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Circadian programming in cyanobacteria

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Prokaryotic cyanobacteria express robust circadian (daily) rhythms under the control of a timing mechanism that is independent of the cell division cycle. This biological clock orchestrates global regulation of gene expression. Competition experiments demonstrate that fitness is enhanced when the circadian period is consonant with the period of the environmental cycle. Mutational analyses have identified three clock genes in the organism, one of which is related to DNA recombinases and helicases. We propose a new model for the core 'clockwork' that implicates rhythmic changes in the status of the chromosome that underly the rhythms of gene expression.

Key words: circadian / biological clock / fitness / competition / cell division

Independence of circadian and cell division timing in cyanobacteria

Circadian rhythms are endogenous biological programs that 'free-run' with a period that is close to 24 h in constant conditions, but will entrain to appropriate environmental cycles of light/dark (LD) or temperature so as to become exactly 24 h in duration. Their free-running period is 'temperature compensated,' that is, it is nearly constant over a range of physiological ambient temperatures. Before 1985, it was believed that circadian programs were exclusively found among eukaryotes.[1] The conclusion that only eukaryotes have circadian oscillators seemed reasonable, because it was assumed that an endogenous timekeeper with a period close to 24 h would not be useful to organisms that divide more rapidly than once every 24 h, as do many prokaryotes. However, two reports in 1986 on circadian rhythms of nitrogen fixation in cyanobacteria 'broke the prokaryotic barrier.'[2,3] We and others extended the studies on circadian programming in cyanobacteria.[4,5] Our first studies used a strain of *Synechococcus elongatus* PCC 7942 (previously named *Synechococcus* sp. strain PCC 7942) transformed with a bacterial luciferase reporter construct that allowed the assay of the promoter activity of the *psbAI* gene encoding the D1 protein of the photosystem II reaction center.[4,5] Because the circadian clock turns this promoter on and off rhythmically, the transformed strain of *S. elongatus* glows rhythmically.

How pervasive is the circadian control over gene expression? A sensitive and comprehensive promoter trap experiment was used to address this question. A promoterless luciferase gene set was randomly inserted throughout the genome; whenever the luciferase gene set inserted into a genomic locus that was correctly positioned and oriented to a promoter, luciferase was expressed and the colonies glowed. The luminescent patterns of 3000 independent colonies were analysed, and the astounding result was that *all* of the glowing colonies displayed circadian rhythms with the same period.[6] Apparently the cyanobacterial clock globally controls gene expression—in this case, the activity of promoters,[6] and in another study, of protein abundances.[7] Moreover, when an *E. coli* promoter (*conIIp*) that can be transcribed in *S. elongatus* is assayed by a similar test (i.e. using a promoter::luciferase construct that is integrated into the chromosome), even the heterologous *conII* promoter was rhythmically active.[8] A model for this global control will be presented below.

Besides gene expression patterns, what else does this circadian pacemaker control in cyanobacteria? Despite the fact that cyanobacteria grow with dou-

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bling times that are significantly faster than once per 24 h, we decided to test whether cell division might be regulated by the circadian clock. We found that—in contrast to eukaryotic unicells—cultures of *S. elongatus* that are growing with doubling times as rapid as one division every 6–10 h continue to exhibit circadian rhythms of *psbAI* gene expression.[9,10] When we maintained cultures in the actively growing state indefinitely by continuously diluting liquid cultures to keep the average cell density constant, we observed an interesting pattern in the cell growth profile [Figure 1(A)]. As expected, in LD cycles, the cells divided in the light and did not divide in darkness, but, under constant illumination, a circadian pattern of division continued, even though the doubling time of the culture was significantly faster than once every 24 h [11.8 h in Figure 1(A)]. To put it another way, even though cells are dividing on average more than twice every 24 h, *when* they divide is still 'gated' by a circadian oscillator.[9] The phase of the slowdown of division rate coincides with the early subjective night. Using flow cytometry, we determined that the DNA replication rate and the growth in size of each individual cell is not rhythmic over the circadian cycle, but it is the timing of division that is controlled by a circadian program in a manner that is independent from the cell division cycle.[9]

The independence of circadian timing from cell division has also been demonstrated by overexpressing the cell septum protein FtsZ in *S. elongatus*.[11] When FtsZ is overexpressed, cell division—but not growth—is suppressed, resulting in green spaghetti-like filamentous cells [Figure 1(B) and 1(C)]. Remarkably, this inhibition of division has no detectable effect on rhythms of gene expression [Figure 1(D) and 1(E)].[11] Therefore, the periodicity of the circadian system is precise and stable whether the cells are dividing rapidly, dividing slowly, or not dividing at all. These differing division states must have an important impact upon the intracellular milieu, but the circadian mechanism appears to be unaltered. Apparently, the circadian clockwork is well buffered and stable against significant changes of the intracellular milieu. Consequently, the circadian clockwork in *S. elongatus* gates cell division and gene expression, but its timing circuit is independent of the cell division cycle.

Enhanced fitness conferred by circadian programming in *S. elongatus*

Based on the earlier work with eukaryotic unicells, selection for circadian organization had seemed reasonable only if the generation time of the cell is as long or longer than a day.[12] To us, however, it seems equally plausible that the advantages of anticipating, for example, the onset of solar illumination with its concurrent benefits and deficits would accrue to all light-dependent organisms, even if they divide more rapidly than the daily cycle. Presumably, the advantage of having a daily biological clockwork is that it organizes a temporal *program* that cues processes to occur at specific phase relationships to the daily environmental cycle. This programming can enable the organism to *anticipate* changes in the environment that are important and enable it to respond appropriately.

But are there any direct tests of a fitness advantage conferred by circadian clocks? We undertook a rigorous test of the adaptive significance of circadian programs in *S. elongatus*. For asexual microbes, differential growth of one strain under competition with other strains is a good measure of reproductive fitness. The circadian phenotypes of the strains used had free-running periods of about 22 h, 25 h (wild-type), and 30 h. In pure culture, the strains grew at about the same rate in constant light and in LD cycles, so there did not appear to be a significant advantage or disadvantage to having different circadian periods when the strains were grown separately.[13] The fitness test was to mix different strains together and to grow them in competition to determine if the composition of the population changes as a function of time.

The results of this competition test were noteworthy. When each of the strains was mixed with another strain and grown together in competition, a pattern emerged that depended upon the frequency of the LD cycle and the circadian period. When grown on a 22-h cycle (11 h light, 11 h dark), the 22 h-period mutant became the dominant cell type in the mixed cultures. On a 30-h cycle (15 h light, 15 h dark), the 30 h-period mutant could vanquish either wild type or the 22 h-period mutant. On a 'normal' 24 h cycle (12 h light, 12 h dark), the wild type strain could defeat either mutant.[13] Under a non-selective condition (in this case, constant light), each strain was able to maintain itself in the mixed cultures. Because the mutant strains could defeat the wild-type strain in LD cycles in

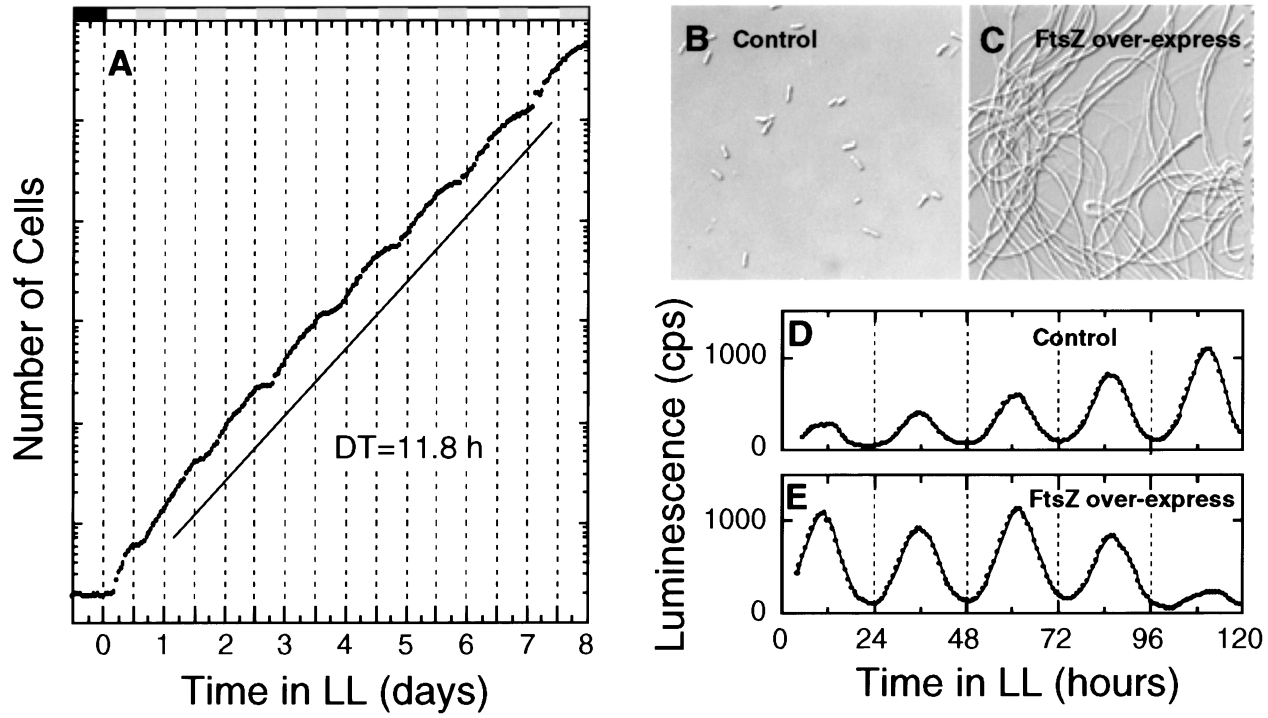

Figure 1. Circadian rhythm of cell division in continuous cultures of rapidly growing *S. elongatus* cells and luminescence rhythms in dividing versus non-dividing cells. (A) A log-phase culture of *S. elongatus* was continuously diluted with fresh medium. The growth curve was calculated from the actual cell density and the dilution rate (dilution rate = 0.057 h^{-1}). (B)–(E) Cell division of growing cyanobacteria is stopped by overexpression of FtsZ. Morphology of: (B) control cells, and (C) cells in which FtsZ is over-expressed. Luminescence rhythms in: (D) control cells, and (E) cells in which division has been halted by FtsZ overexpression. The reporter in (D) and (E) is the *kaiBC* promoter driving bacterial luciferase expression.

which the periods are similar to their endogenous periods, the differential effects that were observed are likely to result from the differences in the circadian clock. A genetic test was also performed to demonstrate that only the clock gene mutation was responsible for the differential effects in the competition experiment.[13] More recent experiments with arhythmic mutants demonstrate that they can compete effectively with wild-type in constant light (non-selective conditions), but lose rapidly in LD cycles (selective conditions; unpublished results). Clearly, for rhythmic strains, the strain whose period most closely matched that of the LD cycle eliminated the competitor. Moreover, the results with arhythmic mutants demonstrate that having a clock is adaptive when cells are in a rhythmic environment.

This is the first rigorous demonstration in any organism of a fitness advantage conferred by a circadian system. But what is the basis of the competitive advantage? In the original publication, two possibilities were introduced: (a) clocks allow optimal utilization of limiting resources such as light, nutrients, carbon dioxide, or (b) cyanobacteria rhythmically secrete diffusible factors that inhibit the growth of other cyanobacterial strains. No physiological data are currently available that distinguish between these hypotheses, but a modelling study favored the 'rhythmic inhibitor' alternative.[14] A likely explanation for the differences in selective advantage is that the phase relationships between biological rhythms and environmental cycles are predictably altered in different combinations of free-running period versus the period of the LD cycle.[13] This conclusion is consistent with the postulate that the circadian system regulates temporal programming of metabolic processes and that fitness is maximized when the phasing of those processes relative to the environmental cycles is optimized.

Genetic insights into the circadian clockwork of cyanobacteria

Without a doubt, the most successful strategy for unveiling circadian components has been screening for mutants with altered phenotypes, cloning the genes that have been mutated and characterizing the expression patterns and products of those genes. Using the luciferase reporter strain in a saturation mutagenesis screen, more than 100 mutants exhibiting various circadian phenotypes, including arhythmia, altered waveforms, and atypical periods (ranging between 14–60 h) were isolated from *S. elongatus*.[15] Most of these mutants grow apparently as well as wild type and exhibit no other obvious phenotype in single-strain cultures besides circadian anomalies. Over 30 of these mutants have been rescued by the introduction of libraries of wild-type *S. elongatus* DNA. DNA fragments from several rescued mutants complemented other mutant phenotypes, including short-period, long-period and arhythmic phenotypes.

These rescue experiments pinpointed a cluster of three adjacent genes, named *kaiA*, *kaiB*, and *kaiC*.[16] Nineteen mutants were rescued by transformation with a plasmid carrying the entire *kaiABC* cluster, and the relevant mutations were mapped by DNA sequencing to the three *kai* genes. All are missense mutants resulting from single nucleotide exchanges. Most of the mutations are recessive (rescue by wild type DNA is complete), but a few are semidominant. Each of the three genes has at least two clock mutations mapped to it, and the largest gene, *kaiC*, has multiple mutations that include many clock phenotypes: short period, long period, low amplitude, and arhythmia. The *kaiABC* cluster appears to be a clock-specific region of the chromosome in cyanobacteria because deletion of the entire cluster or of any one of the *kai* genes separately does not affect viability (in single-strain cultures), but causes arhythmicity. None of the Kai proteins has an obvious DNA-binding motif.

There are putative homologs of KaiC in other bacteria, especially archaebacteria, but no homologs to any of the Kai proteins have yet been discovered in eukaryotes.[5,17] A recent genomic survey claims that KaiC is a member of the bacterial RecA/DnaB family.[18] RecA is an ATP-dependent DNA recombinase, and DnaB is the replication fork helicase in bacteria. The *kaiC* gene appears to be an internally duplicated version of a RecA/DnaB-like gene; it has two parts that are very similar to each other.[19] In each half of the *kaiC* gene, there is a Walker A or 'P-loop' motif. This motif, [G or A]XXXXGK[T or S], is often a ATP/GTP nucleotide binding region. Indeed, KaiC binds ATP [and GTP with a lower affinity, Figure 2(a) and 2(b)], and when the Walker A motifs are mutated, the nucleotide binding is abolished [Figure 2(c)] (also see Reference 20). Furthermore, when the Mg^{2+}-coordinating threonine residues of either motif are mutated to alanine [Figure 2(d)], or the nucleotide-binding lysine residues are mutated to histidine,[20] rhythmicity is severely disrupted or abolished. Therefore, this motif is essential in both parts of the KaiC molecule for rhythmicity.

The rhythmic expression patterns of the *kai* genes are reminiscent of *Drosophila* and *Neurospora* clock genes. Promoter activities were found in the upstream regions of both the *kaiA* and *kaiB* genes. The *kaiA* promoter gives rise to a monocistronic *kaiA* mRNA, whereas the *kaiBC* promoter produces a dicistronic *kaiBC* mRNA.[16] Both *kaiA* and *kaiBC* transcripts are rhythmically abundant.[16,21] Continuous overexpression of *kaiC* repressed the *kaiBC* promoter (negative feedback), whereas *kaiA*-overexpression enhanced it (positive feedback). The abundances of the KaiB and KaiC proteins undergo circadian oscillations in constant light, whereas the pattern of the KaiA protein exhibits little, if any, circadian variation.[22] Pulsatile expression of the *kaiC* gene from an inducible promoter to physiological levels resets the phase of the rhythms.[16,22] Therefore, it appears that the level of KaiC expression (or some form of KaiC) is directly linked to the phase of the oscillation. The original model[16] for the cyanobacterial clockwork proposed that there was specific negative feedback of KaiC on the *kaiBC* promoter. More recently, however, we have found that KaiC expression can be driven by an unrelated promoter (*trcp*, a fusion of $lacI^q$, the -35 region of *trp* and the -10 region of *UV5* promoters followed by lacO of *E. coli*[23]) and still allow the manifestation of rhythmicity (Dr Y. Xu, unpublished observations). An alternative mechanism for the negative feedback of KaiC on its own expression will be proposed below.

A feature of the Kai proteins that is reminiscent of clock proteins in eukaryotes is that Kai proteins appear to interact. Two-hybrid, *in vitro* binding, and resonance energy transfer assays indicate that the KaiA, KaiB, and KaiC proteins interact both homotypically and heterotypically.[19,24] One long period mutant exhibits an altered heterotypic interaction between KaiA and KaiB, suggesting

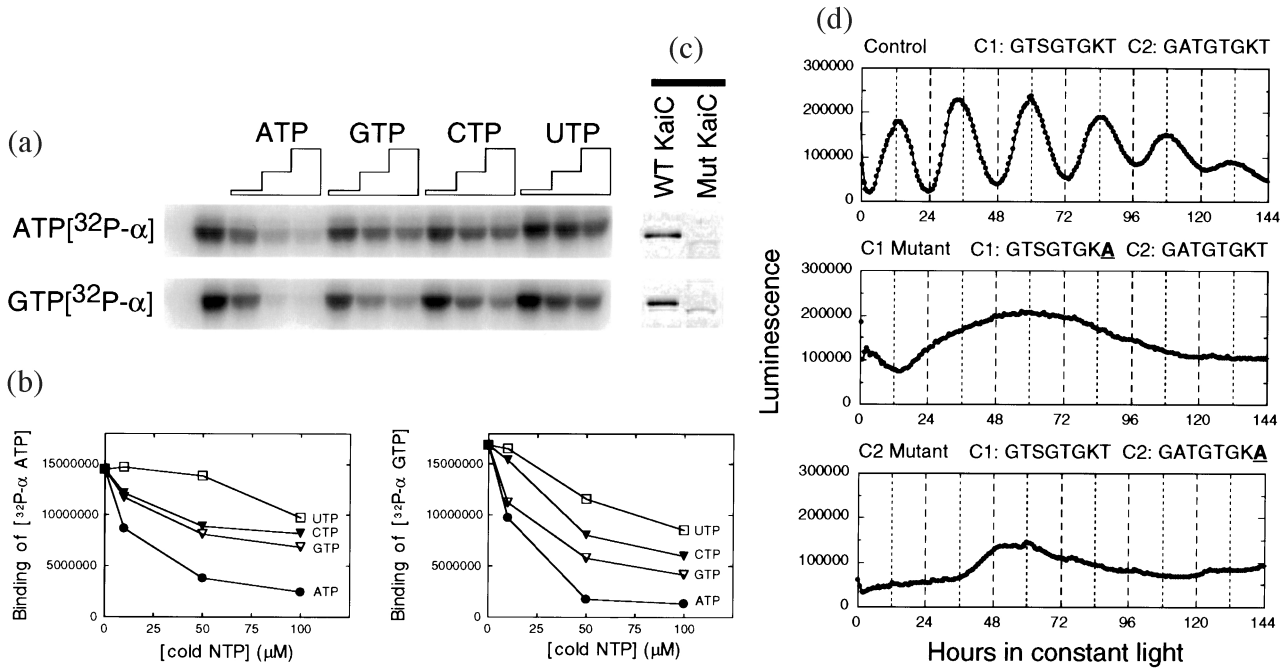

Figure 2. Nucleoside triphosphates bind to the clock protein KaiC. (a) and (b) KaiC protein was incubated with [α-^{32}P]ATP or [α-^{32}P]GTP in binding buffer containing various concentrations of non-radioactive nucleoside triphosphates. Then, the samples were UV treated to cross-link the ATP or GTP to KaiC protein. Cross-linking was quantified after SDS-PAGE.[35] (c) Mutations in both Walker A motifs of KaiC abolish all ATP and GTP binding (WT = wild-type KaiC; Mut = KaiC in which both Walker A motifs have been mutated). (d) Mutations in either Walker A motif of KaiC disrupt rhythms of gene expression. In the *psbAIp* luciferase reporter strain, the threonine residue in either the first or the second Walker A motif of KaiC protein was substituted with alanine (T53A in C1 and T295A in C2 mutants, respectively).

that inter-Kai contact is important to the clock mechanism.[19] Yet another similarity between the cyanobacterial clock system and those of eukaryotes is that phosphorylation events may play a role.[25,26] KaiC appears to be able to autophosphorylate,[20] and KaiC may exist in different phosphorylation states at different phases of the circadian cycle (Drs T. Nishiwaki, H. Iwasaki, T. Kondo, unpublished observations). Furthermore, in a two-hybrid screen for KaiC-interacting proteins, a histidine protein kinase, SasA, was discovered.[21] The N-terminal sequence of SasA is similar to the sequence of KaiB. Disruption of sasA lowers *kaiBC* expression and results in arhythmicity of most genes in moderate to high light intensities. Clearly, SasA is necessary for robust expression of the cyanobacterial clock.[21] Interestingly, low amplitude rhythms were observed in dim constant light, implying that the role of SasA has something to do with light perception or light intensity compensation. Another gene that appears to be involved in light perception by the cyanobacterial clock is the phytochrome-like gene, *cikA*.[27]

A new model: global clock control mediated by chromosomal oscillations

Three lines of evidence lead us to propose a new model for the fundamental mechanism of the clockwork in *S. elongatus*: (1) global circadian regulation of promoters, even heterologous (e.g. *conII*p) promoters; (2) when KaiC expression is driven from a non-specific heterologous promoter (*trcp*) instead of the endogenous *kaiBC* promoter, rhythmicity persists; and (3) the key clock protein KaiC is related to bacterial recombinases and helicases, implying that KaiC might act directly on DNA. These observations tempt us to consider that KaiC might mediate both its own negative feedback regulation[16] and global regulation of the cyanobacterial genome[6] by orchestrating oscillations in the condensation and/or supercoiling status of the entire cyanobacterial chromosome. The chromosome of most bacteria is organized into a 'nucleiod,' which has a highly organized architecture based on condensation and coiling of DNA.[28,29] It is

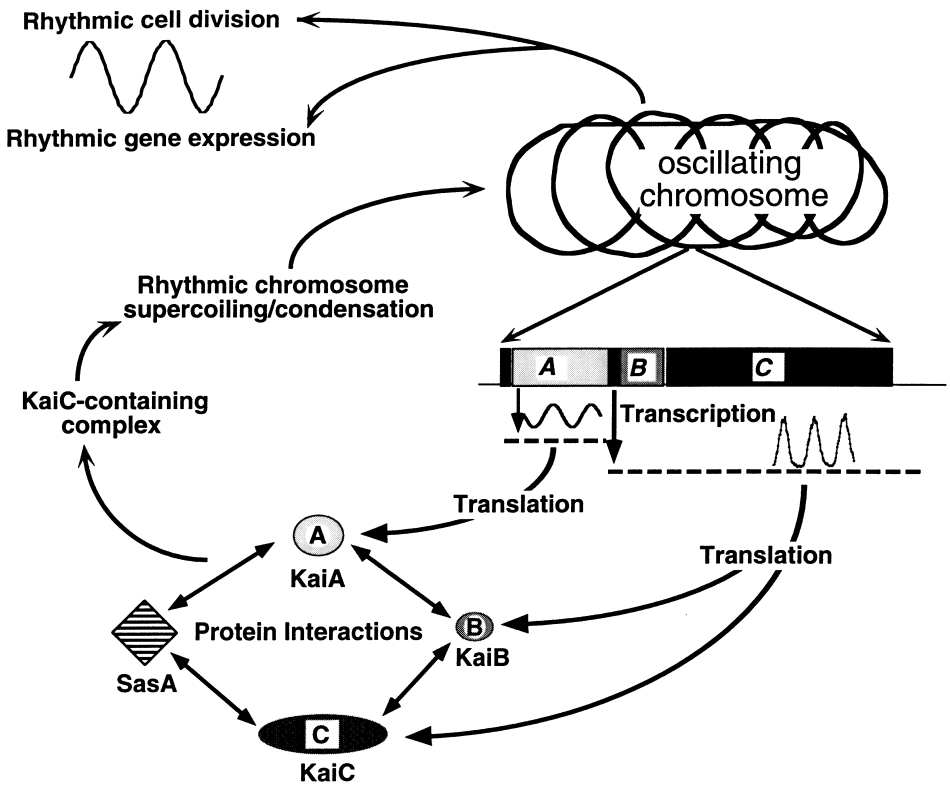

Figure 3. Oscilloid model for the circadian oscillator of *S. elongatus*. The *kaiA* gene is transcribed as a single transcript, while the *kaiB* and *kaiC* genes are cotranscribed. Both the *kaiA* and *kaiBC* promoters are rhythmcally active. Interaction of KaiA, KaiB, KaiC, and SasA proteins form a multimeric KaiC-containing complex that rhythmically regulates the condensation/supercoiling status of the chromosome. Oscillations of chromosomal status globally regulate gene expression.

well known that changes in the local supercoiling status of DNA can affect the transcription rate of genes.[30–32]

We therefore propose that a fundamental component of the cyanobacterial clockwork is that the condensation/supercoiling status of the chromosome rhythmically changes such that it becomes an oscillating nucleoid, or 'oscilloid' [Figure 3]. There is already a precedence for circadian rhythms of supercoiling status in the chloroplast chromosome of the eukaryotic alga *Chlamydomonas*.[33] Based on its endosymbiotic origin, the chromosome of the chloroplast is already thought to be prokaryotic-like, and so the supercoiling rhythms of the *Chlamydomonas* chloroplast chromosome encourage the idea that a similar phenomenon might exist in *S. elongatus*. In cyanobacteria, we postulate that these condensation/supercoiling oscillations promote rhythmic modulation of the transcription rates of all genes, accounting for global regulation of gene expression.[6] Therefore, gene-specific cis-elements that mediate rhythmic gene expression might be (at least partially) responsive to chromosomal status rather than exclusively to trans factors. In addition, heterologous promoters (or *trcp::kaiC* constructs) that are integrated into the chromosome are driven rhythmically since they are also subjected to the oscillating chromosomal status. A minor proportion of the genes oscillate in the opposite phase relationship to the majority of the genes,[6] and this might be due to differential responses in different domains of the chromosome, or to the fact that different promoters react differently to negative versus positive supercoiling.[34] Finally, we suggest that KaiC (or most likely, a KaiC-containing complex) is a key player in regulating these changes of chromosomal status.

Research on circadian programming in cyanobacteria has overturned at least two dogmas—that prokaryotes cannot have circadian clocks, and that organisms cannot express circadian rhythms when they divide

more rapidly than once per 24 h. These investigations have also provided the first rigorous evidence for a fitness advantage conferred by circadian programming, and for global orchestration of gene expression. The study of the central mechanism of the cyanobacterial clock has progressed rapidly within a short time, but the clockwork remains mysterious. We propose a new model to account for global gene regulation and for the role of the essential clock protein KaiC by envisioning an oscillating chromosome, the oscilloid.

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