea *rbcS-3.6* promoter was cloned into the HincII site of pUC18, and its probe was made by cutting the plasmid with HindIII and EcoRI. The tomato *rbcS-3A* from −1098 to −984 was cloned into the HincII site of pUC18, and probes (Figure 4) were made by cutting the plasmid with HindIII and EcoRI (fragment a), EcoRI and SspI (fragment b), and HindIII and SspI (fragment c). The oligonucleotide corresponding to the AT-1 box was synthesized with BglII and BamHI sticky ends, and was cloned into the BglII-BamHI sites of a modified Bluescript vector (where a BglII site had been cloned into the EcoRV site of the polylinker). Probes were made by cutting with HindIII and XbaI. In the case of the control probe used in Figure 1c, the Bluescript polylinker was cut with BglII and XbaI. Labeling was achieved with the Klenow polymerase fragment using [α-^{32}P]dATP and [α-^{32}P]dCTP as the radioactive nucleotides. The labeled probes were purified on a 6% nondenaturing gel.

Gel Retardation Assays

Gel retardation assays were carried out in a final volume of 10 μL containing 10 mM Tris, pH 8.0, 1 mM EDTA, 5% glycerol, 0.025% xylene cyanol, 0.025% bromphenol blue, and 10 μg of salmon sperm DNA (20 ng of salmon sperm DNA when small oligonucleotides containing AT-1 box were used as probes). Extract (0.5 μg to 0.7 μg), containing AT-1, was added and allowed to incubate at room temperature for 5 min, after which the reaction was started with 0.5 ng to 1.0 ng of ^{32}P-labeled probe (20,000 cpm). After 30 min of incubation at room temperature, the samples were electrophoresed on a 6% native acrylamide gel to separate the free DNA from the protein-DNA complexes. Following electropho-

resis, gels were dried and exposed overnight to x-ray films. For competitions, minimal amounts (0.1 μg to 0.2 μg) of extract were used, and the salmon sperm DNA concentration was reduced to 1 μg per assay (10 ng when cloned AT-1 box oligonucleotide was used as the probe). Competitor DNAs were prepared in the same way as the probes except that the nucleotides in the Klenow reaction were not labeled.

Bal31 Delineation of the Binding Site

The pea *rbcS-3.6* promoter fragment −574 to −433 was used for this analysis. The 0.4 M NaCl extract was used, and Bal31 delineation of the binding site was done exactly as described in Jensen et al. (1988).

Phosphorylation and Dephosphorylation Assays

The pea *rbcS-3.6* promoter fragment −574 to −298 was used for these assays. All the AT-1-binding activity in this probe resides in the −574 to −433 region. The 0.4 M NaCl extract was preincubated with 50 mM sodium fluoride (unless otherwise indicated) for 15 min on ice before the addition of Mg-ATP or Mg-GTP (final concentration 20 mM Mg and 2 mM nucleotide triphosphate). In the case of controls, $MgCl_2$ and NaCl were added to a final concentration of 20 mM and 6 mM, respectively. EDTA was used at a final concentration of 50 mM; spermine was at 0.5 mM final concentration. The incubations were carried out at room temperature, and the reaction was stopped by taking an aliquot from the mix and adding it to the gel retardation assay mix containing salmon sperm DNA. The samples were then processed as regular gel retardation assays, outlined above.

ACKNOWLEDGMENTS

We thank Drs. Randolph Hellwig, Mark Hand, and Takashi Ueda for their help during the initial phase of this work. This work was supported by National Institutes of Health grant GM38409 and U.S. Department of Energy grant DE FG02 87ER13680 (both to A.R.C.).

Received July 24, 1989; revised September 19, 1989.

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