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Multiple DNA–Protein Complexes at a Circadian-Regulated Promoter Element

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Higher plant *CAB* genes encode chlorophyll *a/b* binding proteins that are part of light-harvesting complexes in chloroplasts. Transcription of the Arabidopsis *CAB2* (*lhcb1*1*) gene is under the control of a circadian oscillator and exhibits high amplitude diurnal oscillations that persist within a period close to 24 hr in the absence of environmental time cues. Initial deletion studies in transgenic tobacco have demonstrated that the region between −111 and −38 of the *CAB2* promoter sequence confers circadian regulation to a luciferase (*luc*) reporter gene. We dissected this element further and characterized five DNA binding complexes from Arabidopsis whole-cell extracts that bind within this region of the promoter and may be components of the signal transduction pathway for the control of transcription by the circadian clock. The in vivo analysis of *cab2::luc* fusion constructs in transgenic Arabidopsis demonstrated that a circadian-regulated element lies within a 36-bp sequence that overlaps a conserved CCAAT box and contains binding sites for three putative transcription factors.

INTRODUCTION

Many cellular, physiological, and behavioral activities exhibit daily oscillations, adjusting continuously to changes in environment caused by the alternation of night and day. Some of these oscillations persist in the absence of environmental time cues, with a period that only approximates 24 hr. This free-running period is the hallmark of an endogenous oscillator, or circadian clock, which in natural conditions is entrained to an exact 24-hr period by the diurnal light or temperature cycles. This endogenous timer permits the anticipation of daily changes in environmental conditions (Edmunds, 1988).

The molecular basis of circadian rhythms is still poorly understood, although genes that are required for rhythmicity have been cloned from Drosophila and Neurospora (Dunlap, 1993; Hardin and Siwicki, 1995; Loros, 1995). The mechanisms by which a central oscillator controls multiple rhythmic outputs are still to be determined. One early step in the output pathway(s) for physiological rhythms is likely to be the control of transcription of a subset of genes, and it would be of interest to identify those transcription factors that act downstream of the oscillator to modulate gene expression. In Drosophila, the *per* gene product is expressed rhythmically (Hardin et al., 1992) and regulates its own transcription (Zeng et al., 1994). There is no evidence that the PER protein interacts directly with its own promoter, however. In rats, expression of the transcriptional activator DBP in the liver (Wuarin and Schibler, 1990) and of the repressor of cAMP-induced transcription ICER (inducible cAMP early repressor) in the pineal gland (Stehle et al., 1993) has been shown to exhibit circadian rhythmicity. The rhythmic expression of ICER and DBP is thought to reflect rhythmic hormonal input from the central oscillator located in the SCN (suprachiasmatic nucleus), and its regulation may lie several steps downstream from the central oscillator located in the SCN. The pathway for the circadian regulation of transcription may be easier to define in plants, because circadian regulation has been shown to function in protoplasts (Kim et al., 1993) in the absence of systemic signals. The expression of many higher plant genes has been shown to be regulated by the circadian clock, including RNA binding proteins (Carpenter et al., 1994; Heintzen et al., 1994), catalase, nitrate reductase (Pilgrim et al., 1993), and genes that play a role in photosynthesis, such as the small subunit of ribulose bisphosphate carboxylase (*rbcS*) (Pilgrim and McClung, 1993) and chlorophyll *a/b* binding protein (*CAB*) (Kloppstech, 1985; Kellmann et al., 1993).

Transcription of *CAB* genes is tissue specific and is regulated by a network of signals, including plant growth hormones, light, and a circadian clock. A 268-bp fragment of the wheat *CAB1* gene directs phytochrome- and circadian-regulated transcription in transgenic tobacco (Fejes et al., 1990). Similarly, the −111 to −38 region sequence of the Arabidopsis *CAB2* (*lhcb1*1*) promoter has been shown to be sufficient for induction of a luciferase (*luc*) reporter gene by red light in etiolated tobacco plants as well as for rhythmic expression in green tissue (Anderson et al., 1994). Importantly, the phase and the period of the rhythm were identical to those conferred by our pseudo–wild-type *CAB2* fragment (extending from −322 to +1). The −111 to −38 region of the *CAB2* promoter contains a

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CCAAT box and three GATA motifs (Figure 1). The sequence and spacing of these motifs are highly conserved between many *CAB* promoters from several species (Castresana et al., 1987; Gidoni et al., 1989; Mitra et al., 1989; Piechulla et al., 1991; Anderson et al., 1994), suggesting critical roles in the regulation of *CAB* expression. An activity named CGF-1 (for *CAB* GATA factor 1), identified in tobacco nuclear extracts, binds the triple GATA repeat within the −78 to −44 sequence of *CAB2* (Anderson et al., 1994). CGF-1 has been shown to be closely related (or possibly identical) to the GT-1 complex, which plays a role in the regulation of the pea *rbcsS-3A* promoter by the red light photoreceptor phytochrome (Green et al., 1988; Gilmartin and Chua, 1990; Dehesh et al., 1992; Teakle and Kay, 1995). The role of CGF-1 in the phytochrome regulation of *CAB2* expression was also demonstrated by the finding that mutations of the GATA repeats that prevent CGF binding strongly reduce the acute induction of *cab2::luc* expression by light in etiolated seedlings (Anderson and Kay, 1995). Another complex named CUF-1 (for *CAB* upstream factor 1) binds a ACGT (G box–type) sequence in the −124 to −127 region and is not required for circadian or phytochrome regulation.

This article provides a more detailed analysis of the *CAB2* promoter that was conducted with Arabidopsis. Five novel protein complexes that bind the −111 to −55 region were identified in whole-cell extracts. In vivo analysis of a new set of *cab2::luc* fusions demonstrated that a circadian-regulated element lies within the region between −111 and −74 of the promoter sequence that overlaps a conserved CCAAT box and contains binding sites for three putative transcription factors.

RESULTS

Five DNA Binding Complexes Bind the −111 to −38 Region in Arabidopsis Extracts

DNA binding activities in Arabidopsis whole-cell extracts were identified by gel mobility shift assays using a radiolabeled probe comprising *CAB2* sequences from −111 to −38 (Figure 2A). A low electrophoretic mobility complex (a) and another band corresponding to at least two complexes (b) were resolved by this approach. The binding of these complexes to DNA was stimulated by 10 mM $MgCl_2$, a concentration that strongly inhibits the CGF-1 activity previously identified in tobacco extracts using an identical probe (Anderson et al., 1994; Teakle and Kay, 1995).

To facilitate the characterization of comigrating complexes, extracts were fractionated by cation exchange chromatography (see Methods). Two DNA binding activities named CUF-2 and CUF-3 were retained on the column and eluted with 0.15 and 0.25 M KCl, respectively. Three fractions were isolated that corresponded to the peak of CUF-2 activity (CM–CUF-2), the peak of CUF-3 activity (CM–CUF-3), and the flow through of the column (CM-FT). DNA sequences required for binding by these activities were defined by gel shift assays using a set of small overlapping *CAB2* promoter fragments. CUF-2 and CUF-3 bound a −111 to −74 probe (Figure 2B). Their affinity for the −111 to −74 probe was low, however, because the −111 to −74 sequence did not compete for CUF-2 or CUF-3 binding to the −111 to −38 probe. Affinity was increased by additional sequences either upstream or downstream, as demonstrated by competition assays with the −123 to −74 and −111 to −55 fragments (data not shown). Binding was also detected in the flowthrough fraction (data not shown). This may correspond in part to CUF-2 and CUF-3 activities that were not completely retained by the column but may also correspond to the Tac complex (see below) or to yet another activity.

We also tested for binding of partially purified CUF-2 and CUF-3 to the −94 to −65 region (centered on a conserved CCAAT box) and to the −120 to −99 region (centered on a GATA motif). No binding was detected to either of these probes, indicating that the sequences tested are not sufficient for the binding of these complexes (Figures 2C and 2D, lanes containing CM–CUF-2 and CM–CUF-3). In contrast, the −94 to −65 probe identified two other complexes named Tic and Tac (Figure 2C), and the −120 to −99 probe identified another DNA binding activity named Toe (Figure 2D). These three complexes did not bind the CM column but were identified in the CM-FT fraction. The fact that these activities were absent from the CM–CUF-2 and CM–CUF-3 fractions indicates that Tic, Tac, and Toe correspond to distinct biochemical activities from CUF-2 and CUF-3. Competition assays (data not shown) indicated that Tic and Tac do not bind the −120 to −99 probe and

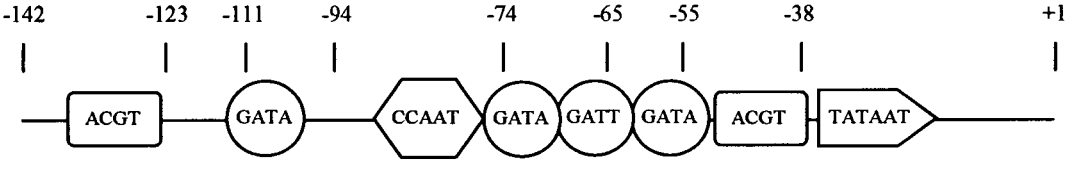


Figure 1. Structure of the *CAB2* Proximal Promoter.

The −94 to −55 region contains a CCAAT box and three GATA repeats, whose sequence and spacing are conserved in many *CAB* promoters. An additional GATA motif is present at position −109 to −106, and two ACGT sequences (G boxes) that are bound by leucine zipper types of transcription factors are present at positions −127 to −124 and −48 to −45, respectively.

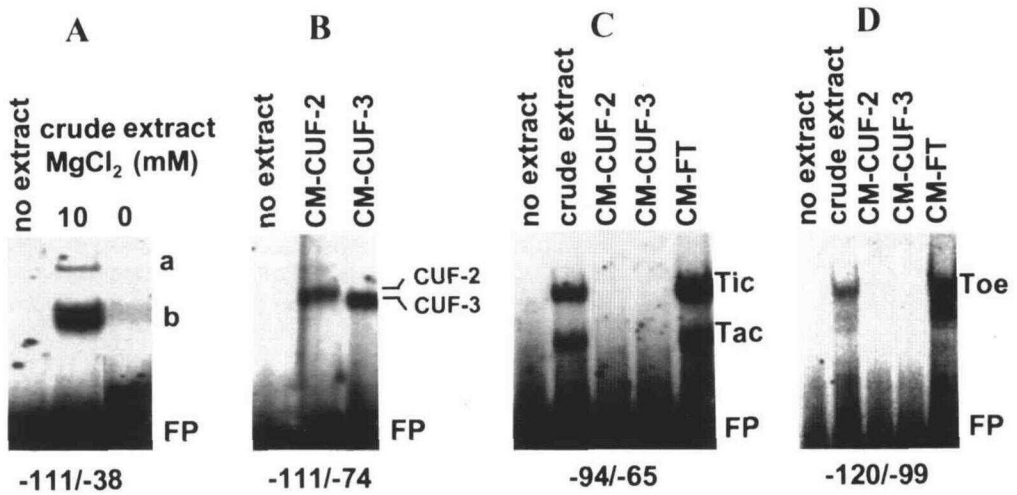


Figure 2. Identification of Five Complexes Binding between Positions −111 and −38 by Gel Shift Assays.

Arabidopsis whole-cell extracts were fractionated by cation exchange chromatography on a CM column. Three fractions that corresponded to the flowthrough of the column (CM-FT) and to the elution peaks for two DNA binding complexes (CM–CUF-2 and CM–CUF-3) were isolated. The position of the DNA–protein complexes and of the free probe (FP) are indicated to the right of the gels.

(A) The −111 to −38 probe identified a slowly migrating complex (a) in the crude extract and at least two others that migrated as a doublet (b).

(B) Both partially purified CUF-2 and CUF-3 bound a −111 to −74 probe.

(C) The −94 to −65 probe identified two complexes, Tic and Tac, that were present in the flowthrough fraction but were absent from the CM–CUF-2 and CM–CUF-3 fractions.

(D) The −120 to −99 probe identified another complex named Toe that was also present in the flowthrough fraction.

that Toe does not bind the −94 to −65 sequence. These results demonstrate that the binding specificity of the Toe complex is distinct from that of Tic and Tac.

The Tic and Tac Complexes Have Distinct DNA Binding Specificities

Both Tic and Tac bind within a 31-bp sequence (−94 to −65) of the *CAB2* promoter. To examine whether Tic and Tac compete for binding to the same site or bind adjacent sequences, we synthesized a set of five −94 to −65 oligonucleotides carrying point mutations, as described in Figure 3A. The effects of the mutations were tested in competition assays using radiolabeled −94 to −65 wild-type oligonucleotide as the probe (Figures 3B and 3C). All of the mutant oligonucleotides competed efficiently for binding when tested at high molar excess concentrations. This result indicated that none of the mutations was fully effective in preventing Tic or Tac binding. However, some of the mutations caused differential decreases in the affinity of Tic and Tac for the oligonucleotide. The AATm mutant oligonucleotide competed for binding of Tac to the labeled probe less efficiently than the wild-type sequence at all concentrations tested, indicating that the corresponding mutation impairs Tac binding. The CCm mutation also reduced competition for Tac binding although to a lower extent. In contrast, competition for Tic binding was severely impaired by the Am and the GATm mutations, which lie farther downstream in the promoter. The TGm mutation had no significant effects on either Tic or Tac binding.

These results indicate that Tic and Tac have distinct binding specificities within the −94 to −65 sequence. Tac binds the CCAAT box and sequences immediately upstream, whereas the binding site for Tic is located 3′ of the CCAAT box and overlaps two GATA repeats in the 3′ half of the −94 to −65 oligonucleotide. Interestingly, the AAT mutation, which most impaired Tac binding, competed for Tic binding more efficiently than did the wild-type probe. Similarly, the Am and GATm oligonucleotides, which have a reduced affinity for Tic, competed for Tac binding more efficiently than did the wild-type probe (Figure 3C). These observations suggest that mutations that decrease binding of one activity favor the binding of the other. The simplest explanation may be that the binding of Tic and Tac is mutually exclusive, possibly due to an overlap between their respective binding sites.

Tic and CGF Are Distinct Activities Whose Binding Is Impaired by Deletion of the Region between −74 and −55

The CGF-1 complex previously identified in tobacco nuclear extracts (Anderson et al., 1994) could not be detected in Arabidopsis extracts using the −111 to −38 probe (data not

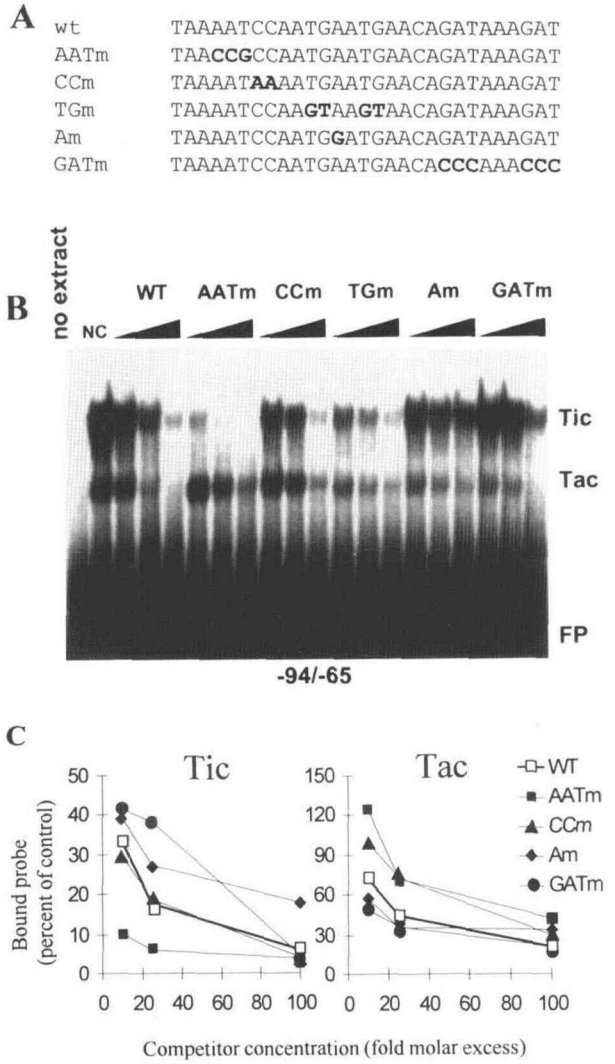


Figure 3. Definition of Tic and Tac Binding Domains.

(A) Set of mutant oligonucleotides used in competition assays in **(B)**.
(B) Gel shift assay using the wild-type (WT) sequence from positions −94 to −65 as a probe and 4 μg of total protein from an Arabidopsis whole-cell extract per lane. Probe sequences are indicated under the corresponding autoradiograms. The position of the DNA–protein complexes and of the free probe (FP) are indicated to the right of the gels. NC indicates assays performed in the absence of competitor DNA. The names of competitors are shown at the top of the gel. Graded triangles indicate the gradient of competitor concentrations (10-, 25-, and 100-fold molar excess).
(C) PhosphorImager quantitation of competition assays shown in **(B)**.

shown). CGF-1 has been shown to be related to GT-1, a transcription factor that plays a role in the induction of *rbcS* gene expression by light (Teakle and Kay, 1995). Cloned, bacterially expressed Arabidopsis GT-1 has been shown to bind the −111 to −38 fragment of the *CAB2* promoter in gel shift assays (Hiratsuka et al., 1994; S.L. Anderson and S.A. Kay, unpublished data), suggesting that these two activities may in fact be identical. However, the affinity of cloned GT-1 for the *CAB2* probe was much lower than that for the *rbcS* box II probe (Lam and Chua, 1990). Thus, our failure to detect CGF-1 (although we can detect GT-1 using a box II probe) may have been due to the lower abundance of GT-1 in Arabidopsis whole-cell extracts.

The fact that Tic binding is stimulated by $MgCl_2$, rather than inhibited as for CGF-1, suggested that Tic and CGF-1 are distinct binding activities, even though both require the presence of GATA motifs for binding. To confirm this hypothesis, we tested the binding specificities of Tic and CGF-1 in competition assays. CGF-1 was assayed in tobacco nuclear extracts (where it is abundant and can be easily detected) in the absence of $MgCl_2$, using the −111 to −38 *CAB2* region as a probe (Figure 4A). Tic was assayed in an Arabidopsis whole-cell extract using the −94 to −65 fragment as a probe in the presence of 200 mM NaCl and 10 mM $MgCl_2$ (Figure 4B). The −123 to −55 sequence competed for binding of both CGF-1 and Tic, but the −123 to −74 fragment did not. Thus, the region between −74 and −55, which overlaps the two 5′ GATA repeats, is necessary for both Tic and CGF binding. The −94 to −65 sequence did not compete for CGF-1 binding, however (Figure 4A), indicating that the two upstream GATA repeats are not sufficient for CGF-1 binding. The −78 to −44 oligonucleotide, which overlaps all three conserved GATA repeats and was originally characterized as the CGF-1 binding site (Anderson et al., 1994), competed weakly but consistently for the binding of CGF-1 to the −111 to −38 probe. Competition was weaker than that with the −123 to −55 sequence, however, suggesting that sequences upstream of position −78 stabilize CGF-1 binding. In contrast, the −94 to −65 fragment did compete for Tic binding, but the −78 to −44 oligonucleotide did not, up to a 50-fold molar excess (Figure 4B). These results indicate that Tic and CGF-1 have distinct binding requirements and that Tic binds 5′ of CGF-1 (because it requires sequences from −78 to −94 for binding).

Toe Is a GATA Binding Complex That Requires Sequences between −120 and −111 for Binding

GATA motifs are found within binding sites for a number of zinc-finger transcription factors from plants and animals (Merika and Orkin, 1993) and have been identified in circadian- and phytochrome-regulated genes from higher plants (Castresana et al., 1987; Gidoni et al., 1989; Gilmartin et al., 1990; Daniel-Vedele and Caboche, 1993). Mutation of a GATA motif at positions −109 to −106 to CCCA strongly reduced the ability of the −120 to −99 oligonucleotide to compete for Toe binding to the wild-type probe (Figure 5A). However, the −111 to −38 sequence did not compete for Toe binding to the −120 to −99 probe (Figure 5B). Thus, sequences between −120 and −111 are also necessary for Toe binding to the *CAB2* promoter. In addition, this result demonstrates that Toe has a distinct bind-

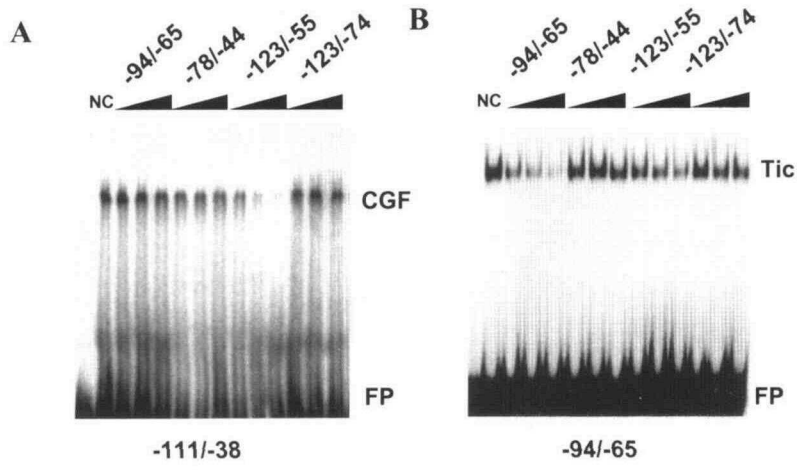


Figure 4. Tic and CGF Have Distinct Requirements for Binding DNA.

(A) CGF-1 was assayed in a tobacco nuclear extract using the *CAB2* sequence from −111 to −38 as a probe in the absence of added $MgCl_2$ and NaCl.

(B) Tic was assayed in an Arabidopsis whole-cell extract using the −94 to −65 sequence as a probe in the presence of 10 mM $MgCl_2$ and 200 mM NaCl, which stimulate Tic binding but severely inhibit CGF binding. Tac was present in very low amounts in this experiment and is not visible at this exposure. Probe sequences are indicated under the corresponding autoradiograms. The position of the DNA–protein complexes and of the free probe (FP) are indicated to the right of the gels. NC indicates assays performed in the absence of competitor DNA. The names of competitors are shown at the top of the gel. Graded triangles indicate the gradient of competitor concentrations (10-, 25-, and 100-fold molar excess).

ing specificity from CGF-1 and Tic, two other GATA binding complexes that bind within the −111 to −38 region.

To test for the function of some of these putative transcription factors, we fused *CAB2* promoter fragments (lacking the TATA box and promoter proximal sequences) upstream of the truncated (−90 to +8) cauliflower mosaic virus 35S promoter and of the *luc* gene (Anderson et al., 1994). The resulting constructs were introduced into Arabidopsis by Agrobacterium-mediated transformation. To help with the interpretation of in vivo effects of promoter deletions, we have summarized the DNA binding activities and the relative locations of their binding sites in a simplified diagram (Figure 6). The largest fragment tested was between −123 and −55. This fragment comprises sequences immediately downstream of the CUF-1 binding site (Anderson et al., 1994) and is truncated 3′ of the conserved GATA repeats (Figure 1). We tested the effect of a 5′ deletion from −123 to −111, shown to impair binding of Toe (Figure 5). A 3′ deletion from −55 to −74 was also constructed that removed binding sites for both Tic and CGF-1 (Figure 4).

Both the −123 to −111 and the −74 to −55 Sequences Overlap Binding Sites for Transcriptional Activators

Millar et al. (1992a) demonstrated that the LUC activity of transgenic plants bearing the −322 to +1 sequence of the *CAB2* promoter fused to *luc* faithfully reports circadian- and light-induced changes in endogenous *CAB2* transcription. In transgenic tobacco, *CAB2* promoter fragments that lacked promoter proximal sequences and were fused upstream of the −90 to +8 sequence of 35S also confer phytochrome and circadian regulation to the *luc* reporter gene (Anderson et al., 1994). Thus, clock- and light-regulated elements within these promoter fragments can function as enhancers to regulate transcription from a heterologous promoter. The −90 to +8 sequence of the 35S promoter contains a binding site for the transcription factor ASF-1 (for activating sequence factor 1), which increases the level of transcription from *CAB2* promoter fragments, thus making possible the detection of regulatory patterns conferred by very short promoter sequences (see below). The amplitude of regulation by the clock and by phytochrome is generally lower (only two- to threefold) with these constructs than with the *CAB2* proximal sequences (five- to 10-fold) (Anderson et al., 1994).

Figure 7 shows the average luminescence levels conferred by the *CAB2* fragments tested. LUC activity was monitored in vivo over a 70-hr period under constant light (LL) in 7- to 9-day-old seedlings from several transgenic lines. Comparison of the −123/−74*cab2*::*luc* and −111/−74*cab2*::*luc* lines indicates that deletion of the −123 to −111 sequence removes a binding site for a transcriptional activator. This transcription factor, which stimulates transcription ∼18-fold, corresponds to the Toe complex identified in vitro using the −120 to −99 sequence as a probe (Figure 5B).

Deletion of the −74 to −55 region also resulted in a 2.6-fold drop in transcriptional activity (compare −111/−55*cab2*::*luc* and −111/−74*cab2*::*luc* lines). This result suggests that Tic, CGF-1, or both may function as transcriptional activators. Interestingly, a mutation of the triple GATA repeat that impairs CGF-1 binding in vitro causes a significantly greater decrease in peak expression levels in transgenic tobacco (32-fold in the

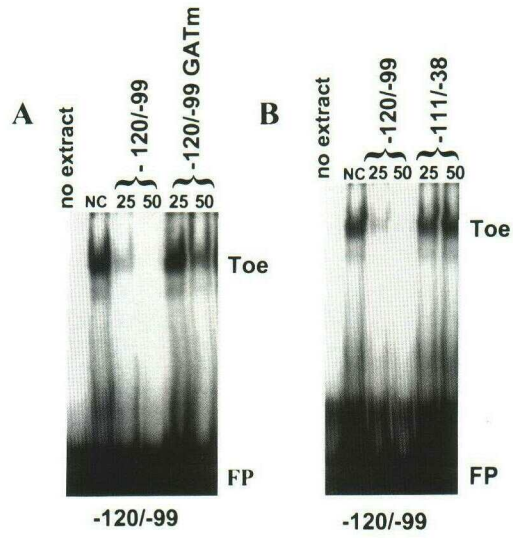


Figure 5. Toe Requires a GATA Motif and Sequences between −120 and −111 for Binding.

Binding requirements for Toe were determined in gel shift competition assays in a crude Arabidopsis extract using the −120 to −99 sequence as a probe, in the presence of 200 mM NaCl and 10 mM $MgCl_2$. The positions of the DNA–protein complex and of the free probe (FP) are indicated to the right of the gels. NC indicates assays performed in the absence of competitor DNA. The names of competitors and competitor concentrations (25- and 50-fold molar excess) are indicated at the top of the gel.

(A) Mutation of the GATA motif at position −109 to −106 (GATm mutation) impairs binding of the Toe complex to the −120 to −99 probe.

(B) The −111 to −38 sequence does not compete for binding of Toe to the −120 to −99 probe.

−199 to +1 context and 100-fold in the −111 to +1 context) (Anderson and Kay, 1995). This discrepancy may be due to interactions between CGF-1 and adjacent DNA binding activities in the longer promoter fragments that are absent in the context of the −123 to −55 sequence. Alternatively, they may reflect different interactions between CGF-1 and the transcriptional machinery in the presence of the *CAB2* or the 35S TATA box. Another possibility is that deleting sequences from −55 to −74 removes both binding sites for a strong transcriptional activator (CGF-1) and for a repressor (Tic).

Thirty-Six Base Pairs Are Sufficient for the Timing of *CAB2* Expression by the Circadian Clock

To test for the presence of circadian-regulated elements within *CAB2* promoter fragments, transgenic plants bearing *cab2::35S::luc* gene fusions were grown for 7 days under 12L/12D (a light/dark cycle composed of 12 hr of light and 12 hr of darkness) and then transferred to LL at the beginning of the light period. LUC activity was monitored in vivo every 1.5 hr using a microtiter plate scintillation counter or, alternatively, a photon-counting camera. The −123 to −74 fragment of the *CAB2* promoter conferred rhythmic expression to the *luc* reporter gene (Figures 8E and 8F). In 12L/12D, the peak of luminescence occurred 4 hr after dawn. Rhythmic LUC expression persisted in LL, with the same phase and with a period slightly greater than 24 hr. The phase and the period of the rhythm were similar to those conferred by the −198 to +1 sequence (Figures 8A, and 8B) shown previously to match those of endogenous *CAB2* expression (Millar and Kay, 1991; Anderson et al., 1994; Millar et al., 1995). The amplitude of the rhythm was lower (twofold as compared with eight- to 10-fold), but this was expected from sequences fused to the heterologous 35S promoter (Anderson et al., 1994). The −111 to −55 sequence conferred patterns of expression similar to those of −123/−74 *cab2::luc* lines, although at a lower level (Figures 8C and 8D), suggesting that the target for regulation of *CAB2* expression by the circadian clock lies within the 36-bp overlapping region, from −111 to −74. Most of the −123/−74 *cab2::luc* and −111/−55 *cab2::luc* lines tested showed clearly rhythmic patterns of LUC expression (seven of eight lines and six of seven lines, respectively). Plants that failed to exhibit detectable rhythmicity were those that had the lowest expression levels, suggesting that the low signal-to-noise ratio as we approach the limits of detection of the system obscured the temporal pattern of luminescence. Despite very low luminescence levels, we were still able to detect circadian variations of LUC expression in two of the brightest −111/−74 *cab2::luc* transgenic lines (Figures 8G and 8H). This oscillation was robust and did not reflect random fluctuations of expression levels, because averaging data from 12 (Figure 8H) or 45 (Fig-

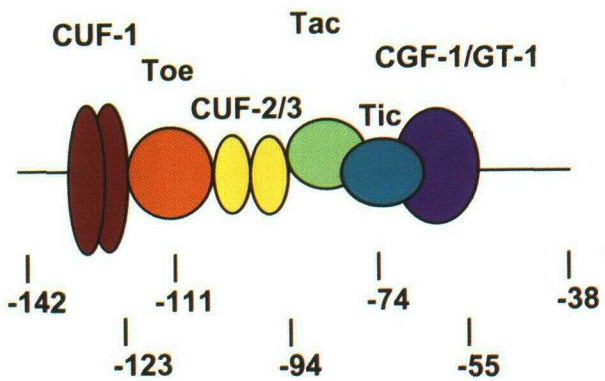
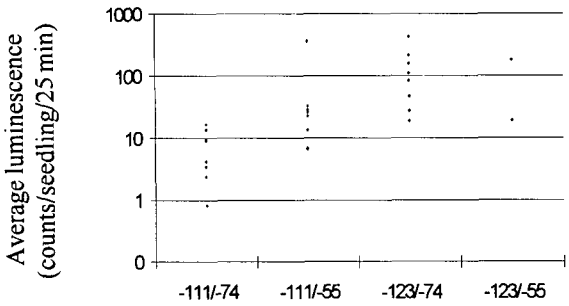


Figure 6. Relative Positions of DNA Binding Activities Identified in the *CAB2* Promoter.

CUF-2 and *CUF-3* are shown as distinct complexes but may in fact correspond to two forms of the same activity. CUF-1 belongs to the leucine zipper family of transcription factors (Anderson et al., 1994) and is shown as a dimer. Other complexes represented as circles may in fact be composed of several subunits.



	-111/-74	-111/-55	-123/-74	-123/-55
mean	7.49	19.47	136.05	100.64
SEM	2.02	3.71	47.89	-
number of lines	8	8	8	2

Figure 7. Expression Levels Conferred by *cab2* Promoter Fragments.

luc expression was monitored in 7- to 10-day-old seedlings, using a photon-counting camera. Luminescence levels were averaged over 70 hr. Each point represents data obtained from an individual transgenic line.

ure 9C) seedlings did not abolish the rhythmic pattern of luminescence.

Previous findings (Millar et al., 1992a, 1992b) indicated that transcription from the full-length 35S promoter does not exhibit significant circadian rhythmicity. A more thorough evaluation using several transgenic lines indicated that the 35S promoter drives a weak but significant transcriptional rhythm (data not shown). Thus, it is possible that a circadian-regulated element is contained within the truncated 35S sequence and is responsible for the rhythmic expression pattern in weakly expressing *cab2*::*35S*::*luc* lines. Data obtained with transgenic tobacco indicated that the −90 to +8 sequence does not drive sufficient levels of expression for detection of the *luc* reporter gene (S.L. Anderson and S.A. Kay, unpublished results). To investigate the effects of this minimal promoter, we inserted a tetramer of the ASF-1 binding site (4XASF-1) upstream of the −90 to +8 sequence in our *luc* expression vector. Figure 9B shows that *luc* expression was rhythmic in plants carrying the 4XASF1::*luc* construct. Under 12L/12D, the phase of the oscillation was similar to that of *CAB2*, and the peak of luminescence occurred 2 to 4 hr after dawn (compare Figures 9A and 9B). The oscillation persisted with a period of ~24 hr after plants were transferred to LL. However, in contrast to the expression pattern of *cab2*::*luc*, the phase of the oscillation was shifted under constant conditions so that the peak of 4XASF-1::*luc* expression now corresponded to the beginning of the subjective night, at a time when *CAB2* expression is minimal. This phase shifting of the oscillation in constant light was observed in all of the nine transgenic lines tested. Thus, the phase of the oscillation conferred by the synthetic 4XASF-1 promoter is clearly distinct from that of *CAB2* under LL. This result indicates that circadian oscillations in *CAB2* and of 35S transcriptional activity occur through distinct mechanisms that promote transcription at different times of the day.

Photon-counting images of the seedlings (data not shown) indicated that most if not all of the luminescence detected by the camera originated from the cotyledons and primary leaves. Thus, the different temporal pattern of luminescence in *cab2*::*35S*::*luc* and in 4XASF-1::*luc* seedlings was not due to expression in different tissues. The fact that *cab2*::*35S*::*luc* lines exhibit a luminescence pattern similar in phase to that of *CAB2* (Anderson et al., 1994; present study) suggests that the circadian output pathway that drives *CAB2* expression is able to override the control of transcription by the −90 to +8 (35S) sequence. A simple explanation may be that the circadian-regulated element from the *CAB2* sequence confers a much greater amplitude than the one present in the truncated 35S promoter. To determine whether the pattern of *luc* expression in −111/−74 *cab2*::*luc* lines reflects timing of transcription by *CAB2* or by 35S control pathways, we imaged −111/−74 *cab2*::*luc* seedlings for 96 hr under LL. Figure 8C indicates that the phase of *luc* expression in these plants was similar to that in the −198/+1 *cab2*::*luc* line (Figure 9A), both under LD and LL conditions. Peaks of expression occurred out of phase with the 4XASF-1::*luc* plants under LL (Figure 9B). These results demonstrate that the −111 to −74 sequence of the promoter defines a clock-responsive element with the same circadian properties as that of the pseudo–wild-type (−198 to +1) *CAB2* promoter.

DISCUSSION

A previous study (Anderson et al., 1994) indicated that the −111 to −38 sequence (73 bp) was sufficient to recapitulate most aspects of *CAB2* regulation, including tissue specificity, regulation by phytochrome, and regulation by a circadian clock. A single transcription factor, CGF-1, was identified that bound within this region. CGF-1 is related to GT-1 (Teakle and Kay, 1995) and plays a role in the acute induction of *CAB2* expression by red light in etiolated seedlings (Anderson and Kay, 1995). This article reports the identification of five novel DNA binding activities within this region (CUF-2, CUF-3, Tic, Tac, and Toe; summarized in Figure 6) that are distinct from CGF-1 and that may participate in other aspects of *CAB2* regulation. We demonstrate that sequences sufficient for the regulation of CAB2 transcription by the circadian oscillator reside within 36-bp region from −111 to −74, which is bound in vitro by three complexes, CUF-2, CUF-3, and Tac.

Characterization of DNA Binding Complexes

Gel shift assays with overlapping DNA probes suggested that both CUF-2 and CUF-3 bind between −111 and −94. We do not know at this point whether these complexes correspond

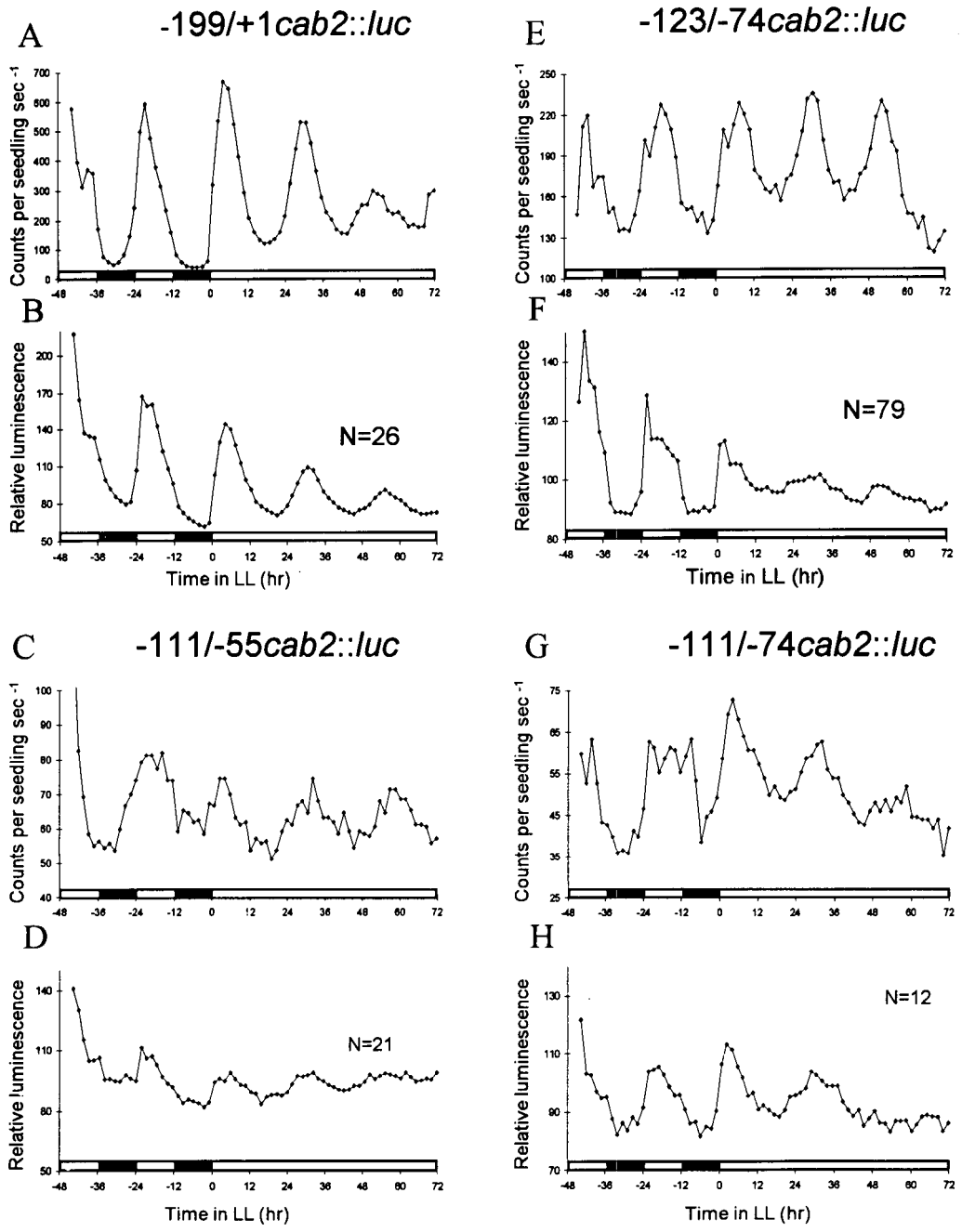


Figure 8. Circadian Regulation of *luc* Expression in *cab2::35S::luc* Transgenic Plants.

Assays for circadian-regulated expression were performed in a Packard TopCount Scintillation counter. Black and white boxes above the x-axis indicate the periods of dark and light.

(A), **(C)**, **(E)**, and **(G)** Expression pattern in single representative seedlings. The background level in these experiments was 25 counts/sec.

(B), **(D)**, **(F)**, and **(H)** Average expression levels in a group of seedlings representing two transgenic lines after normalization of expression levels to individual means. N indicates the numbers of seedlings included in the average.

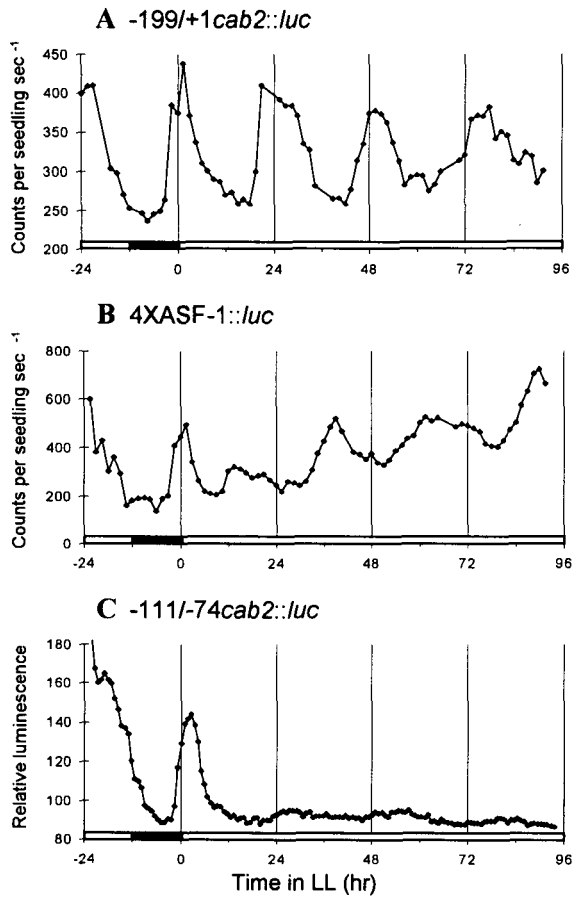


Figure 9. Comparison of the Temporal Pattern of Expression from *CAB2* Promoter Fragments and from CaMV 35S Sequences.

(A) Circadian rhythm of luminescence conferred by the pseudo-wild-type (−198 to +1) fragment of the *CAB2* promoter.

(B) Rhythmic expression from the truncated (−90/+8) 35S promoter. Transcriptional levels were enhanced by the insertion of a tetramer of the binding site for the transcription factor ASF-1 (4XASF-1) upstream of the minimal promoter.

(C) The phase of expression in −111/−74 *cab2::35S::luc* plants matched that in −198/+1 *cab2::luc* controls and was opposite that in 4XASF-1:: *35S::luc* **(B)** under LL. The experiments described in **(A)** and **(B)** were performed side by side using the photon-counting camera, whereas that described in **(C)** was performed independently in a Packard Top-Count scintillation counter.

to nuclear and cytoplasmic forms of the same transcription factor, to distinct isoforms, or to independent activities. The −111 to −94 region includes an A-rich sequence (AAAACA-AAAA) similar to the binding site for the CA-1 complex, a phosphoprotein that binds the Arabidopsis *CAB1* (*lhcb1*3*) promoter. CA-1 has been suggested to play a role in the induction of the Arabidopsis *CAB1* gene by light, because a mutation that impairs CA-1 binding to the *CAB1* promoter suppresses the induction of a β-glucuronidase reporter gene by red light in etiolated seedlings (Kenigsbuch and Tobin, 1995). Neither CUF-2 nor CUF-3 is related to CA-1, however, because their electrophoretic mobilities were unaffected by treatment with alkaline phosphatase (data not shown). Furthermore, both the A2 fragment, which binds CA-1, and a mutated version of the oligonucleotide (A2 mut1), which does not (Sun et al., 1993), competed for binding of CUF-2 and CUF-3 complexes to the −111 to −38 probe (data not shown). CUF-2 and CUF-3 were also distinct from the AT-1 binding activity that binds AT-rich regions within the *CAB-E* promoter (Datta and Cashmore, 1989), because their binding was not modulated by treatment with calf intestinal phosphatase (data not shown).

Toe binds a 21-bp sequence, from −120 to −99. Although this sequence comprises a GATA motif that is required for binding, competition assays (data not shown) indicated that Toe has a binding specificity that is distinct from the GATA binding proteins CGF-1 (Anderson et al., 1994), ASF-2 (for activating sequence factor 2; Lam and Chua, 1989), GAF-1 (for GA factor 1; Gilmartin et al., 1990), GT-1 (Green et al., 1988; Teakle and Kay, 1995), and Tic. In vivo assays of LUC expression in plants bearing *cab2::35S::luc* gene fusions demonstrated that deletion of sequences from −123 to −111 removes a binding site for a strong transcriptional activator, causing an 18-fold drop in expression levels (Figure 7). The deletion did not impair circadian regulation of transcription, however (Figure 8). We show that Toe requires these sequences for binding (Figure 5B), unlike Tic, Tac, CUF-2, CUF-3, and CGF-1, which all bind sequences downstream of −111. These results suggest that Toe plays a critical role in maintaining high levels of *CAB* transcription.

A 10-bp linker mutation spanning the CCAAT box region has been shown to impair phytochrome regulation of the Lemna *CABab19* gene (Kehoe et al., 1994), suggesting that the conserved CCAAT box may play a role in regulation of gene expression by light. Binding of Tac was reduced by mutation of the conserved CCAAT box to AAAAT. However, mutation of an AAT sequence immediately upstream of the CCAAT box reduced the affinity of the Tac complex for the −94 to −65 probe at least as effectively as the CCm mutation (Figures 3B and 3C), indicating that Tac requires more than the CCAAT sequence for binding. Sequences upstream of the CCAAT box are not conserved between *CAB* promoters, suggesting that the function of this conserved motif may be independent of Tac binding. The Tic complex binds immediately downstream of the CCAAT box. The CCAATGAAT sequence contains an 8 out of 9 bp homology with the binding site for the Arabidopsis homeobox leucine zipper binding protein Athb-2, defined by binding site selection (Sessa et al., 1993). Cloned, bacterially expressed Athb-2 did not bind the −94 to −65 *CAB2* probe, and the Athb-2 binding probe BS2 did not compete for Tic binding (data not shown), indicating that Tic and Athb-2 have distinct binding requirements. A single A to G mutation (to CCAATG-GAT) that corresponds to a disruptive mutation in the binding site for the Arabidopsis homeodomain leucine zipper protein Athb-1 (Sessa et al., 1993) impaired binding of Tic to the −94 to −65 probe (Figure 3B, Am). Tic binding was also impaired

by mutation of the GATA repeats to CCCA, although to a lesser extent (Figure 3B, GATm). Thus, Tic requires GATA sequences in addition to a homeobox-type target sequence but is unlikely to be a classical homeobox binding protein.

Identification of a 36-bp Circadian-Regulated Element

The deletion of sequences between −74 and −55 (removing binding sites for CGF-1 and Tic) resulted in a significant (approximately sixfold) drop in expression levels. The −111 to −74 sequence still conferred a low but detectable level of expression to the *luc* reporter gene and a rhythmic pattern of transcription that was similar in phase and period to that of the full-length *CAB2* promoter. This pattern was distinct from the temporal pattern of expression conferred by the 4XASF-1 sequence (Figure 9B).

Interestingly, the rhythmic pattern of expression from the truncated 35S promoter occurred with the same phase as that from the *CAB2* promoter under LD cycles (Figure 9A). A similar, low-amplitude fluctuation of *luc* expression under LD cycles was also detected previously using the full-length 35S promoter (Millar et al., 1992b). However, the phase of the oscillation for the 4XASF-1::*luc* plants became phase shifted under free-running conditions, so that peaks of expression occurred out of phase with those of *CAB2*. One hypothesis to account for this pattern is that expression from the 4XASF-1 promoter is light regulated and is induced at the time of the LD transition, close to the peak of *CAB2* expression. Transcriptional regulation by the circadian clock would then be revealed only under LL and would result in the gating of *luc* expression to the early subjective night. The pattern of *luc* expression in 4XASF-1::*luc* lines grown under 12L/12D suggests that it is not merely induced, however, as the rise of luminescence clearly anticipated dawn by ~1.5 hr in all plants tested (Figure 9B). Alternatively, transcription from *CAB2* and from the 4XASF-1 sequences may be controlled by two different output pathways from the same clock, whose phase relationship changes under free-running conditions. Another possibility is that the transcription from the 4XASF-1 promoter is regulated by a clock distinct from that which regulates *CAB2* and that this second cellular oscillator becomes phase shifted relative to the first one after transfer to constant conditions. However, if one subtracts the endogenous pattern conferred by the −90 to +8 region (as is commonly done in studies of spatial regulation of gene expression), it is clear that the circadian control of transcription from the *CAB2* and from the 35S promoter is mediated by different output pathways that promote transcription at different phases of the circadian cycle.

Altogether, these results point to a role for a 36-bp region surrounding a conserved CCAAT box in the circadian regulation of the *CAB2* gene. They indicate that the DNA binding complexes Toe, Tic, and CGF-1 are not required for the timing of *CAB2* expression by the clock, although they may play a role in modulating the amplitude of circadian oscillations. Finally, they suggest a role for Tac, CUF-2, or CUF-3 in the control of transcription by the circadian oscillator. Gel shift assays in extracts prepared from plants harvested every 3 hr over 3 days in both LL and constant darkness indicated that all of the DNA binding complexes described in this article are present throughout the circadian cycle in both light-grown and dark-adapted plants (data not shown). In tobacco nuclear extracts, the CGF-1 activity was also found to be constant across the circadian cycle (Anderson et al., 1994). This suggests that the regulation of *CAB2* transcription by the clock is mediated by post-translational modifications of these activities that do not affect their DNA binding properties in vitro. Cloning of the polypeptide components of these complexes will be necessary to dissect further the signal transduction pathway for the control of *CAB2* expression by the circadian oscillator.

METHODS

Preparation of Whole-Cell Extracts and Fractionation

Arabidopsis thaliana (ecotype Columbia) were grown for 3 to 4 weeks under greenhouse conditions. Plants were harvested just before bolting and frozen in liquid nitrogen. Whole-cell extracts were prepared from frozen tissue as described by Foster et al. (1992), except that EDTA (12.5 mM) and NaF (10 mM) were added to the extraction buffer to minimize kinase and phosphatase activities during the extraction.

Extracts were fractionated by cation exchange chromatography on Econopac CM cartridges (Bio-Rad). Typically, 120 mg of protein were loaded on two columns coupled back to back and pre-equilibrated with starting buffer (15 mM Hepes, 40 mM KCl, 10 mM NaF, 12.5 mM EDTA, 1 mM DTT, pH 7.5). The flowthrough peak of protein was collected, and the columns were washed until the OD_{280} in the eluant returned to baseline. Bound proteins were eluted by a linear KCl gradient (0 to 0.3 M) in starting buffer. DNA binding activities were assayed by gel retardation using a DNA probe comprising the −142 to −38 sequence of the *CAB2* (chlorophyll *a*/*b* binding protein) promoter. CUF-2 (for CAB upstream factor 2) eluted at 0.15 M KCl (CM–CUF-2 fraction) and CUF-3 at 0.25 M KCl (CM–CUF-3 fraction). CUF-1–type activities were found in the flowthrough fractions, as well as Tic, Tac, and Toe.

Gel Shift Assays

CAB2 promoter fragments from −111 to −38 and from −111 to −74 were generated by polymerase chain reaction and cloned into the BamHI to XhoI sites of pBluescript II KS−. They were then isolated as restriction fragments and purified by agarose gel electrophoresis and electroelution. Oligonucleotides corresponding to sequences from −120 to −99 and from −94 to −65 were synthesized with BamHI and XhoI sites at their 5′ and 3′ ends, respectively. Annealed oligonucleotides were used directly in competition assays; for the generation of radiolabeled probes, they were digested with both restriction enzymes and purified by ethanol precipitation. Probes were end labeled by filling in the restriction sites with the Klenow enzyme fragment of DNA polymerase I using all four α-^{32}P-deoxynucleotide triphosphates.

Gel shift assays were performed as described by Foster et al. (1992),

with some modifications. Briefly, 4 μg of protein from whole-cell extracts was incubated with 7.5 fmol of radiolabeled probe and 3 μg of poly(dI-dC) in NEB buffer (20 mM Hepes-KOH, 40 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM DTT, pH 7.5) containing 0.05% Nonidet P-40. The binding buffer was supplemented with 10 mM $MgCl_2$ for the assay of CUF-2 and CUF-3 and with 200 mM NaCl and 10 mM $MgCl_2$ for the assay of Tic, Tac, and Toe. After a 30-min incubation at room temperature, DNA binding activities were resolved on an 8% acrylamide gel in 40 mM Hepes, 0.2 mM EDTA, pH 7.5, run at 170 V for ~3 hr, with buffer recycling. CGF-1 (for *CAB* GATA factor 1) was assayed in tobacco nuclear extracts, as described by Teakle and Kay (1995).

Dried gels were autoradiographed on x-ray film. Alternatively, gels were imaged using a PhosphorImager (445 Si; Molecular Dynamics, Sunnyvale, CA).

Construction of *cab2::35S::luc* Gene Fusions

The region between −199 and +1 of the promoter was fused upstream of the tobacco mosaic virus translational enhancer Ω and of the firefly luciferase gene (*luc*) and then transferred to the polylinker of the binary vector pMON721 (Anderson et al., 1994). The −123 to −55, −123 to −74, −111 to −55, and −111 to −74 fragments of the *CAB2* promoter were generated by polymerase chain reaction using the −198 to +1 sequence in pBluescript II KS− as the template. BamHI and XhoI restriction sites were introduced at the 5′ and 3′ ends, respectively, to facilitate cloning into pBluescript II KS−. Cloned *cab2* fragments were sequenced and then subcloned into the polylinker of the LRB-90 vector (Anderson et al., 1994), upstream of the truncated (−90 to +8) cauliflower mosaic virus 35S promoter and followed by the tobacco mosaic virus translational enhancer (Ω), the firefly *luc* coding region, and the poly(A) addition sequence from the pea ribulose bisphosphate carboxylase *rbcsS-E9* gene. A tetramer of the ASF-1 (for activating sequence factor 1) binding site, cloned in pBluescript II (Lam and Chua, 1990), was isolated as a HindIII-XhoI restriction fragment and then subcloned into the polylinker of LRB-90 to yield the 4XASF-1 construct.

Generation of Transgenic Plants

Binary vector constructs were transformed into the *Agrobacterium tumefaciens* strain ABI by the freeze–thaw method (An et al., 1988). Agrobacterium-mediated transformation of Arabidopsis (ecotype Landsberg *erecta*) was performed by vacuum infiltration of adult plants (Bechtold et al., 1993). The seed harvested from individual infiltrated plants was plated on solid Murashige and Skoog medium (Sigma) containing 3% sucrose, 50 mg/L kanamycin, and 100 mg/L carbenicillin. Resistant plants were transferred to soil as soon as they grew primary leaves. T_2 seed was harvested and used in imaging experiments. Transformants obtained from different infiltrated plants, or that had more than twofold differences in their expression levels, were considered to result from independent transformation events.

Circadian Regulation of *cab2::luc* Expression in Transgenic Plants

T_2 seeds carrying the *cab2::luc* fusions were sown onto Petri dishes of solid Murashige and Skoog medium containing 3% sucrose and 2% charcoal and stored at 4°C for 7 days. Plants were then grown at 22°C under a light–dark (LD) cycle composed of 12 hr of light (40 μmol m^{-2} sec^{-1} of white light) and 12 hr of darkness (12L/12D). After 7 days, they were transferred to constant light (LL) at the onset of the normal photoperiod. LUC activity was imaged and quantitated every 2 hr using a photon-counting camera (Hamamatsu, Bridgewater, NJ) as described by Millar et al. (1992a, 1992b). Seedlings were sprayed three times with 5 mM D-luciferin (Promega) in 0.01% Triton X-100 within the 16 hr preceding imaging and then with 1 mM D-luciferin immediately before each time point. Alternatively, seedlings were grown for 5 days on Petri dishes and then transferred to opaque, 96-well microtiter plates containing luciferin (10 μL of a 5 mM stock per well), and luminescence was monitored in a scintillation counter (Top-Count; Packard, Meriden, CT). Aeration was provided by piercing pinholes in the plastic film cover. Opaque plates were alternated with clear plates to allow light to reach the seedlings in the stack. Illumination was provided by two banks of four wide spectrum fluorescent bulbs (GTE, New Brunswick, NJ), raised on either side of the plate stacker. Counting time was 0.2 min per well. Data are presented without background subtraction.

Data from groups of seedlings were expressed as "relative luminescence." To remove differences in expression levels from one seedling to another, data from individual plants were first expressed as a percentage of the mean of the data series. The average of these normalized levels was then calculated at each time point for each transgenic line.

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