REVIEW

Circadian clocks go *in vitro*: purely post-translational oscillators in cyanobacteria

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Recent findings about the core of the circadian oscillator in cyanobacteria are challenging the dogma that such clocks are driven through transcriptional–translational feedback regulation. Instead, the master pacemaker is independent of both transcription and translation, and consists of self-sustained oscillations in the phosphorylation status of the KaiC protein *in vivo*. Using a minimal cocktail of three recombinant proteins with adenosine triphosphate, the core clock was even reproduced *in vitro*. The so-born chemical oscillator could reproduce accurately temperature compensation and altered period phenotypes in mutants. This system now provides an ideal playground for rebuilding the circadian clock by adding successive components while understanding every single step with chemical resolution.

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Circadian clocks

Long before 19th century watchmakers from the Jura mountains invented the ‘detached pin lever escapement’, living organisms evolved their own time-keeping devices for adapting to a rhythmically changing geophysical environment. In particular, light/dark and temperature cycles play a distinguished role as Zeitgebers (entrainment cues). Ultimately, circadian (with a period close to 24 h) clocks help organisms schedule their physiological and behavioral activities according to the hourly opportunities and constraints specific to their ecological niches. The almost ubiquitous circadian oscillator is now dissected molecularly in a growing number of genetic model organisms including cyanobacteria (Kondo *et al*, 1997; Iwasaki *et al*, 2002), the fungus *Neurospora crassa* (Dunlap and Loros, 2004), *Arabidopsis thaliana* (Salome and McClung, 2004), *Drosophila melanogaster* (Young and Kay, 2001), mouse (Reppert and Weaver, 2002; Hardin, 2004) and, more recently, zebrafish (Whitmore *et al*,

1998). Strikingly, the designs adopted are surprisingly similar across organisms, consisting of cell-autonomous biomolecular oscillators, in which transcription, translation, translocation and post-translational modifications participate in interlocked positive and negative-feedback loops (Young and Kay, 2001; Cyran *et al*, 2003).

The defining properties of these molecular clocks include their ability to keep ticking indefinitely after external entrainment cues have been removed, often for the animal’s full lifespan. Entrainment cues differ across systems and can be light (the photocycle), temperature or chemical signals like hormones (Schibler *et al*, 2003). Recent single-cell measurements in cell cultures have clarified that ‘entrainment’ acts as resynchronization at the cell population level rather than a restart of arrested individual clocks (Nagoshi *et al*, 2004; Welsh *et al*, 2004; Carr and Whitmore, 2005). Additionally, circadian clocks are temperature compensated, meaning that the period of oscillation is unaffected by temperature changes in the physiological range.

In mammals, the master pacemaker resides in the brain’s suprachiasmatic nucleus (SCN) and coordinates autonomous peripheral clocks located in organs such as liver, lung or kidney (Yoo *et al*, 2004). Oscillations have also been shown to exist in immortalized mammalian cell cultures, noticeably in fibroblasts (Balsalobre *et al*, 1998). Intercellular communication has been shown to play an important role for generating synchrony in SCN slices (Silver *et al*, 1996; Yamaguchi *et al*, 2003). However, intercellular signals were found to be negligible in cyanobacteria (Mihalcescu *et al*, 2004), mouse fibroblast cell cultures (both immortalized (Nagoshi *et al*, 2004) and from dissociated primary tissues (Welsh *et al*, 2004)) or zebrafish cell cultures (Carr and Whitmore, 2005). In the higher eukaryotes, it remains open whether this is a consequence of culture conditions or a fundamental biological difference between central pacemaker and that in peripheral organs. From the modeling perspective, synchronization properties link to a rich mathematical phenomenology (Winfree, 1967; Strogatz, 2003), and it would be interesting to develop model systems (besides the SCN) in which intercellular communication can be flexibly tuned.

The self-sustained oscillator exhibits properties of a stable limit-cycle, mathematically an isolated periodic orbit in a nonlinear differential equation model (Strogatz, 2000). Unlike the familiar (frictionless) pendulum that can swing indefinitely no matter the oscillatory amplitude, stable limit-cycles have fixed amplitude and neighboring trajectories are attracted to the cycle. The limit-cycle character of circadian oscillators was revealed through phase response experiments. For instance, one measures how short light pulses affect the circadian phase in *Drosophila* (Myers *et al*, 1996) or similarly determines the phase-resetting properties of serum shocks in mammalian cell culture systems (Nagoshi *et al*, 2004). Because of their attractor properties, limit-cycle

trajectories are resistant to perturbations and can thus explain the stability properties of circadian oscillators (Barkai and Leibler, 2000). In mathematical models recapitulating the better-characterized biochemical processes (typically using a chemical kinetics description), the nonlinearities leading to limit-cycle mathematics originate in the mass action law, cooperativity and mass conservation (Vilar *et al*, 2002; Leloup and Goldbeter, 2003).

Here, we review recent discoveries showing that the core circadian oscillator in the cyanobacterium *Synechococcus elongates* can freerun independently of transcription and translation (Tomita *et al*, 2005). Furthermore, in what might become a prime example of reductionism in molecular biology, these studies succeeded in recapitulating an elaborate biological mechanism in a test tube using only three recombinant proteins (Tomita *et al*, 2005). Foreseeable biochemistry experiments hold key to cracking the clock's remaining nuts and bolts at the atomistic level.

Prokaryotic post-translational oscillators

The simplest model organism exhibiting circadian rhythms is the cyanobacterium *S. elongates*. Three *kai* genes (*kai* means cycle in Japanese) were shown to be essential for rhythmicity *in vivo*, and the molecular clock is independent of cell division (Ishiura *et al*, 1998). In fact, good rhythmicity is observed during exponential growth where cell division ticks about three times faster than the circadian clock (Kondo *et al*, 1997). Bacteria in the wild are normally exposed to a light–dark (LD) photocycle with a period of 24 h; in most experiments, the entrainment LD schedule consists of 12 h light followed by 12 h darkness. Cyanobacteria being photoautotrophs with virtually stopped metabolism in the dark, freerun is traditionally studied under constant light conditions (LL) under which the clocks continue ticking. In contrast, the canonical freerun condition in metazoan is complete darkness (DD), *Drosophila* being even arrhythmic in LL. Imaging of growing microcolonies suggested that coupling between phases in neighboring colonies plays no significant role. Moreover, the high level of synchrony found within colonies could be explained by the stability properties of single oscillators and the accuracy by which time is passed to daughter cells (Mihalcescu *et al*, 2004).

Phylogenetic profiling indicates that *kaiC* is the oldest among the three clock components also found in protobacteria and archaea (Dvornyk *et al*, 2003) (see also www.microbesonline.org). *KaiB*, together with formation of the *kaiBC* operon, seems to have originated in cyanobacteria and was laterally transferred to protobacteria. Establishing the phylogeny of *KaiA* was not possible due to its limited representation among bacteria. Interestingly, *kaiA* is found only in a subgroup of cyanobacteria, raising the issue about possible *kaiA*-independent oscillators or perhaps yet to be discovered functional substitutes thereof. In cyanobacteria, the selective advantage of a clock tuned to the environment was clearly demonstrated. Indeed, using cocultures with strains having endogenous periods of 22 and 30 h, it was shown that the strain with a period matching that of the external photocycle would rapidly outgrow that with detuned rhythm, hence confirming the adaptive purpose of circadian clocks (Ouyang *et al*, 1998; Woelfle *et al*, 2004).

Transcription translation oscillator model

Until recently, the consensus model in cyanobacteria postulated a clock design orchestrated around transcription-based autoregulatory negative-feedback loops similar to eukaryotic models (Young and Kay, 2001). Some of these eukaryotic models are amenable to detailed mathematical analysis (Leloup and Goldbeter, 2003), and can thereby suggest novel components or missing connections (Locke *et al*, 2005) (Figure 1A). *kaiC* was found through promoter assays to negatively regulate the *kaiBC* operon (Ishiura *et al*, 1998). In conjunction with phase-shifting behavior, *KaiC* was inferred as the state variable of the oscillator, playing similar role as for example the *period* transcriptional repressor in *Drosophila*. Further, the delay between peak mRNA and protein expression levels was also consistent with negative elements in *Drosophila* or mammals (Xu *et al*, 2000). On the other hand, *KaiA* was reported to positively influence *kaiC* expression (Ishiura *et al*, 1998).

Phosphorylation dynamics of the *kaiC* protein was found to be essential for proper circadian function (Nishiwaki *et al*, 2004; Xu *et al*, 2004). For instance, it was found that *KaiC* is capable of both autokinase (Nishiwaki *et al*, 2000; Tomita *et al*, 2005) and autophosphatase (Kitayama *et al*, 2003; Xu *et al*, 2003) activities, and that these activities are modulated by *KaiA* and *KaiB* proteins. Specifically, phosphatase activity of *KaiC* in the absence of *KaiA* switches to kinase activity in the presence of *KaiA*, and *KaiB* can reduce *KaiA*-dependent kinase activity (Iwasaki *et al*, 2002; Kitayama *et al*, 2003). Further the phosphorylation and dephosphorylation kinetics are temperature independent (Tomita *et al*, 2005), hence providing a biochemical basis for the *in vivo* temperature compensation of the period. Site-specific mutagenesis (Nishiwaki *et al*, 2004), NMR (Xu *et al*, 2004) and X-ray (Pattanayek *et al*, 2004) structures discovered three phosphorylation sites in *KaiC* at Ser⁴³¹, Thr⁴³² and Thr⁴²⁶ essential for circadian rhythmicity.

Perhaps the most crucial aspect of *Kai* proteins is their ability to form heteromultimeric complexes with stoichiometries that change dynamically during the circadian cycle, both in LD and LL conditions (Kageyama *et al*, 2003). For example, *KaiA* and *KaiB* directly interact with *KaiC*. Like other members of the RecA/DnaB family, *KaiC* assembles in ring-shaped hexamers and the process is adenosine triphosphate (ATP) dependent (Mori *et al*, 2002; Vakonakis *et al*, 2004). *In vivo*, this complex is most abundant during late or late subjective night when it is also augmented with *KaiA* dimers and later during the night with *KaiB* dimers (Kageyama *et al*, 2003). Interestingly, the formation of this *KaiABC* 500–600 kDa complex called the periodosome is not observed in *KaiC* mutants lacking the above phosphorylation sites. On the other hand, *KaiC* hexamers form independent of the presence of these sites (Nishiwaki *et al*, 2004).

Core clock is not the transcription translation oscillator

Recent breakthrough came from studying cyanobacteria under DD conditions. *S. elongates* being a photoautotroph, transcription and translation are rapidly arrested after transfer into darkness. This trivially implies that cycling of mRNA and

Figure 1 (A) The transcription translation oscillator (TTO model) in *Arabidopsis thaliana* as studied by Locke *et al* (2005). Here, the clock consists in an interlocked negative-feedback system with light as Zeitgeber acting positively on the transcription of *LHY/CCA1* and indirectly on *TOC1* via a hypothetical gene *Y*. *GIGANTEA* was identified as a candidate for *Y* via fitting to experimental data. (B) A hypothetical model for the rotary clock taken from Wang (2005). After hexamer and KaiA/B/C complex assembly (I), the KaiC phosphorylation level increases through rotary autophosphorylation driven by a rotating KaiA dimer inside the KaiC central channel (II and III). The fully phosphorylated KaiC hexamer then dissociates and KaiC levels decrease due to proteasome-dependent degradation (IV). KaiA: blue; KaiB: green; KaiC: gray.

protein levels would stop. However, evidence that some form of oscillation must continue in the dark came from observations that no drastic phase shifts were induced after cycling

colonies were returned to LL after a journey in DD lasting 0–52 h (Xu *et al*, 2000). This led Iwasaki and co-workers to monitor the protein abundance and phosphorylation states dynamically through DD time (Tomita *et al*, 2005). In a series of elegant experiments relying on phospho-specific immunoblots, they were able to disprove the traditional model that the core oscillator required transcription and translation. Instead, they proved that purely post-translational oscillations in the phosphorylation levels of KaiC lied at the heart of the circadian clock. The so-found DD oscillation *in vivo* also exhibited the expected temperature compensation and mutant period phenotype, further supporting that the phospho-cycle is the fundamental circadian pacemaker.

Circadian clocks *in vitro*

After the design of synthetic oscillators in *Escherichia coli* (Elowitz and Leibler, 2000; Atkinson *et al*, 2003), the reductionist approach to circadian biology is now taking another turn. In a surprising follow-up article, the same group went further and recapitulated the KaiC phospho-cycle *in vitro* using physiological ratios of the three Kai proteins homogeneously mixed in the presence of ATP (Nakajima *et al*, 2005). The circadian period was reproduced accurately although the amplitude of the phosphorylation oscillations was reduced in comparison with both LL and DD conditions *in vivo* (Tomita *et al*, 2005). More, temperature compensation and mutant phenotypes closely matched *in vivo* data, providing strong evidence that the three Kai proteins are sufficient to build the circadian core pacemaker (Nakajima *et al*, 2005).

Perspectives

With the developments reviewed above, our understandings of circadian clockworks in bacteria and eukaryotes have taken rather opposite directions. While the complexity of eukaryotic model increases steadily by involving new processes such as heme biosynthesis (Kaasik and Lee, 2004), cellular redox state (Rutter *et al*, 2001; Schibler and Naef, 2005) or new interaction partners for classical clock proteins (Brown *et al*, 2005), the bacterial clock community is embarking on a reductionist path following the discovery of *in vitro* models.

Cracking the chemical oscillator

Even though a large body of work has dissected the mechanics of the Kai proteins biochemically, the *in vitro* experiment offers the unique possibility to push this undertaking even further. It seems unavoidable that a full mechanistic description amenable to mathematical modeling will require that structures, 3D protein modeling, NMR data and possibly mass spectrometry be combined with classical biochemistry to understand the protein mechanics during complex formation and disassembly. Such experiments, facilitated *in vitro*, will allow tackling the nonlinearities responsible for the generation of limit-cycle oscillations in detail. For example, the optimal stoichiometry between KaiA and KaiC is still debated (Pattanayek *et al*, 2004; Wang, 2005) and the knowledge of reaction kinetics is incomplete. Ultimately, this will elucidate how just three

protein molecules with their biochemical repertoire can function as a chemical oscillator. One recent hypothesis mainly relying on structural information about the Kai family uses the similarity of KaiC hexamers and KaiA dimers with the F1-ATPase rotor system (Abrahams *et al*, 1994; Pattanayek *et al*, 2004; Wang, 2005). Although various models disagree as to the site and geometry of KaiA–KaiC binding (Pattanayek *et al*, 2004; Vakonakis *et al*, 2004; Wang, 2005), the crystal structure of the KaiA/C complex suggests a rotary clock model in which a KaiA dimer rotates within the central KaiC hexamer channel, thereby progressively saturating the phosphorylation level of KaiC before complex disassembly (Figure 1B). Future work will clarify to what extent this intriguing model translates into a dynamically realistic scenario.

What about eukaryotic clocks?

Current view in eukaryotes presents the core circadian engine as made of interlocked autoregulatory feedback loops in which transcriptional regulation plays a crucial role. Interestingly though, evidence in *Drosophila* showed that oscillations can be sustained with constitutive expression of *per* and *tim* messengers (Yang and Sehgal, 2001), or even in mutants with forced anti-phase *dClk* expression (Kim *et al*, 2002). Such findings evidently suggest a prominent role for post-translational mechanisms in the generation of sustained oscillations. Moreover, phosphorylation events play crucial roles in modulating the stability and timing of the oscillator (Price *et al*, 1998; Martinek *et al*, 2001). Post-translational modifications in eukaryotes are nevertheless often regarded as a sophistication of a transcription-based system. The reported findings in cyanobacteria present evidence for a somewhat opposite scenario in which the core oscillator, purely post-translational, is reinforced through additional transcription–translation feedback loops. It will be interesting to follow whether a similar play repeats in eukaryotes, or whether such level of simplification can be afforded only by prokaryotes.

Modular clocks

By operating autonomously in a test tube, the discussed *in vitro* system perfectly satisfies the definition of a functional module (Hartwell *et al*, 1999). In fact, the bacterial post-translational clock lends support to the concept that complex biological function can be accomplished through assemblies of weakly coupled autonomous functional entities dubbed modules (Alon, 2003). With this perspective, it will be crucial to study how the *in vitro* model tolerates addition of further parts, for example the TTO loops, or perhaps easier the molecules responsible for phase resetting. Since clocks and host coevolved, there is unique opportunity to study the mechanics, constraints and consequences of coupling with the host machinery. In their conclusion, Nakajima *et al* suggest an analogy with wall clocks in which the core pacemaker, disguised as the pendulum, would be amplified and transmitted to the world through an escapement mechanism consisting of the outer TTO. Future will tell how far this metaphor holds and whether similar developments will occur in other systems.

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