an already left–right asymmetric body plan. Interestingly, in amphioxus, the somites, which form only axial muscle, are asymmetric throughout most of somitogenesis, with left-sided somites arising earlier and hence slightly anterior to those on the right [17]. Perhaps the cephalochordate body plan could tolerate the asynchrony, precluding the requirement for an RA-mediated buffering mechanism. But in vertebrates, where the somites generate both the axial muscle and vertebrae, the left and right somitic primordia must fuse at the midline to provide support and spinal cord protection. Left–right asymmetry here would thus likely have drastic consequences, and it is intriguing to speculate that a RA buffering mechanism arose in the vertebrate lineage to compensate for the ancestral template of asynchronous somitogenesis.

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Circadian Clocks: Translation Lost

One of the big questions in biological rhythms research is how a stable and precise circa-24 hour oscillation is generated on the molecular level. While increasing complexity seemed to be the key, a recent report suggests that circa-24 hour rhythms can be generated by just four molecules incubated in a test tube.

Till Roenneberg[1] and Martha Merrow[2]

The film ‘Lost in Translation’ uses jet lag to construct its story: two Americans in a Tokyo hotel form a melancholic bond as they meet for days on end at odd times during the night, unable to sleep. Sleep propensity is controlled by at least two factors: the amount of time since we last slept, and our circadian clock. The latter is of great interest to both science and society — far beyond the luxury ‘disease’ of jet lag — because it regulates processes from gene expression to behaviour.

One of the burning questions in circadian research is how a limited set of molecules, so-called ‘clock’ genes and proteins, can foster biochemical oscillations with such a long period. Not surprisingly, the models, explaining how circadian rhythms are generated at the molecular level have become highly complex. But a Japanese group [1] has now managed to make the circadian clock of cyanobacteria tick in a test tube, a finding that has great potential for further exploration and understanding of circadian clocks.

After flying across time zones, our biological clock adjusts only slowly to the new local time, needing about one day for each hour time difference. This sluggishness is indicative of a robust, endogenous oscillator, a daily biological program which is not simply triggered by light and darkness. In artificial constant conditions, circadian rhythms cycle (free-run) at their own speed, with a period close to, but not exactly, 24 hours. An active ‘entrainment’ process ensures synchronization to the earth’s 24 hour rotation.

Circadian rhythms have been demonstrated in organisms of all phyla, from pond scum (cyanobacteria) to humans, and their genetic basis has been intensely studied over the past decades. All models of the circadian clock share a common feature: a transcription–translation negative feedback loop (TTL),

whereby clock genes are transcribed and translated into clock proteins which are modified by phosphorylation and act as inhibitors of their own gene expression.

Ever since it was clear that circadian rhythms are a molecular feature of single cells, clock researchers have dreamt of reconstructing a 24 hour oscillation in a test tube. Given our increasing understanding of the molecular complexity, this task seemed far off, if not impossible. Surprisingly, Nakajima *et al.* [1] incubated the purified *Synechococcus* clock proteins KaiA, KaiB and KaiC together with ATP and found that the phosphorylation state of KaiC oscillates with a circa-24 hour period *in vitro*. But how many circadian properties does this clock-in-a-test-tube represent?

Circadian clocks have several, distinguishing features [2]: they are rhythmic in constant conditions, with no or little damping of their oscillation over time; they have a period in the 24 hour range that varies little with ambient temperature (Q_{10}~1, indicating temperature compensation); and they can be entrained by zeitgebers, such as light or temperature cycles. It is remarkable how many of these properties have been reconstructed in the artificial system. The autophosphorylation–dephosphorylation rhythm of KaiC is in the circadian range and not damped, and its period is temperature compensated [1]. When proteins from clock mutants were used, the period of the test tube rhythm mirrored that of the respective mutant *in vivo*, reinforcing the view that period is an inherent quality of the amino acid sequences of clock proteins. Thus, apart from entrainment, which has not been tested (see below), all properties appear to be present in the test-tube-clock.

In vivo, the *Synechococcus* clock has been mainly monitored with the help of a luciferase reporter driven by the *psbA1* or *kaiBC* promoter [3]. Cyanobacteria are photosynthetic organisms, and general transcription in *Synechococcus* is light dependent: in constant darkness (DD), RNA synthesis (including that of the reporter gene) is lowered to background levels, whereas abundant, regulated transcription is observed in constant light (LL) [4]. For these reasons, the *Synechococcus* clock has been predominantly investigated in LL, where the *kaiBC* operon is rhythmically transcribed generating rhythmic concentrations of KaiB and KaiC proteins. KaiC shows an additional rhythm in (auto)phosphorylation (Figure 1, left panels).

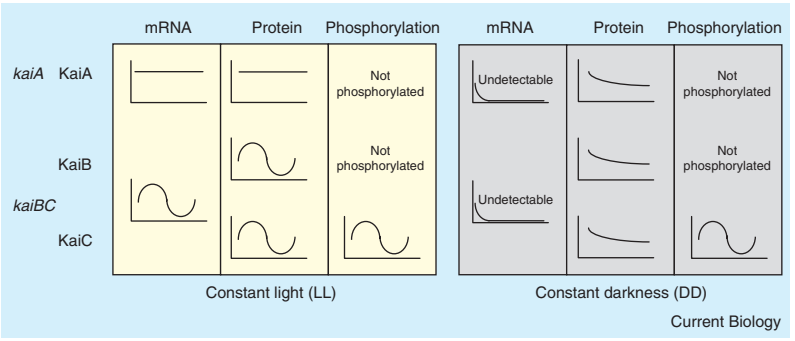

Figure. 1. Kinetics of the three *Synechococcus* clock components KaiA, KaiB, and KaiC in constant light (left) and constant darkness (right).

In LL, *kaiBC* transcription (mRNA) is rhythmic leading to cyclic concentrations of KaiB and KaiC (protein). The phosphorylation state of KaiC is also rhythmic (phosphorylation). In DD, *kai* mRNA levels are undetectable and protein concentrations show no rhythmicity, yet the phosphorylation–dephosphorylation rhythm of KaiC continues. The independence of the phosphorylation rhythm from transcription and translation has now been proven by incubation of the three Kai proteins together with ATP [1].

From the LL data, a model was constructed resembling the TTL hypothesised for the molecular clocks in eukaryotes. Interactions of KaiA, KaiB and KaiC affect phosphorylation of KaiC, regulating KaiC hexamer formation and transcription on a global scale (hence the rhythmicity of transcription) [5–7]. But when KaiC phosphorylation was examined in DD, the TTL model had to be revised (Figure 1, right panels). Although KaiC transcript levels were undetectable, the phosphorylation rhythm of KaiC persisted with a circadian period, even continuing in the presence of transcriptional and translational inhibitors [4]. These results indicated that the circadian rhythm generator in *Synechococcus* is metabolic — independent of rhythmic gene expression, which has now been impressively proven by the clock-in-a-test-tube experiments [1].

It is a pity that Nakajima *et al.* [1] stopped short of investigating in *vitro* entrainment, considering its importance for clock function in real life. Light is the predominant zeitgeber for all clocks, and *in vitro* entrainment by light would require a dedicated photoreceptor, creating a technical problem. But light generally affects transcription in cyanobacteria, so it should be possible to entrain the metabolic oscillator by manipulating the concentrations of KaiB and/or KaiC. It would be interesting to test this in the test tube by simply adding protein at different times and monitoring whether, how much, and in which direction the phase of the phosphorylation rhythm changes.

Temperature also cycles during the course of a day and it entrains the circadian clock in several systems (for example [8]). Circadian entrainment is indicated when the rhythm's phase relationship to the zeitgeber changes systematically with its length [8]. This could be examined in the test tube using temperature cycles of different lengths: if KaiC phosphorylation peaks, in a 24 hour cycle, in the middle of a cold phase, it would move towards the beginning of the cold phase in a longer cycle and towards the end of the cold phase in a shorter cycle. This test would show whether the *in vitro* oscillator has

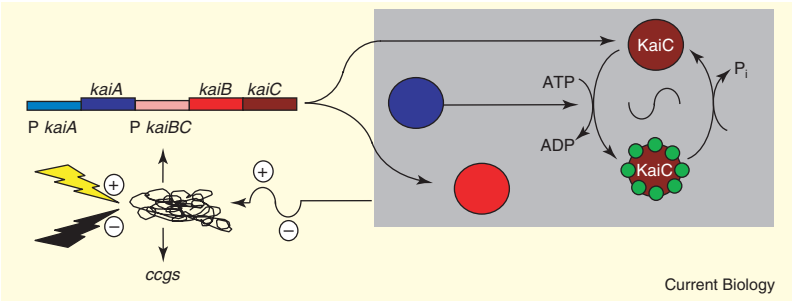

Figure. 2. A model of the *Synechococcus* circadian clock.

The circa-24 hour rhythmicity is generated by the metabolic phosphorylation oscillator which is able to oscillate both in light and in darkness (grey area). KaiA acts positively on KaiC phosphorylation (not shown quantitatively here) which can be down-regulated by KaiB. This metabolic pacemaker regulates general transcription up (+) and down (–), transmitting circadian regulation to the cyanobacterial transcriptome (ccgs, clock controlled genes). The transcriptional level of circadian regulation in *Synechococcus* is thus an output of the clock. Because light up-regulates (+) and darkness down-regulates (–) transcription, gene expression also is an input to the clock by transiently altering the concentrations of the pacemaker components KaiA, KaiB and KaiC.

the robustness of circadian clocks *in vivo* that produce jet-lag. If not, then it will be highly informative which additional components would be responsible for this property.

Nakajima *et al.* [1] are careful to stress that this metabolic, phosphorylation-based pacemaker is unique to the prokaryotic clock and so their results cannot readily be extrapolated to eukaryotic systems. But phosphorylation also plays a key role in the molecular generation of circadian rhythmicity in all eukaryotes, where at least one of the central components is phosphorylated. Phosphorylation, in general, can have effects ranging from determining a protein's half-life, directing sub-cellular localization to modulating transcription factor activity; any of these effects could contribute to clock protein function. Casein kinase mutant mammals free-run with an ultra-short period and begin their days, under entrained conditions, many hours earlier than wild-type animals [9,10]. Casein kinase is also implicated in human timing disorders, apparently involved in an inherited form of severely advanced sleep onset [11].

Several results indicate that the TTL also cannot explain the entire molecular clock machinery in eukaryotes. *Neurospora* clock null mutants, for example, can still produce self-sustained circadian rhythms under certain conditions (see references in [12]), and can still be systematically entrained by temperature cycles, as described above [8,13]. The data obtained by following both physiology and gene expression under entrained conditions indicates dissociation between transcription (showing direct regulation by the external cycle) and clock-regulated outputs (showing systematic entrainment, as described above for temperature entrainment) [14,15]. There is much to be discovered both in prokaryotes and eukaryotes well beyond transcriptional regulation, despite the overwhelming evidence for the involvement of transcription factors in circadian systems.

The findings of Nakajima *et al.* [1], together with the recent work emphasizing the effects of kinases on the circadian system [11], will shift the attention of circadian research beyond transcription and translation for all model systems and may change the concepts that we currently use to model the clock. Many results already indicated that metabolism plays an important part in the generation of circadian rhythms (for references see [12]). The *in vitro* reconstruction with three proteins emphasizes that transcription and translation are necessary, but do not necessarily have to be rhythmic.

The TTL leading to rhythmic accumulation of KaiC in LL may simply represent an output of the oscillator, transducing rhythmicity to gene expression but, as described above, it also functions as an input to the oscillator, transducing signals of the zeitgeber light. TTLs in all circadian model systems are intimately tied to light reception (for references see [16–18]). In mammals, light acts indirectly (via the retino-hypothalamic tract) on the expression of the clock genes *period1* and *period2*; in *Neurospora* it acts directly on the expression of the clock gene *frequency* (via the clock protein and photoreceptor WHITE COLLAR-1); and in *Drosophila* it acts on the degradation of the clock protein Tim. Finally, Cryptochrome, a clock protein in plants and animals, is directly responsible for circadian light reception in plants and flies.

Figure 2 shows the *Synechococcus* TTL as an interlocked loop with the metabolic pacemaker. *Zeitnehmer* ('time taker') loops forming both inputs to and outputs of a rhythm generating pacemaker have been shown in many circadian model systems (for example [19]). A mathematical model predicted that components of TTLs can be components of *zeitnehmer* loops, and as such may be indistinguishable in their mutant phenotypes from components of the pacemaker [2]. It also predicts that *zeitnehmer* loops contribute the important circadian quality of robustness to the oscillation (remember the jet lag) through reinforcement. This theoretical model resembles the new model of the *Synechococcus* clock (Figure 2).

The fact that the metabolic pacemaker oscillates in both light and darkness while the TTL is only rhythmic in light points to another important aspect in circadian physiology, namely measuring changing daylength over the course of the year. The metabolic pacemaker and the TTL may be candidates for a 'morning' and an 'evening' oscillator which have been postulated for several circadian systems [20]. Their

interaction could enable *Synechococcus* to anticipate and adapt to its highly seasonal habitat.

Transcription and translation are surely not entirely lost from any circadian clock, but these new findings should make us critically assess what clock properties they contribute to the circadian system, such as how much are they involved in keeping people awake during jet-lag. The physiology and behaviour that the *Synechococcus* clock regulates via transcription is an output of the clock but it obviously also supports the system via feedback (*zeitnehmer*). We clearly still have to understand much more about how rhythm generation, transcription, translation, inputs and outputs interact before we can put this story to bed.

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DNA Repair: How to PIKK a Partner

In eukaryotes, members of the phosphoinositide-3-kinase-related protein kinase (PIKK) family co-ordinate the cellular response to DNA damage. But how do these important kinases detect DNA damage and relay this information to the DNA repair and checkpoint machinery?

Kevin Hiom

Genomic DNA damage poses a threat to the genetic integrity of any organism. In most cells, detection of DNA lesions elicits a coordinated set of responses with the ultimate aim of faithfully repairing the damaged DNA. In higher organisms, the presence of DNA damage is signalled by a family of proteins known as the phosphoinositide-3-kinase-related protein kinases (PIKKs); these include the Ataxia-telangeictasia mutated (ATM), Ataxia-telangeictasia related (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKcs) proteins. PIKKs are recruited to sites of DNA damage, where they are rapidly 'activated' to phosphorylate a host of downstream substrates involved in the maintenance of genomic integrity [1].

How then do PIKKs detect DNA damage and relay this information? Two recent papers [2,3] shed new light on this process. Falck *et al*. [2] have identified a hitherto unknown protein interaction motif that is important in the damage response, and shown that PIKKs are recruited to sites of DNA damage through a physical association with specific DNA damage-binding proteins. Lee and Paull [3] found that, for ATM at least, recruitment to DNA by the damage-binding Mre11/Rad50/Nbs1 (MRN) complex greatly stimulates its ability to phosphorylate downstream substrates.