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Circadian Regulation of Chloroplast Gene Transcription : A Review

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Abstract

The chloroplast is the plant cell organelle for photosynthesis, but it is also the location of important biogenetic activities. Chloroplasts have their own genetic system that contain ~100 - 250 genes, depending on the species. The functions of these genes are mainly in photosynthesis and genetic functions of the organelle. The expression of chloroplast genes is regulated at every level, including transcription. The regulation of plastid gene expression during the development of etioplasts (or proplastids) into chloroplasts (a.k.a., "greening") has been well studied. Gene regulation is also critical to mature chloroplasts, and this review focuses on the regulation of chloroplast transcription that is driven by the solar cycle and mediated by the circadian clock. Historical, as well as recent, work with vascular plants and *Chlamydomonas* are discussed. We have also included brief summaries on plant circadian systems, and the proteins and promoter elements involved in chloroplast transcription. Finally, possible mechanism(s) for circadian regulation of chloroplast transcription are discussed, as is a perspective for future work.

What are circadian rhythms?

Adaptation and biological organization has been the product of natural selection. Adaptation to an organism's external environment also includes the measurement of time and external cues like sunlight and temperature. In 1729, a French astronomer, Jean Jacques d'Ortuous de Marian, observed that bean plants displayed daily leaf movements even when kept in constant darkness for several days. The plants continued to open their leaves during the subjective daytime, and close them during the subjective night. He concluded the phenomenon was the result of an endogenous rhythm (Edery 2000; Enright 1982).

There are a number of genetic, physiological, and behavioral rhythms that are set by light-dark cycles. Rhythms that peak during the day are diurnal, and those that peak during the night are nocturnal. Some of these rhythms are also

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circadian, i.e. they will continue more or less on schedule and with an approximate 24-hour periodicity for at least one cycle under constant light and/or constant darkness. Circadian rhythms are made possible by a circadian clock or pacemaker, which keeps time via endogenous molecular oscillations. The clock provides organisms with an adaptive advantage by enabling them to anticipate daily changes in the environment, such as the onset of dawn or dusk, that change during the year (Edery 2000; McClung 2001).

Properties of circadian clocks

Response to light: The duration, brightness and intensity of the exposure of the organism to light affect the functioning of the clock, and serves as a synchronizing input stimulus. Light-dark cycles are the most commonly used input stimulus to set the phase, or entrain circadian rhythms.

Response to continuous conditions: When the clock has been entrained by one or more light-dark cycles (L-D), and then the organism is shifted to constant conditions (light or dark), circadian clocks are capable of continuing its output rhythms. This period of time in which the clock is capable of maintaining its schedule in the absence of the entraining stimulus is called a "free-running" period. The free-running period deviates somewhat from an exact 24-hour cycle, and this deviation is species-specific. A rhythm that persists under free-running conditions is the most important indicator of circadian rhythm, and that a particular process is under control of an endogenous pacemaker.

Response to temperature: Circadian rhythms are relatively resistant to fluctuations in temperature so long as the temperature is within the physiological range of the species. This property is important, because the clock must continue to function as the ambient temperature changes. It should be noted, however, that temperature cycles can be used to entrain (or "set") the clock in some species.

Effects of genetic mutations: Single gene mutations can affect the length of circadian periods, produce arrhythmia, or affect the amplitude of a specific rhythm. Thus, there is a genetic basis for clock function. This will be discussed further (below), as genetic approaches in model organisms have led to the identification of clock genes (Dunlap 1999; Edery 2000; Hastings and Sweeney 1957; McClung 2001; Sweeney 1987).

Although the clock is at the heart of a circadian system, they also include photoreceptors to perceive the light signal, input pathways to transduce the signal to the central clock, and output pathways leading from the clock to the observed rhythms. There can also be cross-talk among these pathways. In addition, there is evidence for more than one circadian pacemaker in a single cell

type of a multicellular plant, and in single-celled organisms (Roenneberg and Morse 1993; Sai and Johnson 1999).

Based on studies with several genetic model systems, including animals (mice and *Drosophila)*, a fungus (*Neurospora*), a cyanobacterium (*Synechococcus)*, and an angiosperm (*Arabidopsis*), the central clock comprises at least one protein that controls its own expression via a negative feedback loop. In addition, there are positive factors in the loop, which are usually transcriptional activators that stimulate expression of the repressor, as well as other clock-controlled genes (*ccg*) (Roenneberg and Merrow 2003). Central clock components are not the same in all organisms, indicating that the clock has evolved multiple times (Mittag *et al.* 2004).

Circadian rhythms in algae and plants

Most early studies in algal systems were with populations of single-celled organisms, such as *Euglena* and *Chlamydomonas*, and hence it was formally possible that the circadian rhythms in these organisms were the result of intercellular interactions. The giant cells of *Acetabularia*, however, permitted the detection of circadian rhythms (such as photosynthetic oxygen evolution) in single cells (Sweeney and Haxo 1961). Temperature compensation was also shown with *Acetabularia* (Berger et al. 1992). These experiments first proved that circadian rhythms in algae are properties displayed by individual cells.

Cyanobacteria were the first prokaryotes shown to have a circadian clock, which was demonstrated with rhythms of physiology and transcription. One very practical reason for a circadian system in these organisms is that it maintains the incompatible processes of photosynthesis and nitrogen fixation as opposite-phased rhythms. This is necessary because the *Nitrogenase* enzyme that reduces (fixes) atmospheric nitrogen in *Synechococcus* is inhibited by oxygen. Hence photosynthesis, which evolves oxygen, occurs during the daytime, while nitrogen fixation occurs during the night (Ditty et al. 2003; Suzuki and Johnson 2001). Also, by using a promoterless luciferase gene fusion (*luxAB*) and introducing it randomly into the *Synechococcus* genome, evidence was obtained that indicated the transcription of most genes in this organism is under circadian clock control (Liu et al. 1995). There were two major types of transcriptional phasing observed; one class peaked at subjective dusk and the other at subjective dawn.

In higher plants, a number of processes, including cell elongation, enzymatic activities, leaf movements, stomata opening, and the expression of specific genes, show daily fluctuations that are circadian. Of particular importance is the finding that the efficiency of photosynthesis fluctuates with a diurnal-circadian peak in the early to mid-light period. Interestingly, so does the transcription of nuclear

CAB genes, which encode the chlorophyll *a*/*b*-binding proteins that support the harvesting of light energy (reviewed in McClung 2001).

The *Arabidopsis* Genome Project (The *Arabidopsis* Genome Initiative 2000), together with microarray technology, has enabled a genome-wide analysis of mRNAs whose levels are controlled by the circadian clock. Using an oligonucleotide array, it was shown that mRNAs for at least 2% of the ~25,000 (or ~450) *Arabidopsis* genes are circadian regulated in plants entrained by light-dark cycles and shifted to continuous light (Harmer et al. 2000). These clock-controlled genes are involved in light responses and in metabolic pathways, such as photosynthesis and nitrogen utilization. This analysis also provided insight into clock-controlled promoters; a nine-nucleotide sequence (AAAATATCT), called the 'evening element', occurred frequently in promoters that exhibited nocturnal-circadian activity. Mutagenesis of this element in the *CCR*2 (cold, circadian rhythm RNA binding protein 2) promoter demonstrated that it does function in rhythmic expression (Harmer et al. 2000).

What is the clock in photosynthetic organisms?

Most of the initial studies on the molecular nature of the circadian clock in photosynthetic organisms were carried out with the cyanobacterium, *Synechococcus*. This organism's clock was initially defined by mutagenizing a bioluminescent strain that carried a luciferase gene whose expression was controlled by a strong circadian promoter (Kondo et al. 1993). Mutants with altered bioluminescence periods were mapped to a locus containing three adjacent genes, termed *KAIA-KAIC* (Ishiura et al. 1998). These genes are transcribed from two promoters, one for *KAIA* and the other for *KAIB-KAIC*. On the basis of biochemical and genetic studies, *KAIA* is proposed to be the positive element for expression of *KAIB-KAIC*, while *KAIC* is the negative element that represses *KAIB-KAIC* expression (Ishiura et al. 1998). A revised and extended model of the *Synechococcus* circadian system has been recently published. A revised and extended model of the *Synechococcus* circadian system has been recently published. The model describes proteins involved in signal transduction through input or output pathways (Ditty et al. 2003). The mechanism used by the KAI clock to control global transcription in *Synechococcus* could be highly instructive, and possibly relevant to the exploration of the mechanism of clock control of chloroplast transcription. Although the cyanobacterial mechanism is not yet clear, there is evidence for a role for certain RNA polymerase sigma factors, and possibly the KAIC protein, which can form a hexameric ring that binds to DNA (Mori et al. 2002) in clock control of global transcription.

In higher plants, the clock machinery has been studied genetically in *Arabidopsis*. A model of the *Arabidopsis* clock postulates that it is composed of three main components: *CCA*1 (circadian clock associated protein 1) (Wang and Tobin 1998), which is a transcription factor; *TOC1* (timing of *CAB* expression), a transcriptional regulator (Strayer et al. 2000); and *LHY* (late elongated hypocotyl) (Schaffer et al. 1998). Mutations in *TOC1* and *CCA1* cause alterations in period length, i.e. shorter or longer rhythms, while *LHY* mutants are arrhythmic (Alabadi et al. 2002). LHY and CCA1 proteins have been shown to be functionally redundant (Mizoguchi et al. 2002). Also, according to one model, CCA1 and LHY are transcription factors that stimulate expression of genes in the output pathways during the day, e.g. *CAB*, while simultaneously repressing *TOC1*. As the day progresses, CCA1/LHY levels decrease, and this relieves the repression of *TOC1*, which is a positive element for *CCA1/LHY*. Hence CCA1/LHY indirectly represses its own expression, closing the negative feedback loop (Alabadi et al. 2001).

There are several novel proteins that have been identified to work in conjunction with the clock in *Arabidopsis* (Eriksson and Millar 2003), including three genes that affect flowering time: *GI* (gigantean) (Fowler et al. 1999), *ELF3* (early-flowering 3), and *ELF4* (early-flowering 4) (Doyle et al. 2002). These genes seem to activate *TOC1*. The TOC1 protein is also known to interact with PIF3 (phytochrome interacting factor 3) and PIL1 (PIF3-like 1); these interactions could provide molecular linkage between the clock and the photoreceptor, phytochrome (Eriksson and Millar 2003).

Circadian regulation of chloroplast gene transcription: Preface

Chloroplasts are ubiquitous plant cell organelles whose evolutionary origin is believed to be that of a cyanobacterial-like organism that became an endosymbiont within a proto-eukaryotic cell. Evolution of the endosymbiont's genome has involved massive gene loss. Many of them were transferred to the nucleus, including essentially all genetic regulatory genes. Chloroplasts are the site of photosynthesis, but they are also the site of several other key biosynthetic pathways that are most active during the daytime. These pathways comprise the synthesis of proteins, RNAs, amino acids, lipids, and pigments (Buetow et al. 1980). The understanding of how these processes are restricted to the daytime is highly incomplete; however, the fluctuating energy charge within the organelle is probably involved in most of these pathways.

Many chloroplast proteins are nuclear-encoded, synthesized in the cytoplasm, and post-translationally imported into the organelle. Some prominent examples are the small subunit of Ribulose-1,5-bisphosphate carboxylase (the large subunit is encoded by the chloroplast), subunits of the membrane-bound

complexes that perform the light reactions of photosynthesis (photosystems I and II, cytochrome f/b_6 complex, and the ATP synthetase), and many ribosomal proteins (Rochaix et al. 1998). The genome of the plastid encodes proteins essential for photosynthesis; e.g., the PSAA and PSAB subunits of photosystem I, the D1 and D2 (a.k.a., PSBA and PSBD) polypeptides of photosystem II, and the a and b (a.k.a., ATPA and ATPB) subunits of the ATP synthetase, as well as components of the transcriptional and translational machineries, in addition to RNA polymerase subunits, ribosomal proteins, tRNAs, and rRNAs (Rochaix et al. 1998).The segregation of genes for different subunits of the same complex between the nuclear and organellar genomes presents a fascinating regulatory problem for plant cells, because they must coordinate the synthesis of related proteins in two different compartments.

One way to achieve overall coordination of gene expression in two compartments is to link them to the same, reliable environmental stimulus, such as light or light-dark cycles. A regulatory entity, such as a circadian system that responds to light and keeps time, would have obvious advantages in this regard. There are multiple ways that clock control could be utilized. For example, there could be one central clock located in the nuclear-cytoplasmic compartment controlling gene expression in both the nucleus and in the chloroplast. Alternatively, there could be separate clocks in each compartment controlling genes only in that compartment. Also, it is implicit in the latter model that the respective clocks are similarly synchronized by light-dark cycles; however, it is also possible that the hypothetical chloroplast clock is the "slave" of a nuclear-cytoplasmic or "master" clock, which exerts ultimate control in response to L-D cycles. The evidence to date, as discussed below, seems to support the first model, at least for the rhythm of chloroplast gene expression.

In angiosperms, proplastids in meristematic cells differentiate into one of several types of organ or tissue-specific plastids such as amyloplasts (in roots and seeds), chloroplasts (in shoots under the influence of light), and etioplasts (in shoots grown in the dark). It is well known that these plastid transformations involve changes in organellar gene expression; besides, there is also regulation of plastid genes in mature chloroplasts in response to light-dark cycles (Chen et al. 1995; Nakahira et al. 1998; Riesselmann and Pechulla 1992). This type of plastid gene regulation has been studied more intensively in algae, providing evidence that land plant chloroplasts show daily fluctuations in gene expression of which at least one gene, *psbD*, is controlled by the circadian clock at the transcriptional level.

The circadian regulation of chloroplast-encoded genes occurs mainly at the transcriptional level in *Chlamydomonas,* and encompasses many if not all of the genes in the organelle (Hwang et al. 1996). Translational control was also

originally speculated to be an important part of circadian regulation. The speculation was based on the fact that chloroplast protein synthesis is low during the dark period despite the relative abundance of mRNAs for several key proteins (e.g., *psbA*, *psbD*, and *rbcL*; Herrin et al. 1986). However, when acetate was present (as a reduced carbon source for *Chlamydomonas*) chloroplast translation was strong during the dark and light periods. Also, when L-D entrained cells growing photoautotrophically were placed in constant light (LL), translation in the chloroplast was robust all of the time. Those results indicate that chloroplast translation is not strongly circadian (Lee and Herrin 2002). They also suggest that the principal reason for the drop in translation during the dark period in cells growing photoautotrophically is insufficient energy in the chloroplast. Hence, chloroplast translation is more tightly controlled by energy charge than is transcription, despite the fact they both use ATP.

Circadian regulation of plastid transcription in angiosperms

The RNA polymerases of land plant plastids: The plastid genome in higher plants is transcribed by two types of RNA polymerases, the NEP (or nuclear encoded polymerase) and the PEP (or plastid encoded polymerase) (Hess and Borner 1999). The plastid NEP is actually similar to mitochondrial and bacteriophage RNA polymerases, which are single-subunit enzymes playing an important role in early plant development. The PEP is similar in structure to eubacterial RNA polymerases, and it plays a major role in the expression of genes involved in photosynthesis in mature chloroplasts. The PEP consists of an *E. coli*-like core enzyme that contains three evolutionarily conserved subunits (a, b and b′) encoded by the plastid *rpoA*, *rpoB*, *rpoC1* and *rpoC2* genes; the bacterial *rpoC* gene is split into two genes in higher plants (Hess and Borner 1999). The holoenzyme also requires a specificity factor to initiate transcription at the correct nucleotide. The specificity factor is more commonly called the sigma factor, and is usually encoded by a small gene family (*SIG* genes) in the nucleus (Fujiwara et al. 2000; Kanamaru et al. 1999; Oikawa et al. 2000; Tan and Troxler 1999; Tozawa et al. 1998). The evolutionary transfer of *SIG* genes to the nucleus during chloroplast evolution has thus enabled the control of PEP levels by the nucleus.

Sigma factor genes have been cloned from a number of land plants such as *Arabidopsis thaliana* (*SIGA*, *SIGB*, *SIGC*, *SIGD*, *SIGE*, *SIGF*) (Fujiwara et al. 2000; Isono et al. 1997; Kanamaru et al. 1999; Tanaka et al. 1997), corn (*SIG1*, *SIG2*, *SIG2B* and *SIG3*) (Beardslee et al. 2002; Lahiri et al. 1999; Tan and Troxler 1999), the moss *Physcomitrella patens* (PpSig1, PpSig2, and PpSig5) (Ichikawa et al. 2004), rice (Tozawa et al. 1998), *Sinapis alba* or mustard (Kestermann et al. 1998), tobacco (*SIGA1* and *SIGA2*) (Oikawa et al. 2000), and wheat (Morikawa et al. 1999). All of the nuclear-encoded *SIG* gene products from these plants have chloroplast

targeting signals, and many of them show differential expression during development or in response to light. T-DNA insertions and anti-sense plants in *Arabidopsis* gave no phenotypes for some of the sigma factor genes; however, inhibiting *SIGB* (a.k.a. *SIG2*) resulted in poor chloroplast development and severe losses of certain plastid tRNA genes (Hanaoka et al. 2003; Kanamaru et al. 2001). A T-DNA knock-out of *Arabidopsis SIGE* (a.k.a. *SIG5*) totally blocked plant development at the embryo stage in one study (Yao et al. 2003); in another, a T-DNA insertion in the same gene specifically inhibited *psbD* transcription (Tsunoyama et al. 2004). Finally, the phenotype of a null mutant of the *SIG6* (*SIGF*) gene of *Arabidopsis* suggested that the SIG6 protein acts as a general transcription factor for PEP in early chloroplast development (Ishizaki et al. 2005), possibly making it the first general sigma factor identified in a higher plant.

In angiosperms, there are two developmental/physiological phases that have been found to be associated with differential expression of plastid sigma factors: (1) the development of chloroplasts from proplastids in cotyledons and young leaves, and (2) the maintenance of photosynthetic function in mature chloroplasts. In *Arabidopsis*, *SIGA* and *SIGB* were both abundantly expressed in green tissues. *SIGB* promoter activity was detected earlier than *SIG*A (Kanamaru et al. 1999), suggesting that *SIGB* is more important for the early phase of chloroplast development. In other reports, the expression of all the *Arabidopsis* sigma factor genes was detected in green tissues of seedlings grown in the light (Fujiwara et al. 2000; Tanaka et al. 1997). Using specific antibodies to sigma factors, Privat et al. (2003) showed that SIG3 of *Arabidopsis* was present in seeds and increased on day 4 of imbibition. SIG2 appeared and increased dramatically on day 3 while SIG1 stayed at relatively low levels throughout early development. In tobacco, the *SIGA1 and SIGA2* genes were expressed (mRNAs) mainly in green tissues, suggesting they play a role in the maintenance of the chloroplast (Oikawa et al. 2000). In corn, *SIG1* and *SIG2* also showed high expression (mRNA) levels in green tissues (Tan and Troxler 1999), but *SIG2* mRNA and SIG2 protein were detected in dark-grown etioplasts, suggesting that it plays a role in early plastid development as well as in chloroplast maintenance. Interestingly, another sigma factor of corn, *ZmSig2B*, is apparently targeted to both mitochondria and chloroplasts (Beardslee et al. 2002). Thus, the roles of sigma factors in higher plants can vary with respect to the PEP-dependent genes they transcribe.

Plastid promoters and circadian control in higher plants

NEP and PEP recognize different promoters, as expected. Examples of PEP dependent promoters are *rbcL*, encoding the large subunit of ribulose-bisphosphate carboxylase, *psbA* encoding the D1 protein of the photosystem II

reaction center, and *psbD* encoding D2 which is the partner to D1 in photosystem II. PEP promoters usually have consensus sequences at -35 (TTGACA) and -10 (TATAAT) that are similar to eubacterial promoters (Hajdukiewicz et al. 1997). Plastid genes with NEP-dependent promoters include *accD*, which encodes a subunit of acetyl-CoA carboxylase, ATP synthetase genes *atpA* and *atpB*; the latter genes are actually transcribed by both PEP and NEP (Hajdukiewicz et al. 1997). NEP promoters share a consensus sequence, ATAGAATA/GAA, close to the site at which transcription initiates (Hess and Borner 1999).

A chloroplast PEP-type promoter that is different from the usual promoters recognized by PEP is the *psbD* light-responsive promoter (LRP), which is conserved among higher plants (Hess and Borner 1999). This promoter has been studied in barley (Thum et al. 2001) and wheat (Nakahira et al. 1998). It consists of a core promoter with -10 and -35 elements, an "AAG box" between bp -36 and -64, and a "PGT (plastid GT) box" upstream of the AAG box, between bp -71 to -100. Of key importance here is the fact that the *psbD* LRP has been shown to be regulated by the circadian clock, with the transcription peak occurring in the daytime (Nakahira et al. 1998; Thum et al. 2001). Moreover, *in vitro* and *in vivo* transcription experiments using mutated forms of *psbD* LRP revealed that the boxes upstream of the -10 region were not totally required for a circadian rhythm of transcription, despite the fact that the expression amplitude was substantially reduced when they were deleted. It is not clear if the transcription of other chloroplast genes in higher plants is under circadian control, in part because it is difficult to directly measure chloroplast gene transcription rates *in vivo*, and this is necessary when dealing with mRNAs that are highly stable and might fluctuate only 2-fold.

Since the transcription of most chloroplast-encoded genes may be diurnal, it follows that there should be an important role for light in the expression of these genes. Indeed, there is evidence linking photosynthesis (electron transport and reduced intermediates) with chloroplast gene expression that may have implications for circadian control. Using mustard, Link and associates (Baginsky et al. 1999), have shown that a protein kinase (they call it plastid transcription kinase (PTK) is associated with and phosphorylates PEP, and that this effectively decreases PEP activity. They have also shown that the reduced form of glutathione (a strong reductant) inhibits PTK activity, thereby stimulating PEP activity. On the basis of these results, they propose the following model to explain the effect of light on chloroplast transcription: light, acting through photosynthesis, generates reduced intermediates such as glutathione, which stimulate PEP activity by inhibiting its phosphorylation by PTK (Baginsky et al. 1999; González et al. 2001). This mechanism has not been shown to be involved in circadian control of *psbD*, although it does provide a testable hypothesis for

how the clock might control transcription by regulating a kinase or the redox status of the organelle.

It is reasonable to expect that the transcription of chloroplast genes having PEP-dependent promoters would depend, at least in part, on the expression of a nuclear-encoded sigma factor(s) that initiates transcription at those promoters. Nakahira et al. (1998) provided evidence that the circadian peak of transcription of *psbD* in wheat chloroplasts occurs in the morning. Subsequently, Morikawa et al. (1999) showed that the *SIGA* mRNA in wheat exhibits a circadian oscillation in concert with that of *psbD* mRNA. Since *psbD* transcription depends on PEP, it is reasonable to suggest that *psbD* transcription in wheat might be influenced by *SIGA* expression. Direct experiments to prove this, however, are needed, because wheat is likely to have several sigma factors. The *psbD* LRP promoter in *Arabidopsis* is transcribed by SIG5 (Tsunoyama et al. 2004), but it is not known if the expression of *psbD* or *SIG5* is circadian in *Arabidopsis*. The *SIG2* (*SIGB*) mRNA of *Arabidopsis* has been shown to cycle in a circadian fashion, peaking early in the morning (Harmer et al. 2000). The target of SIG2 includes several tRNA genes, but it has not been shown if these are under circadian control. If they are, that could potentially put plastid translation under circadian control in this organism. There is suggestive evidence that the *SIGA* and *SIGB* genes of *Arabidopsis* (Kanamaru et al. 1999), and the *SIGA1* and/or *SIGA2* genes in tobacco (Oikawa et al. 2000) are under circadian control. In moss, the mRNA for an orthologue of the *Arabidopsis SIG5* gene, *PpSig5*, was shown to be under circadian control, and its expression correlated with the circadian peak of *psbD* mRNA. Interestingly, the time of the expression peak for both mRNAs was advanced in a cryptochrome mutant (Ichikawa et al. 2004).

Circadian regulation of chloroplast gene transcription in algae

The algal chloroplast RNA polymerase: Similar to higher plants, algae have a PEP (the core subunits are chloroplast encoded), but there is a lack of evidence for the presence of a NEP in the plastid (reviewed in Smith and Purton 2002). It thus appears that all plastid genes are transcribed by the PEP enzyme. Also, the *rpoB* gene in *Chlamydomonas reinhardtii* is divided into two coding regions, *rpoB1* and *rpoB2*; hence, the *Chlamydomonas* PEP has RPOA, RPOB1, RPOB2, RPOC1, and RPOC2 subunits (Smith and Purton 2002). Even more surprising, is the occurrence of a single sigma factor in *Chlamydomonas*, encoded by the *RPOD* (= *SIG*) gene in the nucleus (Carter et al. 2004, and references therein). The predicted mature protein, RPOD, is ~80 kDa and contains two motifs not previously recognized in sigma factors, adjacent PEST sequences and a coiled-coil or leucine zipper (LZ) motif near the N-terminus. Both of these motifs are probably involved in protein-protein interactions. PEST sequences have been shown to increase the susceptibility of proteins to proteolytic degradation, and

they could regulate the stability of the RPOD protein (Rechsteiner and Rogers 1996). The coiled-coil LZ domain is known to be involved in dimerization of nuclear transcription factors (Landschulz et al. 1988). However, there are no reports of dimerization of sigma factors, but this possibility cannot be ignored. The LZ domain could also regulate RPOD activity by mediating its interaction with other proteins.

In contrast to the situation in *Chlamydomonas*, the red alga *Cyanidium caldarium* (strain RK-1) possesses at least three nuclear-encoded sigma factors, namely *SIGA*, *SIGB* and *SIGC* (Oikawa et al. 1998). The expression profile of *SIGA* differs from that of *SIGB* and *SIGC*; the level of *SIGA* mRNA was similar throughout the 24-h light-dark cycle, while the levels of *SIGB* and *SIGC* mRNA increased in the light. When a RNAP holoenzyme was reconstituted using the sigma factors from *Cyanidium* and the core enzyme from *E. coli*, the chimeric enzyme recognized conserved *E. coli* promoters, confirming that the proteins were bona fide sigma factors. It is not clear why this organism has multiple sigma factors, whereas *Chlamydomonas* has only one; there has been little study of chloroplast promoters in *Cyanidium*. Also, it is possible that the expression of *SIGB* and *SIGC* is under circadian control, but this remains to be determined.

Plastid promoters and circadian control in algae

In vivo studies of the 16S and *atpB* rRNA promoters of *Chlamydomonas* indicated that there are at least two types of chloroplast promoters in this organism (Klein et al. 1992). The 16S rRNA promoter is similar to bacterial sigma-70 promoters in having -10 and -35 elements that are used *in vivo*. The *atpB* promoter, on the other hand, does not have the -35 element, but was stimulated by a sequence downstream of the -10 element (Klein et al. 1992). The -35 region also was not obvious in several other promoters, including *psbA* (Klein et al. 1992), which is a very strongly transcribed gene. Thus, a -35 region is not essential to achieving a high rate of transcription in *Chlamydomonas* chloroplasts.

As discussed above, there seems to be only one sigma factor gene (*RPOD*) in *Chlamydomonas*, which could, in theory, transcribe both types of promoters; however, there could also be other DNA-binding proteins or factors that modulate (either promote or inhibit) binding of the RNAP holoenzyme, possibly by interacting with RPOD through the LZ or PEST sequences. RPOD could also be modified post-translationally, e.g. by phosphorylation, and this could affect binding to other potential *trans*-acting factors, or to the core subunits of the PEP enzyme.

In *Chlamydomonas* growing in 12 h: 12 h LD cycles, the transcription of all five chloroplast-encoded genes that were examined, as well as total chloroplast transcriptional activity, were shown to undergo circadian fluctuations, peaking

in the early morning (Hwang et al. 1996). The specific genes examined were the *rRNA* genes (16S-23S-5S), the *atpB*, *psbA*, and *psaA* genes of photosynthesis, and the elongation factor Tu gene, *tufA*. Evidence of circadian rhythmicity was observed in LD-entrained cells shifted to continuous light (LL) and continuous darkness (DD). In the latter case, the rhythm of *tufA* mRNA continued for at least three cycles in DD, and in the context of a slow rate of cell division. Moreover, these rhythms were obtained in the presence of acetate, which disrupts the LD pattern of chloroplast translation, indicating that the circadian clock is the dominant regulator of chloroplast transcription in cells growing under LD cycles in nutrient replete conditions.

More recent work by Kawazoe et al. (2000) showed that the daily (or LD) pattern of chloroplast transcription in *Chlamydomonas* is actually the result of two types of transcription that are separable on the basis of sensitivity to short-term treatments with cytoplasmic translation inhibitors. The two types of transcription were basal and clock-induced. This conclusion was based mainly on the transcriptional response to cycloheximide (CH) in both asynchronously and synchronously growing cultures. CH induced a rapid reduction in the transcription of all chloroplast genes examined, i.e. *rRNA, tufA*, *atpB*, *psaA* and *psbA* genes, in asynchronous cultures. The reduction in transcription by CH was not equal for all genes; for example, *psbA* was inhibited only 40%, whereas inhibition was 80% in the case of *tufA* transcription. Interestingly, in synchronously growing cells (i.e., in LD cycles), the inhibition of transcription by CH was only apparent during the circadian peak of transcription, and not during the trough period. This result indicated that the clock-induced transcription requires a short-lived, nuclear-encoded protein(s), but basal transcription does not. Also, the relative contribution of each type of transcription is gene-specific, e.g., *psbA* has a relatively high basal rate (50-60% of total) and less circadian (~40-50% of total) than other genes examined. The identity of the cycloheximide-sensitive factor(s) needed for the circadian peak of chloroplast transcription is unknown, but it should be something that is involved in the transcription of most, if not all chloroplast genes in *Chlamydomonas*. A sigma factor, specifically under circadian control, was one suggested hypothesis (Kawazoe et al. 2000).

In support of a sigma factor as the cycloheximide-sensitive inducer of the rhythmic peak of chloroplast transcription, Carter et al. (2004) showed that there is a daily peak in *RPOD* mRNA that occurs prior to or coincident with the peak in chloroplast transcription. Carter et al. also showed that the expression of *RPOD* mRNA displays a circadian rhythm in LL (continuous light) and DD (continuous dark), with the maximum level occurring in the mid to late dark period, prior to or coincident with, the peak in chloroplast transcription. These results suggested strongly that the circadian expression of *RPOD* contributes to

the rhythmic control of chloroplast transcription. Moreover, the presence of only one sigma factor gene in *Chlamydomonas* facilitates global control of chloroplast transcription by the circadian clock (Hwang et al. 1996). It remains to be explained how one sigma factor could be responsible for both basal and circadian transcription of chloroplast genes.

The amplitude of the circadian peak of the sigma factor (*RPOD*) mRNA is not as great as the peak in transcription of chloroplast genes, suggesting that there is another nuclear-encoded factor, in addition to *RPOD*, that contributes to the circadian peak of chloroplast transcription (Carter et al. 2004). Fig. 1 presents a current model of clock control of chloroplast transcription in *Chlamydomonas*. A logical candidate for the nuclear-encoded factor "X" is a topoisomerase that would contribute to the transcription rhythm by affecting the degree of supercoiling of chloroplast DNA. Salvador et al. (1998) presented evidence that the superhelical topology of chloroplast DNA fluctuates rhythmically, in concert with the rhythm of transcription. However, the topological fluctuation in genome structure could be an effect rather than a cause of the chloroplast transcription rhythm. In support of the causal nature of the topology rhythm, Salvador et al. (1998) analyzed a non-transcribed region of the genome, and obtained evidence that the fluctuation in DNA supercoiling is independent of transcription. Interestingly, Thompson and Mosig (1990) found that the degree of torsional stress of certain regions of chloroplast DNA of *Chlamydomonas* is affected by light and this effect is blocked by cycloheximide.

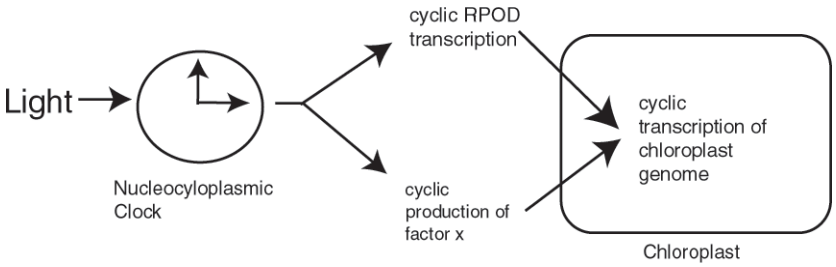

Fig. 1. Working model for circadian regulation of chloroplast transcription in *Chlamydomonas*. ROPOD is the lone sigma factor gene that directs specific transcription in the chloroplast. Factor X is a hypothetical protein(s), possibly a topoisomerase, that promotes supercoiling of chloroplast DNA. These two act together to drive circadian transcription of chloroplast genes.

Biological significance of circadian rhythms in chloroplast transcription

The regulation of chloroplast transcription is circadian, while that of translation seems to be regulated more by energy levels (Lee and Herrin 2002). The fact that mRNA synthesis begins before the light period starts could be advantageous to

the organelle as the process provides ample strongly light-dependent mRNA for translation. Also, in chloroplasts, transcription and translation are not as tightly coupled as in bacteria, and therefore do not require controlling by the same signal. It may be relevant that DNA repair is coupled to transcription in bacteria (Svejstrup 2002), and the clock control chloroplast DNA repair could ensure the repair of the genome each day before the beginning of the new light period. However, this is highly speculative as transcription-coupled repair has not yet been documented in chloroplasts.

Perspectives on future work

Studies on circadian regulation of plastid gene transcription in higher plants have so far been restricted mainly to the *psbD* gene (Nakahira et al. 1998; Thum et al. 2001). It would be useful to know if this control extends to other plastid genes. Also, it is now possible to examine all chloroplast genes at once using microarray technology (Schena et al. 1995). Along these lines, the circadian transcription of most chloroplast genes in *Chlamydomonas* has yet to be proven, and it would be useful to extend the study to all plastid genes (Maul et al. 2002).

Both higher plants and *Chlamydomonas* use PEP for circadian plastid transcription. The sigma factor in *Chlamydomonas*, and at least one sigma factor in higher plants, is under circadian control, and the expression peak is consistent with a role in circadian chloroplast transcription (Carter et al. 2004; Harmer et al. 2000; Ichikawa et al. 2004; Morikawa et al. 1999). However, it has not yet been shown that the levels of the proteins fluctuate, i.e., that they are unstable proteins. Alternatively, they may be stable proteins whose activity is down regulated by a post-translational mechanism, such as phosphorylation - dephosphorylation. With the plethora of nuclear-encoded sigma factor genes in higher plants, it may be easier to understand this regulation in *Chlamydomonas*. Other interesting things to explore would be to determine how the clock regulates *SIG/RPOD* expression, i.e., what transcription factors control *SIG/RPOD* genes, and how they are controlled by the clock.

With regard to topological changes in chloroplast DNA, linked to circadian fluctuations in chloroplast transcription in *Chlamydomonas* (Salvador et al. 1998), it would be interesting to extend this analysis to higher plants. The experiments with *Chlamydomonas* suggested that the topological changes were independent of transcription because they occurred in a non-transcribed region immediately downstream of the transcribed gene; and hence long-range effects could have influenced the results. The question of whether the cyclic topological changes in chloroplast DNA are the result of cyclic transcription needs to be investigated further, perhaps by adding rifampicin to block transcription (Surzycki 1969) and then determining if the cyclic topological change persists on schedule.

The working of the clock, as well as its components has been partially deciphered in *Arabidopsis*, which is the model system for higher plants (Alabadi et al. 2001, 2002; Eriksson and Millar 2003). However, central clock components have not been identified in *Chlamydomonas*, which is the model system for algae. *Chlamydomonas* does not seem to have close homologues of the *Arabidopsis* or *Synechococcus* clock genes (Mittag et al. 2004; Herrin et al. (unpublished). However, not all of the nuclear genome has been sequenced (JGI version 2.0), and thus there is a small possibility that the unsequenced portion contains such clock genes.

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