

# Transcriptional Regulation by a Circadian Rhythm

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**Transcription of genes encoding the light-harvesting chlorophyll *a/b* protein (Cab) was shown previously to be regulated by light and a number of developmental factors. I show that a circadian rhythm in transcriptional activity is superimposed on these other regulatory programs. In vitro transcription measurements in isolated maize leaf nuclei demonstrated that changes in transcription are not coincident with light-dark transitions and that diurnal changes in transcription continue in continuous light and disappear in complete darkness. Light intensity influences the amplitude of transcriptional changes but has no effect on periodicity. Major diurnal changes in Cab mRNA are evident not only in developing seedling leaves, but also in mature, fully expanded leaves. Genes encoding phosphoenolpyruvate carboxylase and the cytosolic form of aldolase show no diurnal changes in transcription.**

## INTRODUCTION

The light-harvesting chlorophyll *a/b* apoprotein (CAB) of photosystem II is a major component of chloroplast thylakoid membranes. The prevalence of the apoprotein and its mRNA has made it an attractive candidate for the study of gene regulation in higher plants. Consequently, a fair amount is known about the factors that separately regulate the accumulation of Cab mRNA and apoprotein.

Although the accumulation of both Cab mRNA and apoprotein are induced by light, the mechanisms are quite different. Several lines of evidence suggest that stable insertion of the apoprotein into the thylakoid membrane requires the binding of chlorophylls *a* and *b*; hence, chlorophyll controls the accumulation of the apoprotein (Apel and Kloppstech, 1980; Bennett, 1981; Harpster et al., 1984).

The accumulation of Cab mRNA appears to be regulated exclusively at the transcriptional level, with a number of factors affecting Cab gene transcription. Light acting through phytochrome and a blue light receptor plays a major role in regulating transcriptional activity (Tobin and Silverthorne, 1985; Fluhr et al., 1986). Analyses of chimeric Cab gene constructions in transgenic plants have identified positive and negative regulatory elements that respond to light (Simpson et al., 1986a; Nagy et al., 1987; Castresana et al., 1988).

Cab gene expression is also regulated by organ-specific, cell-specific, and developmental-stage-specific factors. Cab mRNA has been detected only in green organs such as leaves (Muller et al., 1980; Eckes et al., 1985; Lamppa et al., 1985) and developing fruit (Piechulla et al., 1986). Its prevalence is influenced by the stage of cell development (Lamppa et al., 1985; Martineau and Taylor, 1985; Piechulla et al., 1986). Cell-specific localization is evident

in C4 plants such as maize, where Cab mRNA is localized primarily in mesophyll cells (Broglie et al., 1984; Schuster et al., 1985; Sheen and Bogorad, 1986a). Each gene within the Cab gene family exhibits a specific program of expression that differs from other family members in its light response and developmental program (Lamppa et al., 1985; Stayton et al., 1989).

An additional factor that plays a major role in regulating Cab gene expression is a chloroplast signal (Mayfield and Taylor, 1984; Batschauer et al., 1986; Simpson et al., 1986b). The signal originates at an early stage of chloroplast development and its continuous synthesis is required for optimal Cab gene transcription (Oelmüller et al., 1986; Burgess and Taylor, 1988).

I show that a circadian rhythm is superimposed on these regulatory programs, causing major diurnal changes in the transcription of Cab genes and hence changes in the prevalence of Cab mRNA. Similar effects have been reported for Cab mRNA in developing tomato fruit (Piechulla and Gruissem, 1987) and a wheat Cab gene in wheat leaves and in transgenic tobacco leaves (Nagy et al., 1988).

## RESULTS

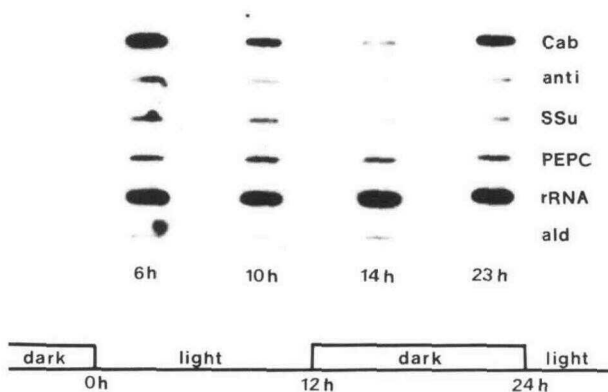
### Periodic Changes in Transcription

Our previous studies on the role of a chloroplast signal in Cab transcription gave the first hint of diurnal transcriptional changes (Burgess and Taylor, 1988). Maize seedlings were grown for 8 days in dim light on a 12-hr photoperiod and then transferred to continuous light at a  $10^4$ -fold higher fluence rate. Nuclei from leaves sampled at

14 hr after the lights turned on showed a dramatic decrease in the apparent transcription rate of Cab genes (see Figure 5 in Burgess and Taylor, 1988).

The time course of changes in the rate of Cab transcription was determined by growing seedlings for 9 days after germination in a growth chamber on a 12-hr photoperiod of normal intensity white light ( $96 \mu\text{mol photon/m}^2/\text{sec}$ ). Nuclei were prepared from leaves harvested at 6 hr, 10 hr, 14 hr, and 23 hr on the 9th day. The lights turned on at 0 hr and off at 12 hr. Figure 1 shows the hybridization of in vitro labeled RNA from these nuclei to slot blots of single-stranded DNA probes for the indicated mRNAs. Major changes in the hybridization signal are evident for the Cab transcript, indicating major changes in the apparent rate of Cab transcription. These changes in Cab transcription are, however, not coincident with light-dark transitions.

The labeling of rRNA is constant. Saturation hybridization to excess DNA demonstrated that the rate of rRNA synthesis is unchanged between sampling times (data not shown). Although the hybridization signal for the transcript encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSu) appears to vary, this is most likely an experimental artifact. SSu mRNA, and presumably transcription, is limited to bundle sheath cells in maize, a C4 plant (Broglie et al., 1984; Martineau and Taylor, 1986; Sheen and Bogorad, 1986b). The yields of transcriptionally competent nuclei from the tough bundle sheath cells of maize leaves are low and variable. Cab transcription occurs in mesophyll cells, which are much easier to lyse. Another mesophyll-specific transcript, phosphoenolpyruvate carboxylase (PEPC) (Broglie et al., 1984; Martineau and Taylor, 1986), shows no significant variation between sampling times, as is the case for transcripts encoding the cytosolic form of aldolase.



**Figure 1.** Diurnal Changes in Cab Transcription.

In vitro labeled RNA from nuclei isolated at the indicated time points from 9-day-old seedling leaves were hybridized to single-stranded probes for Cab, SSu, PEPC, rRNA and aldolase (ald) mRNAs, and antisense (anti) Cab mRNA.

### Light Intensity Influences Transcription Rates

Seedlings were again grown for 9 days after germination in a growth chamber on a 12-hr photoperiod, but in dim white light ( $0.011 \mu\text{mol photon/m}^2/\text{sec}$ ). Nuclei were prepared from leaves taken on the 9th day at the same times as in Figure 1. Histograms of densitometric tracings of autoradiographs are presented in Figure 2. Although seedlings grown in normal and dim light exhibit a similar time course of Cab transcriptional rate changes, there are significant differences in the relative rates of transcription. The rate of Cab transcription relative to rRNA is fairly similar for leaves taken at 14 hr. The  $10^4$ -fold higher photon fluence rate of the normal light treatment is reflected by a sixfold to eightfold higher relative rate of Cab transcription in leaves during the light period at 6 hr and 10 hr. The fact that the quantitative differences in transcription rates are 3 orders of magnitude less than the differences in photon fluence rate is not surprising, given that Cab transcription has been shown to be induced by much lower fluences than the dim light used in this experiment (Kaufman et al., 1984).

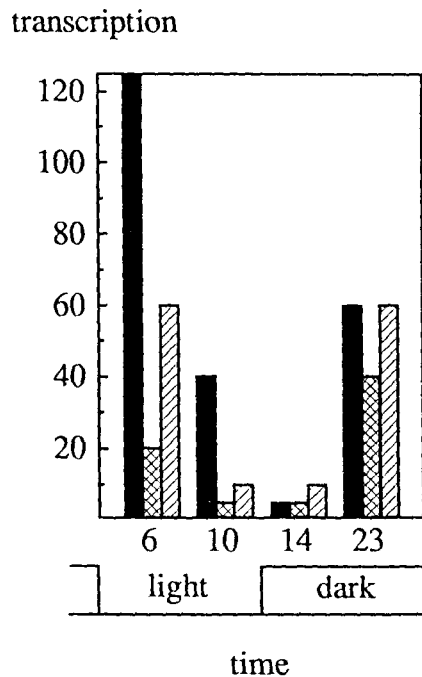
The in vitro labeling of Cab mRNA relative to rRNA was found to be quite reproducible. Different transcription reactions performed on the same preparation of nuclei gave similar hybridization signals (data not shown). The variability between nuclei preparations was not tested.

In both normal and dim light, Cab transcription decreases prior to the light-dark transition at 12 hr (Figure 2). Because these seedlings have been grown in growth chambers with no variation in light quantity or quality during the 12-hr light period, the decrease in transcription at 10 hr cannot be due to any external light cues. A more dramatic demonstration of this point comes from an experiment in which seedlings were grown for 8 days after germination on a 12-hr photoperiod of dim light, and then transferred at 0 hr on the 9th day to continuous light at the higher fluence rate. Figure 2 shows that the same decreases in Cab transcription occurred at 10 hr and 14 hr, followed by an increase at 23 hr, despite the fact that the lights remained on throughout the leaf sampling period.

The relative rates of Cab transcription in this experiment were more similar to those of the higher fluence rate photoperiod than the dim light photoperiod. The relative rate of Cab transcription, therefore, reflects the fluence rate of the immediate or most recent period of illumination.

### Transcription Decreases in Continuous Darkness

Seedlings were grown for 8 days after germination on a photoperiod of 12 hr in dim light and then transferred to a dark room. Leaf samples were taken on the 9th day in complete darkness at the same times as in previous experiments. Figure 3 shows that the relative rate of Cab transcription decreased at 10 hr and 14 hr, as in all of the



**Figure 2.** Diurnal Changes in Cab Transcription Persist in Varying Light Treatments.

Nuclei were isolated from 9-day-old leaves at the indicated times. In vitro labeled RNA was hybridized to slot blots of probe DNAs and the resulting autoradiographs scanned by a microdensitometer. Dark bars represent data from Figure 1; cross-hatched bars represent leaves from plants grown 9 days on 12-hr cycles of dim light; right-hatched bars represent plants grown for 8 days on 12-hr cycles of dim light and then transferred to higher intensity light for the last day.

previous experiments, but that the 23-hr sample failed to show the increase that seedlings subjected to periodic or constant illumination show (compare Figures 2 and 3). Despite the absence of light on the 9th day, the relative rate of Cab transcription at 6 hr was remarkably similar to that at 6 hr in seedlings grown in dim light.

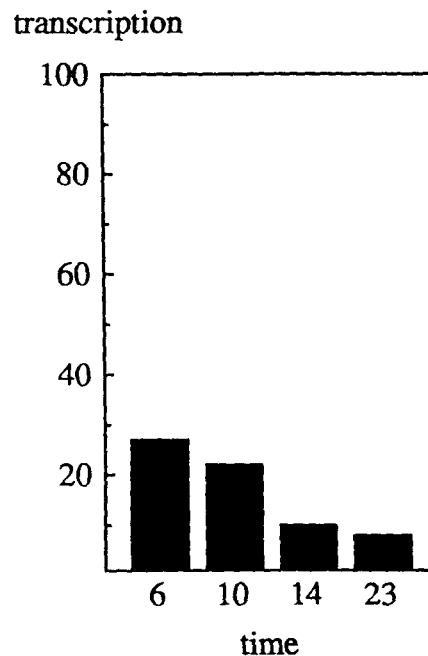
#### Diurnal Changes in Cab mRNA Persist in Mature Leaves

Most of the measurements of periodic changes in transcription rate and mRNA prevalence have been done on seedling leaves, where high rates of chlorophyll synthesis are accompanied by high rates of CAB apoprotein synthesis and accumulation. To determine whether these periodic changes are only found during the assembly of light-harvesting complexes, RNA was isolated from samples of mature leaves. Fully expanded leaves from glasshouse-

grown plants maintained on a constant 16.5-hr photoperiod were used. The fifth leaf below the tassel was sampled at various times 1 week prior to tassel emergence. This tissue was taken prior to senescence but long after leaf cell expansion had ceased.

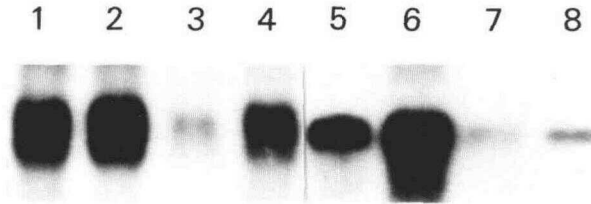
Figure 4 shows that, even in this mature leaf tissue, there were dramatic changes in the relative quantity of Cab mRNA (lanes 5 to 8). The magnitude of these changes was similar to that seen for Cab mRNA in seedling leaves (compare lanes 1 to 4). Although transcription was not measured in mature leaves, it is reasonable to assume that similar period changes in Cab transcription persist in maize leaves through to maturity.

The seedling leaf RNA preparations were made from the same leaf samples as those used for the nuclei preparations in Figure 1. Comparison of the relative rates of Cab transcription with the prevalence of Cab mRNA (compare Figures 1 and 4) shows that changes in Cab mRNA prevalence lag behind changes in the rate of Cab transcription. The rapid decrease in mRNA between 10 hr and 14 hr (Figure 4, lanes 2 and 3) is consistent with the observed decrease in transcription rate and a short half-life of the mRNA. Previously, we have estimated the half-life of maize Cab mRNA to be less than 1 hr (Burgess and Taylor, 1988).



**Figure 3.** Diurnal Transcription Changes Disappear in Complete Darkness.

Seedlings were grown for 8 days on 12-hr cycles of dim light and then transferred to complete darkness for the last day. Transcription quantitated as in Figure 2.



**Figure 4.** Diurnal Changes in Cab mRNA in Both Seedling and Mature Leaves.

Seedlings were grown for 9 days on 12-hr cycles of normal intensity light. RNA was isolated from leaves at 6 hr (lane 1), 10 hr (lane 2), 14 hr (lane 3), and 23 hr (lane 4) after the lights turned on. Sections of mature leaves were isolated as described in "Methods." Samples were taken at 3.5 hr (lane 5), 6.5 hr (lane 6), 14.5 hr (lane 7), and 18.5 hr (lane 8) after the lights turned on. The lights turned off at 16.5 hr.

## DISCUSSION

Several lines of evidence indicate that the diurnal changes in Cab gene transcription are due to a circadian rhythm. Increases or decreases in Cab transcription are not coincident with light-dark transitions, but rather precede the turning on or off of the lights by several hours. Because these experiments were conducted in growth chambers, the turning on or off of the lights was not preceded by changes in light quantity or quality that might provide regulatory cues, such as occur in the real world. More convincing evidence comes from the experiment in which seedlings placed in continuous light continued to show a diurnal fluctuation in transcription (Figure 2). Seedlings placed in complete darkness also showed the same diurnal fluctuations (Figure 3), but as the level of transcription decreased in the dark, quantitative changes became difficult to measure. Nagy et al. (1988) measured quantitative changes of one specific wheat Cab mRNA and found that diurnal fluctuations continued in complete darkness or continuous light. Because their plants had been subjected to higher intensity light during the previous photoperiods, they were able to measure mRNA fluctuations over several days in the dark.

Nagy et al. (1988) demonstrated that plants do not require a prior photoperiod to establish the circadian rhythm. Wheat seedlings grown in continuous white light from germination exhibited a 24-hr periodicity in the expression of a specific Cab mRNA.

Light appears to interact with the circadian rhythm in two ways. Light acts to quantitatively induce Cab transcription to levels high enough that the effect of the endogenous circadian rhythm can be measured. Photoperiods differing by  $10^4$  in fluence rates only affected the amplitude of the diurnal fluctuations (Figure 2). The periodicities of the fluctuations were identical, as was the lowest level of transcription during the dark period. Nagy et al.

(1988) showed the phytochrome was responsible for determining the level of Cab gene expression within the diurnal cycle. A change in the photoperiod can influence the periodicity of Cab mRNA fluctuations. Stayton et al. (1989) grew petunia plants on an extended day length of 16 hr and found that the periodicity of Cab mRNA fluctuations adjusted to the longer light and shorter dark periods. The mature maize leaf samples used in Figure 4 (lanes 5 to 8) were taken from plants grown on a 16.5-hr day length. The Cab mRNA levels in this experiment appear similar to the samples taken from 12-hr daylength seedlings (Figure 4, lanes 1 to 4). In both cases mRNA levels were highest at 6 hr and lowest at 14 hr after the lights turned on, irrespective of the time at which the lights turned off. However, this experiment was not designed to measure the effects of a different photoperiod and not enough samples were collected to derive any meaningful conclusions.

To what extent the circadian rhythm affects the expression of other nuclear and plastid genes is not clear. Kloppstech (1985) reported that the levels of pea mRNAs coding for Cab, SSu, and an unidentified light-induced protein were all influenced by a circadian rhythm. Piechulla and Gruißem (1987) found small diurnal changes in the levels of SSu mRNA and two plastid mRNAs, *rbcL* and *psbA*, in developing tomato fruit. The level of Cab mRNA showed more dramatic diurnal changes. Nagy et al. (1988), however, found no diurnal changes in the level of wheat SSu mRNA. The transcriptional activity of PEPC, another light-induced gene family (Nelson et al., 1984; Thomas et al., 1987), also showed no diurnal fluctuations (Figure 1). Whether these differences reflect differences between monocots and dicots or between plant species or are due to differences in sensitivity of experimental technique remains to be seen.

A major question raised by all of these findings concerns the functional role of transcriptional regulation by a circadian rhythm. The effect of the rhythm on Cab gene transcription is found universally in monocots and dicots, in leaves and green fruit, and at all stages of organ development. The rhythm acts at the level of transcription, having no effect on the turnover rate of Cab mRNA (Nagy et al., 1988). Gehring et al. (1977) determined that the capacity for chlorophyll synthesis is also controlled by a circadian rhythm. Because net accumulation of the CAB apoprotein is dependent both on its rate of synthesis and on the binding of chlorophylls *a* and *b*, one might propose, as Nagy et al. (1988) have done, that a functional role of circadian regulation is to coordinate the biosynthesis of apoprotein and chlorophyll. Although this may be true in developing leaves, the circadian regulation of Cab transcription continues in mature leaf tissue (Figure 4). The fact that greening and leaf expansion were completed long before samples of mature leaves were taken suggests that there was little, if any, assembly of new light-harvesting complexes in these leaves. Although the turnover rate of

CAB apoprotein has never been determined accurately to my knowledge, there is no evidence that it has a rapid turnover rate (Staehelin and Arntzen, 1983). The fact that the apoprotein survives extensive photooxidative damage suggests that it is quite stable (Mayfield et al., 1986).

A consequence of these findings is that the design of experiments involving Cab gene expression or Cab promoter activity in transgenic plants must take into account the major effect of the circadian rhythm.

## METHODS

### Plant Material

Seeds of the *Zea mays* L. inbred B73 (Pioneer H-Bred International) were grown on soil covered with vermiculite in a growth chamber. Leaf tissue was harvested at the indicated times and immediately frozen in liquid N<sub>2</sub>.

Mature leaf tissue came from sibling 62-day-old maize plants that had been grown in a glasshouse with supplemental lighting to extend the day length from 5:30 AM until 10:00 PM. The distal 25 cm of the fifth leaf below the tassel was used. The 10 cm of the leaf tip was cut off prior to freezing in liquid N<sub>2</sub>, even though there was no evidence of senescence at the tip.

### In Vitro Transcription

Nuclei were isolated and used for in vitro transcription measurements as described by Burgess and Taylor (1988). A number of controls indicated that this in vitro transcription system reflected in vivo transcription rates.

Quantitative comparisons were made by scanning autoradiographs with a Joyce Loebel microdensitometer. The intensity of Cab hybridization was normalized to that of rRNA hybridization.

### RNA Blot Hybridizations

Total RNA was isolated as described by Burgess and Taylor (1987). RNA was isolated from pooled leaf samples of 9-day-old seedlings or from single leaves of mature plants. Formaldehyde gel electrophoresis, blotting, and hybridization were performed as described by Burgess and Taylor (1988). The probe was nick-translated pZLH5, a maize cDNA Cab clone.

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