SCIENTIFIC VERSES

The chemistry behind circadian clocks

Anand Vaidya,
Dept. of Chemistry and Chemical Biology, Cornell University, Ithaca, New York, United States.

Imagine a tree that sheds leaves in the summer and grows them in the winter. What would be the fate of birds if they forget when to migrate? How would a human survive if he/she is awake only during the nights? All these organisms will be extinct as they lack the ability to take clues from the environment and/or fail to tune their daily or seasonal needs with changes in nature. To precisely predict the daylight or temperature, helps organisms to anticipate the availability of food, predation, migration, hibernation, reproduction, etc. which are essential for survival. In humans it is important for sleep-wake cycle, food habits, work habits, drug administration and on a broader note to maintain order in society. The mechanisms of entrainment are diverse and can result from changes in nutrient levels, temperature, light or alternative environmental cues. Thus, species with a robust circadian rhythm gain an evolutionary edge.

The word circadian was coined by Franz Halberg and is derived from two Latin words circa (round) and diem (day), which literally means 'around a day'. Every day activities such as sleep, hunger, bowel movements, hormonal regulation in humans, as well as seasonal activities such as migration, mating/reproduction, hibernation in animals reflect circadian rhythms. Circadian rhythms are not confined to the animal kingdom. Conidiation, the process of forming spores in fungus, and the seasonal shedding, reappearance of leaves in plants are some everyday examples. Cyanobacteria, a photosynthetic bacterium, also have a circadian rhythm.

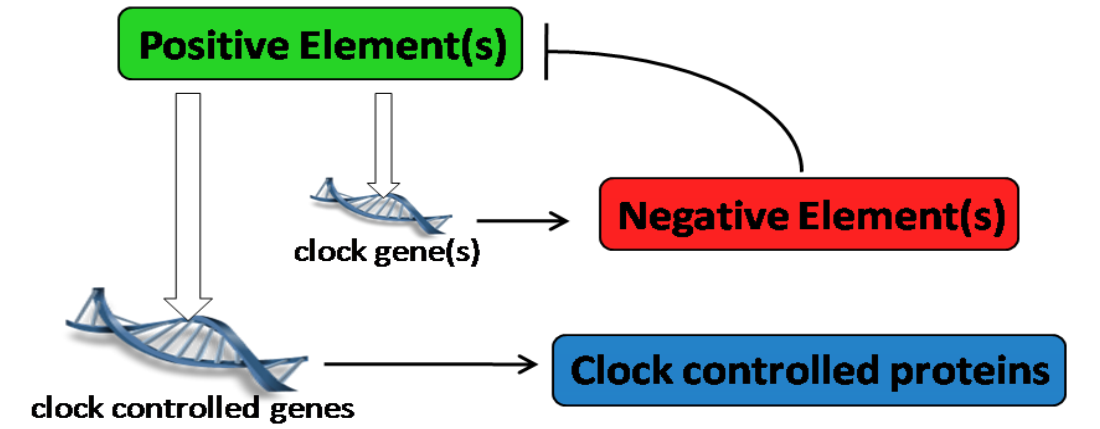


Fig. 1: Common elements in the circadian feedback loops.

The mechanism of circadian rhythm involves positive and negative feedback loops composed of interacting macromolecules (proteins, DNA, RNA) within the cell (Fig. 1). The core of the clock is a protein (or a group of proteins) called the positive element(s), which transcriptionally activates certain genes. These genes can be divided into two types-the clock genes, which are involved in the feedback loop and the clock controlled genes (ccgs), which control the rhythmic activities of the entire organism.

The positive element initiates transcription of the clock gene(s), leading to the respective mRNA(s) in the nucleus, and then transported to the cytoplasm where it is translated into protein(s). The protein, called negative element, blocks (directly or indirectly) the transcription activity of the positive element and prevents the activation of it's own gene along with the clock controlled genes (ccgs). This reduces the mRNA levels of the clock genes and the levels of clock protein (negative element), which leads to the activation of the transcription factor (positive element). Thus, the cyclic increase-decrease in the levels of clock gene mRNA, ccgs, clock protein, clock controlled protein and transcription activator forms the basis of circadian rhythm of an organism. Though the numbers of players and the complexity varies in different organisms, the core mechanism remains the same. The proteins involved in the feedback loops of different organisms are listed in table 1.

Neurospora crassa is a species of Fungi and is encountered on a day-to-day basis as red bread mold. It is used as a model organism as it is easy to grow and the entire genome has been sequenced. Because Fungi a eukaryote, an understanding of it's fundamental processes can be applied, at least partially, to humans. It has a circadian rhythm of 22 hr. and conidiation is used as a marker for it's rhythm. The fungal proteins are produced in E. coli using the recombinant DNA technique for in vitro analysis and interesting mutants are introduced into the fungi to study the effect on conidiation (ultimately the circadian rhythm). The proteins Frequency (FRQ), White Collar-1 (WC-1) and White Collar-2 (WC-2) are the core clock proteins. WC-1 and WC-2

Organism	Gene	Protein	Clock role
Neurospora	*wc-1*	White collar-1	Positive element
	wc-2	White collar-2	Positive element
	frq	Frequency	Negative element
Drosophila	*Clk*	dClock	Positive element
	cyc	Cycle	Positive element
	dbt	Double time	Facilitating element
	per	Period	Negative element
	tim	Timeless	Negative element
Mouse	*Clock*	mClock	Positive element
	Bmal1/mop3	BMAL1/MOP3	Positive element
	tim1	mTimeless1	Facilitating element
	Per1	mPeriod1	Negative element
	Per2	mPeriod2	Negative element
	Per3	mPeriod3	Negative element

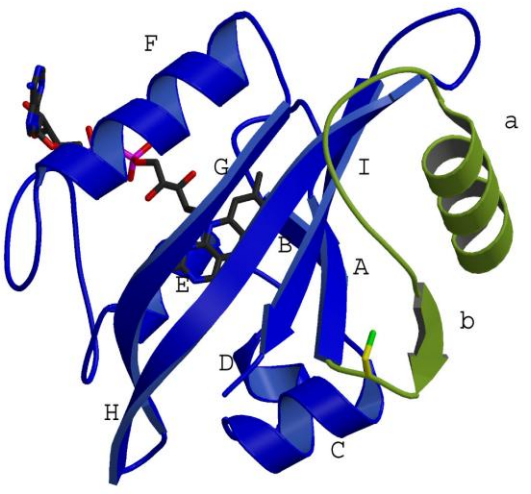
Table 1: The circadian clock proteins and their roles in different organisms.

form a White Collar complex (WCC) which is the positive element and initiates the transcription of frq and other ccgs. FRQ is the negative element which inactivates the WCC and completes the feedback loop. The extent of phosphorylation of the WCC varies rhythmically over the day and determines it's activity: hypo-phosphorylated WCC is more active than the hyper-phosphorylated WCC.

The following is the approximate daily routine of Neurospora, assuming sunrise at 6.30am:

- 05.30am-10.00am: WCC is dominantly hypophosphorylated and the mRNA frq level gradually rises.
- 11.00am-12.00pm: frq mRNA level peaks.
- 04.00pm-05.00pm: FRQ protein level peaks and WCC hyperphosphorylation is on the rise and WCC is inhibited from initiating transcription.
- 08.00pm-09.00pm: FRQ is phosphorylated, leading to peak degradation.
- 11.30pm-12.30am: Dephosphorylation of WCC begins.

If the circadian clock is strictly endogenous, then how are the rhythms synchronized to external environment? Light, temperature, food intake are three major external cues or Zeitgebers, of which photo-entrainment is the most important. The WC-1 is activated by light (specifically, the blue wavelength) leading to the rapid association of the WCC to their target genes and initiation of the cycle. Vivid (VVD), another fungal blue-light photoreceptor protein forms another negative feedback loop with the WCC and acts as a negative element. VVD



blocks the transcriptional activity of WCC, which affects the other loops and the overall rhythmicity of the organism. VVD renders the WCC (specifically, the WC-1) less sensitive to light and adapts the clock to constant daylight, thereby prevents resetting. The light sensing ability of WC-1 and VVD is attributed to the **PAS** domain, which is named after the first three proteins assigned to this protein domain: Period (**P**ER), Arylhydrocarbon nuclear translocation protein (**A**RNT), Single minded protein (**S**IM).

The PAS domain is the sensory motif of many signaling proteins and plays an important role in all kingdoms of life. Different types of PAS domains regulate phototropism in plants and photo-entrainment in animals by sensing light, control ion-channels in humans by sensing the voltage and result bacterial nitrogen fixation by sensing the oxygen levels. The PAS domain is a simple mix of α/β type protein structure, with α-helices on either side of a central five-stranded anti-parallel β-sheet (Fig. 2). One of the faces of the β-sheet forms a hydrophobic pocket with the α helical element and the other face, due to it's hydrophobic nature, is often involved in protein:protein interactions. The domain topology involves two β-strands (Aβ, Bβ), followed by a series of short α-helices (Cα, Dα, Eα, Fα) and three anti-parallel B-strands (Gβ, Hβ, Iβ). Many PAS domains contain N-terminal/C-terminal extensions (called the Ncap/Ccap), which directly interact with the hydrophobic region of the core β-sheet. Generally, PAS domains bind to co-factors such as flavin adenosine diphosphate (FAD), flavin adenosine monophosphate (FMN), heme, citrate, etc.; those binding flavin moieties belong to a

family of light-oxygen-voltage (LOV)

sensing domains. In response to an external stimulus such as chemical ligand, light or redox potential, the PAS domain undergoes conformational changes in the variable regions which ultimately allow the interaction with new partners.

Vivid (VVD), the photoreceptor protein involved in the Neurospora crassa circadian clock, is a 186 amino acid long, FAD containing, single LOV domain protein with N-terminal and C-terminal extensions. As it is considerably smaller than most LOV-containing proteins and can be studied as a full length molecule, it has served as a model system for light signaling in LOV domain photoreceptors. VVD undergoes reversible structural changes on exposure light, which stems from the interaction of light with the flavin. Exposure to light results in the formation of adduct between the sulphur of the conserved Cys108 and the C4a carbon of flavin (Fig. 3). The mechanism of adduct formation is not clear, but a free-radical mechanism seems more probable than an ionic mechanism. The UV-vis spectrum of the dark state protein has absorption maxima at 380 and 450nm, with vibrational fine structure at 428 and 478nm, while the light state has a single peak at 392nm. The light

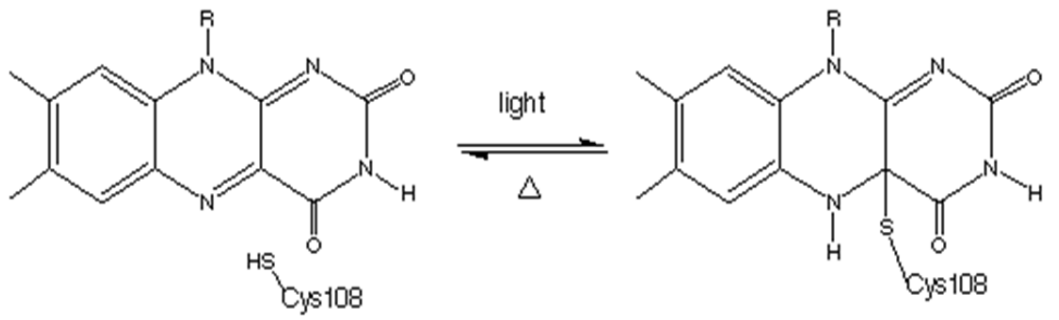


Fig. 3: Photocycle of VVD.

induced bleaching of the 380, 450 and 478nm peaks is used to study the reverse kinetics (light to dark) of VVD and it's mutants, which helps in understanding the effect of different residues on adduct formation. A Cys108 mutation completely abolishes the photo-activity, due to the loss in the ability of adduct formation. The crystal structure of VVD showed that the adduct formation at the active centre led to comformational changes at the N-terminus via rearrangement of a few hydrogen bonds that respond to protonation of the flavin in the adduct state.

X-ray diffraction analysis of the dark state crystals and the photo-bleached crystals reveal the conformational changes induced by light. Formation of the adduct reduces the flavin ring and protonates the N5 position, which leads to the flipping of the Gln182 amide, to maintain a hydrogen bond with the new protonated N5. The Gln182 flip leads to the formation of a hydrogen bond between the Ala72 carbonyl and the Gln182 amide, resulting in the rotation of the Cys71 thiol to interact with the peptide nitrogen of the Asp57. This altered interaction of Cys71 (Fig. 2) corresponds to a bβ (Ncap) shift of 2.0 Å towards the core. Photo-bleaching of dark grown crystals cannot reveal large conformational changes as the protein is confined by the crystal lattice. Currently, a VVD mutant with slow recovery kinetics (light to dark state) has been crystallized and preliminary data suggests conformational changes in the Ncap region are essential to generate the signaling state. The light state structure would be very interesting as it would reveal the mechanism by which life-forms sense light and tune their activities to gain an evolutionary edge.

The mechanism of adduct formation is still unclear and the immediate targets of VVD are still unknown. VVD lacks a nuclear localization signal (NLS), but it regulates the nucleus bound White Collar complex (WCC) by an unknown pathway. The structures of the WCC and it's components are yet to be worked out. Many clock controlled genes and their regulatory functions are yet to be characterized. Do the different clocks, from different organs, of the human body talk to each other? If so, then how and where does this happen? If the grasshopper had a non-functional circadian clock protein, then the ant got more credit than it deserved.

References:

1. M. Brunner, K. Káldi, *Mol. Microbiol.* **68**, 255 (2008).
2. J. C. Dunlap, *Cell* **96**, 271 (1999).
3. M. W. Young, S. A. Kay, *Nat. Rev. Genet.* **2**, 702 (2001).
4. B. D. Zoltowski *et al*, *Science* **316**, 1054 (2007).
5. W. R. Briggs, J. L. Spudich, *Handbook of photosensory receptors*, Wiley-VCH, 2005.

