

# Phase-Specific Circadian Clock Regulatory Elements in Arabidopsis<sup>1</sup>

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We have defined a minimal Arabidopsis *CATALASE 3* (*CAT3*) promoter sufficient to drive evening-specific circadian transcription of a *LUCIFERASE* reporter gene. Deletion analysis and site-directed mutagenesis reveal a circadian response element, the evening element (EE: AAAATATCT), that is necessary for evening-specific transcription. The EE differs only by a single base pair from the CIRCADIAN CLOCK ASSOCIATED 1-binding site (CBS: AAAAAATCT), which is important for morning-specific transcription. We tested the hypothesis that the EE and the CBS specify circadian phase by site-directed mutagenesis to convert the *CAT3* EE into a CBS. Changing the *CAT3* EE to a CBS changes the phase of peak transcription from the evening to the morning in continuous dark and in light-dark cycles, consistent with the specification of phase by the single base pair that distinguishes these elements. However, rhythmicity of the CBS-containing *CAT3* promoter is dramatically compromised in continuous light. Thus, we conclude that additional information normally provided in the context of a morning-specific promoter is necessary for full circadian activity of the CBS.

The circadian clock enables an organism to specifically partition aspects of its biology to precise times over the day (Dunlap, 1999). Although the circadian clock is, by definition, endogenous and continues to run in the absence of external time cues, environmental stimuli such as light and temperature act to entrain the internal processes of an organism both to the exact external daily period and in a defined relationship, or phase angle, to the diurnal cycle. For example, in Arabidopsis, light and temperature information are integrated to partition physiological activities such as circadian-regulated leaf movement, stomatal opening, and gene expression to distinct times of day or phases (McClung et al., 2002).

A central theme that has emerged in circadian biology is that the core oscillator is composed of a negative feedback loop grounded in positive and negative transcriptional regulation (Dunlap, 1999). It has recently been demonstrated that the Arabidopsis circadian clock entails such a transcriptional feedback loop (Alabadí et al., 2001) that includes at least three components: TIMING OF CAB EXPRESSION 1 (*TOC1*; also called Arabidopsis PSEUDO-RESPONSE REGULATOR 1, *APRR1*; Millar et al., 1995; Makino et al., 2000), CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*; Wang and Tobin, 1998), and LATE ELONGATED HYPOCOTYL (*LHY*; Schaffer et al., 1998).

*CCA1* and *LHY* are single-Myb domain transcription factors, and DNA-binding activity of *CCA1* to a *CCA1*-binding site (CBS: AAAAAATCT) has been characterized (Wang et al., 1997). The hypothesized role of *TOC1* as a transcription factor is based on similarity to *CONSTANS*, although DNA binding by *TOC1* has not been experimentally established (Strayer et al., 2000). However, *TOC1* (*APRR1*) has been shown to bind to PHYTOCHROME-INTERACTING FACTOR 3 (*PIF3*), a Myc-related basic helix-loop-helix transcription factor, and to the related *PIF3-LIKE 1* (*PIL1*; Makino et al., 2002). Expression of each of the three clock components, *TOC1*, *CCA1*, and *LHY*, is circadian regulated (Schaffer et al., 1998; Wang and Tobin, 1998; Matsushika et al., 2000; Strayer et al., 2000). *TOC1* (*APRR1*) and *CCA1/LHY* make up a feedback loop in which *TOC1* acts as a positive regulator of *CCA1* and *LHY*, which in turn are negative regulators of *TOC1* (Alabadí et al., 2001). *CCA1* and *LHY* bind to the *TOC1* promoter in vitro at a CBS-related motif called the evening element (EE: AAAATATCT), and overexpression of either *LHY* or *CCA1* results in nonoscillating, low-level accumulation of *TOC1* mRNA, indicating that both *CCA1* and *LHY* are negative regulators of *TOC1* (Alabadí et al., 2001; Matsushika et al., 2002). In plants homozygous for the strong loss-of-function *toc1-2* allele, oscillations of *LHY* and *CCA1* mRNA exhibit both the short period characteristic of *toc1* mutations (Millar et al., 1995; Somers et al., 1998) and greatly reduced *CCA1* and *LHY* mRNA abundance, consistent with a role of *TOC1* as a positive regulator (Alabadí et al., 2001). *TOC1* (*APRR1*) overexpression disrupts rhythmic expression of many genes, including *CCA1* and *LHY*, but the results are not entirely consistent with the simple explanation of *TOC1*

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(APRR1) acting directly as a positive regulator at the promoters of *CCA1* and *LHY* (Makino et al., 2002).

It has been demonstrated in Arabidopsis, cyanobacteria, fruitfly (*Drosophila melanogaster*), and mammals and that the oscillations in the mRNA abundance of circadian-regulated transcripts peak at many unique phases that span the entire day (Liu et al., 1995; Harmer et al., 2000; Claridge-Chang et al., 2001; Grundschober et al., 2001; McDonald and Rosbash, 2001; Akhtar et al., 2002; Duffield et al., 2002). Included among these are genes encoding a number of key clock components, such as *CCA1*, *LHY*, and *TOC1*, that function within the circadian oscillator. Among other clock-controlled genes are a number of additional transcription factors, which leads to the simple and attractive hypothesis that the phasing of transcription of clock-controlled genes to specific times of day emerges through the interaction of a specific clock-controlled transcription factor with its cognate DNA target. Genes transcribed at a specific times of day share a promoter motif that binds a specific transcription factor whose activity peaks at that time of day, and genes transcribed at other times of day possess different promoter motifs that interact with distinct clock-regulated transcription factors.

Two elements implicated in circadian control of transcription, the EE and CBS (also called the *lhc* motif), were originally identified in the promoters of clock controlled genes (Carré and Kay, 1995; Wang et al., 1997; Harmer et al., 2000). The CBS and EE are closely related with a difference of only 1 bp (AAAaATCT versus AAAtATCT). The similarity of CBS and EE, coupled with their specific association with genes phased to morning and evening, respectively (Carré and Kay, 1995; Wang et al., 1997; Harmer et al., 2000), suggests that phase may be specified by the 1-bp difference that distinguishes the two motifs. To test this directly we used the promoter of the Arabidopsis *CATALASE 3* (*CAT3*) gene, which oscillates with an evening-specific peak in circadian-regulated mRNA abundance (Zhong and McClung, 1996). Deletion analysis and site-directed mutagenesis of the *CAT3* promoter reveals that the EE is necessary for evening-specific transcription. Converting the *CAT3* EE to a CBS (aaaTatct to aaaAatct) renders the promoter substantially arrhythmic when examined in continuous light (LL), whereas in continuous dark (DD) conditions or in entraining conditions of 12 h light and 12 h dark (12/12 LD), this promoter confers morning-specific rhythmicity. These results reinforce the centrality of the CBS/EE in circadian transcription and demonstrate that the single base pair difference between these elements is sufficient to specify the time of day at which transcription occurs. However, our results also make it clear that additional promoter elements provide critical contextual information that is essential for complete circadian regulation.

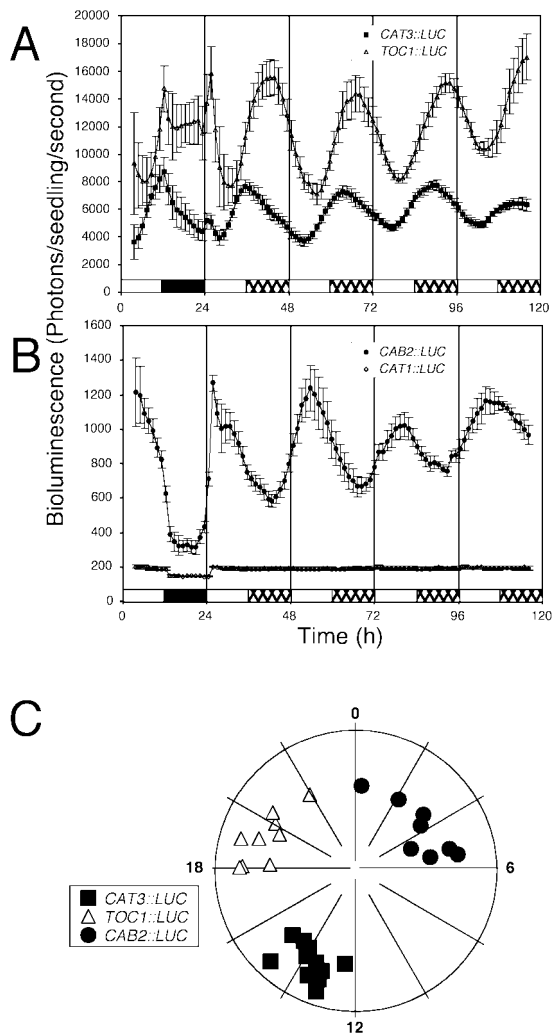
## RESULTS

### Circadian Evening-Specific Transcription of the *CAT3* Promoter

The circadian clock regulates *CAT3* mRNA abundance with a peak at dusk and a trough at dawn (Zhong and McClung, 1996). *CAT3* promoter::*LUCIFERASE* fusions (*CAT3::LUC*) were constructed and transformed into ecotype Columbia (Col) plants to address whether circadian regulation is at the level of transcription. T<sub>2</sub> plants containing *CAT3::LUC* were grown in entraining conditions of a 12/12 LD cycle at 22°C for 7 d. Seedlings were moved to a luminometer (TopCount, Packard, Meriden, CT), entrained in LD for 3 d, and then released into LL at 22°C. Figure 1A shows that, in LL, luciferase activity of *CAT3::LUC* seedlings oscillates with a period of about 24 h and with an evening-specific phase (period =  $24.85 \pm 0.19$  h; phase =  $13.78 \pm 0.22$  circadian time [CT] h;  $n = 12$ ). In contrast, neither a *CAT1::LUC* fusion (Fig. 1B) nor the promoterless *LUC* gene alone (data not shown) demonstrated oscillations in luciferase activity in LL. Therefore, we conclude that circadian clock regulation of *CAT3* transcription contributes to the circadian oscillation previously described for *CAT3* mRNA abundance (Zhong and McClung, 1996). Similar period and phase results were obtained for the ecotypes Rschew (RLD), Wassilewskija (WS), Landsberg *erecta* (*Ler*), and Cape Verde Islands (Cvi; data not shown). When *CAT3::LUC* seedlings were entrained to different photoperiods (long days: 16/8 LD or short days: 8/16 LD), there was no significant difference in period or phase compared with plants that were entrained to 12/12 LD cycles (data not shown). The evening-specific phase of transcription of the maize (*Zea mays*) *CAT3* ortholog similarly has been shown to be insensitive to photoperiod (Abler and Scandalios, 1994).

In entraining LD conditions, clock-controlled reporters like *CAB2::LUC*, *CAT3::LUC*, and *TOC1::LUC* display sinusoid circadian rhythms with clear anticipation of dawn and dusk, respectively (Fig. 1). In addition, *TOC1::LUC* shows pronounced acute responses to the lights on signal at dawn and to the lights off signal at dusk. In contrast, during LD cycles both *CAT1::LUC* (Fig. 1B) and promoterless::*LUC* (data not shown) demonstrate driven rhythms as seen by “square waves,” in which LUC activity increases and decreases in direct response to lights on and lights off, with no evidence of anticipation of either dawn or dusk. This may reflect altered plant metabolism in light and dark affecting basal luciferase activity.

The phase of peak *CAT3::LUC* transcription is distinct from that of other clock-regulated genes. For example, *CAB2::LUC*, a well-documented clock-regulated gene fusion (Millar et al., 1992), cycles with a mid-day-specific phase (period =  $24.61 \pm 0.52$  h;

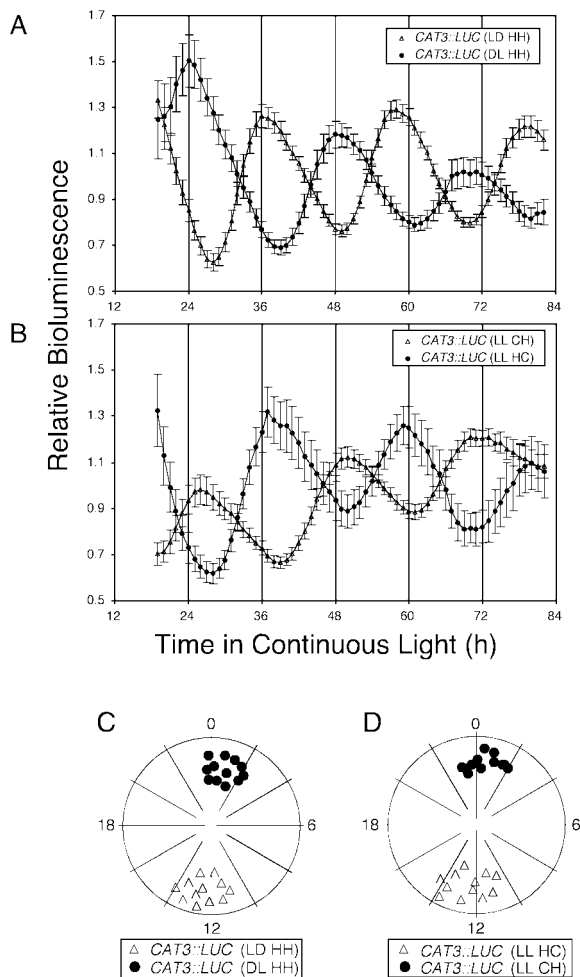


**Figure 1.** Transcription of a *CAT3::LUC* transgene is regulated by the circadian clock. Plants were grown under 12-h light/12-h dark photoperiod at 22°C for 7 d. Plants were moved to a Packard TopCount luminometer and further entrained for 3 d in the LD cycle before being released into LL. The LD regime is indicated by the bars beneath the traces, with day (light) indicated by white bars, night (dark) indicated by black bars, and subjective night (dark of the entraining cycle) indicated by hatched bars. A, Traces present average values ( $\pm$  SE,  $n = 12$ ) from individual seedlings expressing *CAT3::LUC* (squares) or *TOC1::LUC* (triangles). B, Traces present average values ( $\pm$  SE,  $n = 12$ ) from individual seedlings expressing *CAB2::LUC* (circles) and *CAT1::LUC* (diamonds). C, Phase plot in which phases of individual seedlings are plotted against the strength of the rhythm. Phase is expressed in CT (phase/period  $\times$  24 h) around the circumference of a 24-h clock face. Strength of the rhythm is expressed as relative amplitude error (RAE), where a perfect sine wave is defined as 0 and a value of 1 defines the weakest rhythm considered to be statistically significant. The strength of the rhythm is plotted along the radius with the strongest rhythms (RAE = 0) at the outer edge of the circle and weakest rhythms (RAE = 1) at the center. *CAT3::LUC*, squares; *CAB2::LUC*, circles; *TOC1::LUC*, triangles.  $[-221/-103]_2$  *CAT3::LUC* seedlings are depicted because of their highly reproducible and accurate representation of endogenous *CAT3* circadian-regulated transcription. Similar results have been obtained with all other rhythmic *CAT3::LUC* fusions tested.

phase =  $4.39 \pm 0.75$  CT h;  $n = 12$ ) and *TOC1::LUC* cycles with a midnight-specific phase (period =  $24.67 \pm 0.32$ ; phase =  $18.89 \pm 0.55$  CT h;  $n = 12$ ) in LL (Fig. 1). The *TOC1::LUC* phase lags by about 6 h that reported by Alabadí et al. (2001; phase approximately 12 CT h). One possible explanation is that Alabadí et al. (2001) describe a translational fusion in which the 5'-untranslated region of *TOC1* is present ( $-834/+1$  from the ATG), whereas the *TOC1::LUC* fusion described in this study is a transcriptional fusion that includes only promoter elements upstream of the transcriptional start ( $-890/-381$ ). We suspect that the distinct phases of these two constructs results from different regulatory elements provided in the two fusion constructs. It is worth noting that *TOC1* transcript abundance displays biphasic peaks, one at approximately CT12 and another at approximately CT18 (Makino et al., 2000; Strayer et al., 2000); possibly the transcriptional and translational *TOC1::LUC* fusions separate two bouts of transcriptional activity that contribute to this biphasic pattern of mRNA abundance. Others have shown that *CCR2* and *ELF3* promoters confer circadian transcription with afternoon- and late evening-specific phases (CT approximately 10 and approximately 16, respectively; Staiger and Apel, 1999; Strayer et al., 2000; Covington et al., 2001). To highlight phase differences between *CAT3::LUC*, *TOC1::LUC* and *CAB2::LUC*, phase was plotted against the strength of the rhythm (Fig. 1C). Strong rhythms are plotted close to the outer edge of the circle, whereas weaker rhythms are plotted near the center of the circle (see "Materials and Methods" for details).

LD cycles entrain the circadian rhythm in *CAT3::LUC* activity (Fig. 2, A and C). Although light serves as a major external entrainment stimulus in plants, temperature cycles have also been shown to entrain the circadian clock (Heintzen et al., 1994; Somers et al., 1998). Consistent with this, *CAT3::LUC* expression is entrained by temperature cycles of 12-h hot (22°C) and 12-h cold (18°C) in LL (LL HC), where 22°C acts as a "day" signal and 18°C acts as a "night" signal. After entrainment to LL HC, *CAT3::LUC* activity peaks at the beginning of the subjective cold period (Fig. 2, B and D), whereas *CAB2::LUC* activity has been shown to peak in the middle of the subjective hot period (Somers et al., 1998). Either light (Fig. 2, A and C) or temperature (Fig. 2, B and D) cycles provided 180° out of phase can be used to entrain two populations of seedlings antiphase to one another; *CAT3::LUC* expression is always phased to the beginning of the subjective dark or cold period. Both light and temperature cycles provide strong entraining stimuli that can override previous time-of-day information that the plant may have received.

Rhythmic oscillation of *CAT3::LUC* (all fusions discussed in this study) persists in DD with evening-specific phase and 24-h period (Fig. 3; data not shown). This is interesting because *CAT3* mRNA os-



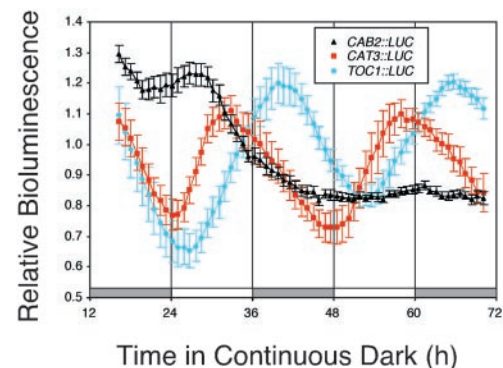
**Figure 2.** *CAT3::LUC* expression can be entrained by light or temperature cycles. **A**, Plants were grown at 22°C either under a 12/12 LD photoperiod (LD HH; triangles) or under a 12-h/12-h dark-light (DL HH; circles) photoperiod for 7 d before release into LL at  $T = 0$ . Traces present average values ( $\pm$  SE,  $n = 12$ ) from individual independent transgenic lines. Data are normalized to the average luciferase activity of the individual seedling and are presented as relative bioluminescence. **B**, Plants were grown in LL either under a 12-h hot (22°C)/12-h cold (18°C) thermoperiod (LL HC; circles) or under a 12-h cold (18°C)/12-h hot (22°C) thermoperiod (LL CH; triangles) for 7 d before release into constant temperature (22°C) and LL at  $T = 0$ . Traces present average values ( $\pm$  SE,  $n = 12$ ) from individual independent transgenic lines. **C** and **D**, Phase plots as described in the legend to Figure 1C for multiple seedlings from **A** and **C**, respectively.  $[-221/-103]_2$  *CAT3::LUC* seedlings are depicted, but similar results have been obtained with all other *CAT3::LUC* fusions tested, except those constructs that have lost rhythmicity.

oscillations damp to constitutively high levels in DD (Zhong et al., 1997). That *CAT3* mRNA abundance oscillations damp in DD while transcription continues to oscillate suggests posttranscriptional control in mRNA abundance; either *CAT3* mRNA becomes stabilized in DD or *CAT3* mRNA abundance is destabilized in the light. Of course, it is also possible that the *CAT3::LUC* fusions do not completely recapitulate endogenous *CAT3* transcriptional activity. The per-

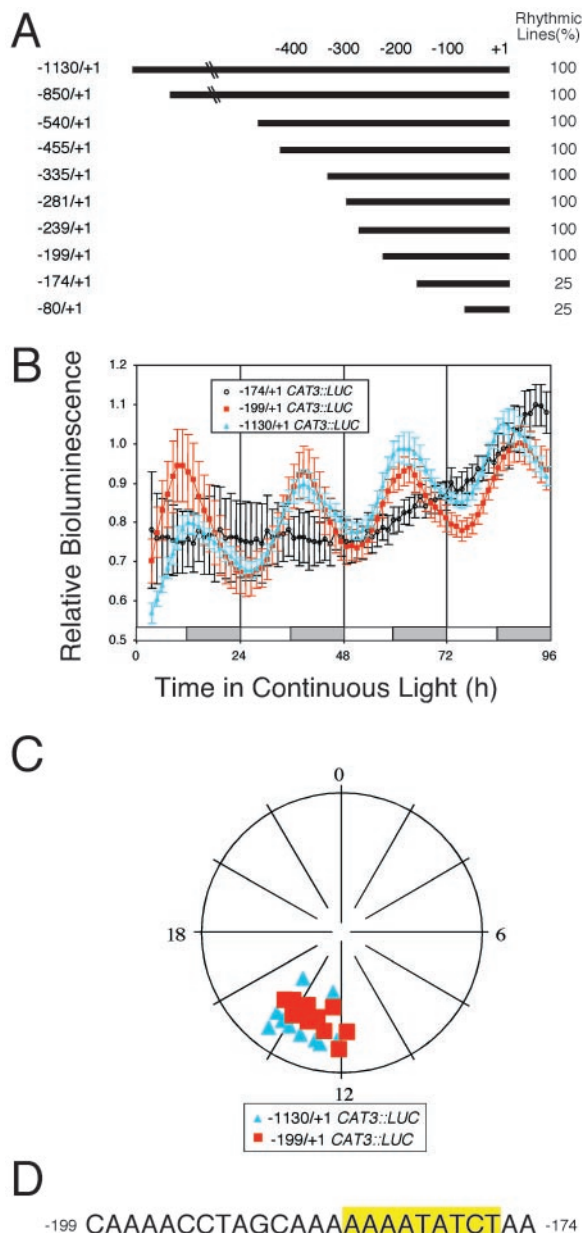
sistence of robust circadian oscillations in *CAT3* transcription in DD contrasts strikingly with the rapid damping seen in *CAB2* transcription in DD (Fig. 3; Millar et al., 1992). However, transcription as measured with transcriptional *LUC* fusions has been shown to oscillate in DD for several genes in addition to *CAT3*, including *CCR2* (Strayer et al., 2000), *TOC1* (Strayer et al., 2000), *EARLY FLOWERING 3* (Covington et al., 2001), *PHYTOCHROME (PHY) A*, *PHYB*, *PHYD*, *PHYE*, *CRYPTOCHROME 1*, and *CRYPTOCHROME 2* (Hall et al., 2001; Tóth et al., 2001). Moreover, overexpression of tobacco (*Nicotiana tabacum*) ZGT allows sustained oscillation of *CAB2* transcription in extended dark (Xu and Johnson, 2001).

### *CAT3* Promoter Deletion Series Reveals That an EE Is Necessary for Evening-Specific Transcription

Progressive deletion of the *CAT3* promoter from  $-1,130$  to  $-199$  yielded a series of eight promoter fragments that conferred similar evening-specific rhythmicity with a period of about 24 h (Fig. 4). The strength of the promoter fragment, as indicated by absolute *LUC* activity, was correlated with the size of the promoter fragment (data not shown), suggesting the presence multiple additive positive elements. At least nine independent lines of  $T_2$  seedlings were tested for each construct, and the vast majority ( $>85\%$ ) of the seedlings for any given line were rhythmic (Fig. 4A). In contrast, transgenic lines carrying the two shortest *CAT3* promoter fragments tested,  $-174/+1$  and  $-80/+1$ , were substantially arrhythmic (Fig. 4A). From these results, we conclude that an element necessary for evening-specific circadian transcription lies in the 25-bp region between  $-199$  and  $-174$  of the *CAT3* promoter (Fig. 4D).



**Figure 3.** *CAT3::LUC* activity continues to oscillate in DD. Plants grown as described in the Figure 1 legend and released into DD conditions instead of LL. Traces present average values ( $\pm$  SE,  $n = 12$ ), normalized as described in the legend to Figure 2, from *CAB2::LUC* (black triangles), *CAT3::LUC* (red squares), and *TOC1::LUC* (blue circles) seedlings. The LD regime is indicated by the bars beneath the traces, with subjective day indicated by white bars and subjective night indicated by gray bars. As discussed in the Figure 1 legend,  $[-221/-103]_2$  *CAT3::LUC* seedlings are depicted.



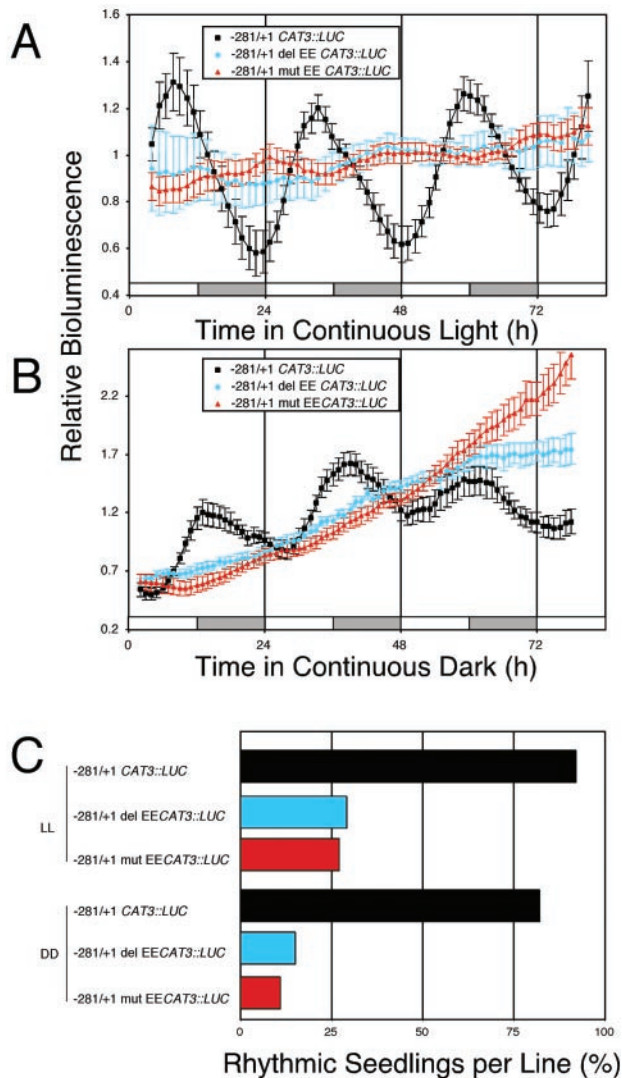
**Figure 4.** Deletion analysis of the *CAT3::LUC* promoter reveals an EE that is necessary for evening-specific circadian transcription. **A**, Summary of the *CAT3::LUC* promoter resection indicating the proportion of independent transgenic lines expressing evening-specific circadian LUC activity in LL. **B**, Plants were grown as described in the Figure 1 legend and released into LL. The LD regime is indicated by the bars beneath the traces, with subjective day indicated by white bars and subjective night indicated by gray bars. Traces present average values ( $\pm$  SE,  $n = 12$ ), normalized as described in the legend to Figure 2, from  $-1,130/+1$  *CAT3::LUC* (blue triangles),  $-199/+1$  *CAT3::LUC* (red squares), and  $-174/+1$  *CAT3::LUC* (black circles) seedlings. **C**, Phase plots of 12 seedlings from single transgenic lines carrying either the  $-1,130/+1$  *CAT3::LUC* (blue triangles) or the  $-199/+1$  *CAT3::LUC* (red squares) constructs. **D**, Nucleotide sequence of the 25-bp *CAT3* promoter region between  $-199$  and  $-174$ , which is required for rhythmicity and contains the EE, AAAAATATCT (highlighted), and the *lhc* motif, CAN<sub>2-4</sub>ATC (underlined; Piechulla et al., 1998).

Located between  $-199$  and  $-174$  of the *CAT3* promoter is an EE (AAATATCT; Fig. 4D; see Harmer et al., 2000) that is similar to the CBS (AAAAATCT, Wang et al., 1997) or the closely related *lhc* motif (Piechulla et al., 1998). To determine whether this EE is necessary for evening-specific circadian LUC activity, we performed two loss-of-function experiments. In the context of the  $-281/+1$  *CAT3::LUC* construct, deletion of a 40-bp region from  $-194$  to  $-153$  that contains the EE ( $-281/+1$  delEE *CAT3::LUC*) or mutation of three positions (AAATATCT to AtATAgCg;  $-281/+1$  mutEE *CAT3::LUC*) previously shown by to be important for CCA1 binding to the CBS (Wang et al., 1997) rendered LUC activity substantially arrhythmic ( $<25\%$  rhythmic seedlings) in both LL and DD conditions (Fig. 5, A–C). Therefore, we conclude that the EE is necessary for evening-specific circadian transcription of the minimal *CAT3* promoter, as has been previously demonstrated for the *CCR2* and *TOC1* promoters (Harmer et al., 2000; Alabadí et al., 2001).

Is the EE sufficient to confer evening-specific circadian transcription? A dimerized 118-bp fragment of the *CAT3* promoter encompassing the EE ( $[-221/-103]_2$  *CAT3::LUC*) is sufficient to confer robust evening-specific circadian rhythmicity on the *LUC* reporter, consistent with the other *CAT3* fusions (Fig. 6, A and B). However, monomers of 41 ( $-203/-163$ ) or 20 bp ( $-192/-173$ ), or a dimer of 14 bp ( $-190/-177$ ), each centered on the EE, failed to confer rhythmic *LUC* transcription (Fig. 6; data not shown).

#### The T to A Difference between CBS and EE Determines Circadian Phase in DD and LD

The EE (AAATATCT) is related to the CBS (AAAAATCT; Wang et al., 1997), and both have been shown in vitro to be the targets of the single MYB domain transcription factors CCA1 and LHY (Wang et al., 1997; Alabadí et al., 2001). Functional studies indicate that the EE is important for evening-specific transcription of *CCR2* (Harmer et al., 2000) and that the CBS is important for mid-morning-specific transcription of the *CAB2* (Carré and Kay, 1995). Because of the difference of only 1 bp between the EE and the CBS, we hypothesized that it is the difference at this single position that is responsible for the distinct phase properties of promoters carrying the two elements. To test this hypothesis, we changed the EE into a CBS (AAATATCT to AAAAATCT) in the  $-199/+1$  *CAT3::LUC* context ( $-199/+1$  CBS *CAT3::LUC*). In LL,  $>85\%$  of seedlings carrying the intact EE ( $-199/+1$  *CAT3::LUC*) expressed robust evening-specific circadian oscillations, whereas plants carrying the EE to CBS mutation ( $-199/+1$  CBS *CAT3::LUC*) were substantially arrhythmic ( $<25\%$  of the plants rhythmic; Fig. 7, A and D). Similar results were obtained when we changed the T to an A in the  $-333/+1$  *CAT3::LUC* and  $-281/+1$

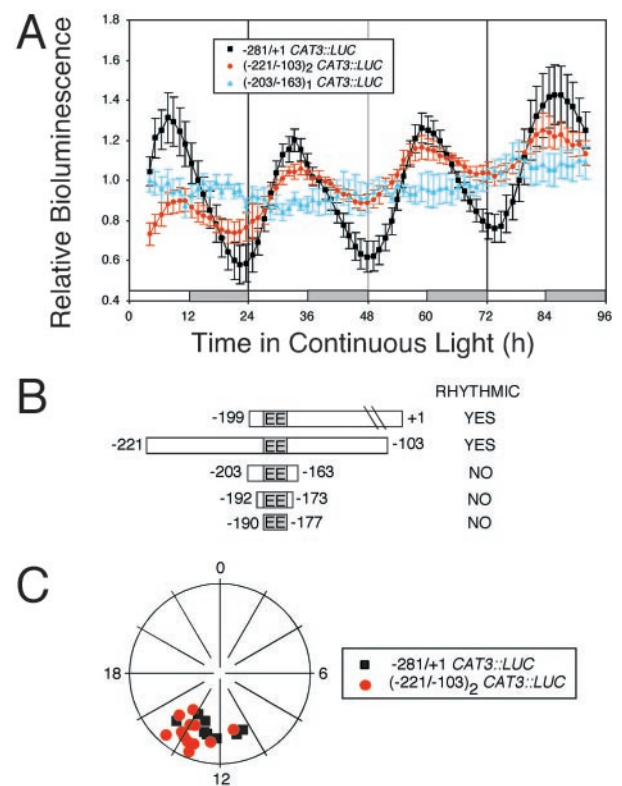


**Figure 5.** Deletion and site-directed mutagenesis show that the EE is necessary for circadian-regulated transcription of *CAT3::LUC*. Plants were grown as described in the Figure 1 legend and released into LL (A) or DD (B). The LD regime is indicated by the bars beneath the traces, with subjective day indicated by white bars and subjective night indicated by gray bars. A, Traces present average values ( $\pm$  SE,  $n = 12$ ), normalized as described in the legend to Figure 2, from  $-281/+1$  *CAT3::LUC* (black squares),  $-281/+1$  delEE *CAT3::LUC* (blue circles), and  $-281/+1$  mutEE *CAT3::LUC* (red triangles) seedlings assayed in LL. B, Traces present average values ( $\pm$  SE,  $n = 12$ ), normalized as described in the legend to Figure 2, from  $-281/+1$  *CAT3::LUC* (black squares),  $-281/+1$  delEE *CAT3::LUC* (blue circles), and  $-281/+1$  mutEE *CAT3::LUC* (red triangles) seedlings assayed in DD. C, Average proportion (%) of seedlings per each independent transgenic line exhibiting circadian rhythmicity in LL and DD.

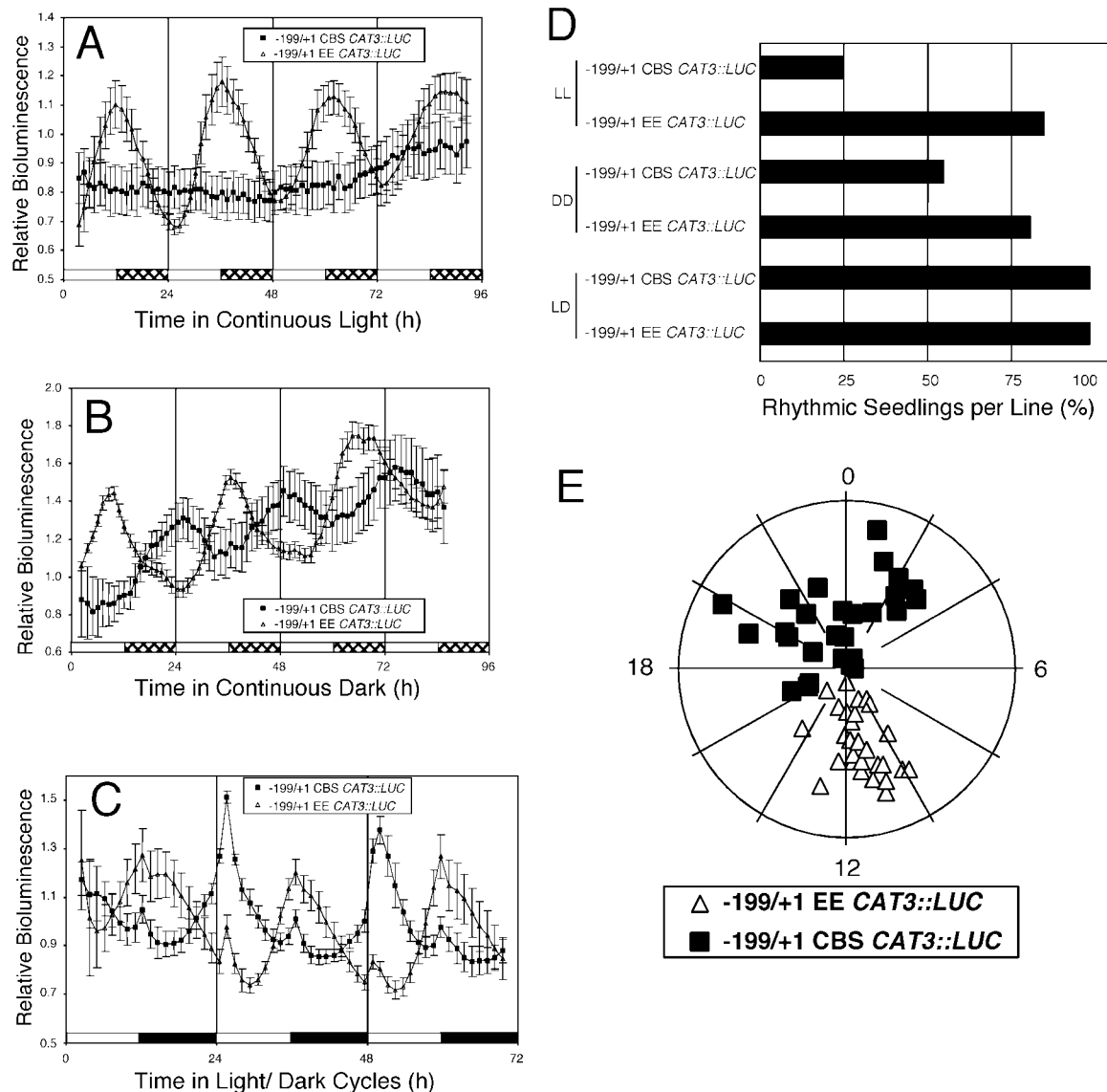
*CAT3::LUC* constructs (data not shown). In contrast, the circadian dysfunction resulting from the T to A substitution was less pronounced in DD conditions; 55% of the  $-199/+1$  CBS *CAT3::LUC* seedlings were rhythmic. Moreover, it is important to note that these rhythmic seedlings displayed the morning-specific

phase characteristic of the CBS (Fig. 7, B and E). This is in contrast to the  $-281/+1$  del EE *CAT3::LUC* or the  $-281/+1$  mut EE *CAT3::LUC* seedlings, which were arrhythmic in DD conditions (Fig. 5C). Therefore, the morning-specific expression of the  $-199/+1$  CBS *CAT3::LUC* in DD cannot be simply attributed to loss of EE function. These results suggest that the CBS cannot function properly in the context of the *CAT3* promoter in LL but exhibits morning-specific activity in DD.

Furthermore, we hypothesized that if the clock confers morning-specific activity to the  $-199/+1$  CBS *CAT3::LUC* in DD, then the circadian clock should drive morning-specific transcription during LD cycles also. In LD, >90% of the  $-199/+1$  EE *CAT3::LUC* plants display driven circadian rhythms with dusk anticipation. That is, LUC activity increases throughout the light period, peaks at dusk, and declines throughout the dark period, as expected for an evening-specific promoter (Fig. 7C). A small



**Figure 6.** Gain of function experiments show that a 118-bp region from the *CAT3* promoter is sufficient to confer evening-specific circadian LUC activity. Plants were grown as described in the Figure 1 legend and released into LL. A, Traces present average values ( $\pm$  SE,  $n = 12$ ), normalized as described in the legend to Figure 2, from  $(-221/-118)_2$  *CAT3::LUC* (red circles),  $-281/+1$  *CAT3::LUC* (black squares), and  $(-203/-163)_1$  *CAT3::LUC* (blue triangles) seedlings. The LD regime is indicated by the bars beneath the traces, with subjective day indicated by white bars and subjective night indicated by gray bars. B, Cartoon comparing *CAT3* promoter fragments used in gain-of-function experiments. C, Phase plots of 12 seedlings from one transgenic line for each of the two rhythmic constructs shown in A.



**Figure 7.** The CBS and EE are phase-specific motifs. Plants were grown under a 12/12 LD photoperiod at 22°C for 7 d. Plants were grown as described in the Figure 1 legend and released into LL (A) or into DD (B) or retained in LD cycles (C). Traces present average values ( $\pm$  SE,  $n = 12$ ), normalized as described in the legend to Figure 2, from  $-199/+1$  EE *CAT3::LUC* (triangles) and  $-199/+1$  CBS *CAT3::LUC* (squares) seedlings. The LD regime is indicated by white bars and subjective night indicated by hatched bars. C, The entraining LD cycle is indicated with white and black bars, respectively. D, Average proportion (%) of seedlings per each independent transgenic line exhibiting circadian rhythmicity in LL, DD, and LD cycles. E, Phase plots of all rhythmic seedlings, assayed in DD, from five transgenic lines of  $-199/+1$  EE *CAT3::LUC* (triangles) and for eight transgenic lines of  $-199/+1$  CBS *CAT3::LUC* (squares).

acute response at both dawn and dusk is observed in all promoter luciferase fusions assayed in LD regardless of the promoter used (data not shown). In contrast, >90% of the  $-199/+1$  CBS *CAT3::LUC* plants exhibit dawn anticipation where LUC activity increases throughout the dark period, peaks at dawn, and declines throughout the light period (Fig. 7C). This demonstrates that the  $-199/+1$  CBS *CAT3::LUC* plants are responding to circadian clock control.

## DISCUSSION

Resection of the *CAT3* promoter has revealed that the EE is required for evening-specific circadian clock-regulated *CAT3* transcription. Deletion of 40 bp centered on the *CAT3* EE or mutation of 3 bp in the *CAT3* EE renders *CAT3::LUC* expression substantially arrhythmic. The EE had been implicated previously in evening-specific clock-regulated transcription of *AtGER3* (Staiger et al., 1999) and *AtGRP7* (also

called CCR2; Staiger and Apel, 1999). The EE was also identified through sequence analysis of the promoters of 31 genes that exhibited circadian oscillations in mRNA abundance that peaked in the evening (Harmer et al., 2000). Our study confirms the necessity of this element through site-directed mutagenesis and is consistent with the results of loss of function mutation of the EEs in the *TOC1* and *CCR2* minimal promoters (Harmer et al., 2000; Alabadi et al., 2001).

The available data strongly suggests that the EE is a phase-specific circadian clock response element that is necessary to confer not only circadian-regulated transcription but also time-of-day (phase) information. It is quite striking that the EE is closely related to the CBS, which has been identified in morning-specific promoters (Carré and Kay, 1995; Liu et al., 1996; Piechulla et al., 1998; Kellmann et al., 1999) and which differs from the EE by one base (CBS, AAAAATCT, and EE, AAAATATCT). Conversion of the *CAT3* EE into a CBS within the context of a *CAT3* minimal promoter dramatically reduces rhythmicity in LL; the low frequency of rhythmic plants (<25%) is similar to that seen when EE activity is eliminated by deletion or by site-directed mutation at three positions. Thus, we conclude that the EE to CBS mutation results in a loss of circadian promoter activity in LL. However, conversion of the *CAT3* EE into a CBS shifts the phase of transcription from evening to morning in LD and DD. This represents the first attempt to define the mechanism by which the circadian clock imparts time-of-day-specific information to the transcriptional apparatus. Our results reinforce the centrality of the CBS/EE in circadian transcription in *Arabidopsis* and clearly establish that phase may be modulated through the 1-bp difference between the CBS and EE.

The CBS in the context of the *CAT3* promoter functions as a morning-specific element in DD and LD, but fails to impart circadian control in LL. It is reasonable to suppose that the complement of proteins recruited to the promoter differs in light versus dark. For example, mRNA accumulation of *CAB2* and *CCA1* damps dramatically in the dark, which has been attributed to the depletion of phytochrome in the Pfr form (Kay and Millar, 1993). In contrast, the core clock components *LHY* and *TOC1* robustly oscillate in DD conditions. Because there exist significant differences in the abundance and activity of transcription factors between light and dark (Terzaghi and Cashmore, 1995), it should not be surprising that the activity of the CBS or EE may differ in either condition, reflecting the altered milieu at the promoter environment surrounding the EE/CBS.

Although the EE is necessary for transcription of *CAT3*, a number of lines of evidence have established that the presence of an EE is insufficient to confer circadian-regulated transcription. For example, the 500-bp *CAT1* promoter fragment contains one con-

sensus EE (−124 AAAATATCT −132), yet transcription of the *CAT1::LUC* construct displays no circadian rhythm. Monomers of 41 or 20 bp, and a 14-bp dimer centered on the *CAT3* EE are insufficient to confer robust circadian regulation. Furthermore, the −687/+1 *TOC1::LUC* retains an EE (−25/−39), yet is substantially arrhythmic (Alabadi et al., 2001). These findings collectively suggest that the EE and the CBS require additional contextual information to confer circadian-regulated transcription. Although a 41-bp fragment of the *CAT3* promoter, centered on the EE, is insufficient to drive circadian-regulated transcription of the LUC reporter gene, a 118-bp *CAT3* dimer is sufficient to confer robust circadian transcription with wild-type period and evening-specific phase. The implication is that additional information is contained in the additional 78 bp of this larger construct that is essential for the circadian activity of the EE. A minimal promoter consisting of the −199/+1 region of the *CAT3* promoter similarly retains rhythmicity, as do minimal *CCR2* and *TOC1* promoters of 130 and 190 bp, respectively (Harmer et al., 2000; Alabadi et al., 2001). It seems reasonable to hypothesize that there are additional binding activities associated with these promoters that are necessary for circadian transcription. These activities are, themselves, insufficient for circadian transcription because deletion or mutation of the EE eliminates circadian activity. Rather, they provide a permissive context within which the EE can function.

Similar conclusions have been reached regarding circadian transcription in fruitfly. A 69-bp circadian regulatory sequence (CRS) from the *period* (*per*) promoter was initially identified as sufficient to confer circadian-regulated transcription (Hao et al., 1997). The CRS is sufficient to confer normal spatial and temporal expression on a *per* transgene and to drive *per* expression sufficient to restore normal behavioral rhythms to a *per*-null mutant (Hao et al., 1999). At the heart of the CRS is the E-box (CACGTG), which binds the dCLOCK-CYCLE heterodimer to drive rhythmic transcription (Darlington et al., 1998; Gekakis et al., 1998; Jin et al., 1999). In mammals, the E box plays a similar role and is bound by heterodimers of the mammalian orthologs, CLOCK and BMAL (Darlington et al., 1998; Gekakis et al., 1998; Jin et al., 1999). However, mutation of the core E-box of either the *per* or *timeless* (*tim*) genes, allows the retention of rhythmic transcription, although transcript levels are reduced (Hao et al., 1997; McDonald et al., 2001). Mutation of other *per* CRS sequences outside the E-box affects spatial and temporal expression and impairs the restoration of behavioral rhythms to *per*-null mutants by the driven *per* transgene (Lyons et al., 2000). Thus, the context of the E-box within the CRS is critical for fully functional spatial and temporal *per* transcription. The most parsimonious interpretation is that the interaction of other binding activities with dCLOCK-CYCLE bound to the E-box is necessary for



wild-type *per* expression (Darlington et al., 2000; Kyriacou and Rosato, 2000; Lyons et al., 2000). Analysis of the *tim* promoter identified two non-canonical E-boxes as well as other elements, at least one of which is also found in the *per* promoter, that each contribute to robust rhythmic transcription (McDonald et al., 2001).

Although elements other than the canonical E-box contribute to rhythmic transcription of both *per* and *tim*, a tetramer of an 18-mer centered on the *per* E-box (and including 6 bp on either side) drives reduced rhythmic *per*-like LUC expression that displays partial spatial overlap with the pattern conferred by the intact CRS (Darlington et al., 2000). It is thought that multimerization enhances the strength of the element, compensating for the lack of the flanking elements provided in the context of the full CRS (Darlington et al., 2000; Kyriacou and Rosato, 2000). Although the multimerized E-box will drive rhythmic *per*-like LUC expression, it is not known whether this construct will rescue *per*-null flies when driving *per* expression (Darlington et al., 2000; Kyriacou and Rosato, 2000). Moreover, a single E-box is insufficient to drive transcription (Lyons et al., 2000), consistent with our observations that monomers up to 41 bp centered on the *CAT3* EE are insufficient to confer circadian-regulated transcription. As with the *per* E-box, a tetramer of a 36-bp sequence including the *CAB2* CBS is sufficient to drive robust morning-specific circadian transcription (Carré and Kay, 1995). It is worth noting that this 36-bp sequence binds at least four distinct factors that do not exhibit circadian oscillation in binding activity (Carré and Kay, 1995; Wang et al., 1997) but that may be providing contextual information.

Thus, we conclude that the EE/CBS are cis-acting elements central to the generation of rhythmic transcription in *Arabidopsis* and may be analogous to the E-box of fruitfly and mammals. Of course, we would not preclude the possibility of other motif/transcription factors interactions imparting clock regulation to other genes. Like the E-box, the EE and CBS are found in promoters both of clock component genes and of clock-controlled genes that function purely on circadian output loops. Although the EE and CBS have been defined as critical to evening- and morning-specific transcription of some genes, it is clear that the *Arabidopsis* circadian clock transcribes clock-controlled genes at multiple phases that span the entire day-night cycle (Harmer et al., 2000; Schaffer et al., 2001). There might be a DNA element and a cognate-binding factor for each distinct phase, but it seems more likely that additional information provided by the promoter context modulates activity at the CBS and EE. Combinatorial regulation of promoter activity is well established in light-regulated gene expression (Menkens et al., 1995; Puente et al., 1996; Chattopadhyay et al., 1998) and combinatorial

interactions might contribute to the specification of circadian phase-specific promoter activity.

We suggest that one role of the contextual information provided by sequences surrounding the EE/CBS may be to modulate the phase at which the EE/CBS is transcribed. For example, we note that the *CAT3* and *TOC1* promoter elements described in this study each contain a single EE, yet drive transcription at distinct phases (CT14 versus CT19, respectively). Sequences flanking the *CAT3* and *TOC1* EEs apparently include an element or elements that function as "phase modifiers." Alone, these phase modifiers are insufficient to confer rhythmicity but, instead, modulate activity of the EE/CBS to confer distinct phases seen with the two promoters. These phase modifiers might function constitutively to establish a stable phase that is distinct from that inherent in the interaction of the element with its clock-controlled-binding factor (e.g. *CAT3* versus *TOC1*), but also might provide targets to integrate other environmental or developmental information with clock regulation. For example, the phase of both *CAB2* and *TOC1* transcription is modulated by daylength (Millar and Kay, 1996; Matsushika et al., 2000), which suggests that activities of the CBS and EE in the *CAB2* and *TOC1* promoters, respectively, are modulated by light- and/or photoperiod-sensitive phase modifiers. The *CAB2* and *TOC1* promoters both contain the Hexamer (Hex) element (TGACGTGG), a relative of the light-mediated motif, the G-box (CACGTG, curiously identical to the E-box of flies and mammals) that binds G-box-binding factor 1 (Schindler et al., 1992; Menkens et al., 1995). Both the Hex element and G-box are candidates for light-specific phase modifiers. It may be pertinent that casein kinase 2 phosphorylates G-box-binding factor 1 (Klimczak et al., 1992, 1995) in addition to CCA1 and LHY (Sugano et al., 1998, 1999).

Interestingly, a motif related to both the Hex motif and the E-box, the cAMP response element (CRE: TGACGTCA), has also been implicated in circadian transcription of mammalian *c-fos* and *Arg vasopressin* genes (Robertson et al., 1995; Iwasaki et al., 1997). Multimers of the CRE confer circadian-regulated transcription in both the mouse SCN (Obrietan et al., 1999) and fruitfly (Belvin et al., 1999). CRE elements are present in both the *per* and *tim* promoters, although their contribution to circadian-regulated transcription remains unclear (Kyriacou and Rosato, 2000). It is possible that the CRE acts as a phase modifier, or perhaps modulates promoter activity in response to environmental or developmental cues.

The circadian transcriptional machinery must be responsive to environmental and developmental change. Combinatorial regulation in which the activity of core clock components is modulated through interaction with other factors recruited to clock-controlled promoters provides an important mechanism to integrate circadian control of gene expression

with other levels of control (Kyriacou and Rosato, 2000). It is thought that interlocked feedback loops contribute to the robustness and stability of the circadian oscillator itself (Glossop et al., 1999; Lee et al., 2000; Shearman et al., 2000). It seems equally reasonable to posit that combinatorial control of rhythmic transcription is also likely to add to the stability of circadian transcription both of core oscillator components and of clock output circuits.

## MATERIALS AND METHODS

### *CAT3::LUCIFERASE (CAT3::LUC) Constructs*

*CAT3* promoter fragments (−1,130, −850, −540, −455, −335, −281, −239, −199, −174, −80 to +1, where +1 denotes the transcriptional start site of *CAT3* [Zhong and McClung, 1996]) were isolated from BAC T10F14 and subcloned into pZP $\Omega$ Luc<sup>+</sup> (Schultz et al., 2001). The [−221/−103]<sub>2</sub> *CAT3::LUC* construct was created by digesting the 118-bp fragment from the *CAT3* promoter and ligating into pZP $\Omega$ Luc<sup>+</sup>; the resultant clone carried two tandem copies of the 118-bp fragment inserted in the reverse orientation. −281/+1 delEE *CAT3::LUC* was created by removing bases −194/−153 by restriction digestion, and religating the resulting *CAT3* promoter fragments. The −281/+1 mutEE *CAT3::LUC* was created using the site-directed mutagenesis primer 5′-GCCCCACTTCGCTATTATTTT-GCTAGGTTTTG-3′ (where the mutated EE is underlined and shown in the inverse orientation). The *CAT3::LUC*, −335/+1 CBS *CAT3::LUC*, −281/+1 CBS *CAT3::LUC*, and −199/+1 CBS *CAT3::LUC* were made with overlapping primers containing the mutated base. The *CAT1::LUC* and *TOC1::LUC* transcriptional fusions contained 500 bp (starting 78 bp upstream of the ATG) and 509 bp (starting 381 bp upstream of the ATG) of their promoter regions, respectively. All constructs were sequenced to confirm fidelity and to check for mutations and/or unwanted DNA fragments introduced by the subcloning process.

### Arabidopsis Transformation

Floral dip transformation was performed on different ecotypes (Col, COL CS933; Rschew, RLD CS913; WS, WS CS915; *Ler*, *LER* CS20; and *Cvi*, *Cvi* CS902) with slight modifications (Clough and Bent, 1998). *Agrobacterium tumefaciens* strain GV3101 was used in all transformations. T<sub>0</sub> seeds were collected, and resistant seeds were selected on 1% (w/v) agar Murashige and Skoog (1962) plates with 70  $\mu$ L mL<sup>−1</sup> gentamicin and 150  $\mu$ L mL<sup>−1</sup> carbenicillin. T<sub>1</sub> seedlings were collected and allowed to self, and T<sub>2</sub> seeds were collected and analyzed for luciferase activity.

### Luciferase Assays

T<sub>2</sub> plants containing *CAT3::LUC* constructs were analyzed using a Packard TopCount luminometer and scintillation counter (Packard) as described (Carré and Kay, 1995). Seeds were vapor-phase sterilized (Clough and Bent, 1998) and plated on 1% (w/v) agar Murashige and Skoog media containing 70  $\mu$ L mL<sup>−1</sup> gentamicin. Seeds were stratified 3 d in the dark at 4°C and then transferred into 12-h white light (70  $\mu$ mol m<sup>−2</sup> s<sup>−1</sup>)/12-h dark (LD) cycle for 7 d at 22°C. For temperature experiments plants were grown in 12-h 18°C/12-h 22°C in constant white light (70  $\mu$ mol m<sup>−2</sup> s<sup>−1</sup>). Seedlings were transferred to black microtiter plates (Dynex Technologies, Chantilly, VA) containing, per well, 200  $\mu$ L of 0.8% (w/v) agar Murashige and Skoog medium plus 2% (w/v) Suc and 35  $\mu$ L of 0.5 mM luciferin (Biosynth AG, Staad, Switzerland). Microtiter plates were covered with clear plastic TopSeal (Packard) in which holes were placed above each well for seedling gas exchange. Plates were moved to the Packard TopCount and interleaved with four clear plates to allow light diffusion to the seedlings. Seedlings were entrained in white light (15–25  $\mu$ mol m<sup>−2</sup> s<sup>−1</sup>) for 3 d with 12/12 LD cycles. Luciferase activity was measured every 1 h by integrating photons emitted by seedlings during a 10-s sampling period. DD experiments were conducted as above with the exception that they received DD after they were entrained on the Packard TopCount.

## Data Analysis

Data were formatted using Import and Analysis Excel software (Plautz et al., 1997; Strayer et al., 1999). Rhythms were analyzed by fast Fourier transform-nonlinear least squares analysis (Plautz et al., 1997; Zhong et al., 1997). Except in Figure 1, all data were normalized to the average luciferase activity of the individual seedling and are presented as relative bioluminescence. Seedlings were determined to be rhythmic if their period was between 20 and 28 h, the peak signal strength exceeded 100 photons seedling<sup>−1</sup> s<sup>−1</sup>, and the RAE, a measure of the strength of the rhythm, was <1.0. A perfect noise-free cosine wave would return an RAE = 0, because the analytical estimate of rhythmic amplitude would be determined with practically no error. A rhythmic component assessed to have an RAE approaching 1 is contrarily approaching the limit of statistical significance (i.e. RAE = 1 is the limit of statistical significance for any given rhythmic amplitude). For all experiments, between nine and 24 independent T<sub>2</sub> lines were tested in a minimum of two independent experiments. All lines, except −174/+1 and −80/+1 *CAT3::LUC*, contained a proportion of plants that were rhythmic. Because lines varied in the proportion of seedlings that were rhythmic, we established the cutoff that 50% of the seedlings in a given line must be rhythmic for that line to be called “substantially rhythmic.” If fewer than 50% of the seedlings in that line were rhythmic, that line was considered to be “substantially arrhythmic.” All values are presented as mean  $\pm$  SE. CT (phase  $\times$  24-h period) allows the normalization of rhythms with different period to ascertain how phase compares in constant conditions. To compare phase of different genes or constructs, phases of individual seedlings are plotted against the strength of the rhythm. Phase (CT) is plotted around the circumference of a 24-h clock face. The strength of the rhythm is plotted along the radius with the strongest rhythms (RAE = 0) at the outer edge of the circle and weakest rhythms (RAE = 1) at the center.

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