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 vitro. 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 biochemical experiments hold key to cracking the clock’s remaining nuts and bolts at the atomic 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 were 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 no OA, the 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 archa (Dyrowsky 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; Woelffe 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, phosphorylase 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
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 phoso-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 phoso-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 phosphate-cycle in vitro using physiological ratios of the three Kai proteins homogenously 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; Wang, 2005). Although various models disagree as to the site and geometry of KaiA–KaiC binding (Pattanayek et al., 2004; Yokota 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; Martinez 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 escape 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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