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Control of *lhc* gene transcription by the circadian clock in *Chlamydomonas reinhardtii*

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Abstract

Transcription of nuclear *lhc* genes has been shown to be under circadian clock control in angiosperms, but many aspects of this regulation have not been elucidated. Unicellular organisms, such as the green alga *Chlamydomonas reinhardtii*, offer significant advantages for the study of cellular clocks. Therefore, we have asked whether *lhc* gene expression is regulated by a circadian clock in *C. reinhardtii*. The mRNA for a photosystem I chlorophyll *a*/*b* apoprotein showed a strong diurnal rhythm in cells growing under 12 h/12 h light/dark (LD) cycles; the mRNA accumulated and then declined during the light period reaching very low levels at mid-dark. A similar diurnal pattern was documented for *rbcS* mRNA. In LD-grown cells shifted to continuous light, the ca. 24 h rhythm of *lhca1* mRNA continued for at least 2 cycles. In LD-grown cells shifted to continuous darkness the rhythm of *lhca1*, but not *rbcS2*, mRNA also continued, although at lower absolute levels than in LD-grown cells. Also, in the cells shifted to continuous dark, the *lhca1* mRNA rhythm persisted in the absence of significant cell division. Pulse-labelling with $^{32}PO_4$ and sensitivity to actinomycin D demonstrated that control of *lhca1* (and *rbcS*) is mainly transcriptional. However, it was also shown that the half-life of *lhca1* mRNA (and *rbcS2*) is short (1–2 h) and may also vary somewhat during a cycle. We conclude that a cellular, circadian clock regulates *lhca1* transcription in *C. reinhardtii*.

Introduction

A circadian rhythm is a biological rhythm which shows a periodicity of ca. 24 h. Although the rhythm can be reset by environmental stimuli (e.g. light), it persists when the stimuli are absent; therefore, a circadian rhythm is controlled by an endogenous biological clock [36]. Circadian rhythms are apparently universal in eukaryotes, and have also been reported in cyanobacteria [22, 33]. In recent years, it has become clear that genes encoding the apoproteins of the light harvesting complexes of photosystem II (*lhcb*) and photosystem I (*lhca*) are under circadian control in angiosperms [14, 31, 38, 40, 42, 52, 54]. It is not clear, however, whether they are under similar control in lower plants, since no evidence was found for a circadian rhythm of *lhcb* expression in

a gymnosperm [1], or the green unicellular alga *Chlamydomonas eugametos* [13].

A circadian rhythm can be dissected into 3 parts: the input pathway(s), the endogenous clock, and the output pathway(s) [36]. The input pathway includes both the means to perceive environmental stimuli, and a mechanism to transduce the signal to the clock. The clock can be reset, and therefore multiple clocks synchronized, via the input pathway. Among various environmental stimuli, sunlight is the most effective cue for circadian rhythms. In angiosperms, the photoreceptor, phytochrome, seems to be involved in the synchronization (or resetting) of the *lhcb* mRNA rhythm [40, 53]. In addition to light, temperature shifts can act as a 'Zeitgeber' [32, 42]; however, nothing is known about the input pathway used by temperature cycles. The second part of the input pathway, the transduction mechanism, has not been at all elucidated.

The second component of a circadian rhythm, the clock, controls the rhythm via the output pathway. The nature of the circadian clock is a mystery, although many hypotheses, including molecular, network, tape-reading, and membrane models, have been proposed (see refs. in [10]). It may be a turning point that the cloned genes *per* in *Drosophila* and *frq* in *Neurospora* are reported to be components of the clock [9, 44, 51].

The output pathway of the circadian system is a transduction step which transfers messages from the clock and results in expression of the rhythm. It has been shown that, in angiosperms, the *lhc* mRNA rhythm is due primarily to periodic transcription. Furthermore, a clock-responsive 5′ *cis*-element has been identified for a wheat *lhcb* gene [12]. Circadian control of transcription has also been documented in non-plant systems [35]; however, the clock may also control gene expression at other levels [39, 55].

Unicellular organisms provide excellent, well-defined systems for the study of circadian rhythms [10]. Two major advantages of these systems are the ability to obtain homogeneous populations of cells, and the fact that rhythms are inherently cellular, as opposed to those in multicellular organisms that might be tissue-derived and/or involve intercellular communication. In the unicellular green alga *Chlamydomonas reinhardtii*, rhythms of phototaxis [3], stickiness to glass [50], chemotaxis to ammonium [8], methyl ammonium uptake [8], and, depending on growth conditions, cell division [7] have been reported to be controlled by the circadian clock. In addition, *C. reinhardtii* has been used to study the nature of the circadian clock, and is one of the few organisms for which clock mutants have been isolated [4–6]. Circadian rhythms of gene expression in *C. reinhardtii* have not yet been clearly shown, but evidence of endogenous rhythms of gene expression can be found in the literature [27, 34, 45]. In this study, we show that *lhc* gene expression in *C. reinhardtii* is controlled by the circadian clock at the transcriptional level, and that such control is independent of any cell cycle controls.

Materials and methods

Strains, culturing, and cell manipulations

The wild-type *2137* mt+ (CC-1021) strain, and the *per2* mt+ (CC-1118), *per4* mt+ (CC-1119), *per4* mt− (CC-1120), and *per6* (CC-1123) mutants of *C. reinhardtii* were obtained from the *Chlamydomonas* Genetics Center (Duke University, Durham, NC). The cells were grown in Tris-acetate-phosphate medium [16] with shaking at a light flux of ca. 20 μE m^{-2} sec^{-1} and 23 °C. Synchronization was obtained by growing cells under 12 h light/12 h dark cycles for at least 3 photoperiods. For the continuous dark treatments, cells were transferred to flasks wrapped with black tape or doubly wrapped in aluminum foil. When necessary, manipulations were performed under a green fluorescent light (20 W, General Electric). Cells were harvested during the exponential phase of growth ($1–6 \times 10^6$ cells/ml) for analyses. For the drug treatments, cycloheximide and actinoymcin D were added to cultures from concentrated stock solutions in ethanol; the final ethanol concentration in the culture was less than 1%. Cell counts were performed with the aid of a hemacytometer, and all cells, including those not hatched, were counted.

RNA isolation, northern blot hybridization, and mRNA quantification

Isolation of total RNA and northern blot hybridizations were performed as described [18, 19]. The RNA blots were stained with methylene blue to check for equal loading and transfer [18]. After hybridization, the blots were washed to high stringency, 0.1× SSPE, 0.5% SDS at 65 °C [24]. The specific DNA probes were *C. reinhardtii* genes and were as follows: the *Eco* RI-*Hin*c II fragment of *lhca1* cDNA [24]; the *Pst* I fragment of a *rbcS* cDNA clone, pCS2.1 [15]; the *Xba* I insert of a chloroplast *psbA* gene clone, pGEMR14.2 [20], the insert of a β-tubulin cDNA clone, pcf9–12 [48]; and the insert of a *lhcb* genomic clone, pKG11a [21]. DNA probes were gel-purified restriction fragments ^{32}P-labelled by the random-primed method [11]. Autoradiographs of RNA gel blots were quantified by densitometry using a gel scanner (ISCO Inc.); multiple exposures of each blot were made and scanned to achieve signals within the linear range of the film.

Northern blots of RNA isolated from cells which had been treated with actinomycin D to block transcription were used to estimate mRNA half-lives. The values obtained from densitometric quantification of the autoradiographs were plotted (versus time) on semi-log paper.

In vivo *pulse-labelling and dot-blot hybridization*

[^{32}P]Orthophosphate labelling was performed as previously described [21]. After isolating total ^{32}P-RNA [18], poly(A) RNA was purified using poly(U)-Sephadex (batch method) as instructed by the manufacturer (Gibco-BRL). For dot-blot hybridization, excess amounts of plasmid DNAs containing specific inserts were applied to a Zetaprobe membrane (BioRad) using the Bio-Dot apparatus as described by the manufacturer (Bio-Rad). Hybridization of ^{32}P-poly(A) RNA to the dot-blot was performed as for the northern hybridization described above.

All experiments were repeated at least twice and with similar results.

Results

Diurnal rhythms of selected nuclear mRNAs

Previously, we characterized a *C. reinhardtii* cDNA clone (*lhca1*), which encodes a 20 kDa photosystem I light-harvesting chlorophyll *a*/*b*-binding protein [24]. The *lhca1* cDNA hybridized to a single nuclear gene under stringent conditions [24]. Thus, this gene-specific probe was used to determine if *lhca1* gene expression is under circadian control in *C. reinhardtii*. However, it was first necessary to characterize the diurnal rhythm of *lhca1* mRNA under these growth conditions (see Materials and methods).

Cells were grown for three 12 h/12 h light/dark (LD) cycles and then gene expression and cell number monitored during the fourth and fifth cycles, which will be referred to as the first and second cycles (after synchronization). Figures 1 and 2 (LD) show that the steady-state level of the 1.1 kb *lhca1* mRNA, as monitored by northern blot analysis, shows a strong diurnal oscillation; the highest level (h 42 = L6 of the second cycle) was ca. 50-fold greater than the lowest level (h 30 and h 54 = D6 of first and second cycle). Since the amplitude of the second cycle (ca. 50-fold) was greater than the first cycle (ca. 30-fold), but the lowest expression levels (h 30 and h 54) were similar, the amplitude of each oscillation is dependent on the maximum expression level.

For comparison, and as an internal control, mRNA levels for another prominent nuclear-encoded chloroplast protein, *rbcS* (the small subunit of ribulose 1,5-bisphosphate carboxylase), were examined. In *C. reinhardtii*, there are two *rbcS* genes, which produce two distinguishable transcripts [15]. The smaller transcript (ca. 0.85 kb) is derived from the *rbcS2* gene, is more abundant under these conditions, and the only one we quantified. Figures 1 and 2 (LD) show that steady-state levels of *rbcS2* mRNA also fluctuate diurnally, but the amplitude is much smaller (ca. 2.5-fold) compared to *lhca1* mRNA (30–50-fold).

The diurnal expression of a β-tubulin gene and the *lhcb* gene family were also examined. The

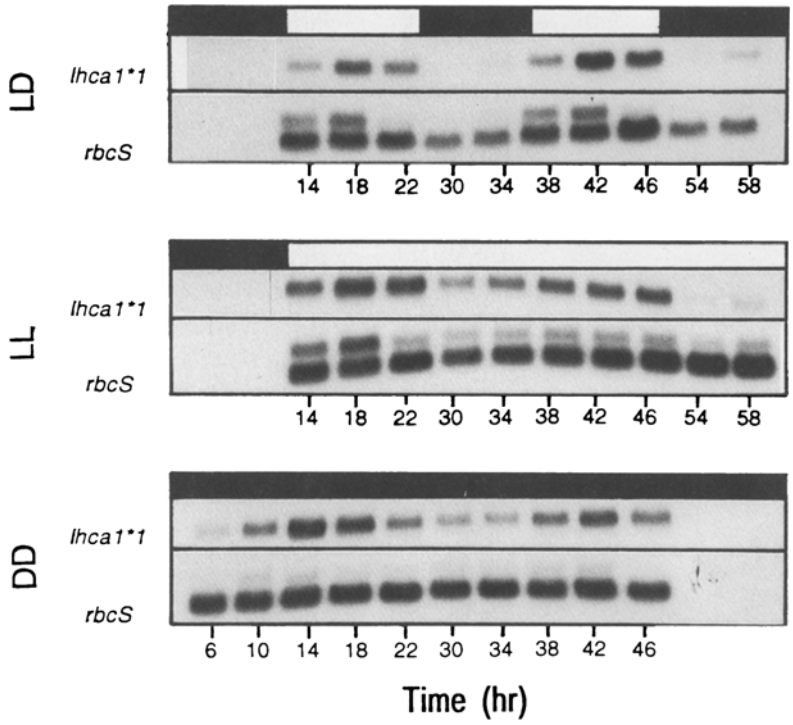

Fig. 1. Northern blot analysis of *lhca1* mRNA levels. After 3 cycles of growth in 12 h/12 h light/dark cycles, the culture was split and aliquots were: (1) retained in the light-dark cycling condition (LD), (2) shifted to continuous light at the first light period (LL), or shifted to continuous dark (DD) (i.e. the lights were not allowed to come on for the fourth light period). 5 μg of total RNA, extracted from cells at the indicated time points, was electrophoresed, blotted, and hybridized with the *lhca1*-specific cDNA probe (see Materials and methods). The same blots were probed with the *rbcS* cDNA after removing the *lhca1* probe. The autoradiographs of LD and LL RNA were prepared by exposing the blots to X-ray film for 3 h, while the DD RNA blot was exposed for 12 h. The h-46 time point from LD-grown cells was overloaded by ca. 30%. Time is measured beginning with the third dark period and refers to h in continuous conditions. RNA was analyzed only at the time points indicated below each box. Open rectangles, light periods; filled rectangles, dark periods.

β-tubulin transcript showed a diurnal rhythm that peaked in the early to mid-dark period, similar to the pattern reported previously [2]. The *lhcb* family of transcripts varied diurnally with a pattern that was essentially identical to the *lhca1* mRNA rhythm (data not shown).

It is well established that phototrophic growth of *C. reinhardtii* under LD cycles results in synchronous growth and division, with cytokinesis occurring in the dark period, e.g. [17]. Figure 2 shows that cell division (in this wild-type strain) was also synchronous with these mixotrophic (light plus acetate) conditions, as indicated by the step-wise increases in cell number in the dark period.

Persistence of the lhca1 *mRNA rhythm under continuous conditions*

To determine whether the diurnal fluctuation of *lhca1* mRNA reflects an underlying circadian rhythm, LD-entrained cells were shifted to continuous light and steady-state levels of *lhca1* mRNA examined for at least 2 cycles (Figs. 1 and 2, LL). The oscillation of *lhca1* mRNA levels continued, and with the same phase as in the normal LD cycle, although with less amplitude. Decreased amplitude of *lhc* mRNA levels under LL has also been observed in higher plants, e.g. [29]. In contrast, the diurnal oscillation of *rbcS2* mRNA did not persist in continuous light, al-

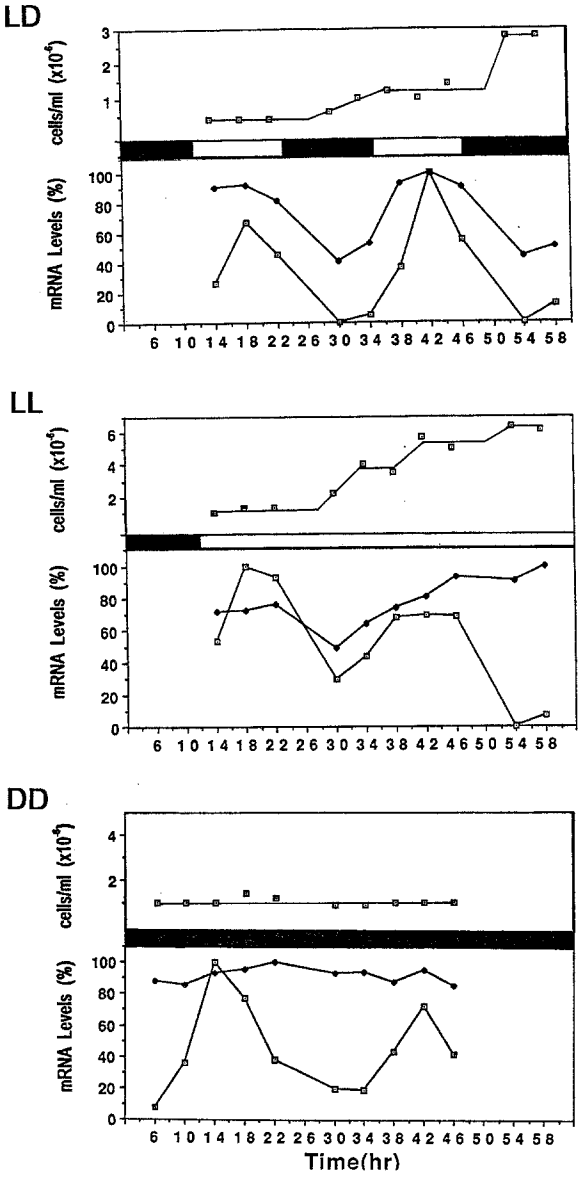

Fig. 2. Cell number and quantification of the northern blots in Fig. 1. At each time point that RNA was extracted in Fig. 1, cell number was determined and plotted as cells/ml $\times 10^{-6}$. Levels of mRNA were quantified as described in Materials and methods; maximum levels were arbitrarily set to 100%. Overloading (ca. 30%) of the h-46 time point in LD (see Fig. 1) was taken into account for the quantification. Open rectangles, light periods; filled rectangles, dark periods; filled diamonds, *rbcs2* mRNA levels; squares, *lhca1* mRNA levels.

though it appears that it may have persisted for 1 cycle (Figs. 1 and 2, LL). The larger *rbcS* transcript, *rbcS1*, showed a rhythm of accumulation in both cycles, however it was more difficult to quantify by northern blotting. It also should be noted that, in continuous light, the increases in cell number were more frequent than every 24 h (Fig. 2).

LD-entrained cells were also shifted to continuous dark, and mRNA levels, as well as cell number, were monitored (Figs. 1 and 2, DD). Levels of *lhca1* mRNA showed a robust rhythm although with a slightly longer period (ca. 28 h). The apparently longer period is due to the peak of the first cycle occurring a few h earlier in the DD cells compared to the LD-grown cells. In contrast, *rbcS2* mRNA levels did not significantly oscillate in continuous darkness. The *rbcS1* transcript was at too low levels to quantify. It is also important to note that cell number did not increase in constant darkness (Fig. 2, DD). Thus, we can conclude that *lhca1* mRNA, but not *rbcS2* mRNA, is under strong circadian control, and this control is independent of cell division.

When the same LL and DD blots from Fig. 1 were probed with the β-tubulin and *lhcb* DNAs a clear rhythm was seen only for the *lhcb* transcripts and it was similar to the *lhca1* rhythm (data not shown, [23]). Thus, it seems that *lhcb* transcripts are also under circadian control in *C. reinhardtii.*

The data of Figs. 1 and 2 also provide some indication of the extent to which light directly stimulates *lhca1* mRNA accumulation. The northern blots from the continuous dark grown cells (DD) were exposed 4 times longer than the blots of RNA from LD or LL grown cells.

Analysis of transcription by pulse-labelling with $^{32}PO_4$

The circadian rhythm of *lhca1* mRNA could result from a rhythm in transcription rate, mRNA stability, or both. To answer this question, we initially attempted to utilize a nuclear run-off transcription assay [28]; however, *lhca1* was poorly transcribed in nuclei isolated from the cell-wall mutant *cw15*, too low for reasonable estimates of relative transcription rates (unpublished results). Moreover, we wished to retain the use of wild-

type cells. Thus, pulse-labelling with $^{32}PO_4$ was employed. The pulse-labelling was carried out with cells entrained by 3 LD cycles and then released into continuous light (LL); cells were removed and pulse-labelled at L4 and subjective D4 (h 16 of a 24 h cycle). Figure 3 shows the autoradiographs (A) and the quantified results (B) of the hybridization of pulse-labeled RNA with DNA dot-blots. Transcription of *lhca1* is ca. 15-fold greater at L4 than at subjective D4 (LL).

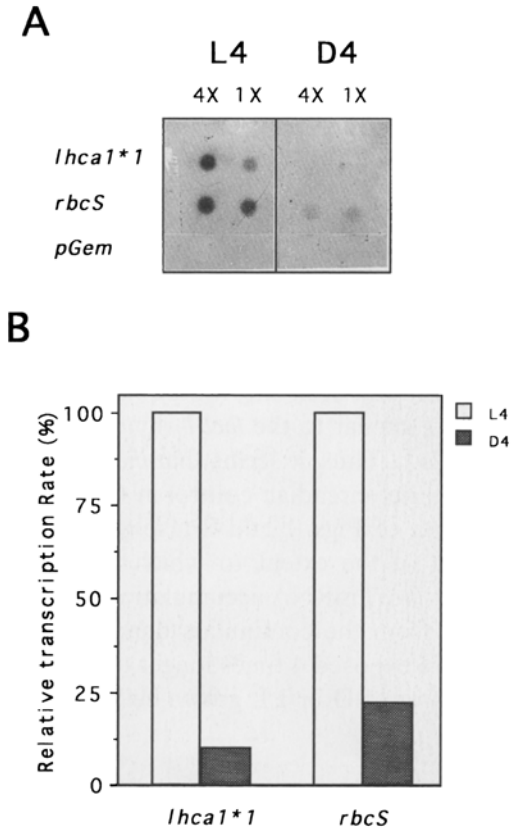

Fig. 3. Transcription rates of selected nuclear genes at L4 and D4 (subjective dark) in continuously illuminated cells. Cells were shifted to continuous light (LL) at the first light period after synchronization for 3 LD cycles. At 4 h (L4) and 16 h (subjective D4) after shifting to LL, cells were labelled with the ^{32}P-orthophosphate for 20 min and poly(A) RNA prepared. A. The purified ^{32}P-mRNA fractions (equal cpm) were hybridized to dot blots of *lhca1*, *rbcS*, and pGem (control vector) DNAs. 1× = 1 μg of DNA; 4× dots contained 4 times the DNA of the 1× dots. B. The autoradiograph of the 4× dots were densitometrically scanned and used to generate the histogram. The transcription rate at L4 was set to 100%.

This is 2–3-fold greater than the difference in *lhca1* mRNA levels at these times in the experiment of Fig. 2, thus raising the possibility that mRNA stability could play a role in *lhca1* regulation. However, it should also be noted that the amplitude of the mRNA oscillations can vary considerably (see Fig. 2).

The relative transcription rate of *rbcS* was also measured and found to be ca. 4-fold less at subjective D4 than L4. The lower rate of *rbcS* transcription at subjective D4 compared to L4 is consistent with the fact that *rbcS* mRNA levels decline during the first (but not the second) subjective dark period after entrainment (see Figs. 1 and 2). Thus, these results indicate that *rbcS* expression is also regulated at the transcriptional level.

Stability of lhca1 *mRNA*

Although Fig. 3 indicates that transcription is important in *lhca1* regulation, it was possible that the stability of *lhca1* mRNA might also be regulated [21]. Thus, we used the transcription inhibitor, actinomycin D, to estimate *lhca1* mRNA half-life at different times. First, however, several experiments were performed to establish an effective concentration of actinomycin D for these studies. Figure 4 shows that 50 μg/ml actinomycin D was sufficient to inhibit the increase in *lhca1* mRNA that occurs during the early light period. The data in Fig. 4 also show that increasing the drug concentration beyond 50 μg/ml does not produce a further or more rapid decrease in *lhca1* mRNA levels. Microscopic examination of cells treated with 50 μg/ml actinomycin D for several h showed no evidence of cell death, and moreover, the ribosomal RNA was intact; this was also true for cells taken at several other points of the light/dark cycle (data not shown). Thus, the lower concentration of 50 μg/ml actinomycin D was chosen for most experiments, since it was effective in blocking transcription of *lhca1* and other genes (data not shown), and in order to minimize possible deleterious effects of the drug on other cellular processes.

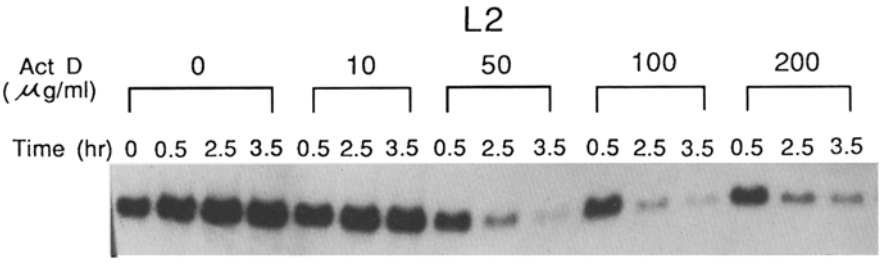

Fig. 4. Dose-response relationship for the transcription inhibitor actinomycin D and the accumulation of *lhca1* mRNA. At L2 of the fourth light-dark cycle, actinomycin D was added to cells at the indicated final concentrations. RNA was extracted at L2 and at successive times (in h) from control (0 μg/ml) and actinomycin D treated cells. 5 μg of total RNA was blotted, and hybridized with the *lhca1* cDNA probe.

The actinomycin D-chase protocol was employed to estimate the stability of *lhca1* mRNA at 3 selected time points (L2, L5, and L8) in a diurnal cycle (Figs. 5 and 6A). These times were chosen because they correspond to periods when *lhca1* mRNA levels are either increasing (L2), near-maximal (L5), or declining (L8). Figure 5 shows the autoradiographs that were obtained and Fig. 6A shows a semi-log plot of the *lhca1* mRNA decay data. The chloroplast-encoded *psbA* mRNA was used as an internal control in the actinomycin D chase experiment of Fig. 5, in part because it has a long half-life. The persistence of intact *psbA* mRNA indicates that the rapid decay of *lhca1* mRNA is not caused by grossly deleterious effects of actinomycin D on RNA metabolism or other cellular processes. In addition, the rRNAs (visualized on the blots using methylene blue staining) were intact and did not decay during the drug treatment (not shown).

Figure 6A shows that the kinetics of decay of *lhca1* mRNA at L2 and L5 were similar, but not identical, with the mRNA decaying at a slightly slower rate at L5 ($t_{1/2}$ of ca. 2.5 h) versus L2 ($t_{1/2}$ of ca. 2 h). In the actinomycin chase at L8, however, the rate of *lhca1* decay increases ca. 2-fold during the latter part of the 3 h chase. These data could indicate a significant decrease in *lhca1* mRNA stability at the end of the light period. Although it should be noted that a lack of linearity in semi-log plots of mRNA decay is not unusual and could represent, for instance, some secondary effect of the drug treatment which kicks in after the first hour of drug administration.

In order to determine if the stability of *lhca1* mRNA does decrease significantly at the end of the light period, the actinomycin D chase protocol was performed at another later point in the light, L10 (Fig. 6B). The rate of decay of *lhca1* mRNA was also measured at L6 and, in this experiment, at subjective D2 (the cells were kept

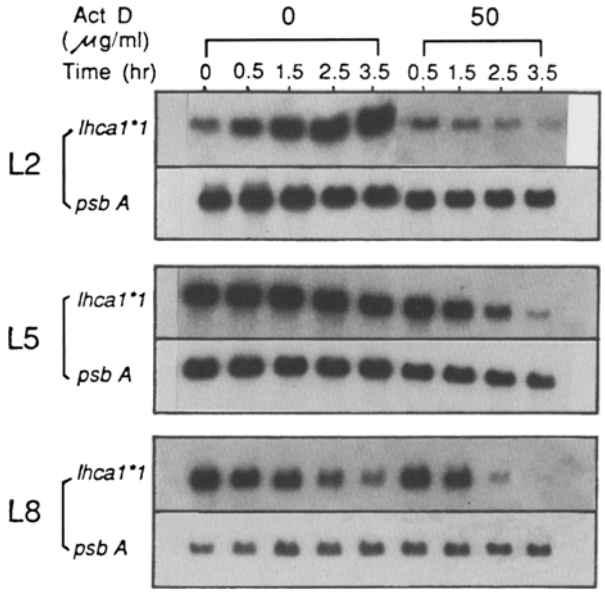

Fig. 5. Northern blot analysis of *lhca1* mRNA decay in the presence of actinomycin D at different times. At L2, L5, and L8 of a diurnal (LD) cycle, actinomycin D (f.c. 50 μg/ml) was added and total RNA extracted at the indicated intervals after drug administration. Total RNA was also extracted at the indicated time points from cells that did not receive actinomycin D (0 μg/ml). 5 μg of total RNA per lane were analyzed as in Fig. 1. After probing for *lhca1* mRNA, the blots were stripped and hybridized with a probe for *psbA* mRNA.

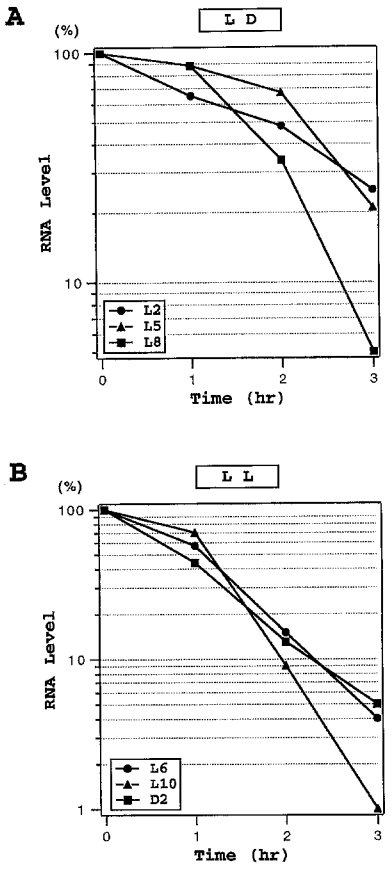

Fig. 6. Semi-log plots of the kinetics of *lhca1* mRNA decay at different times. A. Plots of the data obtained from quantification of the blots shown in Fig. 5. The *lhca1* mRNA level at the time the drug was added (zero time) was set to 100%; L2, L5, and L8 refer to the time of the LD cycle when the cells were withdrawn and chased with actinomycin D. B. A similar analysis of *lhca1* mRNA half-life at 3 different times, except in this case the cells were in the first cycle of continuous light (LL); thus, the D2 time point is in the subjective dark period.

in continuous light). Figure 6B shows that the kinetics of decay of *lhca1* mRNA are quite similar at all three times, although in the L10 chase there does appear to be a greater rate of decay after the first h of the chase, similar to Fig. 6A.

The half-life of *lhca1* mRNA differs by a factor of ca. 2 between the experiments described in Fig. 6A versus 6B; however, different cell cultures were used for these experiments. Results from several additional experiments confirmed that *lhca1* mRNA as well as *rbcS* showed culture-to-culture variation in half-life (the range was 1–2.5 h for *lhca1* mRNA). It should be emphasized, however, that we never observed dramatic (i.e. >1.5-fold) changes in *lhca1* mRNA half-life in the same culture over the course of an experiment; the reason(s) for the culture-to-culture variation in these nuclear mRNA half-lives are not known.

Salvador *et al.* [45] reported that chloroplast-encoded mRNAs have a shorter half-life in the light than in the dark in *C. reinhardtii*. We looked for an effect of light on *lhca1* mRNA stability by determining the half-life in a culture at the dark-light transition period (D11-L3) compared to an aliquot of the same culture kept in darkness (DD); there was no significant difference in the rates of decay of *lhca1* mRNA in the two conditions [23].

In the actinomycin D-chase experiments, we assumed that the decay of *lhca1* mRNA is the indirect result of inhibiting transcription of the *lhca1* gene; however, it is conceivable that actinomycin D might affect the half-life of *lhca1* mRNA more directly by depleting the cells of a protein which normally stabilizes the mRNA. Of course, this hypothesis would also require that the putative protein have a high turnover rate, and be encoded by a short-lived mRNA. In order to test this hypothesis, the translation inhibitor, cycloheximide, was used. If continuous synthesis of an mRNA-stabilizing factor is necessary for *lhca1* stability, then it can be expected that the decay of *lhca1* mRNA should be accelerated by a combined cycloheximide/actinomycin D treatment compared to actinomycin D alone. The rationale being that cycloheximide, by inhibiting the translation of preexisting mRNA, should deplete the cells of any mRNA-stabilizing factor(s) more rapidly than actinomycin D (which would inhibit production of the factor indirectly by halting synthesis of new 'factor' mRNA). As shown in Fig. 7, however, pretreating cells with cycloheximide did not shorten the half-life of *lhca1* mRNA, and in fact it was slightly longer than in actinomycin D alone (69 min versus 57 min). This result indicates that actinomycin D does not destabilize *lhca1* mRNA, at least not by depleting the cells of a *lhca1* mRNA-stabilizing protein factor. Similar

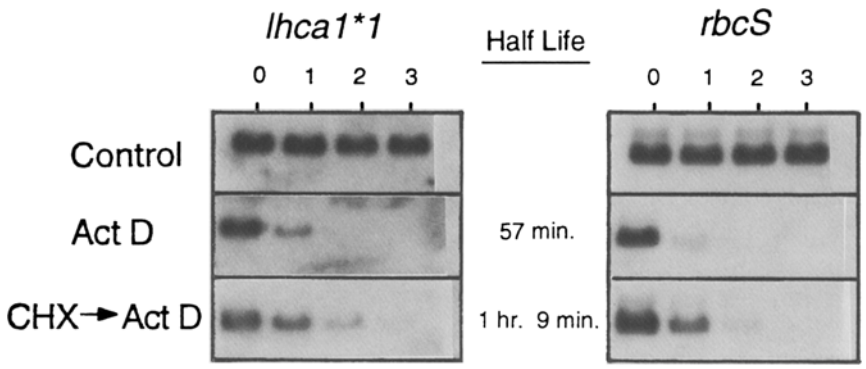

Fig. 7. Effect of cycloheximide on the half-life of *lhca1* mRNA. Cells were shifted to continuous light at the first light period after 3 light-dark cycles. The drugs were added at ca. L8 of the second 24 h cycle in LL and RNA extracted at the indicated times after addition of actinomycin D. For the dual drug treatment, cycloheximide (f.c. 10 μg/ml) was added 30 min before actinomycin D (f.c. 50 μg/ml). 5 μg of each RNA was blotted, hybridized with *lhca1* and *rbcS* cDNA probes. The half-lives of *lhca1* mRNA, which are indicated between the blots, were estimated from semi-log plots (as in Fig. 6). The half-life of *rbcS2* mRNA was not indicated in the figure.

results were obtained for *rbcS* mRNA (Fig. 7), which also has a short half-life (ca. 60 min).

Discussion

Circadian control of lhc *in* C. reinhardtii

We have shown that transcription of the *lhca1* gene is under circadian clock control in *C. reinhardtii*. The diurnal rhythm of *lhca1* mRNA reported previously [24] is shown to persist for at least two cycles (and begins the third on schedule) under continuous light, or continuous dark conditions. In these experiments, cells entrained for three LD cycles were used, ensuring a highly synchronized population of cells. We also included acetate in the medium so that the dark-incubated cells would not be starved for energy; evidence had been presented previously that the energy status of the cells can affect expression of a *lhcb* gene [30]. The end result was a robust rhythm of *lhca1* expression, even after two days in darkness. This contrasts with higher plants, where *lhc* genes are poorly expressed in the second cycle in continuous dark [e.g. 29]. It should be noted, however, that the absolute levels of *lhca1* mRNA were 3–4-fold lower in the dark-incubated cells compared to those in LD conditions. This result is also consistent with previous work which indicated a direct role for light in expression of a *lhcb* gene in *C. reinhardtii* [27, 30].

All of the experiments above used wild-type cells, however, we have also monitored the rhythm of *lhca1* mRNA accumulation in several *per* mutants of *C. reinhardtii* in order to determine if the same clock that controls phototaxis also controls *lhca1* transcription. The *per2*, *per4* and *per6* mutants, which all have a longer than normal period of the phototaxis rhythm, ca. 28 h versus ca. 24 h [5], were examined. Cells were grown in LD cycling conditions, then released into LL or DD, and the *lhca1* mRNA rhythm followed for at least two cycles. A *lhca1* mRNA rhythm was present in these mutants, but a clearly longer period was not evident (data not shown). In the case of *per6*, however, the rhythm lasted only 1 cycle in LL (data not shown). These data raise the possibility that the clock that controls *lhca1* transcription is different from the clock that controls phototaxis. Evidence for more than one cellular clock has been obtained [43]. However, it should be pointed out that it is possible that the *per* mutants we analyzed had reverted or become suppressed, since we did not attempt to verify the period length of the phototaxis rhythm in these strains. Although it seems unlikely that all three mutants would have reverted to a wild-type periodicity.

By tracking cell division under the different light/dark regimes, we have also shown that the clock controls *lhc* gene expression independently of the cell division cycle. This is important because of previous data which suggested that the circadian clock might control cell division [7]. The continuous dark experiments (Fig. 1) were particularly illuminating in that they showed that the clock can drive transcription of an *lhc* gene in the absence of cell division. This finding has implications for clock-driven transcription of *lhc* genes in higher plants where the relationship between cell growth and division, and *lhc* transcription has not been elucidated.

We have also confirmed the report by Spudich and Sager [49] that LD-synchronized cells shifted to continuous darkness do not efficiently enter the cell division cycle for at least 48 h. The present study differs from Spudich and Sager's in that acetate was present in the culture media. Apparently, the acetate is not utilized sufficiently in the dark to allow efficient growth and division (although sufficient to allow a rhythm of *lhca1* expression). This is particularly interesting considering the fact that acetate supports vigorous growth of *C. reinhardtii* in the dark in unsynchronized cultures. Although these data would indicate that the circadian clock doesn't control cell division, it should be mentioned that in some dark experiments we did observe a fraction of the cells divide on schedule. Thus, further experiments under a variety of growth conditions are needed to determine conclusively if cell division in *C. reinhardtii* is controlled by the clock.

These studies also showed that *lhca1* mRNA regulation by the circadian clock is primarily, but maybe not exclusively, transcriptional. Unlike previous studies of circadian regulation of gene expression in plants, we directly looked for evidence of control of mRNA stability. The evidence suggests that changes in half-life may contribute to the oscillating pattern of *lhca1* mRNA steady-state levels, but this contribution is minor at best. Somewhat surprisingly, the half-lives of *lhca1*, *rbcS* (Fig. 7), and the *lhcb* mRNAs (unpublished results) are all quite short, 1–2 h at most. Although more genes need to be examined, these data suggest that mRNAs in *C. reinhardtii* may be generally unstable, as they are in yeast [46], but in contrast to soybean [47]. Finally, it should be noted that transcriptional regulation has also been shown to be important in the stimulation of *lhcb* gene expression by light in *C. reinhardtii* [26].

Recently, Gagné and Guertin [13] reported that the *lhcb* mRNA family in *Chlamydomonas eugametos* showed a diurnal but not circadian rhythm. Their continuous-condition experiment, however, used only constant darkness. Unlike *C. reinhardtii*, *C. eugametos* apparently does not utilize organic carbon sources with any efficiency [55]; thus, the cells may have been severely starved for energy in the dark treatments. Interestingly, Gagné and Guertin [13] did obtain evidence for circadian control of a *lhc*-like gene. Based on run-off transcription analyses in permeabilized cells, they also concluded that the *lhc*-like gene is regulated mainly at a post-transcriptional level. Given the relatively poor homology (ca. 30%) of this protein to *lhc* genes, however, it is of considerable interest to know if this is in fact an *lhc* protein (that binds chlorophyll, etc.), or if it has some other function. Also, it has not been shown that nuclear run-off transcription in permeabilized cells is a valid measure of *in vivo* nuclear transcription. If we assume that it is, then there may be significant differences between *C. eugametos* and *C. reinhardtii* in the regulation of *lhc* gene expression.

Evolutionary implications of circadian regulation of lhc *genes in* C. reinhardtii

In addition to the study of Gagné and Guertin [13] mentioned above, Alosi *et al.* [1] presented evidence that *lhcb* gene expression in a gymnosperm (Douglas-fir) is *not* under circadian clock control. Together, those data suggested that the clock does not control *lhc* transcription in lower plants, and thus might be a recently evolved trait. Our results, however, clearly show that transcription of at least one *lhca* gene, and probably the major *lhcb* genes, are under circadian control in *C. reinhardtii*. Recently, a robust rhythm of a *lhcb*

mRNA in *C. reinhardtii* was reported by Jacobshagen and Johnson [25]. Thus, it seems likely that linkage of *lhc* gene expression to the circadian clock is an evolutionarily conserved trait. It will be interesting to see if other algae, especially some of the more primitive species, also control their *lhc* genes via a circadian clock. The finding of clock control of *lhc* expression in such diverse organisms as angiosperms and a unicellular green alga suggests that this regulatory mechanism provides some selective advantage, presumably by helping the cells to optimize the time of *lhc* protein production.

Finally, these data raise a number of interesting questions that can be efficiently addressed in *C. reinhardtii*, such as whether there is circadian control of *lhc* protein synthesis and/or pigment synthesis, and if such clock control provides an advantage to the cells. It will also be of interest to identify the cis-acting elements that mediate circadian control of *lhc* genes in *C. reinhardtii* and to compare them to the higher-plant derived elements [12].

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