Published as: *Science*. 2013 May 10; 340(6133): 737–740.

Robust circadian oscillations in growing cyanobacteria require transcriptional feedback

Shu-Wen Teng¹, Shankar Mukherji^{1,*}, Jeffrey R. Moffitt^{2,*}, Sophie de Buyl^{3,*}, and Erin K. O'Shea^{1,†}

¹Howard Hughes Medical Institute, Faculty of Arts and Sciences Center for Systems Biology, Departments of Molecular and Cellular Biology and of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, USA

²Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, USA

³Department of Physics, Harvard University, Cambridge, MA 02138, USA

Abstract

The remarkably stable circadian oscillations of single cyanobacteria enable a population of growing cells to maintain synchrony for weeks. The cyanobacterial pacemaker is a post-translational regulation (PTR) circuit that generates circadian oscillations in the phosphorylation state of the clock protein, KaiC. Layered on top of the PTR is transcriptional-translational feedback regulation (TTR), common to all circadian systems, consisting of a negative feedback loop in which KaiC regulates its own production. We demonstrate that the PTR circuit is sufficient to generate oscillations in growing cyanobacteria. However, in the absence of TTR individual oscillators were less stable and synchrony was not maintained in a population of cells. Experimentally-constrained, mathematical modeling reproduced sustained oscillations in the PTR circuit alone and the importance of TTR for oscillator synchrony.

Circadian clocks are present in all forms of life and are crucial in coordinating physiology with the day and night cycle (1, 2). Clocks can maintain oscillation phase, frequency, and amplitude for many cycles even in the absence of external cues (3). In the cyanobacterial species *Synechococcus elongatus*, circadian oscillations exhibit remarkable temporal stability with a correlation time of many weeks in constant environmental conditions (4, 5).

Circadian oscillations in *S. elongatus* are generated by a network architecture that consists of two regulatory loops: a post-translational regulation (PTR) circuit whose output is circadian oscillations in the phosphorylation state of KaiC, an enzyme that catalyzes its own phosphorylation and dephosphorylation in a manner modulated by the accessory proteins KaiA and KaiB (6–9); and a transcriptional-translational feedback regulation (TTR) circuit in which the activity of the *kaiBC* promoter is under circadian control (10, 11). The general architecture of coupled PTR and TTR loops is shared by circadian circuits in many species (1, 12, 13).

The role of the TTR and PTR in establishing and stabilizing circadian oscillations has been previously studied both experimentally (9, 14–17) and theoretically (18). The PTR is required for circadian rhythms – abrogation of this circuit results in damped oscillations at the population level (9); however, the persistence of these oscillations is a subject of debate

[†]To whom correspondence should be addressed. erin_oshea@harvard.edu.

*These authors contributed equally to this work.

(9, 14). By contrast, the TTR appears to be dispensable under some conditions (15, 16). The PTR circuit alone can generate remarkably stable oscillations *in vitro* in a reconstituted system (17). Similarly, *in vivo* circadian oscillations are stable in dark conditions, where transcription is repressed and cells are not growing (6, 19). Theoretical work has suggested that the TTR may be involved in generating robust circadian rhythms during growth (18).

To address the role of the TTR and PTR circuits *in vivo* in growing cells, we initially constructed two strains (Fig. 1A): (i) a wild-type (WT) strain in which both the TTR and PTR circuits are intact; and (ii) a PTR-only strain in which the TTR circuit was abrogated by making expression of the *kaiBC* operon constitutive and at a level similar to the mean expression of *kaiBC* in the WT strain (Fig. S1). A strain in which *kaiBC* expression is under the control of an inducible promoter was previously characterized but is not a true PTR-only strain – we and others observed residual circadian dependence of *kaiBC* expression in this strain (Fig. S1) (11, 20). We monitored the state of the clock by measuring fluorescence derived from expression of a yellow fluorescent protein (YFP) gene under the control of the *kaiBC* promoter (21, 22). YFP intensity reflects the abundance of the phosphorylated form of KaiC that is active in promoting circadian transcription (21).

We measured circadian oscillations over a wide range of growth rates in constant conditions with control of light and composition of the medium using a microfluidics device that limits cellular crowding and replenishes spent nutrient buffer (Fig. 1B and Fig. S2), permitting long duration measurements in a chemostatic environment (23). After two days of entrainment in a test tube to generate a population of cells oscillating with the same phase, we loaded cells into this device and used automated methods to collect phase contrast (Fig. 1C) and fluorescence images (Fig. 1D) every 20 min for five days.

The population-averaged YFP fluorescence (Fig. S3), as well as the signal in individual WT cell lineages (Fig. 2A), oscillated with circadian periodicity throughout the duration of the measurement. Moreover, a polar plot of the amplitude and phase for each cell lineage confirmed that the initial synchronization of this population, produced by entrainment, persisted throughout the five-day measurement (Fig. 2B). The PTR-only strain also showed clear circadian oscillations for both the population average (Fig. S3) and for individual cells (Fig. 2C). After one day in constant light conditions, the variation in phase and amplitude within individual PTR-only cell lineages was comparable to that observed for the WT strain; however, after four days, the variation in the phase of these oscillations between individual PTR-only cell lineages increased (Fig. 2D). Thus, without the TTR circuit, the ability of the PTR-only strain to maintain the phase established by the original entrainment decreases and cell populations lose synchrony.

To quantify the phase stability as a function of time, we calculated the synchronization index (SI) (24). This index is based on the Shannon entropy of the phase distribution (see Supporting Online Text) and varies from one for a completely synchronized population — where all cells oscillate with the same phase — to zero for an unsynchronized population — in which cells oscillate with random phases. The SI for the WT strain starts high and decreases ~25% over the 5 day measurement (Fig. 2E). The SI for the PTR-only strain starts at approximately the same value as the WT strain, indicating that disruption of the TTR circuit does not affect entrainment of this strain to light and dark cycles. However, once these external cues were removed, the population of PTR-only cells quickly and dramatically desynchronized, dropping roughly 70% in SI over 5 days (Fig. 2E). The PTR-only strain desynchronized more quickly when it was growing faster (one division every 14–16 hours; Fig. S4H) than the circadian period than when it was growing more slowly (one division every 72 hours; Figs. 2 and S4G). Thus, the TTR had a more important role in stabilizing circadian oscillations for cells with faster growth rates, in qualitative agreement with the

predictions of both the model we describe below (Fig. S5) as well as a previous modeling study (18).

To explore the properties of the TTR circuit we quantified the dynamics of a mutant $KaiC^{S431E/T432E}$ strain lacking the PTR but containing an intact TTR circuit (the ‘TTR-only strain’, in which the potential for feedback regulation of *kaiBC* expression exists) (Figs. 3A and S6) (9, 14). This strain showed an initial decrease in fluorescence intensity after transfer to constant light conditions that then increased and stabilized on the first day to an average expression level around which individual cells fluctuated (Fig. 3B). Similar behavior was observed for faster growth rate (Fig. S7). The initial dip appeared to be set by the entrainment protocol (Fig. S6D) and was not affected by the transfer of cells to the microfluidics device.

To determine which strains exhibit circadian oscillations, we calculated the average of the power spectrum of the fluorescence intensity of individual cell lineages (Fig. 3C). The WT and PTR-only strains had nearly identical power spectra with a prominent peak at the expected one day period. A comparable peak was not present for the TTR-only strain, confirming that there were no circadian oscillations in this strain. This result is consistent with population studies that reported damped oscillations of a clock transcriptional reporter in the $KaiC^{S431E/T432E}$ strain (9). Thus, the role of the PTR circuit is to establish the oscillations and the role of the TTR circuit is to stabilize these oscillations in growing cells.

To explore the interplay of the PTR and TTR circuits, we created a mathematical model of *in vivo* circadian oscillations in *S. elongatus* that consisted of a PTR pacemaker and a TTR controller (Fig. 3D). The PTR portion of the model was based on a model of the *in vitro* oscillator (7) with addition of terms to account for loss of protein due to degradation and dilution (25). The TTR portion of the model consists of a simple negative feedback loop in which the clock protein KaiC represses its own mRNA synthesis. We performed least squares fitting to our experimental data from the TTR-only strain to derive model parameters that constrain the negative feedback describing the TTR circuit and found that the experimentally observed dynamics of the reporter in the TTR-only strain could be accurately represented by this simple model (Fig. 3E). This model constrained by parameters determined from experiments (Table S1) also reproduced the sustained oscillations observed in the population average data for the WT and PTR-only strains (Fig. 4A and S3).

To determine whether our model could recapitulate circadian oscillations of the WT versus PTR-only strains in individual cell lineages, we performed stochastic simulations of the model using parameters determined from experiments (Table S1) (26). We find that the WT model produces circadian oscillations (Fig. 4B) that desynchronize only weakly with time (Fig. 4C), though not as weakly as observed experimentally, presumably due to simplifications in the model such as treating KaiC as a monomer rather than a hexamer. The PTR-only model, however, produces circadian oscillations (Fig. 4D) that desynchronize far more rapidly than the WT model and that desynchronize more quickly with increased growth rate (Fig. 4E, F and Fig. S5), as observed experimentally. Repression of *kaiBC* transcription by U-KaiC and S-KaiC (see legend to Fig. 1 for details of the PTR circuit) creates a peak of *kaiBC* mRNA prior to the peak of U-KaiC in the PTR circuit. As a result, newly synthesized U-KaiC accumulates coincidentally with the rise of U-KaiC from the PTR cycle, and this synchronization of new and existing U-KaiC enhances the stability of the PTR cycle, increasing the robustness of the oscillator (18).

To characterize the extent to which the TTR circuit enhances robustness of the circadian oscillator to variability in clock components and cellular growth, we performed a systematic

sensitivity analysis of the WT and PTR-only models. We varied the values of parameters common to both the WT and PTR-only models, numerically integrated the WT and PTR-only model differential equations, and analyzed the power spectra of the resulting time trajectories to detect circadian oscillations. We find that the volume of parameter space that supports circadian oscillations in the WT system (Fig. 4G) is far larger than that of the PTR-only model (Fig. 4H). Furthermore, within the space of parameters supporting circadian oscillations, the WT system experiences much smaller changes in period upon parameter variation than the PTR-only system (Fig. 4G, H, S7S8, and) (see Supporting Online Text). Thus, the TTR circuit is able to buffer the circadian oscillator against stochastic fluctuations in clock components, and also against sustained changes in parameters such as the cellular growth rate and KaiC translation and degradation rates, all of which cause profound changes in the PTR-only system when varied (Fig. S8).

We demonstrate that the PTR can generate oscillations *in vivo* in the absence of TTR feedback. However, without TTR feedback, populations of cells lose synchrony due to phase drift of individual oscillators. Coupling of the PTR pacemaker to the TTR controller generates robustness – insensitivity of period and phase to changes in parameters – that enables the clock to maintain accurate oscillations in the absence of external cues for long durations and that explains the remarkable stability of the cyanobacterial clock. This architecture may represent a general solution used in other circadian circuits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank P. Cluzel for the development of the microfluidics platform upon which the device used in this paper is based (funded via NIH/NIAID award 1R21AI094363-01A1). We thank B. Goetze, and J. Wailes for assistance with microscopy; D. Zwicker, P. R. ten Wolde, and A. Subramaniam for stochastic simulation; J. Markson, A. Gutu, V. Vijayan, and A. Puszyńska for strain construction and western blotting; P. Cluzel, D. Gonze and P. de Buyl for helpful discussion; and B. Stern, J. Markson, V. Vijayan, A. Rizvi, M. Rust, and A. Subramaniam for comments on the manuscript. This work was supported by the Helen Hay Whitney Postdoctoral Fellowship (J.R.M.), Fonds National de la Recherche Scientifique Belgium (Postdoctoral Researcher) (S.D.B.), Wallonie-Bruxelles International (S.D.B.), and the Howard Hughes Medical Institute (E.K.O.).

References and Notes

1. Dunlap JC, Loros JJ, Decoursey PJ, Dunlap JC, Loros JJ. Chronobiology: Biological timekeeping. *Chronobiology: Biological Timekeeping*. 2004; i-xx:1–406.
2. Harmer SL, Panda S, Kay SA. Molecular bases of circadian rhythms. *Annu. Rev. Cell Dev. Biol.* 2001; 17
3. Dunlap JC. Molecular bases for circadian clocks. *Cell*. 1999; 96
4. Mihalcescu I, Hsing WH, Leibler S. Resilient circadian oscillator revealed in individual cyanobacteria. *Nature*. 2004; 430:81–85. [PubMed: 15229601]
5. Amdaoud M, Vallade M, Weiss-Schaber C, Mihalcescu I. Cyanobacterial clock, a stable phase oscillator with negligible intercellular coupling. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:7051–7056. [PubMed: 17438272]
6. Tomita J, Nakajima M, Kondo T, Iwasaki H. No transcription-translation feedback in circadian rhythm of KaiC phosphorylation. *Science*. 2005; 307:251–254. [PubMed: 15550625]
7. Rust MJ, Markson JS, Lane WS, Fisher DS, O'Shea EK. Ordered phosphorylation governs oscillation of a three-protein circadian clock. *Science*. 2007; 318:809–812. [PubMed: 17916691]
8. Markson JS, O'Shea EK. The molecular clockwork of a protein-based circadian oscillator. *FEBS Lett.* 2009; 583:3938–3947. [PubMed: 19913541]

9. Qin XM, Byrne M, Xu Y, Mori T, Johnson CH. Coupling of a Core Post-Translational Pacemaker to a Slave Transcription/Translation Feedback Loop in a Circadian System. *Plos Biology*. 2010; 8
10. Ishiura M, et al. Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. *Science*. 1998; 281:1519–1523. [PubMed: 9727980]
11. Nakahira Y, et al. Global gene repression by KaiC as a master process of prokaryotic circadian system. *Proc. Natl. Acad. Sci. U. S. A.* 2004; 101:881–885. [PubMed: 14709675]
12. Young MW, Kay SA. Time zones: A comparative genetics of circadian clocks. *Nature Reviews Genetics*. 2001; 2
13. Gallego M, Virshup DM. Post-translational modifications regulate the ticking of the circadian clock. *Nat. Rev. Mol. Cell Biol.* 2007; 8:139–148. [PubMed: 17245414]
14. Kitayama Y, Nishiwaki T, Terauchi K, Kondo T. Dual KaiC-based oscillations constitute the circadian system of cyanobacteria. *Genes Dev.* 2008; 22:1513–1521. [PubMed: 18477603]
15. O'Neill JS, Reddy AB. Circadian clocks in human red blood cells. *Nature*. 2011; 469:498-U70. [PubMed: 21270888]
16. O'Neill JS, et al. Circadian rhythms persist without transcription in a eukaryote. *Nature*. 2011; 469:554–558. [PubMed: 21270895]
17. Nakajima M, et al. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. *Science*. 2005; 308:414–415. [PubMed: 15831759]
18. Zwicker D, Lubensky DK, ten Wolde PR. Robust circadian clocks from coupled protein-modification and transcription-translation cycles. *Proc. Natl. Acad. Sci. U. S. A.* 2010; 107:22540–22545. [PubMed: 21149676]
19. Hosokawa N, et al. Circadian transcriptional regulation by the posttranslational oscillator without de novo clock gene expression in *Synechococcus*. *Proc. Natl. Acad. Sci. U. S. A.* 2011; 108:15396–15401. [PubMed: 21896749]
20. Xu Y, Mori T, Johnson CH. Cyanobacterial circadian clockwork: roles of KaiA, KaiB and the kaiBC promoter in regulating KaiC. *EMBO J.* 2003; 22
21. Chabot JR, Pedraza JM, Luitel P, van Oudenaarden A. Stochastic gene expression out-of-steady-state in the cyanobacterial circadian clock. *Nature*. 2007; 450:1249–1252. [PubMed: 18097413]
22. Yang Q, Pando BF, Dong G, Golden SS, van Oudenaarden A. Circadian Gating of the Cell Cycle Revealed in Single Cyanobacterial Cells. *Science*. 2010; 327
23. Moffitt JR, Lee JB, Cluzel P. The single-cell chemostat: an agarose-based, microfluidic device for high-throughput, single-cell studies of bacteria and bacterial communities. *Lab on a Chip*. 2012; 12
24. Tass P, et al. Detection of $n : m$ phase locking from noisy data: Application to magnetoencephalography. *Phys. Rev. Lett.* 1998; 81
25. Gonze D, Halloy J, Goldbeter A. Robustness of circadian rhythms with respect to molecular noise. *Proc. Natl. Acad. Sci. U. S. A.* 2002; 99
26. Gillespie DT. Exact Stochastic Simulation of Coupled Chemical-Reactions. *J. Phys. Chem.* 1977; 81

Fig. 1.

Long-term measurement of circadian oscillations in *S. elongatus*. (A) Genetic circuit diagrams of the wild-type strain (WT) and the post-translational-regulation-only strain (PTR-only). KaiC has two phosphorylation sites and transits through four different phosphorylated forms in the PTR circuit during the circadian cycle: unphosphorylated (U-KaiC, represented by 'U'); phosphorylated only on T432 (T-KaiC, 'T'); phosphorylated on both S431 and T432 (ST-KaiC, 'ST'); and phosphorylated only on S431 (S-KaiC, 'S') (7, 8). In the WT strain both the KaiC PTR (gray arrows) and TTR (purple arrows) circuits are intact. In the PTR-only strain the *kaiBC* operon is under the control of a constitutive promoter, abrogating transcriptional regulation. In both strains, a yellow fluorescence protein (YFP) reporter is under the control of the *kaiBC* promoter, whose activity is regulated by the phosphorylation state of KaiC (the PTR). (B) Schematic of growth in the patterned agarose micro-environment. Cells are trapped at the interface between a coverglass and a patterned agarose gel and flow of fresh medium and constant light maintain a chemostatic environment. (C) *S. elongatus* cells growing in the microfluidics device imaged using phase-contrast microscopy. (D) Fluorescence image collage of subsequent frames of a time-lapse movie of WT cells.

Fig. 2.

Requirement of transcriptional regulation to maintain synchronization in a population of oscillating cells. **(A)** Fluorescence intensity derived from the YFP reporter measured in individual cell lineages from the WT strain (~400 cell lineages). Two single-cell traces are highlighted for clarity. **(B)** Polar plot of the phase (angular axis) and amplitude (radial axis) of oscillations of individual entrained WT cells at days 1 (left) and 4 (right) following transfer to constant light conditions. **(C)** Fluorescence intensity derived from the YFP reporter measured in cell lineages from the PTR-only strain (~900 cell lineages). **(D)** Phase and amplitude of oscillations of entrained cells from the PTR-only strain at days 1 and 4

following transfer to constant light conditions. **(E)** Synchronization index for the ensemble of cells as a function of time for the WT (green) and the PTR-only (blue) strains (see Supplemental Online Text for details). Error bars are derived by the bootstrap method.

Fig. 3.

Insufficiency of transcriptional feedback in the absence of a PTR circuit to generate circadian oscillations. **(A)** Genetic circuit diagram of the transcriptional-translational-regulation-only strain (TTR-only). **(B)** Fluorescence intensity derived from the YFP reporter measured in individual entrained cell lineages of the TTR-only strain (~400 cell lineages). Two single-cell traces are randomly selected and highlighted in pink. Cells are dividing once every 24 hours. **(C)** The average of power spectra of single-cell fluorescence traces from the WT strain (green), PTR-only strain (blue), and TTR-only strain (red). **(D)** Diagram of the model of the WT circuit. Oscillations are generated by the PTR circuit (pacemaker in the

blue dashed box), and abundance of newly made KaiC is controlled by the TTR circuit (controller in the red dashed box). “ $\emptyset$ ” represents degradation. Two equations describe the controller dynamics as negative feedback, in which KaiC (C) regulates its own mRNA (M) synthesis in a Hill repression function (see Supplemental Online Text for details). (E) The average YFP expression (red solid line) for the ensemble of cells from the TTR-only strain. The purple solid line shows the average of the stochastic simulated traces generated from the equations in (D) and the purple dashed lines indicate the standard deviation of the simulated traces.

Fig. 4. Requirement of transcriptional feedback for robustness of *in vivo* oscillations. (A) Deterministic simulations of circadian oscillation of KaiC phosphorylation for the WT (green) and PTR-only (blue) circuits. The simulated period of the PTR-only strain is 6% shorter than that of the WT strain. (B) Stochastic simulated traces for the WT strain. One hundred traces are displayed and two were randomly selected and highlighted. (C) Phase versus amplitude plot of simulated traces for the WT strain at days 1 and 4. (D) Stochastic simulated traces (100 traces) of circadian oscillation of KaiC phosphorylation for the PTR-only strain. (E) Phase versus amplitude plots for simulations of the PTR-only strain. (F) Synchronization index for the stochastic simulated traces as a function of time for the WT (green) and the PTR-only (blue) strains. (G, H) Sensitivity analysis of the oscillation period. Models of the WT (G) and PTR-only (H) systems were numerically solved for dilution and translation rates simultaneously varied over 4 orders of magnitude. Solutions with periods ± 5 hours around the circadian period of the model output were classified as circadian (black), and other solutions yielding stable oscillations outside this range were denoted non-circadian (orange). We selected ± 5 hours because the same relative range of periods is observed in the measured WT system power spectrum in Fig. 3C.