Minireview

From biological clock to biological rhythms

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Abstract

The genetic and molecular analysis of circadian timekeeping mechanisms has accelerated as a result of the increasing volume of genomic markers and nucleotide sequence information. Completion of whole genome sequences and the use of differential gene expression technology will hasten the discovery of the clock output pathways that control diverse rhythmic phenomena.

Circadian rhythms are fluctuations in physiological and behavioral activities that occur over a period of about 24 hours. Although these rhythms parallel environmental cycles of light and dark, they are not simply a reaction to environmental fluctuations, but are generated by an endogenous timekeeping mechanism called the circadian clock. The ability of the clock to persist in the absence of environmental cues provides internal temporal organization so that rhythmic activities can occur at characteristic times during the circadian cycle. In addition, two other clock properties, entrainment (that is, setting the clock to local time with respect to environmental cycles) and temperature compensation (that is, the ability of the clock to run at the same rate at different temperatures) ensure synchrony with the environment. The importance of the circadian clock is underscored by its ubiquity; clocks are present in organisms ranging from prokaryotic and eukaryotic microbes to plants, insects and mammals.

The circadian clock has been conceptualized as a series of three components: an 'entrainment pathway' that transmits environmental (usually light) signals to the timekeeping apparatus; a timekeeping apparatus, or 'oscillator', which operates in the absence of environmental cues and is the core component of the circadian clock; and 'output pathways' that are activated at specific times of the circadian cycle by the circadian oscillator. This framework has enabled us to concentrate on the mechanisms that interconnect these components to form an effective timekeeping system. Naturally, a great deal of effort has been focused on identifying genes that encode pieces of the oscillator - also known as 'clock genes'. The increasing volume of genomic and expressed sequence tag (EST) sequences has rapidly increased the pace of clock gene discovery, particularly in mammals; and the abundance of newly identified clock genes has given rise to detailed models of the oscillator mechanism. In contrast, relatively little effort has been put into identifying genes within output pathways, even though these pathways provide the critical links to rhythmic physiology and behavior. New tools that are now available for expression screening should enable rapid advances in the identification of output genes.

Circadian oscillator mechanisms

On the basis of studies in cyanobacteria, *Neurospora*, *Drosophila* and mice, the core circadian oscillator mechanism is thought to consist of one or more transcriptional feedback loops [1]. Despite this conservation of oscillator mechanisms, oscillator components in cyanobacteria and *Neurospora* bear little sequence similarity to each other or to those in *Drosophila* and mice. In contrast, oscillator components from *Drosophila* and mice show a high degree of sequence similarity, a property that has fueled recent advances in our understanding of the oscillator.

Essentially all components of the *Drosophila* and mammalian circadian oscillators have been discovered (directly or indirectly) as a result of genetic screens for rhythm mutants. In this review, I will describe the various clock

genes, how they were isolated and how they function within the oscillator (Table 1, Figure 1). The first clock mutants to be discovered identified the *period* (*per*) gene from *Drosophila* [1]. This gene encodes a protein, Per, that contains a PAS domain (a protein interaction interface) [1]. It feeds back as part of a heterodimer with the *timeless* (*tim*) protein (Tim) to inhibit *per* and *tim* transcription and activate a gene required for *per* and *tim* activation, called *Drosophila Clock* (*dClk*) [2]. This differential effect of Per-Tim heterodimers on transcription gives rise to high levels of *per* and *tim* mRNA at dusk and high levels of *dClk* mRNA at dawn [2]. After many years of searching, PCR-based molecular screens and EST searches uncovered three mouse *per* orthologs (*mPer1*, *mPer2* and *mPer3*) [3]. Although all three mPer proteins are capable of inhibiting *mPer1* transcription in cell culture [4,5], genetic analysis suggests that mPer2 also leads to the activation of *Bmal1*, a gene required for *mPer1* activation [6]. These divergent regulatory functions lead to peaks in *mPer1* transcription during the day and peaks in *Bmal1* transcription at night [1,7,8].

The protein encoded by *tim*, the second clock gene identified in *Drosophila*, dimerizes with, and stabilizes, phosphorylated Per, promotes Per localization to the nucleus and is degraded in response to light [9]. These functions of Tim allow for an approximately 6–8 hour delay in the accumulation of Per protein (with respect to *per* mRNA) and entrainment of the oscillator to light. Mammalian *tim* isologs (related by sequence) of human (*hTim*) and mouse (*mTim*) have been isolated through EST database searches [1,10]. Although the role of mTim in the mouse oscillator is uncertain, it does not promote nuclear localization of the mPers or mediate entrainment to light as Tim does in *Drosophila* [5,10]. Searching the *Drosophila* genome database has, however, led to the discovery of another *tim* gene, thus opening up the possibility that *mTim* is not the true homolog of the *Drosophila tim* clock gene [6].

The first mammalian clock gene identified as a result of genetic screening for rhythm mutants was mouse *Clock*. This gene, which was isolated by positional cloning and transgenic

Table 1

Circadian clock genes and their functions in *Drosophila* and mice

Organism	Gene	Protein function	Clock function
Drosophila	*per*	Binds to dClk-Cyc as a heterodimer with Tim	Inhibits dClk-Cyc-dependent transcription
	tim	Stabilizes phosphorylated Per Promotes Per nuclear localization Binds to dClk-Cyc as a heterodimer with Per Degraded in response to light	Contributes to delayed Per accumulation Acts with Per to inhibit dClk-Cyc-dependent transcription Mediates phase-resetting in response to light
	dClk	Forms a heterodimeric complex with Cyc	Activates *per* and *tim* transcription and inhibits *dClk* transcription
	Cyc (*Bmal1*)	Forms a heterodimeric complex with dClk	Activates *per* and *tim* transcription and inhibits *dClk* transcription
	dbt (*CK1*ε)	Phosphorylates Per	Destabilizes Per
	cry	Photoreceptor element that binds to Tim	Initiates light signaling required for phase resetting
Mouse	*mPer1*	Interacts with Clock-Bmal1?	Moderate inhibition of Clock-Bmal1-dependent transcription
	mPer2	Interacts with Clock-Bmal1?	Activation of Bmal1 transcription Moderate inhibition of Clock-Bmal1-dependent transcription
	mPer3	Interacts with Clock-Bmal1?	Moderate inhibition of Clock-Bmal1-dependent transcription
	mTim	Interacts with Clock-Bmal1?	Moderate inhibition of Clock-Bmal1-dependent transcription
	Clock	Forms a heterodimeric complex with Bmal1	Activates *mPer1-3* and *mCry1*
	Bmal1	Forms a heterodimeric complex with Clock	Activates *mPer1-3* and *mCry1*
	CK1ε (*tau*)	Phosphorylates mPer1 and mPer2	Destabilizes mPers
	mCry1	Dimerizes with mPer1-3 Binds to Clock-Bmal1 Promotes mPer1-3 nuclear localization	Strongly inhibits Clock-Bmal1-dependent transcription
	mCry2	Dimerizes with mPer1-3 Binds to Clock-Bmal1 Promotes mPer1-3 nuclear localization	Strongly inhibits Clock-Bmal1-dependent transcription

Gene symbols are as described in the text. Protein functions are derived from *in vivo* and/or *in vitro* experiments. Clock function is derived from mutant phenotypes and/or *in vivo* experiments. The '?' for mPer1-3 and mTim protein function indicates that the indicated activity is presumed on the basis of the clock function of these proteins. *Cyc* is the *Drosophila* homolog of mouse *Bmal1* and *dbt* is the homolog of mouse CK1ε and hamster *tau*.

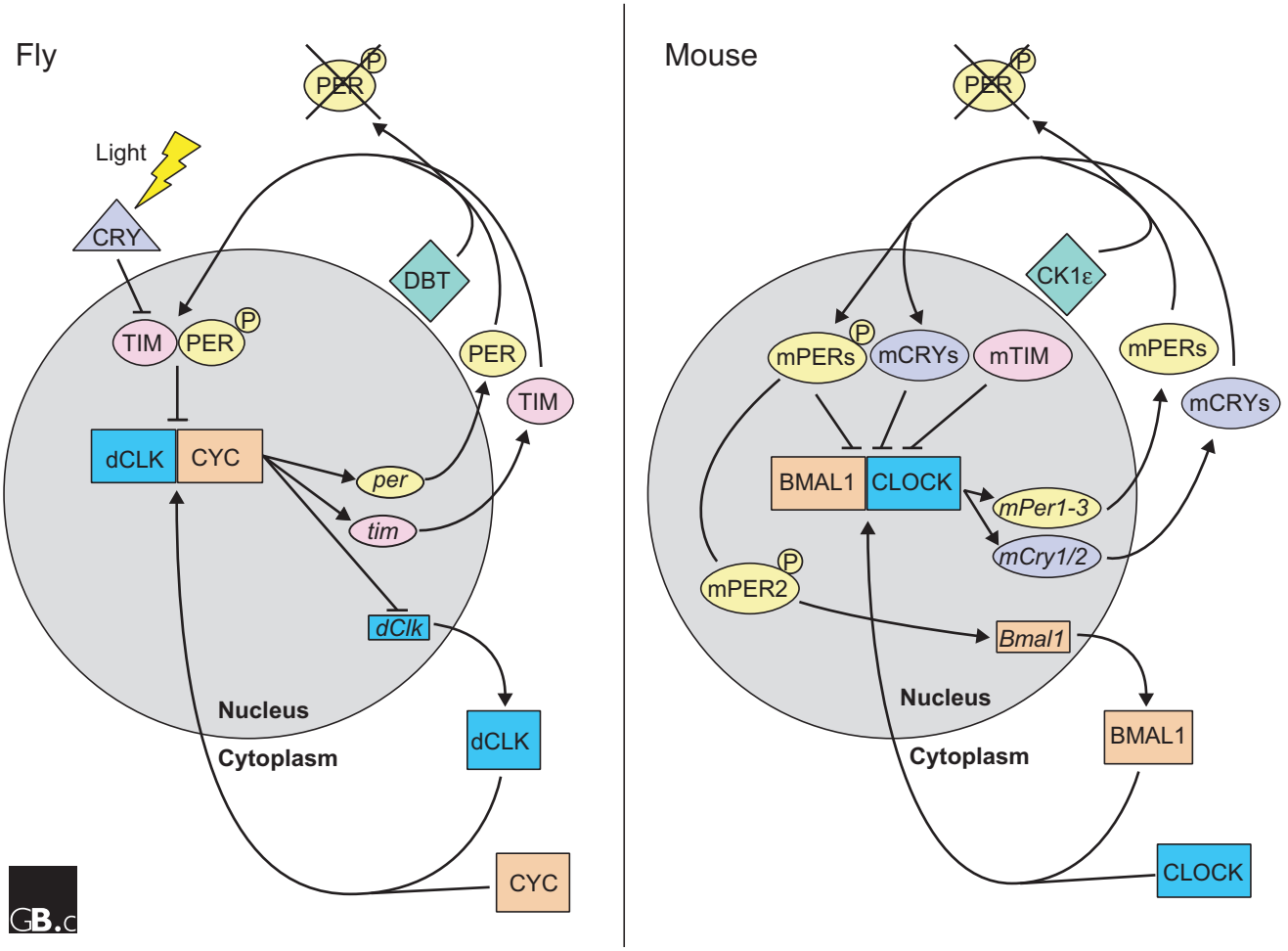

Figure 1
The circadian oscillator mechanisms from flies and mice: models depicting the regulatory interactions within the *Drosophila* and mouse feedback loops. Arrows indicate positive regulation and lines ending in bars denote negative regulation. Gene symbols are as described in the text. The 'P' on Per and mPer represents phosphorylation due to Dbt/CK1ε. The lightning bolt represents light acting on *Drosophila* Cry.

bacterial artificial chromosome (BAC) mutant rescue [3], encodes a basic-helix-loop-helix-PAS (bHLH-PAS) protein (Clock) which is required for activating the transcription of other clock genes (*mPer1-3*, and the genes for mouse cryptochromes 1 and 2) and of an output gene (for vasopressin) [3,5]. Clock mediates transcriptional activation as part of a heterodimeric complex with Bmal1 - a bHLH-PAS protein discovered in a yeast two-hybrid screen for proteins that interact with Clock [3]. Although *Bmal1* mRNA levels cycle with a peak early at night and *Clock* mRNA levels are constant [3,7,8], the levels of Bmal1 and Clock proteins have not been characterized. Genetic, DNA cross-hybridization and EST database screens revealed *Drosophila* homologs of *Clock* and *Bmal1*, termed *dClk* and *Cycle* (*Cyc*), respectively [1]. As in mammals, the dClk and Cyc proteins form heterodimers that activate *per* transcription. This heterodimeric complex also activates *tim* transcription and inhibits *dClk* transcription, however, thereby accounting for the antiphase cycling of these transcripts [2]. In contrast to the situation in mammals, *dClk* mRNA cycles with a peak at dawn and *Cyc* mRNA levels are constant [2]. The dClk and Cyc protein levels parallel *dClk* and *Cyc* mRNA levels: dClk peaks during early day and Cyc does not vary [11].

Perhaps the only mammalian clock genes that were not discovered (directly or indirectly) by genetic screening are the mouse cryptochrome genes, *mCry1* and *mCry2* [12]. Although these genes were initially thought to encode circadian photoreceptors, on the basis of their similarity to plant blue-light receptor cryptochromes, their protein products (mCry1 and mCry2) in fact function within the oscillator to strongly inhibit transcriptional activation by Clock-Bmal1 and to mediate mPer nuclear localization [5,12]. As *mCry1* and *mCry2* are activated by Clock-Bmal1 heterodimers, high levels of the mCry1 and mCry2 inhibitors at dusk feed back to mediate rhythms in the abundance of *mCry1* and *mCry2*

transcripts which, like *mPer* transcripts, peak during the day [5, 12]. In *Drosophila*, a single *cryptochrome* (*cry*) gene was identified through PCR-based screening for *cry* homologs and genetic screening for mutants that alter *per*-promoter-driven *luciferase* reporter-gene cycling [13]. Unlike its mammalian counterparts, *Drosophila* Cry is involved in receiving light signals and transmitting these signals to the clock by forming a complex with Tim [14].

In addition to the intricate network of factors involved in transcriptional control, factors that mediate post-transcriptional regulatory events are also required for circadian oscillator function (Figure 1). One component of the post-transcriptional regulatory mechanism in *Drosophila* is Doubletime (Dbt), a casein kinase Iε (CKIε) ortholog that was identified by genetic screening [9]. Dbt phosphorylates, and consequently destabilizes, Per, which in turn prevents Per from accumulating until it forms a heterodimer with Tim [9]. This post-transcriptional regulation leads to a substantial (approximately 6 hour) lag between *per* mRNA and Per protein accumulation. The gene corresponding to the first identified mammalian rhythm mutant, called *tau*, was recently isolated from hamsters via a groundbreaking positional syntenic cloning approach, and found to be a *dbt* homolog [15]. Like Dbt, hamster Tau-encoded CKIε acts to phosphorylate Per proteins (at least mPer1 and mPer2 *in vitro*) [15]. The defective *tau*-mutant CKIε leads to a premature rise in hamster Per1 levels, which not only explains the short-period phenotype of *tau* mutants but also suggests that CKIε destabilizes hamster Per1.

This explosion of clock gene discovery has also enhanced oscillator analysis in non-mammalian vertebrates such as zebrafish and *Xenopus*. In zebrafish, homologs to *Bmal1* (*Bmal1* and *Bmal2*), *Clock* and *mPer3* have been isolated using low-stringency hybridization and degenerate PCR [16-18]. Homologs of other clock genes (*Per* and *Cry*) present in zebrafish EST databases and new clock mutants (G. Cahill, personal communication) should provide a detailed picture of the oscillator in this species. Likewise, the *Xenopus Clock* gene was recently described [19] and should fuel the analysis of the frog oscillator mechanism. Finally, several rhythm mutants have been identified in plants [20]. In several cases, the corresponding genes have been isolated, but they are not similar to any known clock genes and do not appear to be part of the core oscillator mechanism. Analysis of additional plant clock mutants should reveal whether oscillator components in plants bear any resemblance to those in other model systems.

Clock output pathways

Significant progress has been made in determining how the circadian oscillator keeps time and maintains synchrony with the environment. Relatively little is known, however, about how the oscillator controls rhythmic events so that they occur at particular times of day. A key to understanding how the oscillator carries out this temporal program is the identification and analysis of output pathways. As rhythmic transcription is a prominent feature of the oscillator, mechanisms that regulate rhythmic transcription within the oscillator could also be used to control rhythmic expression within output pathways.

Rhythmically expressed output genes have been identified in many organisms. In some organisms, such as cyanobacteria, rhythmic expression is the rule rather than the exception [1]. In most organisms, however, rhythmically expressed genes are found only infrequently. Several output genes, including the transcription factors *dbp*, *tef* and *icer* from mice, have been found fortuitously during careful analysis of gene expression [21]. Other clock-regulated genes (such as that for vasopressin) were identified because their protein products were expressed in central clock tissues such as the mammalian suprachiasmatic nucleus, and were needed to produce compounds that affect the clock (for example the enzyme serotonin *N*-acetyltransferase, which is required for the production of melatonin), or were involved in diurnally regulated processes such as photosynthesis (for example *cab* and *catalase* in plants).

Screens for rhythmically expressed mRNAs have accelerated the discovery of clock-regulated genes. The first such screen was carried out in *Neurospora* using subtractive hybridization [1]. This identified two clock-controlled genes (CCGs): *ccg-1* and *ccg-2*; *ccg-2* was later found to be involved in the development of conidia, which are reproductive structures produced in a circadian fashion [1]. Different subtractive hybridization techniques have also been used to isolate CCGs from *Drosophila*, including 20 Drosophila rhythmically expressed genes (dregs) [22], *Crg-1* [23], and *takeout* [24], and CCGs from plants, including *sagrp1* and *sagrp2* [25]. Another technique that has been used to identify CCGs is differential display reverse-transcription PCR (DD RT-PCR). This technique was used to identify several CCGs in *Xenopus* [26], the *vrille* gene in *Drosophila* [27] and a set of 17 CCGs from *Arabidopsis* [28]. The advent of DNA microarray technology should enable researchers to identify a vast number of CCGs via differential screening of cDNAs or predicted genes from whole genome sequences. This volume of CCGs should reveal a multitude of clock output pathways and should provide clues to the identity of the physiological and behavioral phenomena they control.

Searches for CCGs have so far focused mainly on rhythmically expressed mRNAs. In a few cases, however, rhythms in protein abundance are seen in the absence of underlying mRNA rhythms, indicating that potent clock-dependent post-transcriptional mechanisms are operating. Examples include luciferase-binding protein (LBP), which contributes to bioluminescence rhythms in dinoflagellates [29], and White collar 1 (Wc1), which is an integral component of the

oscillator in *Neurospora* [30]. As rhythms in protein abundance are generally not investigated unless the corresponding mRNA cycles in abundance or the protein contributes to a rhythmic phenomenon, it is possible that other cycling proteins emanate from non-cycling mRNAs. By coupling proteomics technology and mass spectrometry-based protein sequence analysis, particularly in organisms whose genomes have been sequenced, it will be possible to detect and identify proteins that cycle in abundance. Once a comprehensive collection of clock outputs is available, we will be better able to understand how the biological clock orchestrates the temporal program that governs the coordinate expression of biological rhythms.

References

1. Dunlap JC: **Molecular bases for circadian clocks.** *Cell* 1999, **96:**271-290.
2. Scully AL, Kay SA: **Time flies for *Drosophila*.** *Cell* 2000, **100:**297-300.
3. King DP, Takahashi JS: **Molecular genetics of circadian rhythms in mammals.** *Annu Rev Neurosci* 2000, **23:**713-742.
4. Sangoram AM, Saez L, Antoch MP, Gekakis N, Staknis D, Whiteley A, Fruechte EM, Vitaterna MH, Shimomura K, King DP, *et al.*: **Mammalian circadian autoregulatory loop: a *timeless* ortholog and *mPer1* interact and negatively regulate CLOCK-BMAL1-induced transcription.** *Neuron* 1998, **21:**1101-1113.
5. Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, Reppert SM: **mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop.** *Cell* 1999, **98:**193-205.
6. Shearman LP, Sriram S, Weaver DR, Maywood ES, Chaves I, Zheng B, Kume K, Lee CC, van der Horst GT, Hastings MH, Reppert SM: **Interacting molecular loops in the mammalian circadian clock.** *Science* 2000, **288:**1013-1019.
7. Honma S, Ikeda M, Abe H, Tanahashi Y, Namihira M, Honma K, Nomura M: **Circadian oscillation of *BMAL1*, a partner of a mammalian clock gene *Clock*, in rat suprachiasmatic nucleus.** *Biochem Biophys Res Commun* 1998, **250:**83-87.
8. Oishi K, Sakamoto K, Okada T, Nagase T, Ishida N: **Antiphase circadian expression between *BMAL1* and *period* homologue mRNA in the suprachiasmatic nucleus and peripheral tissues of rats.** *Biochem Biophys Res Commun* 1998, **253:**199-203.
9. Edery I: **Role of posttranscriptional regulation in circadian clocks: lessons from *Drosophila*.** *Chronobiol Int* 1999, **16:**377-414.
10. Hastings M, Maywood ES: **Circadian clocks in the mammalian brain.** *BioEssays* 2000, **22:**23-31.
11. Bae K, Lee C, Hardin PE, Edery I: **dCLOCK is present in limiting amounts and likely mediates daily interactions between the dCLOCK-CYC transcription factor and the PER-TIM complex.** *J Neurosci* 2000, **20:**1746-1753.
12. Hardin PE, Glossop NR: **The CRYs of flies and mice.** *Science* 1999, **286:**2460-2461.
13. Devlin PF, Kay SA: **Cryptochromes - bringing the blues to circadian rhythms.** *Trends Cell Biol* 1999, **9:**295-298.
14. Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ, Kay SA: **Light-dependent sequestration of TIMELESS by CRYPTOCHROME.** *Science* 1999, **285:**553-556.
15. Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS: **Positional syntenic cloning and functional characterization of the mammalian circadian mutation *tau*.** *Science* 2000, **288:**483-492.
16. Whitmore D, Foulkes NS, Strahle U, Sassone-Corsi P: **Zebrafish *Clock* rhythmic expression reveals independent peripheral circadian oscillators.** *Nat Neurosci* 1998, **1:**701-707.
17. Cermakian N, Whitmore D, Foulkes NS, Sassone-Corsi P: **Asynchronous oscillations of two zebrafish CLOCK partners reveal differential clock control and function.** *Proc Natl Acad Sci USA* 2000, **97:**4339-4344.
18. Delaunay F, Thisse C, Marchand O, Laudet V, Thisse B: **An inherited functional circadian clock in zebrafish embryos.** *Science* 2000, **289:**297-300.
19. Zhu H, LaRue S, Whiteley A, Steeves TD, Takahashi JS, Green CB: **The *Xenopus clock* gene is constitutively expressed in retinal photoreceptors.** *Brain Res Mol Brain Res* 2000, **75:**303-308.
20. Murtas G, Millar AJ: **How plants tell the time.** *Curr Opin Plant Biol* 2000, **3:**43-46.
21. Brown SA, Schibler U: **The ins and outs of circadian timekeeping.** *Curr Opin Genet Dev* 1999, **9:**588-594.
22. Van Gelder RN, Bae H, Palazzolo MJ, Krasnow MA: **Extent and character of circadian gene expression in *Drosophila melanogaster*: identification of twenty oscillating mRNAs in the fly head.** *Curr Biol* 1995, **5:**1424-1436.
23. Rouyer F, Rachidi M, Pikielny C, Rosbash M: **A new gene encoding a putative transcription factor regulated by the *Drosophila* circadian clock.** *EMBO J* 1997, **16:**3944-3954.
24. Sarov-Blat L, So WV, Liu L, Rosbash M: **The *Drosophila takeout* gene is a novel molecular link between circadian rhythms and feeding behavior.** *Cell* 2000, **101:**647-656.
25. Heintzen C, Melzer S, Fischer R, Kappeler S, Apel K, Staiger D: **A light- and temperature-entrained circadian clock controls expression of transcripts encoding nuclear proteins with homology to RNA-binding proteins in meristematic tissue.** *Plant J* 1994, **5:**799-813.
26. Green CB, Besharse JC: **Use of a high stringency differential display screen for identification of retinal mRNAs that are regulated by a circadian clock.** *Brain Res Mol Brain Res* 1996, **37:**157-165.
27. Blau J, Young MW: **Cycling *vrille* expression is required for a functional *Drosophila* clock.** *Cell* 1999, **99:**661-671.
28. Kreps JA, Muramatsu T, Furuya M, Kay SA: **Fluorescent differential display identifies circadian clock-regulated genes in *Arabidopsis thaliana*.** *J Biol Rhythms* 2000, **15:**208-217.
29. Morse DS, Fritz L, Hastings JW: **What is the clock? Translational regulation of circadian bioluminescence.** *Trends Biochem Sci* 1990, **15:**262-265.
30. Lee K, Loros JJ, Dunlap JC: **Interconnected feedback loops in the *Neurospora* circadian system.** *Science* 2000, **289:**107-110.