Minireview

From biological clock to biological rhythms

Paul E Hardin

Address: Department of Biology and Biochemistry, University of Houston, Houston, TX 77204-5513, USA.
E-mail: phardin@uh.edu

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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 recovered 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**

| 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                                                     | Contributes to delayed Per accumulation            |
|          |       | Promotes Per nuclear localization                                                | Acts with Per to inhibit dClk-Cyc-dependent transcription |
|          |       | Binds to dClk-Cyc as a heterodimer with Per                                       | Mediates phase-resetting in response to light      |
|          |       | Degraded 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 (CK1c) | 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                        |
|          | CK1c (tau) | Phosphorylates mPer1 and mPer2                                                | Destabilizes mPers                                 |
|          | mCry1 | Dimerizes with mPer1-3                                                           | Strongly inhibits Clock-Bmal1-dependent transcription |
|          |       | Binds to Clock-Bmal1                                                             |                                                    |
|          |       | Promotes mPer1-3 nuclear localization                                           |                                                    |
|          | mCry2 | Dimerizes with mPer1-3                                                           | Strongly inhibits Clock-Bmal1-dependent transcription |
|          |       | Binds to Clock-Bmal1                                                             |                                                    |
|          |       | Promotes mPer1-3 nuclear localization                                           |                                                    |

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 CK1c and hamster tau.
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/CK1c. 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 dCik and Cycle (Cyc), respectively [1]. As in mammals, the dCik and Cyc proteins form heterodimers that activate *per* transcription. This heterodimeric complex also activates *tim* transcription and inhibits *dCik* transcription, however, thereby accounting for the antiphase cycling of these transcripts [2]. In contrast to the situation in mammals, *dCik* mRNA cycles with a peak at dawn and *Cyc* mRNA levels are constant [2]. The dCik and Cyc protein levels parallel dCik and Cyc mRNA levels: dCik 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 (Bmalt 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.

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