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Transcription of *tufA* and other chloroplast-encoded genes is controlled by a circadian clock in *Chlamydomonas*

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ABSTRACT **Levels of mRNA for the chloroplast-encoded elongation factor Tu (*tufA*) showed a dramatic daily oscillation in the green alga *Chlamydomonas reinhardtii*, peaking once each day in the early light period. The oscillation of *tufA* mRNA levels continued in cells shifted to continuous light or continuous dark for at least 2–3 days. Run-off transcription analyses showed that the rate of *tufA* transcription also peaked early in the light period and, moreover, that this transcriptional oscillation continued in cells shifted to continuous conditions. The half-life of *tufA* mRNA was estimated at different times and found to vary considerably during a light–dark cycle but not in cells shifted to continuous light. Light–dark patterns of transcription of several other chloroplast-encoded genes were examined and also found to persist in cells shifted to continuous light or dark. These results indicate that a circadian clock controls the transcription of *tufA* and other chloroplast-encoded genes.**

Circadian rhythms have a period of ≈24 h and are characterized by the fact that they are reset by environmental stimuli, particularly light, and that they continue in the absence of the entraining stimulus (i.e., under constant conditions). This latter property indicates that circadian rhythms are controlled by an endogenous clock (1). The nature of circadian clocks is largely unknown, although genes that encode putative clock components have been isolated from *Neurospora* (2) and *Drosophila* (3).

Regulation of specific genes is one mechanism by which the clock exerts its control over cellular functions. In green plants, the transcription of certain nuclear genes have been shown to be under circadian control (for review, see ref. 4). Some of these genes encode chloroplast proteins that function in photosynthesis and are primarily expressed during the light period of a diurnal cycle. Thus, chloroplast biogenesis is, at least to some extent, regulated by the clock (e.g., ref. 5).

A quantitatively minor but important fraction of chloroplast proteins are encoded within the organelle itself (6). However, it is not clear whether transcription of chloroplast-encoded genes is controlled by a circadian clock. Previous studies with higher plants suggested that chloroplast-encoded genes are not under such control (4); however, only steady-state levels of a few highly abundant stable mRNAs were examined. A recent study of chloroplast gene expression in *Chlamydomonas reinhardtii* indicated a degree of endogenous control that might be circadian (7). Those data, however, were incomplete, since less than half of a cycle under a single continuous condition was examined. The demonstration of persistence under continuous conditions is critical to the identification of a circadian rhythm (1). In the present study, we show that the chloroplast-encoded *tufA* gene in *C. reinhardtii* exhibits a robust circadian rhythm of mRNA accumulation and, moreover, that this rhythm is primarily transcriptional. Evidence is also provided that transcription of many, if not most, chloroplast-encoded genes is under circadian clock control.

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MATERIALS AND METHODS

Cell Strains, Culturing, and Manipulation. The wild-type 2137 mt^+ (CC-1021) strain of *C. reinhardtii* was grown mixotrophically in flasks of Tris/acetate/phosphate medium (8) with shaking (≈200 rpm) at 23°C. Cells were pregrown for at least three 12-h light/12-h dark cycles to a density of 0.5×10^6 cells per ml (early exponential phase); the light intensity during the light period was ≈40 μE per m^2 per sec. Under these conditions, the cells divide synchronously in the mid-dark period (8, 9); cell division was monitored microscopically and by counting cells with a hemacytometer. For the continuous-condition experiments, culture aliquots were removed and transferred to light (at the same intensity as above) or darkness at 23°C. Care was taken in the handling of dark-period cells to minimize exposure to light by working quickly and by using a dim (<0.1 μE per m^2 per sec) green fluorescent light.

RNA Isolation, Northern Blot Hybridization, and mRNA Quantification. The isolation of total RNA, Northern blot hybridization, and ^{32}P labeling of the DNA probe have been described (9). The blots were stained with methylene blue prior to hybridization to check for equal loading and transfer. The DNA probe was the *Pst* I–*Eco*RI fragment of the *tufA* gene (10), which was labeled to a specific activity of 1×10^9 dpm/μg. The autoradiographs were scanned as described below.

Run-On Transcription in Permeabilized Cells. Run-on transcription in toluene-permeabilized cells was performed as described (11, 12). The transcription reaction mixtures contained 5×10^6 cells (2×10^8 cells per ml) and 30 μCi of [α-^{32}P]UTP (specific activity ≈3000 Ci/mmol; 1 Ci = 37 GBq) as label and were incubated at 25°C for 15 min. The specific radioactivity of the RNA was measured by using DE-81 filters. The transcriptional activity of individual genes was assessed by hybridizing equal amounts of RNA (10 μg/ml in hybridization solution) to DNA dot blots (0.5–2 μg per dot) for 72 h (13). The DNA probes for exon 1 of *psaA1* and the *psbA* gene have been described (12). Other plasmid DNA probes were pR03R141, which contains the 3′ two-thirds (≈1200 bp) of the *C. reinhardtii tufA* gene (14); pBC7, which contains Bam5 and the *C. reinhardtii atpB* gene (15); p228, which contains the 16S gene and the spacer between the 16S and 7S–3S–23S *rrn* genes (16); and vectors pGEM3zf(+) and pUC18 as controls. After stringent washing (13) and exposure to x-ray film, the films were quantified as described below. Hybridization to the vector controls was negligible under these conditions.

Estimates of mRNA Half-Life. Actinomycin D (50 μg/ml) and rifampicin (250 μg/ml) (8) were used to estimate the half-life of *tufA* mRNA. After each drug was administered,

Abbreviations: DD, continuous darkness; LD, light–dark cycles; LL, continuous light.

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culture aliquots were removed at different times (up to 4 h), total RNA was isolated, and Northern blot hybridizations were performed as described above.

Quantitation of Autoradiographs. X-ray films, exposed to obtain signals within the linear-response range of the film, were scanned by using a RealTech 800 scanner (and Adobe Photoshop) and quantified by using NIH IMAGE (version 1.5.2). The reliability of the scanner was verified by using known amounts of [^{32}P]RNAs to generate standard curves. Semilogarithm plots were used to calculate mRNA half-lives with the aid of CRICKETGRAPH III (version 1.01, Claris).

RESULTS

Diurnal and Circadian Rhythms of *tufA* mRNA. Fig. 1 shows a Northern blot analysis of *tufA* mRNA levels in light–dark (LD) conditions. The ≈1.7-kb RNA, which is the major transcript from the *tufA* gene (14), was quantified. The level of this RNA fluctuated dramatically, peaking early in the light period (≈L2 or ≈4 h and 28 h of continuous time), decreasing to a very low level in the early dark period, and then increasing again at the end of the dark period. The maximum amplitude of the *tufA* mRNA oscillation in LD-growing cells was ≈20-fold.

Fig. 1 also shows *tufA* mRNA levels over the same time course but in cells that had been shifted to continuous light (LL) in the light period prior to sampling. The steady-state level of *tufA* mRNA continued to oscillate, with a period of ≈24 h (peak to peak), but with somewhat less amplitude (≈8-fold). The second cycle in LL showed a broader peak than in LD, mostly because the mRNA level began increasing a few hours early; such changes under continuous conditions are not unusual for circadian rhythms (1). It should also be noted that, under these conditions, cell division becomes largely asynchronous after the first 24 h in LL, with cell concentration increasing faster than in LD (9). In Fig. 1, the cell concentration was ≈25% higher in LL (3.0×10^6 cells per ml versus 2.4×10^6 cells per ml) by the end of the sampling period. Nonetheless, the *tufA* mRNA rhythm continued on schedule for at least two cycles in LL.

The regulation of *tufA* mRNA levels was also examined in LD-entrained cells shifted to continuous darkness (DD). To examine *tufA* expression, the first cycle in DD was skipped, and RNA was extracted during the second, third, and beginning of the fourth cycles in DD. Fig. 2 shows that *tufA* mRNA accumulated to high levels in DD. Moreover, the mRNA abundance rhythm persisted through the third cycle, and part of the fourth, and with a period of 22–26 h. The maximum amplitude in DD (≈5-fold) was less than in LD or LL and was principally due to higher minimums of expression. It should also be noted that in DD, cell division slows considerably (9). In Fig. 2, the cell concentration had increased less than 2-fold

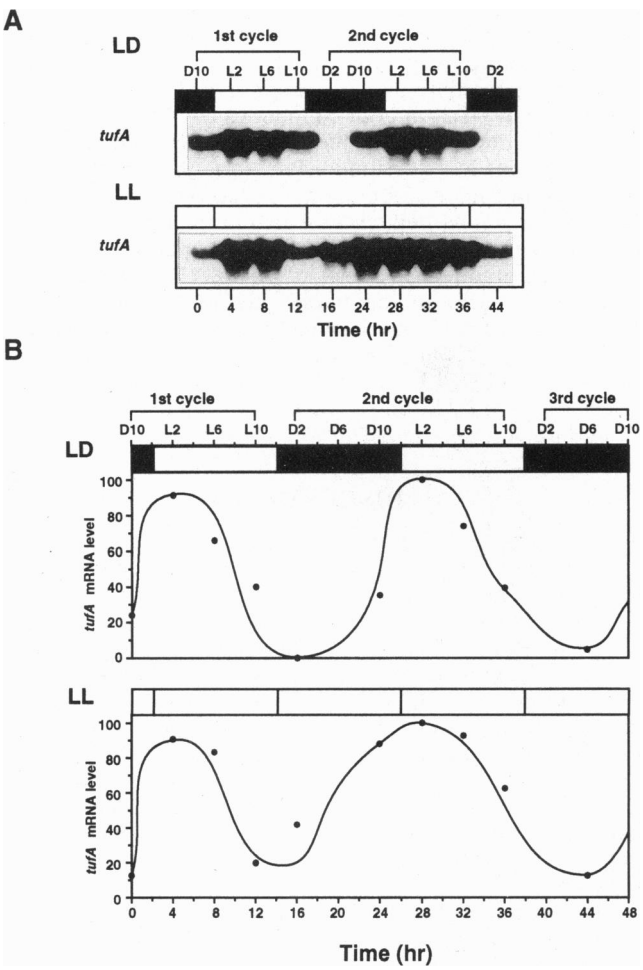

FIG. 1. Levels of *tufA* mRNA in LD and LL conditions. (*A*) Autoradiograph of Northern blots. After entrainment by three 12-h/12-h LD cycles, the culture was split at the beginning of the fourth light period, and aliquots were retained in LD or transferred to LL. RNA (5 μg), extracted from cells at the indicated time points, was examined by Northern blot analysis with the *tufA*-specific probe. The cycle designations refer to the first and second cycles, etc., after entrainment (and shifting in the case of LL). The blots were exposed to film for ≈12 h. (*B*) Graphical presentation of the quantification of the Northern blots in A. Shorter exposures (2–4 h) of the blots were used for quantification, and maximum mRNA levels were arbitrarily set to 100%. Continuous time is also indicated and was measured from the beginning of the sampling period. Open boxes, light periods; solid boxes, dark periods.

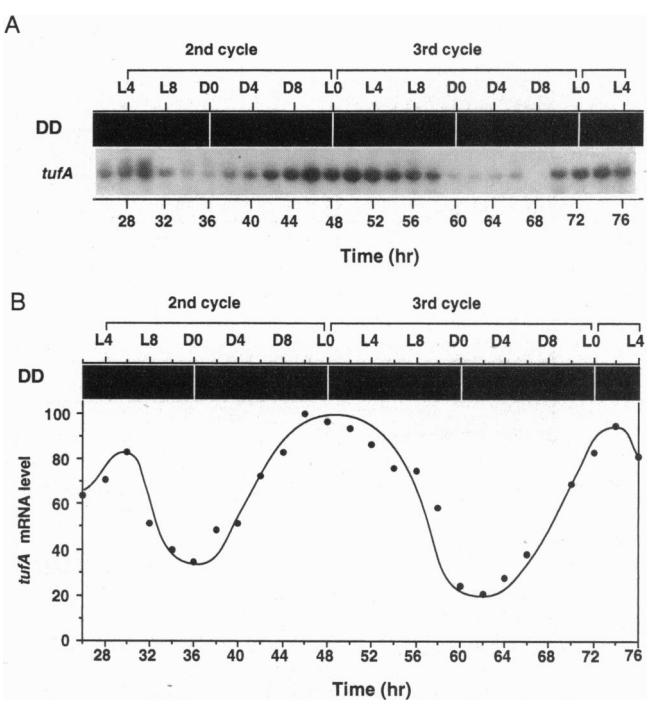

FIG. 2. Levels of *tufA* mRNA in DD. (A) Autoradiograph of the Northern blot. After three LD cycles, cells were transferred to DD, and total RNA was extracted at the indicated time points. The cycle designations refer to the cycle number in DD. Time is also indicated in continuous time, which begins from the first subjective light period in DD. The RNA blot (5 μg per lane) was hybridized with the *tufA* probe and exposed to film for ≈12 h (as in Fig. 1). No RNA was loaded at 68 h. (*B*) Graph of the quantification of the Northern blot in *A*. Shorter exposures (2–4 h) were used for the quantification. The maximum mRNA level was arbitrarily set to 100%.

(from 0.5×10^6 cells per ml to 0.8×10^6 cells per ml) by the end of the extended DD yet the rhythm of *tufA* mRNA remained robust. Thus with the LL and LD data above, these data indicate that the steady-state level of *tufA* mRNA is under circadian clock control.

Regulation of tufA mRNA Stability. Salvador *et al.* (7) reported that the half-life of *tufA* mRNA was relatively brief compared to some other chloroplast mRNAs and that it was shorter in the light. To assess the potential contribution of mRNA stability changes, the half-life of *tufA* mRNA was estimated by using actinomycin D/rifampicin–chase protocols. Actinomycin D blocks the transcription of both nuclear and chloroplast genes, whereas rifampicin specifically inhibits chloroplast transcription in *C. reinhardtii* (8, 9, 11, 13). Both drugs were used to verify that the results were not drug-specific. Fig. 3*A* shows the effectiveness of the drugs in blocking *tufA* mRNA accumulation early in the light period of LD-growing cells. The estimated half-life of the mRNA (1–1.5 h) is similar to that obtained by using a pulse–chase analysis (7).

Fig. 3*B* shows that the half-life of *tufA* mRNA varied ≈3-fold during a LD cycle, being longer in the dark period, and shorter throughout much, but not all, of the light period. This result is consistent with the data of Salvador *et al.* (7), although those authors measured the half-life at only one point in the light period. These data show that *tufA* mRNA stability changes continuously in the light period, indicating that it might represent an endogenous rhythm.

Under LL conditions, however, a different picture emerged. Fig. 3*B* shows that the half-life of *tufA* mRNA was somewhat shorter, and changed comparatively little, although there was a slight reduction in the subjective light period with the actinomycin D–chase experiment (ActD line in Fig. 3*B*). With rifampicin, however, the dip in the subjective light period was even less obvious; its significance is, therefore, doubtful. These data indicate that changes in *tufA* mRNA stability play a role in the diurnal pattern of *tufA* mRNA levels, but not in the circadian pattern, at least not in LL.

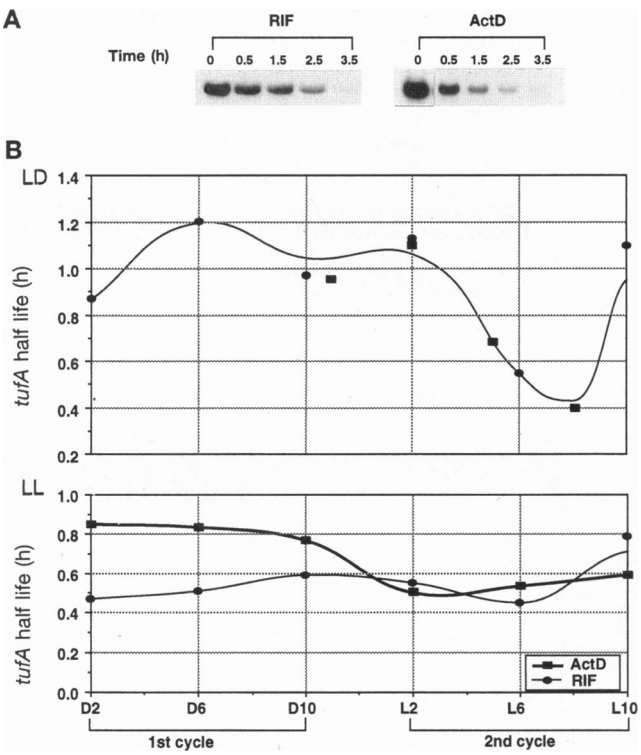

FIG. 3. Stability of *tufA* mRNA in LD and LL. (*A*) Autoradiographs of Northern blots. Actinomycin D (ActD), or rifampicin (RIF), was added to aliquots of two LD-growing cultures at the onset of the light period. RNA was isolated at the indicated times after drug administration and subjected to Northern blot analysis as in Fig. 1. (*B*) Graphical presentation of *tufA* mRNA half-lives estimated at different times over an ≈24-h period in LD or LL. The cycle designations refer to the cycle number after entrainment, and the experiments were begun 2 h into the subjective dark period (i.e., D2). The data points are the time when the drug was administered. Measurements with actinomycin D are solid rectangles; rifampicin-based measurements are solid circles.

Chloroplast Transcriptional Activity. To directly assess the contribution of transcriptional control, run-on transcription in permeabilized cells was used to estimate rates of transcription of *tufA* and other selected chloroplast genes, including rRNA (*rrn*), a photosystem II gene (*psbA*), a photosystem I gene (*psaA* exon 1), and a subunit of the ATP synthetase (*atpB*). Also, since >95% of the [^{32}P]UTP incorporation in this system is chloroplast derived (ref. 11 and D.L.H., unpublished results), estimates of overall plastid transcription activity were obtained. Transcription was analyzed over a 24-h period in LD, LL, and DD conditions after entrainment, and the results are shown in Fig. 4. In LD, *tufA* transcription showed a single sharp peak ≈2 h into the light period, with the rate varying ≥10-fold. A similar result was obtained for the other genes, except *psbA*, which showed a very broad peak and a variation of only ≈3-fold. Total chloroplast transcription in LD also showed a sharp peak at ≈L2. In both continuous conditions, this basic pattern persisted; however, the peaks were broader and there was somewhat less amplitude. For *tufA*, the amplitude was reduced only slightly in LL but was reduced to ≈4- to 5-fold in DD. These results demonstrate that transcriptional control plays a central role in *tufA* expression, particularly under continuous conditions. The data also show that transcriptional oscillations are not unique to *tufA* but are common to other chloroplast genes. Finally, it should be noted that the exposure times and signals in Fig. 4*A* were similar, although not identical, for LD, LL, and DD, indicating a relatively small effect of light on chloroplast transcription, at least under these conditions.

DISCUSSION

These results have shown that expression of the chloroplast-encoded *tufA* gene is under strong circadian control in *C. reinhardtii*. The diurnal oscillation of *tufA* mRNA levels continued for at least two cycles in LL and at least three cycles in DD. Thus, the *tufA* mRNA rhythm appears to be at least as robust as the previously described rhythms of nuclear *lhca* and *lhcb* genes (9, 17). Direct analyses of transcription rates and mRNA stability showed that both of these processes are involved in the diurnal regulation of *tufA* expression [consistent with previous work of Salvador *et al.* (7)] but that transcriptional regulation is more important. However, under circadian conditions, transcriptional regulation seems to be the only factor responsible for the rhythmic pattern of *tufA* expression.

Measurements of *tufA* mRNA half-lives revealed a substantial fluctuation in the stability of *tufA* mRNA in a LD cycle, which apparently contributes to the diurnal regulation of *tufA* mRNA levels. This periodicity, however, did not clearly persist in LL, indicating a diurnal but not circadian rhythm. The *tufA* mRNA half-lives *in vivo* were estimated by blocking transcription with specific inhibitors. Two different inhibitors, actinomycin D and rifampicin (which is specific to the chloroplast), were used to mitigate against possible side effects of either drug. The half-lives of *tufA* mRNA obtained with the two drugs were similar to each other and to that determined by pulse–chase analysis (7). Actinomyin D has been shown to abolish circadian rhythms in the alga, *Gonyaulax*; however, much longer treatments than those used here were needed (18). Moreover, if actinomycin D was a general and rapid inhibitor of the clock, and *tufA* mRNA stability was under clock control,

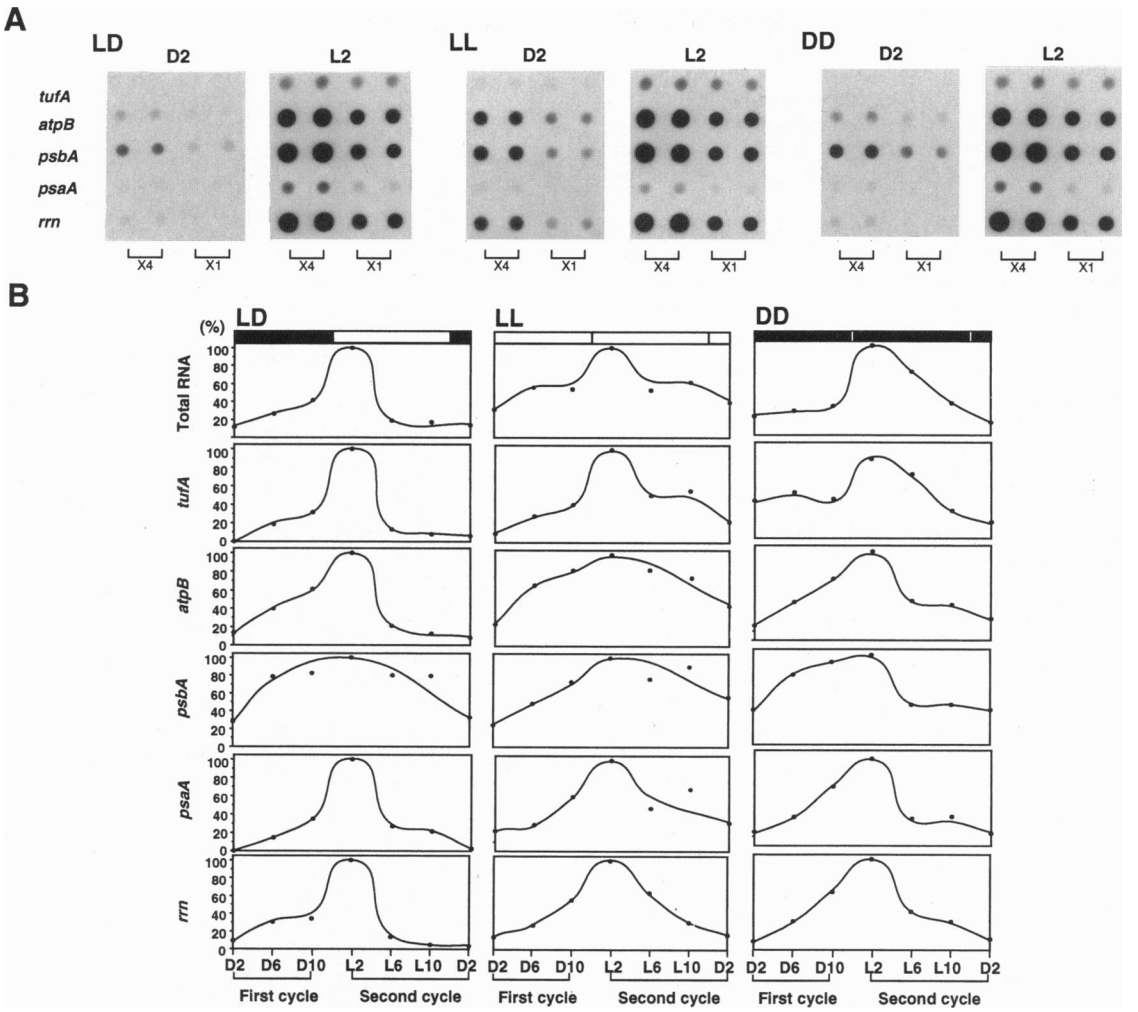

FIG. 4. Rates of transcription of chloroplast-encoded genes in LD, LL, and DD. Cells were removed at the indicated times of the first and second cycles after entrainment, permeabilized, and used for run-on transcription. For analysis of specific genes, equal amounts of [^{32}P]UTP-labeled RNAs were hybridized to DNA dot blots; the dots (in duplicate) contained 2 μg (4×) or 0.5 μg (1×) of DNA. (*A*) Selected autoradiographs of dot blots hybridized with [^{32}P]UTP-labeled RNA from two points in a 24-h cycle, one near the maximum (L2) and the other near the minimum (D2). The blots were exposed to film for ≈24 h. (*B*) Graphical presentation of total and specific gene transcription rates over the 24-h period that began at 2 h into a subjective dark period (i.e., D2). For specific genes, multiple exposures of each dot blot were made, and the signal from the duplicate 4× dots was quantified; each point represents an average of the duplicates, which varied no more than 15%. The maximum values were arbitrarily set to 100%. The cycle designations refer to the cycle number after entrainment. Open boxes, light periods; solid boxes, dark periods.

we should not have obtained a fluctuating pattern in LD. Finally, although we did not directly analyze *tufA* mRNA stability in DD, the close correlation between mRNA levels and transcription rates in DD indicates that the *tufA* mRNA half-life does not fluctuate significantly in DD. Thus, we conclude that *tufA* mRNA stability is not under circadian clock control.

Direct analyses of transcription of several other chloroplast genes that have diverse functions in photosynthesis and translation indicate that circadian control of chloroplast transcription is not unique to *tufA*. Although the peaks for some genes (i.e., *psbA*) were quite broad, the diurnal patterns of transcription of these genes, as well as total plastid transcription activity, persisted in continuous conditions for at least one cycle. Direct transcription measurements have not been extended beyond that time, so it is not yet known whether these rhythms will prove to be as robust as *tufA*, although given the tendency for chloroplast genes to be collectively regulated (13, 19, 20), it seems likely that they will.

Regardless of how robust the transcription rhythms of the other genes turn out to be, however, these results indicate that the diurnal patterns of chloroplast transcription in LD as described previously (7, 13, 20) are primarily due to circadian clock control. A direct effect of light on transcription of chloroplast-encoded genes, at least under these mixotrophic growth conditions, was not readily apparent. This contrasts with the nuclear *lhca1* gene, which showed an obvious 3- to 4-fold lower level of expression in the first subjective dark period of DD (9). However, more detailed analyses of transcription rates at the dark–light transition, and in asynchronous cultures, are needed before we can conclude there is no direct effect of light on chloroplast transcription in wild-type *Chlamydomonas*. In this regard, we note that preliminary experiments indicate a direct role for light (in addition to the clock) in modulating chloroplast transcription in cells growing autotrophically (N. Deshpande and D.L.H., unpublished results).

It was recently shown that a *psbA* gene of the cyanobacterium *Synechococcus* is under circadian clock control (21). Since it is generally believed that endosymbiotic cyanobacterial-like organisms are the evolutionary precursors to chloroplasts (see ref. 6), it is intriguing to speculate that the chloroplast circadian system might be of prokaryotic origin. However, an alternative hypothesis is that the endosymbiont's circadian system was lost during plastid evolution, and a circadian system derived from the nuclear progenitor controls transcription in the organelle. The cloning and sequencing of components of

the circadian system in these and other plants should answer this question (22, 23). It is probably apparent that the discovery of a robust rhythm of a chloroplast-encoded gene raises the possibility of using a reporter gene, such as bacterial luciferase (21), to produce an organelle-based luminescence rhythm. If successful, this approach could lead to the isolation of mutations affecting the clock (22, 23) or possibly the output pathway for this rhythm.

Finally, the fact that direct transcription analyses revealed a fairly strong rhythm in the transcription of an RNA as abundant and stable as rRNA (which would probably not have been detected by analyzing steady-state RNA levels) suggests that it may be worthwhile to reexamine the question of circadian control of chloroplast-encoded genes in higher plants with a similar approach.

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1. Bunning, E. (1973) *The Physiological Clock: Circadian Rhythms and Biological Chronometry* (Springer, Berlin), 3rd Ed.
2. Dunlap, J. C. (1990) *Trends Genet.* **6,** 159–165.
3. Rosbash, M. & Hall, J. C. (1989) *Neuron* **3,** 387–398.
4. Kay, S. A. & Millar, A. (1992) in *The Molecular Biology of Circadian Rhythms*, ed. Young, M. (Dekker, New York), pp. 73–89.
5. Beator, J. & Kloppstech, K. (1993) *Plant Physiol.* **103,** 191–196.
6. Gillham, N. (1994) *Organelle Genes and Genomes* (Oxford Univ. Press, New York).
7. Salvador, M. V., Klein, U. & Bogorad, L. (1993) *Plant J.* **3,** 213–219.
8. Harris, E. (1989) *The Chlamydomonas Sourcebook* (Academic, San Diego).
9. Hwang, S. & Herrin, D. L. (1994) *Plant Mol. Biol.* **26,** 557–569.
10. Baldauf, S. & Palmer, J. (1990) *Nature (London)* **344,** 262–265.
11. Guertin, M. & Bellemare, G. (1979) *Eur. J. Biochem.* **96,** 125–129.
12. Herrin, D. L. & Schmidt, G. W. (1988) *J. Biol. Chem.* **263,** 14601–14604.
13. Herrin, D. L., Michaels, A. S. & Paul, A.-L. (1986) *J. Cell Biol.* **103,** 1837–1845.
14. Silk, G. W. & Wu, M. (1993) *Plant Mol. Biol.* **23,** 87–96.
15. Woessner, J. P., Gillham, N. W. & Boynton, J. E. (1986) *Gene* **44,** 17–28.
16. Harris, E. H., Burkhart, B. D., Gillham, N. W. & Boynton, J. E. (1989) *Genetics* **123,** 281–293.
17. Jacobshagen, S. & Johnson, C. H. (1994) *Eur. J. Cell Biol.* **64,** 142–154.
18. Karakashian, M. W. & Hastings, J. W. (1962) *Proc. Natl. Acad. Sci. USA* **48,** 2130–2137.
19. Mullet, J. (1993) *Plant Physiol.* **103,** 309–313.
20. Leu, S., White, D. & Michaels, A. (1990) *Biochim. Biophys. Acta* **1049,** 311–317.
21. Kondo, T., Strayer, C. A., Kulkarni, R. D., Taylor, W., Ishiura, M., Golden, S. S. & Johnson, C. H. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5672–5676.
22. Kondo, T., Tsinoremas, N. F., Golden, S. S., Johnson, C. H., Kutsuna, S. & Ishiura, M. (1994) *Science* **266,** 1233–1236.
23. Millar, A. J., Carré, I. A., Strayer, C. A., Chua, N.-H. & Kay, S. A. (1995) *Science* **267,** 1161–1163.