The Plant Cell, Vol. 5, 109–121, January 1993

CA-1, a Novel Phosphoprotein, Interacts with the Promoter of the *cab*140 Gene in Arabidopsis and Is Undetectable in *det*1 Mutant Seedlings

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We have identified and partially purified a DNA binding protein from Arabidopsis that interacts specifically with the phytochrome-responsive promoter of the Arabidopsis *cab*140 gene. Promoter deletion analyses in transgenic tobacco showed that, if a region that includes the sequence interacting with this protein was deleted, both expression and phytochrome responsiveness were lost. The protein protected a cytosine- and adenine-rich region from DNase I digestion, and therefore it has been called CA-1. CA-1 was shown to be a phosphoprotein, and dephosphorylation changed the migration of the protein–DNA complex in DNA mobility shift assays. The data suggested that the protein has an apparent molecular weight of 70,000. The CA-1–protected region of the *cab*140 promoter included an ACGT motif that has been found in the target sequences of a number of bZIP transcription factors, but the binding behavior of CA-1 differed from those factors. CA-1 binding activity was present in plants grown in either white light or darkness, and no differences in the binding activity were detected in the dark-grown plants after short red or white light treatments. However, the CA-1 binding activity was not detectable in extracts of seedlings bearing the *det*1 mutation grown in the dark and given the same illumination treatments as wild type. In contrast to wild type, the mutant seedlings express *cab* RNA at a high level when grown in complete darkness, and we found no further increase in *cab*140 mRNA in response to brief red illumination. The lack of CA-1 activity in the *det*1 mutant suggests that it may function as a transcriptional repressor regulating the expression of the *cab*140 gene in Arabidopsis.

INTRODUCTION

Light plays an important role in regulating plant growth and development, and its effects are mediated by several different photoreceptors. These include phytochrome, several blue light photoreceptors, and at least one sensitive to UV irradiation. Changes in transcription of specific genes are among the responses to the actions of these photoreceptors (Thompson and White, 1991), and we have been particularly interested in understanding the signal transduction pathway leading from the absorption of light by phytochrome to changes in transcription.

In studying light-regulated genes, including genes encoding the light-harvesting chlorophyll *a/b*-proteins (*cab* genes), the small subunit of ribulose bisphosphate carboxylase/oxygenase (*rbcS* genes), and genes encoding phytochrome apoproteins (*phy* genes), a number of different motifs have been identified in the promoters. In some instances, these motifs have been shown to be important for expression of the gene and to bind protein factors that may be involved in the regulation of transcription of the gene. For example, multiple protein factors, including GBF, GA-1, GC-1, AT-1, and GT-1, have been found to interact with DNA sequence motifs in the tobacco *cab*E promoter (Schindler and Cashmore, 1990). The G-box, which includes the sequence ACGT and is bound by GBF, has been shown to be an important quantitative element for the expression of the Arabidopsis *rbcS*-1A gene (Donald and Cashmore, 1990). Similar ACGT-containing elements occur in many other plant genes, including ones not regulated by light (Giuliano et al., 1988; Katagiri et al., 1989; Lam et al., 1989; Guiltinan et al., 1990; Schmidt et al., 1990; Singh et al., 1990; Oeda et al., 1991; Tabata et al., 1991; Weisshaar et al., 1991; Schindler et al., 1992a; Williams et al., 1992). Interestingly, at least one member of the GBF gene family is differentially expressed in light-grown and dark-grown plants (Schindler et al., 1992a).

Two other factors, LRF-1, an activity binding to a *Lemna rbcS* gene (Buzby et al., 1990), and ABF-1, an activity binding to a pea *cab* gene (Argüello et al., 1992), have been identified and found to differ in abundance in light-grown and dark-treated plants. Regions similar to the GT-1 binding site (first identified as box II by Fluhr et al., 1986) have been shown to play an important role in the light-regulated expression of the pea *rbcS*-3A gene (reviewed by Gilmartin et al., 1990) and to act as a quantitative element in the oat and rice *phyA* genes (Bruce

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et al., 1989; Dehesh et al., 1990). An element important for negative phytochrome regulation was also found downstream of this GT element in the oat *phyA* gene and called RE-1 (Bruce et al., 1991).

Another approach to understanding the light-regulatory pathways has been to isolate mutants in which the normal regulation is altered. Two interesting categories of such mutants in Arabidopsis include the *det* mutants (Chory et al., 1989) and the *cop* mutants (Deng et al., 1991). These mutants develop in the dark in many ways that are similar to wild-type development in the light. In particular, they have expanded cotyledons, make leaves, and have a substantially developed chloroplast thylakoid membrane system when germinated and grown in complete darkness. Furthermore, they express high levels of *cab* RNA in the dark, suggesting that the normal repression of transcription of this gene that occurs in darkness is missing in the mutant (see review by Chory, 1991).

We have studied the regulation of the expression of *cab* genes in Arabidopsis and found that one of three linked *cab* genes, *cab*140 (Leutwiler et al., 1986), is especially responsive to the action of phytochrome in etiolated seedlings (Karlin-Neumann et al., 1988). The *cab* genes are also regulated by a number of other environmental and endogenous signals (see references in Thompson and White, 1991; Brusslan and Tobin, 1992), and there is evidence that there are multiple promoter elements involved in their extensive and complex regulation (Schindler and Cashmore, 1990). Ha and An (1988) have examined a set of 5′ deletions of the Arabidopsis *cab*140 gene (called *cab*1 in that paper) fused to a chloramphenicol acetyltransferase (CAT) reporter gene in transgenic tobacco. They found differences in expression of the introduced constructs in plants grown in white light or after dark treatments,

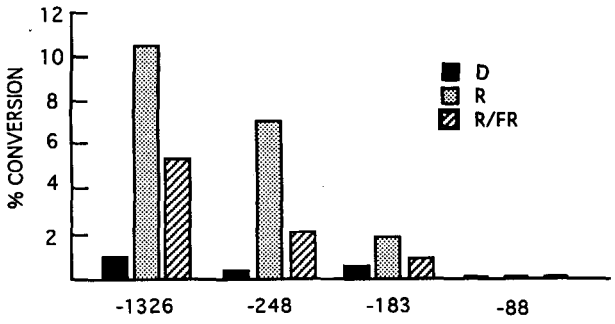

Figure 1. Expression and Phytochrome Responsiveness of *cab*140 5′ Deletion Constructs Fused to the CAT Reporter Gene.

Seeds from tobacco transformed with the indicated constructs (Ha and An, 1988) were germinated and grown for 8 to 11 days in complete darkness (D) or with 2 min of red light every 8 hr (R) or 2 min of red light followed immediately by 10 min of far-red light every 8 hr (R/FR). The distance in nucleotides from transcription start is shown for each construct. CAT enzyme activity was determined for an appropriate aliquot, as described in Methods. The amounts of protein used in the assays for each construct were: −1326, 2 μg; −248, 20 μg; −183, 200 μg; −88, 200 μg.

and showed that the region from −183 to −88 from transcription start (as determined in Karlin-Neumann et al., 1988) was necessary for higher levels of expression in the light. We have used a set of their constructs to test for promoter regions of the *cab*140 gene that are involved in its phytochrome responsiveness. We have looked for proteins that interact with these regions. One such DNA binding protein that can interact specifically with a unique cytosine- and adenine-rich region of the Arabidopsis *cab*140 gene was identified. Its characteristics and the sequence motif with which it interacts are unique among previously reported plant transcription factors. Furthermore, its apparent absence in seedlings carrying the *det*1 mutation supports the idea that it may act as a repressor of transcription and that it may play a role in the light regulation of this gene.

RESULTS

Phytochrome Regulation Is Maintained in 5′ Deletion Constructs That Include 183 bp of the *cab*140 Promoter

Transgenic tobacco plants with 5′ deletion constructs of the Arabidopsis *cab*140 promoter and a substantial part of the coding region fused to a CAT reporter gene were previously tested for differences in expression in white light and darkness by Ha and An (1988). Seeds from these plants were germinated, and the seedlings were tested for phytochrome responsiveness, as described in Methods. The reporter gene expression and phytochrome responsiveness of four of these 5′ deletion constructs are shown in Figure 1. A representative data set from one of two to three individual replicate experiments is shown for each construct. Constructs deleted to −1326, −251, and −183 relative to transcription start were all able to direct CAT expression in a phytochrome-regulated manner: the dark levels of CAT activity were low, red light treatment resulted in substantially increased CAT activity, and a smaller increase was seen if far-red light immediately followed each red light treatment. There was no detectable CAT expression in the −88 construct, consistent with the results of Ha and An (1988). Additional deletions terminating between −1326 and −251 (Ha and An, 1988) were also tested and showed similar responsiveness to phytochrome action (R. A. Doxsee and E. M. Tobin, unpublished data). Although the overall activity level found for each construct differed greatly (see Figure 1 legend), no conclusion about the relative promoter strengths of the constructs can be drawn from these experiments. However, the measurement of relative levels of CAT activity after the various light treatments given to a single population of seedlings allowed us to test each construct for phytochrome responsiveness. These results demonstrated that the sequence from −183 to −88 is necessary, at least as a quantitative element, for phytochrome-regulated expression of the reporter gene and

that sequences involved in phytochrome responsiveness must be located somewhere downstream of −183.

Identification of a DNA Binding Activity Specific for a Region of the *cab*140 Promoter

DNA mobility shift assays were used to identify proteins capable of interacting with the region of the promoter necessary for phytochrome regulation. The region from +12 to −248, designated A, was found in preliminary experiments to be shifted by binding activities found in nuclear extracts, but this fragment was too large for further characterization of these activities. Therefore, the fragment was subcloned into three fragments, designated A1 (+12 to −72), A2 (−73 to −160), and A3 (−161 to −248), and each of these fragments was tested with the nuclear extracts. The major binding activity was found to interact with the A2 fragment; therefore, this fragment was used in subsequent characterizations. Figure 2 shows protein binding to the radioactively labeled A2 DNA fragment. Competition for this binding by unlabeled A, A1, and A3 DNA is also shown. The A fragment competed for the binding (lanes 3 to 5), but the A1 and A3 fragments failed to compete significantly (lanes 6 to 11). Although there may well be interactions of other proteins with these fragments, under our conditions for plant growth, protein extraction, and gel mobility shift assays, this interaction with the A2 fragment was the major binding activity observed. These data demonstrated that the binding was specific for the A2 fragment.

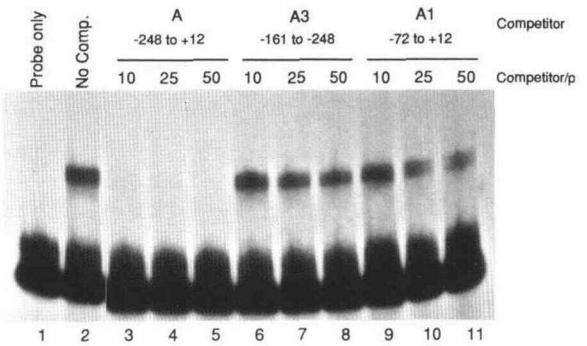

Figure 2. Binding Factor Specificity Shown by Binding Competition Assays.

All DNA fragments (A1, A2, A3, and A) were derived from the *cab*140 gene: A1, −72 to +12; A2, −73 to −160; A3, −161 to −248; A, −248 to +12. The A2 fragment was radioactively labeled and used as the probe. One microgram of Arabidopsis whole cell proteins fractionated through heparin-agarose chromatography was used in lanes 2 to 11. The number on top of each lane represents the molar ratio of competitor to probe, and the number ranges above the ratios indicate locations of the competitors in the *cab*140 promoter.

The Binding Activity Interacts with a CA-Rich Sequence Motif

Several approaches were used to characterize the promoter sequences involved in binding this activity. The results of these experiments are shown in Figure 3. Figure 3A shows protection of the upper strand of the A2 fragment from DNase I digestion. The major protected region spanned −111 to −138, and the −125 adenine residue appeared to be a hypersensitive site. Two smaller protected regions, from −86 to −90 and from −73 to −76, were also observed. The sequences of these protected regions are indicated by overlining in the context of the entire A2 fragment in Figure 3B.

The importance of the major protected region for the binding of the activity was assessed using binding competition experiments. We first compared binding to an oligonucleotide dimer of the longest protected region (−111 to −138) and to the A2 fragment. Figure 3C shows that two protein–DNA complexes could be detected with the radioactively labeled A2 probe and partially purified proteins (lane 2). The unlabeled oligonucleotide dimer DNA could compete for the formation of both complexes, although the unlabeled A2 DNA competed more effectively (lanes 1 to 4). In the reciprocal experiment, the A2 DNA was able to compete with the dimer probe (lanes 6 to 9). The A3 fragment failed to compete with either probe (lanes 5 and 10). Furthermore, the A2 probe showed higher affinity than the dimer probe (compare lane 2 with lane 7). This experiment suggested that the 28-bp DNase I protected region contains sufficient information for this activity to bind specifically, but additional sequence information contained in the A2 fragment is required for higher affinity binding.

Because the dimer of the major footprinted region (−111 to −138) did not bind as well as the A2 fragment and because the A-rich region downstream of −111 was not susceptible to DNase I digestion even in the free DNA, we used a larger fragment that included nucleotides between −138 and −99 to test the effect of various changes in this region on the binding activity. These experiments are shown in Figure 3D. Competition for binding was substantially reduced by changing 9 bp in the A2 fragment (m-1), as shown. Two subsets of these changes in the context of the −99 to −138 fragment (m-2 and m-3) showed substantial, but not complete, competition for the binding activity at the 300-fold excess molar concentration used. The three A residues at positions −100, −102, and −105 could affect the binding affinity. These experiments demonstrated that the sequence from −99 to −138 plays an important role in the binding. Because of the high cytosine and adenine content in this region, the binding activity has been designated CA-1.

CA-1 Differs from Factors That Recognize the ACGT Motif

The CA-1 major footprint includes several interesting sequence motifs shown in Figure 4A. The ACGTAAGC sequence from

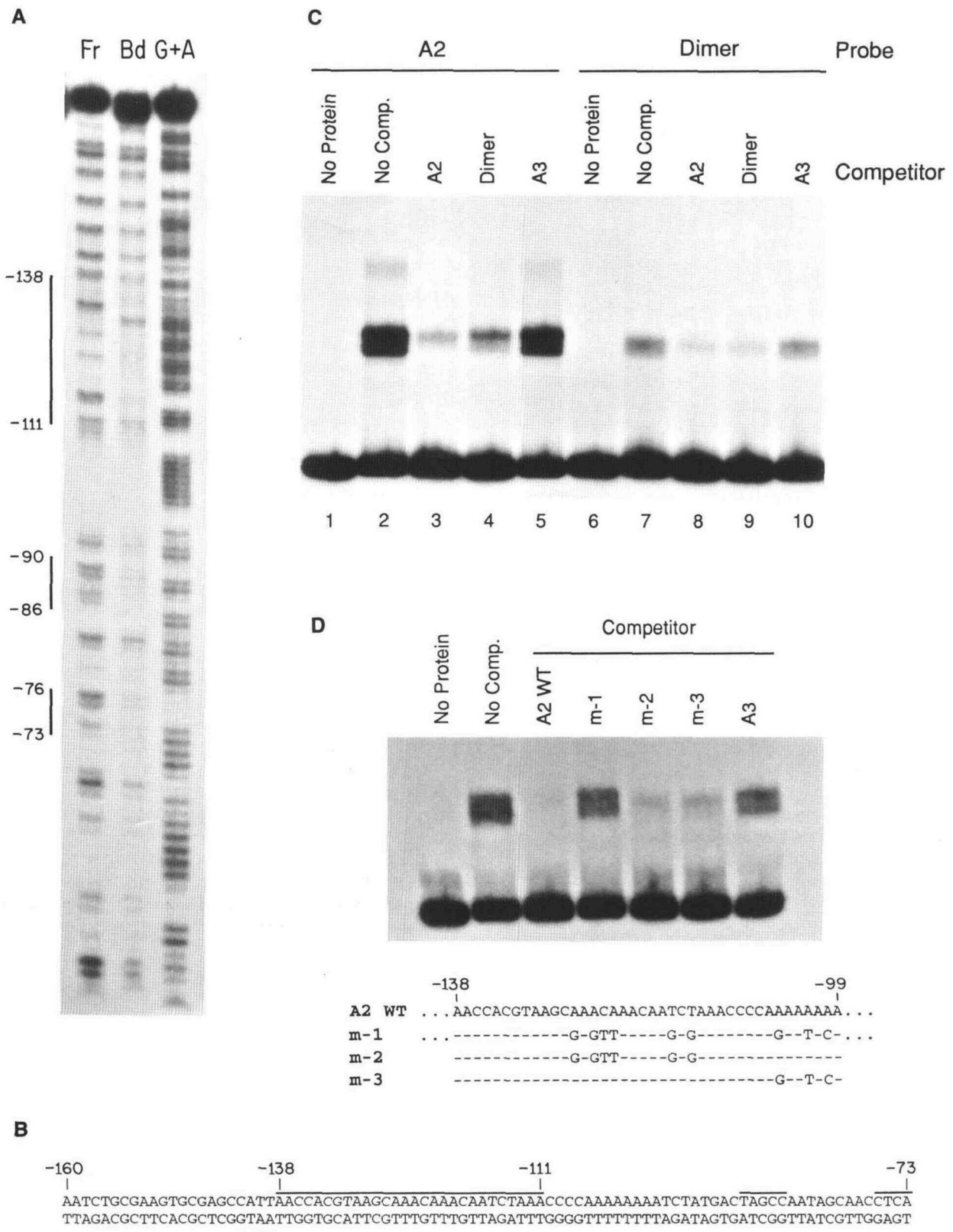

Figure 3. *Binding* Activity Interacts with a CA-Rich Motif.

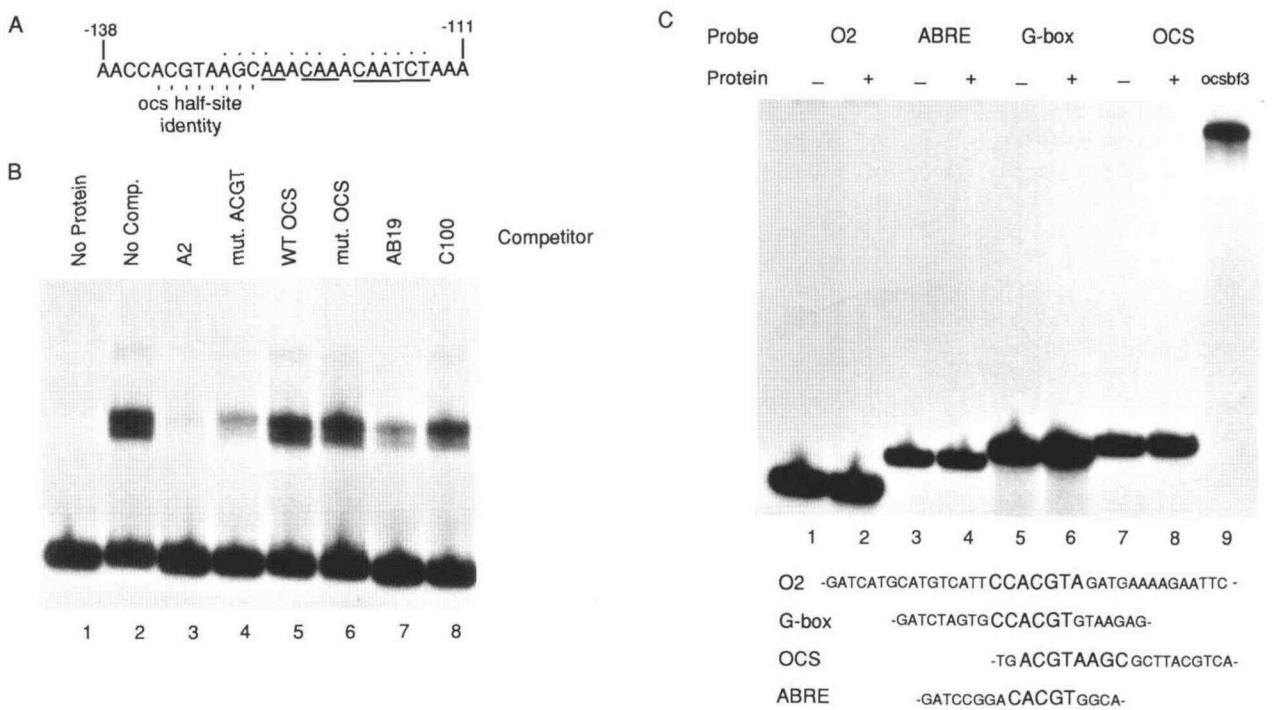

Figure 4. CA-1 Does Not Require an ACGT-Containing Sequence for Binding.

(A) Sequence similarities of the CA-1 footprint to the octopine synthase (OCS) element and to the *Lemna* AB19 gene promoter. The *cab*140 sequence (−138 to −111) was taken from Leutwiler et al. (1986). Underlined and dotted residues represent sequence identities to the promoter of the *Lemna cab*AB19 gene, from −138 to −127 and −167 to −152, respectively.

(B) Binding competitions. Each lane contained 0.5 ng of radioactively labeled A2 probe. Lanes 2 to 8 each contained 0.25 μg partially purified CA-1. Fifty nanograms of competitor was used in lanes 3 to 6, and 8. Forty-five nanograms of competitor was used in lane 7. The competitor types are indicated on top of the lanes and described in Methods.

(C) DNA mobility shift assays using four different ACGT-containing probes. One-quarter microgram of partially purified CA-1 was used in lanes 2, 4, 6, and 8. ACGT-containing sequences of the probes are shown, with larger letters representing sequence motifs also found in the A2 fragment. ocsbf3, in vitro transcribed and translated cloned OCS binding factor 3 (Foley et al., 1993) was used in place of the Arabidopsis extract.

−127 to −134 is found in the palindromic element of the octopine synthase gene promoter in the Ti plasmid. This motif serves as the binding site for a number of bZIP transcription factors binding to the OCS element and called OCS binding factors (OCSBFs) (Singh et al., 1990). This sequence includes an ACGT core that has been found in the target sequences of a number of transcription factors (Katagiri and Chua, 1992). The CCACGT sequence from −131 to −136 is present in many G-box–related elements (Schindler et al., 1992b). The region from −129 to −114 includes 13 of 16 and 10 out of 12 bp identical to two separate regions of the *Lemna gibba cab*AB19 gene (Karlin-Neumann et al., 1985). These *cab*AB19 sequences are

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Figure 3. (continued).

(A) DNase I protection of the upper strand of the A2 fragment. Fr, free probe; Bd, probe + protein extract (see Methods); G+A, Maxam-Gilbert G+A sequencing reaction. The protected regions are indicated by lines, and the numbers indicate the positions relative to transcription start.

(B) Complete sequence of the 88-bp A2 fragment. The numbers indicate distance from transcription start. The nucleotides protected from DNase I digestion [see **(A)**] are overlined.

(C) Binding of CA-1 to the A2 probe and a cloned synthetic oligonucleotide probe corresponding to a head-to-tail dimer of the longest footprinted region (−111 to −138). Lanes 2 to 5 and 7 to 10 each contained 0.25 μg partially purified CA-1 (Figure 5). Each binding reaction contained 0.5 ng of radioactively labeled probe. Lanes 3 to 5 and 8 to 10 contained 25 ng of the competitors, as indicated on top of each lane.

(D) Mutations in the A2 sequence can affect the binding affinity of CA-1. Competition for binding of partially purified CA-1 (0.5 μg protein/reaction) to the radioactively labeled A2 fragment (0.2 ng/reaction). No Protein, free probe; No Comp., no additional DNA competitors added; Competitor lanes, 25 ng of the indicated competitor, as diagrammed at the bottom. Dashes in the mutant sequences (see Methods) represent nucleotides identical to the corresponding position in the A2 fragment.

both located in a 70-bp fragment of this *Lemna* promoter that has been shown to be necessary for phytochrome regulation (D. M. Kehoe and E. M. Tobin, unpublished data).

Figures 4B and 4C show the results of experiments designed to investigate whether CA-1 could bind to these closely related sequences. Both the wild type and a mutated version of the OCS element failed to compete for CA-1 binding with the A2 probe (Figure 4B, lanes 5 and 6). A cloned oligonucleotide in which the CA-1-protected region of the *cab*140 promoter was mutated by changing the ACGT to GTAC showed substantial competition (Figure 4B, lane 4). A 170-bp promoter fragment of the *Lemna cab*AB19 gene containing the homologous motifs was also able to compete (Figure 4B, lane 7). These data showed that the binding behavior of CA-1 differed from that of the ACGT binding factors, for which the ACGT core is essential for binding.

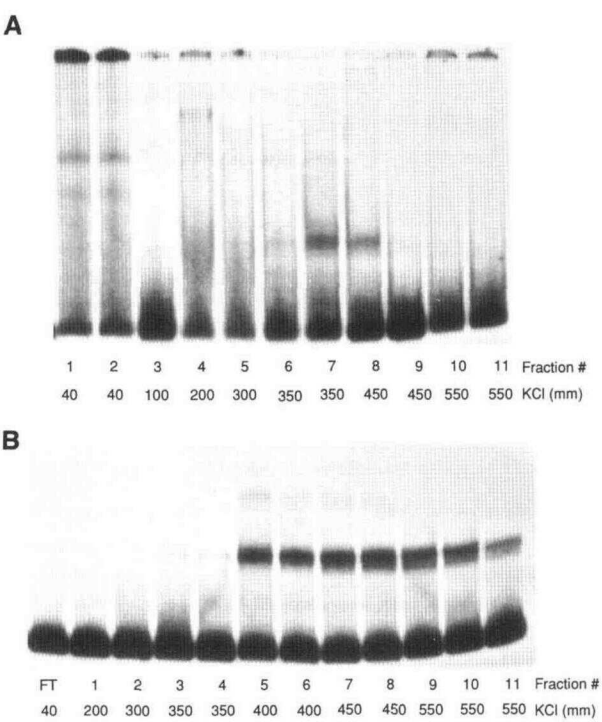

Figure 5. Partial Purification of CA-1.

(A) DNA mobility shift assays of fractions from a heparin-agarose chromatography column. The A2 fragment was radioactively labeled and used as the probe (0.5 ng per reaction). One and a half milliliters of whole cell protein extract (20.5 mg/mL) was loaded onto a 1.5-mL heparin-agarose column. Stepwise KCl elutions were carried out as indicated. Two microliters from each fraction was assayed.

(B) DNA mobility shift assays of fractions from a Bio-Rex 70 chromatography column. Radioactively labeled A2 fragment was used as the probe (0.5 ng per reaction). Peak CA-1–containing fractions from two heparin-agarose columns were pooled and loaded onto a Bio-Rex 70 column. Stepwise KCl elutions were carried out as indicated. Two microliters from each fraction was assayed.

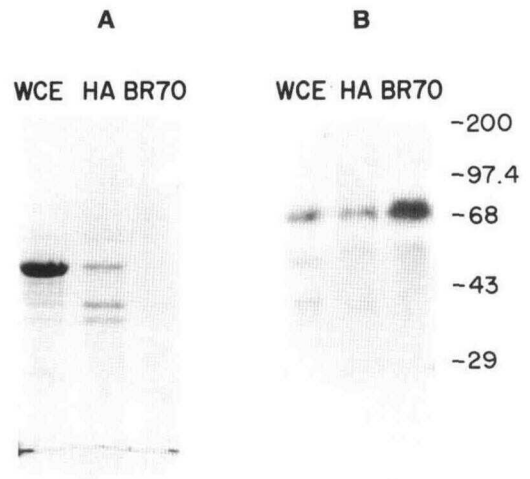

Figure 6. CA-1 May Be a 70-kD Protein.

Five micrograms of 50% ammonium sulfate precipitated whole cell protein extract (WCE lanes), 5 μg of heparin-agarose chromatography enriched CA-1 (HA lanes), or 3 μg of Bio-Rex 70 (BR70 lanes) partially purified CA-1 was electrophoresed on an SDS–polyacrylamide gel. Numbers on the right show the position and size in kilodaltons of molecular weight standards.

(A) Coomassie blue–stained gel.

(B) Autoradiogram of the gel after transferring to nitrocellulose membrane and probing with radioactively labeled A2.

This conclusion is further supported by the experiment shown in Figure 4C. A group of radioactively labeled probes containing the ACGT core, including the opaque-2 (O2) binding site (Schmidt et al., 1990), the abscisic acid–responsive element (ABRE; Guiltinan et al., 1990), a G-box (Giuliano et al., 1988), and the OCS element (Singh et al., 1990), could not bind to CA-1 (Figure 4C, lanes 1 to 8), whereas the OCS element bound to its cognate activity OCSBF3 (Figure 4C, lane 9). Furthermore, under identical conditions, CA-1 bound the A2 probe strongly (Figure 4B, lane 2). From these experiments, we conclude that CA-1 is not an ACGT binding factor and that a region of sequence similarity to the *Lemna cab*AB19 promoter plays an important role for CA-1 binding.

Partial Purification of CA-1

We used column chromatography to partially purify CA-1 from whole cell protein extracts prepared from light-grown plants. Figures 5A and 5B show DNA mobility shift assays of the fractions from the heparin-agarose and Bio-Rex 70 columns, respectively. CA-1 eluted at ~ 350 to 450 mM KCl from the heparin-agarose column and at ~ 400 to 500 mM KCl from the Bio-Rex 70 column.

We analyzed the proteins at the different stages of purification by SDS–polyacrylamide gel electrophoresis and by probing the protein blot with a radioactively labeled A2 fragment. Figure 6A shows the Coomassie blue–stained gel of

samples of the extracts taken after ammonium sulfate precipitation (WCE lane), the heparin-agarose column (HA lane), and the Bio-Rex 70 column (BR70 lane). Figure 6B is the autoradiograph of an identical gel blotted and probed with the radioactively labeled A2 DNA. A 70-kD protein was detected in all samples, but the amount of this protein was substantially enriched after the Bio-Rex 70 column (compare Figure 6B, BR70 lane, 3 μg protein, with WCE and HA lanes, 5μg protein each). This enrichment was achieved while other proteins were separated away, as shown in Figure 6A. Two minor bands with higher mobilities can also be seen in Figure 6B. However, no enrichment of these proteins was observed, suggesting that they do not represent specific binding. Thus, these data suggest that the 70-kD protein may represent CA-1.

Analysis of a Mutant Suggests That CA-1 May Be a Transcriptional Repressor

The mutant *det*1 of Arabidopsis is a photomorphogenic mutant that develops a light-grown morphology and expresses high levels of *cab* mRNA in the absence of light (Chory et al., 1989). We examined the expression of the *cab*140 gene in dark-grown seedlings of this mutant in comparison with the wild-type seedlings with regard to phytochrome regulation. A typical phytochrome-regulated pattern of *cab*140 mRNA levels was not detected in the *det*1 seedlings: no red light induction over the already substantial dark level was seen, as shown in Figure 7.

To examine whether the *det*1 mutation affects CA-1 binding activity, we compared protein extracts prepared from *det*1 and wild-type seedlings. Figure 8 shows that CA-1 DNA binding activity was not detected in the *det*1 seedlings grown in darkness with or without red light or white light treatment (*det*1 lanes). By contrast, CA-1 was present in wild-type seedlings grown under identical conditions (WT lanes). Additionally, no substantial difference in CA-1 binding activity was seen in the wild-type seedlings grown in complete darkness (D), given 1 min of red light 2 hr before harvesting (R), or given 2 hr of white light before harvesting (WL). As a control, a box II probe derived from the pea *rbcS*-3A gene was used to detect a GT-1–type activity, which was shown not to be affected by light in pea (Green et al., 1987). This activity was present in both the *det*1 and wild-type seedlings, indicating that the absence of CA-1 binding activity in *det*1 was not due to protein degradation during the extraction procedure. Interestingly, the GT-1 protein–DNA complex of *det*1 migrated faster than that of the wild type, which may reflect differences in protein modification. The absence of the CA-1, but not the GT-1, activity in the *det*1 seedlings, in combination with the high level of expression

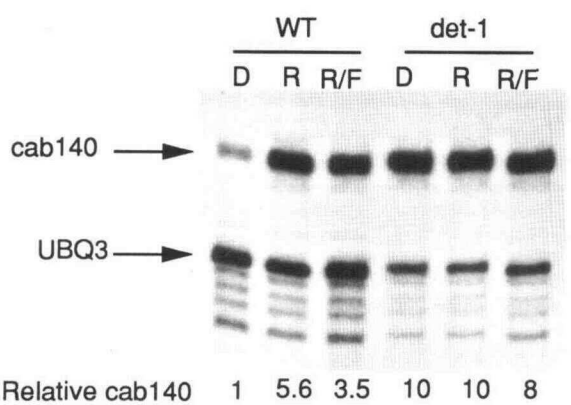

Figure 7. RNase Protection Assays Measuring *cab*140 and Ubiquitin mRNA Levels in Wild-Type and *det*1 Seedlings.

Total RNA was isolated from seedlings grown in complete darkness for 6 days and given the following treatments 2 hr before harvesting: D lanes, no light treatment given; R lanes, 1 min red light; R/F lanes, 1 min red light followed immediately by 10 min far-red light. Five micrograms RNA was used in each reaction. The number below each lane represents the relative *cab*140 mRNA level normalized to the ubiquitin mRNA level, as measured by radioactivity in each protected band.

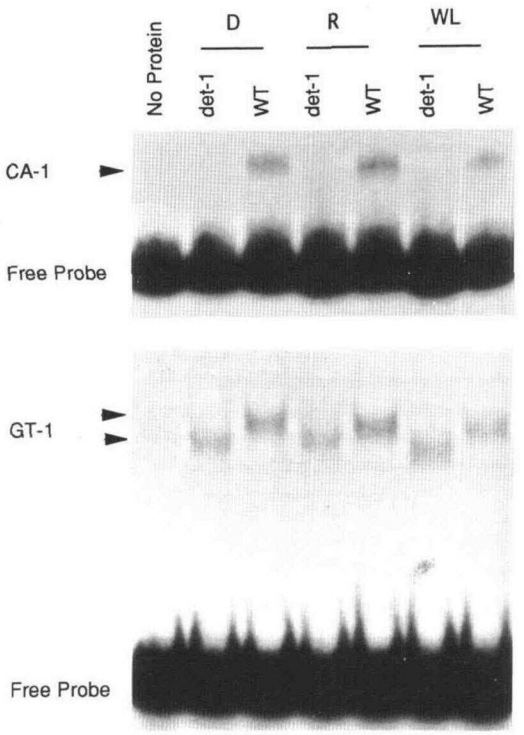

Figure 8. DNA Mobility Shift Assays Measuring CA-1 and GT-1 Activities in Wild-Type (WT) and Mutant (*det*1) Seedlings.

Whole cell protein extracts (50% ammonium sulfate precipitated) were isolated from seedlings grown in complete darkness for 6 days. D lanes, no light treatment given; R lanes, 1 min red light given 2 hr before harvesting; WL lanes, 2 hr white light given before harvesting. Radioactively labeled A2 or box II (Green et al., 1987) probes and 10 μg of protein were used in each reaction.

of the *cab*140 gene in dark-grown *det*1 seedlings, suggests that CA-1 may act as a transcriptional repressor.

Dephosphorylation Changes the Mobility of CA-1–DNA Complexes

The difference in migration of the GT-1–DNA complexes from wild-type and *det*1 seedlings suggested the possibility that the apparent absence of CA-1 binding activity in *det*1 plants might be due to protein modification. We therefore examined whether the phosphorylation state of CA-1 could affect its binding to the A2 DNA. Figure 9 shows the binding of alkaline phosphatase–treated CA-1 to the A2 DNA fragment in comparison with that of the nontreated CA-1. In the absence of phosphatase treatment, CA-1 formed two major slower migrating complexes (indicated by a and b) with the DNA (lane 9). However, after the partially purified proteins were treated with the phosphatase, these complexes were converted to a faster migrating complex (indicated by c) and the extent of the conversion was dependent on the enzyme concentration (lanes 1 to 4). The conversion was inhibited by incubation of the enzyme with 50 mM NaF prior to the addition of the partially purified proteins (lanes 5 to 8). NaF alone did not have any impact on CA-1 binding (data not shown). The binding of the dephosphorylated CA-1 to A2 remained sequence specific, as indicated by binding competitions shown in lanes 10 and 11, in which the proteins were treated with phosphatase before unlabeled competitor DNA was added. The A2 DNA fragment competed for binding to the dephosphorylated CA-1, whereas the A3 DNA fragment failed to compete (compare lanes 10 and 11 to lane 1, in which no competitor was present). These data demonstrated that dephosphorylation can increase the mobility of the CA-1/A2 complexes without loss of sequence specificity.

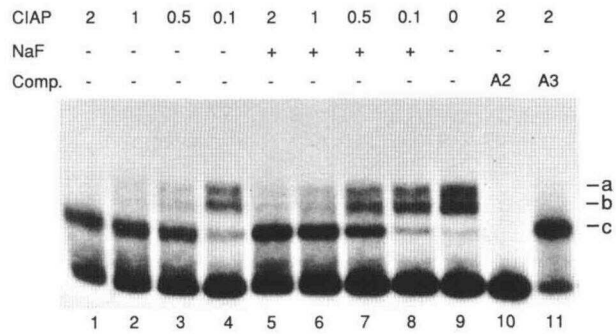

Figure 9. Dephosphorylation Alters the Migration of CA-1 in DNA Mobility Shift Assays.

Partially purified CA-1 was treated with (lanes 1 to 8, 10 and 11) or without (lane 9) calf intestine alkaline phosphatase (CIAP) in the absence (lanes 1 to 4 and 9 to 11) or presence (lanes 5 to 8) of 50 mM NaF. Then DNA binding reactions were carried out. The numbers above each lane indicate the amount of CIAP used in mg/mL. One milligram of solid CIAP equals 1.9 units of enzyme (Sigma). Twenty-five nanograms of unlabeled A2 (lane 10) or A3 (lane 11) was used as a competitor; 0.41 μg of partially purified CA-1 and ~ 0.1 ng of radioactively labeled A2 fragment were used in each reaction. The complexes representing the phosphorylated forms (a and b) and the nonphosphorylated form (c) are indicated on the right.

DISCUSSION

What Functional Role May CA-1 Play in Regulating *cab*140 Gene Expression?

Several lines of evidence suggest that CA-1 functions in regulating the expression of the *cab*140 gene. First, the site to which it binds is located in the region of the promoter that was required for reporter gene expression in transgenic plants (Figure 1). The deletion analyses also showed that the region downstream of −183 was necessary for phytochrome regulation. Second, the CA-1 footprinted region has sequence similarities to promoters of other light-regulated *cab* and *rbcS* genes, including the phytochrome-responsive promoter of a *Lemna cab* gene (AB19) and an octamer repeat region involved in the light regulation of a rice *cab* gene (Luan and Bogorad, 1992). There is also some homology to the heptamer motif identified by Argüello et al. (1992) in a pea *cab* gene. A promoter fragment of the *Lemna cab*AB19 gene that contained the region with sequence similarity competed with the *cab*140 A2 fragment for CA-1 binding (Figure 4B). Furthermore, when a 70-bp region including the sequence identities to the CA-1 footprinted region was deleted from the *Lemna cab*AB19 promoter, phytochrome responsiveness was abolished while significant expression levels were retained (D. M. Kehoe and E. M. Tobin, unpublished data).

Finally, in dark-grown seedlings of the photomorphogenic mutant *det*1, the CA-1 binding activity was not detectable (Figure 8). Because this mutation releases repression of the expression of light-regulated genes and allows the development of a light-grown morphology when seedlings are grown in the dark (Chory et al., 1989), it is possible that CA-1 may be involved in repression of *cab*140 gene expression. This notion is supported by the fact that in the absence of CA-1 binding activity in the dark-grown *det*1 seedlings, the *cab*140 transcript level was 10 times higher than that in the wild-type seedlings (compare the D lanes in Figure 7A). Together, these data suggest a functional relatedness of the CA-rich motifs in these promoters.

The CA-1 Target Sequence Differs from ACGT-Containing and GT Elements

We noted in the *cab*140 A2 fragment the existence of two sequence motifs with homology to previously identified elements

that have been shown to be the target sequences of a number of transcription factors. These include the $^{-136}$CCACGTAAGC^{-127} and the $^{-133}$GTGGTTAAT^{-141} motifs.

The $^{-136}$CCACGTAAGC^{-127} motif contains an ACGT core, has sequence homology to the G-box, and includes a perfect match to half of the 16-bp OCS palindromic element (Ellis et al., 1987). Several plant transcription factors that recognize sequences containing an ACGT core have been identified. These include the G-box binding factors (Giuliano et al., 1988; Schindler et al., 1992a), OCS binding factors (Singh et al., 1990), EmBP-1 (Guiltinan et al., 1990), O2 (Schmidt et al., 1990), HBP-1a, 1b (Tabata et al., 1991), CPRFs (Weisshaar et al., 1991), TAF-1 (Oeda et al., 1991), ASF-1 (Lam et al., 1989), and TGA-1a, 1b (Katagiri et al., 1989). A common feature of these factors is that the ACGT core is essential for binding. We investigated whether this sequence core was also essential for CA-1 binding. The data in Figure 4 demonstrated that the ACGT core was not essential for CA-1 to bind. Further, an oligonucleotide containing the sequence CCACGTAA, which corresponds to the binding site of CUF-1, a factor that interacts with the Arabidopsis *cab*165 gene (S. Kay, personal communication), failed to compete for CA-1 binding (L. Sun and E. M. Tobin, unpublished data). We concluded that CA-1 binding behavior differs from these factors. It should be noted, however, that the ACGT motif is located in such close proximity to the CA motif that binding of CA-1 also protected it from DNase I digestion (Figure 3A), indicating that CA-1 may sterically interfere with DNase I digestion of this region.

The $^{-133}$GTGGTTAAT^{-141} motif in the *cab*140 gene is contained in box II (GTGTGGTTAATATG) of the pea *rbcS*-3A gene that interacts with the GT-1 factor (Green et al., 1987). The GGTTAA core within this motif is critical for GT-1 binding (Green et al., 1988). cDNA clones that encode GT-1–related proteins have been isolated recently (Gilmartin et al., 1992; Perisic and Lam, 1992). This GT motif, present in inverted orientation in the *cab*140 A2 fragment, does not, however, appear to play an important role in CA-1 binding in vitro. This is evidenced by the ability of a mutated version of the CA-1 footprinted region lacking the GT motif to interact with CA-1 (Figure 4B, lane 4). Additionally, a mutated version of the *cab*140 A2 fragment that kept the GT motif intact failed to bind to CA-1 (Figure 3D).

It is intriguing to note the existence of these motifs in close proximity in the *cab*140 promoter. The interactions of factors that bind to these motifs may be functionally important in vivo for regulating the expression of the *cab*140 gene.

CA-1 Can Bind to A2 in Vitro in Both Its Phosphorylated and Dephosphorylated Forms

Unlike many other transcription factors whose DNA binding activities are affected by phosphorylation, CA-1 binding activity in vitro was neither inhibited nor stimulated by phosphorylation or dephosphorylation. Rather, the migration of the protein–DNA complexes was affected, whereas the binding affinity and specificity remained unchanged (Figure 9). Phosphorylated CA-1 formed two protein–DNA complexes that migrated more slowly than the one formed by the dephosphorylated CA-1. Phosphorylation has been shown to result in shifting of such complexes (e.g., Sarokin and Chua, 1992). It is also possible that the dephosphorylated complex may consist of CA-1 monomers complexed with the DNA, whereas the phosphorylated complexes could represent either multimeric forms of CA-1 or additional protein(s) associated with CA-1. The fact that the SDS-denatured proteins could bind to the DNA as demonstrated in the protein blotting analysis (Figure 6) suggests that no additional proteins are required for CA-1 to interact with the DNA.

A possible functional role of CA-1 phosphorylation is not yet known. We did not find differences in CA-1 phosphorylation in extracts prepared from seedlings grown in complete darkness and given different light treatments (Figure 7B). Dephosphorylation did not affect the binding affinity or specificity of CA-1 in the in vitro assay. Thus, the lack of CA-1 activity in the *det*1 mutant could not easily be explained by a change in its phosphorylation state. We cannot rule out the possibility that the phosphorylation state of CA-1 in vivo might affect its stability.

A Model for CA-1 Function in Regulation of *cab*140 Gene Expression

Phytochrome-regulated transcription can be achieved through two possible mechanisms. The first may involve red light–stimulated activation of a positive regulatory protein whose interactions with the promoter and/or other proteins would result in elevated transcription. The other mechanism may involve red light–stimulated inactivation of a repressor that would

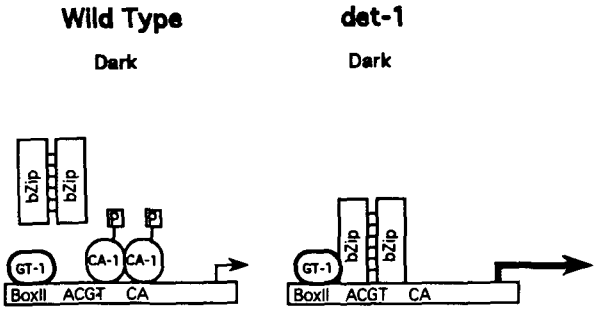

Figure 10. Schematic Representation of a Model for CA-1 Function as a Repressor.

bZIP, GT-1, and CA-1 represent transcription factors. P represents a phosphate group. The horizontal rectangle represents the *cab*140 promoter with sequence elements identified as box II, ACGT, and CA. The arrows indicate transcription, and the thickness and length of these arrows indicate the relative amount of transcription for the wild-type and *det*1 plants grown in darkness. See text for full explanation.

otherwise repress transcription in complete darkness. The inactivation of such a repressing mechanism would then allow elevated transcription to occur. These two mechanisms would not be mutually exclusive.

Our studies suggest that CA-1 qualifies as a candidate for such a repressor because the lack of CA-1 binding activity coincided with elevated *cab*140 gene expression in the *det*1 seedlings (Figures 7 and 8). Figure 10 shows a representation of a model for how CA-1 may act in conjunction with transcriptional activators in regulating *cab*140 gene expression. In dark-grown wild-type seedlings, phosphorylated CA-1 would be bound to the promoter, possibly in a multimeric form. This binding would prevent binding of an activator, such as a bZIP factor, that could recognize the nearby ACGT sequence. In addition, it might prevent a productive interaction between such a factor and a GT-type factor. Thus, transcription would only occur at a low level. In *det*1 seedlings, CA-1 activity is absent, and the other factors would be able to enhance transcription of the gene.

The presence of the ACGT and box II–like motifs in close proximity to the CA-1 binding site would provide a physical basis for transcriptional repression by CA-1. In fact, in vitro CA-1 binding to the A2 fragment protected the ACGT motif from DNase I digestion (Figure 3A). Box II and some ACGT-containing sequences have been shown to activate gene expression (for example, Donald et al., 1990; Lam and Chua, 1990). Consistent with our model's prediction that GT-like and bZIP factors interact is the finding that box II, when fused to the cauliflower mosaic virus 35S promoter, requires the presence of the as-1, an ACGT-containing element, to function (Lam and Chua, 1990). In addition, GT-1 has been shown to be expressed in darkness as well as in the light (Green et al., 1988), and a similar activity is present at equivalent levels in *det*1 and wild-type Arabidopsis seedlings (Figure 8).

According to this model, we might expect that the amount of CA-1 binding activity would vary in dark-grown and light-treated plants. However, equivalent amounts of CA-1 binding activity were present in extracts prepared from 6-day-old wild-type seedlings grown in darkness, or darkness plus short red or white light treatments. CA-1 is also present in 3-week-old wild-type plants grown in continuous white light. We are not yet able to provide evidence for a mechanism by which CA-1 might function in vivo. However, the absence of CA-1 activity in the *det*1 mutant suggests that it might play an important role in the light regulation of the *cab*140 gene in Arabidopsis.

METHODS

Plant Material

The wild-type seeds of *Arabidopsis thaliana* (Heynh) Columbia strain were obtained from plants grown in our greenhouse from February 1988 through April 1988. The *det*1 and transformed tobacco seeds were kindly provided by Dr. J. Chory and Dr. G. An, respectively.

Protein Extract Preparation and Chromatography

Arabidopsis whole cell protein extracts were prepared according to the method of Green et al. (1989). Whole cell protein extracts were fractionated through heparin-agarose (Bio-Rad, Richmond, CA) chromatography and eluted stepwise with 100, 200, 300, 350, 400, 450, and 550 mM KCl in nuclear extraction buffer (NEB; 25 mM Hepes-NaOH, pH 7.2, 40 mM KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol, 10% glycerol; Green et al., 1989). Peak fractions were pooled and further fractionated through ion exchange chromatography (Bio-Rex 70; Bio-Rad) and eluted stepwise with 200, 300, 350, 400, 450, and 550 mM KCl in NEB. Peak fractions were pooled and concentrated through a Centricon-30 microconcentrator (Amicon, Beverly, MA) for small-scale preparations. For large-scale preparations, a stir cell using a YM10 membrane (Amicon) was used. Protein concentrations were determined by the Bradford assay (Bio-Rad).

DNA Mobility Shift and DNase I Footprinting

*Cab*140 5′ regulatory regions were subcloned into pBluescript KS−. The probes used for gel shift and DNase I footprinting were derived from a subclone containing the A2 fragment (−160 to −73). Plasmid DNA was digested with desired restriction enzymes and labeled with α-^{32}P-dNTPs by end-filling with Klenow (Bethesda Research Laboratories, Gaithersburg, MD). The probe was then purified through gel electrophoresis.

DNA binding reactions were carried out in 0.5-mL Eppendorf tubes in 10-μL NEB containing 0.2 to 1 μg partially purified proteins or 5 to 15 μg whole cell proteins, 0.1 mg/mL BSA, 0.1 to 0.5 ng labeled probe, and 0.5 to 2 μg poly (dIdC)/μg protein. After a 15-min incubation at 30°C, the samples were separated on an 8% polyacrylamide gel (1:29 bis/acrylamide) in low ionic strength buffer (Ausubel et al., 1987). The gel was then dried and autoradiographed.

DNase I footprinting was done according to a modification of the method of Green et al. (1989). DNA binding reactions were carried out as described above. One microgram partially purified proteins from the Bio-Rex 70 column (or no protein for the control), 1.5 μg poly (dIdC), and 0.2 ng probe were used in a 10-μL reaction. The samples were then treated with 0.5 μg/mL DNase I (Worthington) for exactly 1 min at room temperature in the presence of 2 mM $CaCl_2$ and 10 mM $MgCl_2$. The reaction was stopped by addition of 5 μL of DNase I stop mix (1:1 mix of 200 mM EDTA and 10 mg/mL proteinase K in NEB) and incubation for 20 min at 37°C. Thirty-five microliters of extraction buffer (6 M urea, 0.36 M NaCl, 1% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 8.0), 6 μL of 7.5 M ammonium acetate, and 1 μL of 10 mg/mL tRNA were mixed with the sample, and the mix was extracted with 70 μL of phenol-sevag (phenol/chloroform/isoamylalcohol 25:24:1) once and precipitated with 2.5 volumes ethanol. Samples were then pelleted, resuspended in formamide loading buffer, and separated on a 6% denaturing polyacrylamide gel.

Probes and DNA Fragments

Fragments A, A1, A2, and A3. Promoter fragments (−248 to +12, +12 to −72, −73 to −160, −161 to −248, respectively) of the *cab*140 gene were subcloned into the BamHI (A) or SmaI sites (A1, A2, A3) of pBluescript KS−. DNA fragments were prepared according to standard procedures.

Dimer. Two tandem copies of a synthetic oligonucleotide corresponding to the DNase I protected region (−111 to −138; Figure 3A) and with BglII and BamHI linkers were cloned into the BamHI site of pBluescript KS−. The DNA fragment was prepared according to standard procedures.

m-1. Oligonucleotides of the A2 fragment (Figure 3B) with nine base substitutions, as shown in Figure 3D, were synthesized. The two complementary strands were annealed and used in the competitions.

m-2 and m-3. Synthetic oligonucleotides corresponding to the −99 to −138 region of the A2 fragment with mutations are shown in Figure 3D. Complementary strands were annealed and used in competitions.

mut ACGT. Mutated oligonucleotides changing ACGT to GTAC in the DNase I footprinted region (−111 to −138) were synthesized. Three copies were cloned into the BamHI site of Bluescript KS− and the insert sequenced. One of the three copies had deletions at positions −137, −132, and −120. A DNA fragment consisting of the three copies was used as a competitor for binding.

WT OCS and mut OCS. DNA fragments contain the wild type and a mutated version of the OCS element (Singh et al., 1990).

AB19. A 170-bp fragment is from the promoter of the *Lemna cab* gene (−70 to −240), AB19 (Karlin-Neumann et al., 1985).

C100. A cloned 100-bp fragment is from the region 3′ to the *Lemna cab* gene AB30. It was used as a nonspecific competitor.

O2, ABRE, G-Box, and OCS. Radioactively labeled probes contain the O2 binding site (Schmidt et al., 1990), the abscisic acid–responsive element (Guiltinan et al., 1990), a G-Box (Giuliano et al., 1988), and the OCS element (Singh et al., 1990), respectively.

Assay of Phytochrome Responsiveness of *cab*140 Deletion Constructs in Transformed Tobacco Seedlings

Five prime promoter deletion constructs of the *cab*140 gene (including a substantial part of the coding region) fused to the CAT gene were analyzed in seeds collected from individual primary transformants (Ha and An, 1988). Samples of ~ 300 seeds were germinated and grown in compete darkness (D) or with 2 min of red light every 8 hr (R) or 2 min of red light followed immediately by 10 min of far-red light every 8 hr (R/FR). Seedlings were harvested after 8 to 11 days and analyzed for CAT activity essentially as described by An (1987) with some modifications. Extraction buffer/tissue was 0.5 μL/mg. Fifty nanocuries ^{14}C-chloramphenicol (CAP; Du Pont–New England Nuclear) with a specific activity of 60 mCi/mmol was used in each assay. Aliquots of the extracts containing 1 to 200 μg protein were used. After quantitation by scintillation counting of the separated chloramphenicol and the acetylated products, the results were expressed as percentage of the ^{14}C-CAP acetylated. Bacterial extract containing pBR325 was used as the control; 100 μg total bacterial protein resulted in 99% conversion. To make valid comparisons between light treatments, the linear range of the reaction was determined for each of the constructs, as each had different amounts of activity per milligram of total protein. This was accomplished by performing the assay with various amounts of total protein, ranging from 0.5 to 200 μg. The amounts used for a given construct are indicated in the legend to Figure 1.

Protein Gel Electrophoresis and Blot Analysis

Protein extracts were separated by discontinuous SDS-PAGE, 4.1% stacking gel, and 8% separating gel. Prestained protein molecular standards were from Bethesda Research Laboratories (high range) and Sigma (MW-SDS-70L). The gel was stained with Coomassie Blue R-250 and electroblotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a protein transfer device (Bio-Rad). Protein blot analysis was done according to the method of Miskimins et al. (1985) with modifications. All of the following steps were carried out at room temperature and with 50 rpm shaking. Briefly, the membrane was blocked with 5% nonfat milk in NEB containing no glycerol (25 mM Hepes-NaOH, pH 7.2, 40 mM KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol; Green et al., 1989) for 1.5 hr. The membrane was washed with NEB containing 0.25% nonfat milk for 5 min, and the membrane was incubated for 3.5 hr in NEB containing 2.25 × 10^5 cpm/mL radioactively labeled A2 probe, 50 μg/mL herring sperm DNA. After briefly rinsing the membrane with NEB, two washes were given in NEB containing 0.1% Triton-X 100, one for 30 min and the other for 5 min. The membrane was air dried and exposed to x-ray film.

Alkaline Phosphatase Treatments

Alkaline phosphatase treatments were performed according to the method of Sarokin and Chua (1992) with modifications. The desired amount (2, 1, 0.5, or 0.1 mg/mL) of calf intestinal alkaline phosphatase (CIAP, 1 mg of solid equals 1.9 units; Sigma) was incubated on ice for 15 min in the presence or absence of 50 mM NaF. Partially purified CA-1 (0.41 μg) was added and incubated for 10 min at room temperature before the radioactively labeled A2 probe was added. Then DNA mobility shift assays were carried out as described above.

RNase Protection

RNase protection using a probe specific for the *cab*140 gene and the ubiquitin 3 gene was performed as described by Brusslan and Tobin (1992).

ACKNOWLEDGMENTS

This research was supported by National Institutes of Health Grant No. GM-23167. We are grateful to Dr. Gynheung An (Washington State University) for providing us with seeds of tobacco transformed with his *cab*::CAT deletion constructs, to Dr. Joanne Chory (Salk Institute) for providing the *det*1 mutant, and to Drs. Nam-Hai Chua and Steve Kay (The Rockefeller University) for the box II–cloned oligonucleotide. Dr. Kay also suggested the use of the Bio-Rex 70 column. We thank

Dr. Karam Singh for the O2, ABRE, OCS, and G-box probes and the OCS BF3 protein; Dr. David Kehoe for the *Lemna cab*AB19 fragment and C100 DNA; Lu Huang for help with the Arabidopsis plants; and Dr. Sharlene Weatherwax for stimulating and helpful advice and discussions.

Received November 19, 1992; accepted November 30, 1992.

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