Circadian rhythms: **Partners in time**

Russell N. Van Gelder* and Mark A. Krasnow†

The *timeless* gene is a second essential component of the circadian clock in *Drosophila*; its product interacts physically with the only other known clock component, the *period* gene product. Together they control the daily cycle of expression of their own and other loci.

Addresses: *Department of Ophthalmology and Visual Sciences, Washington University Medical School, St. Louis, Missouri 63131, USA. †Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305, USA.

Current Biology 1996, Vol 6 No 3:244–246

 ISSN 0960-9822

In 1729, DeMarain published a short note in the *Journal of the French Academy of Sciences* describing daily rhythmic opening and closing of the leaves of a heliotrope plant. Remarkably, DeMarain noted, this rhythm persisted in the absence of light. He closed his paper with the following: "The progress of true science, which is the experimental, cannot be but most slow". In the ensuing quarter of a millennium, DeMarain's prophesy held for the study of the rhythms he discovered. Circadian rhythms — self-sustained, nearly 24 hour rhythms of behavior and physiology that persist in the absence of external time cues — have been found to be a nearly ubiquitous feature of eukaryotic life. These internal rhythms are a major influence on the temporal organization of behavior and physiology, from the sleep–wakefulness cycle in vertebrates to pupal eclosion in insects. Despite intensive analysis of the physiological properties of circadian rhythms, no clear insight into their molecular mechanisms was apparent until recently. In the last few years, however, a spate of papers has begun to define the genetic and molecular basis of circadian time-keeping in the fruitfly *Drosophila melanogaster*. These findings, along with important progress in dissecting circadian rhythms of the fungus *Neurospora crassa* [1], begin to reveal the essential inner workings of the circadian clock.

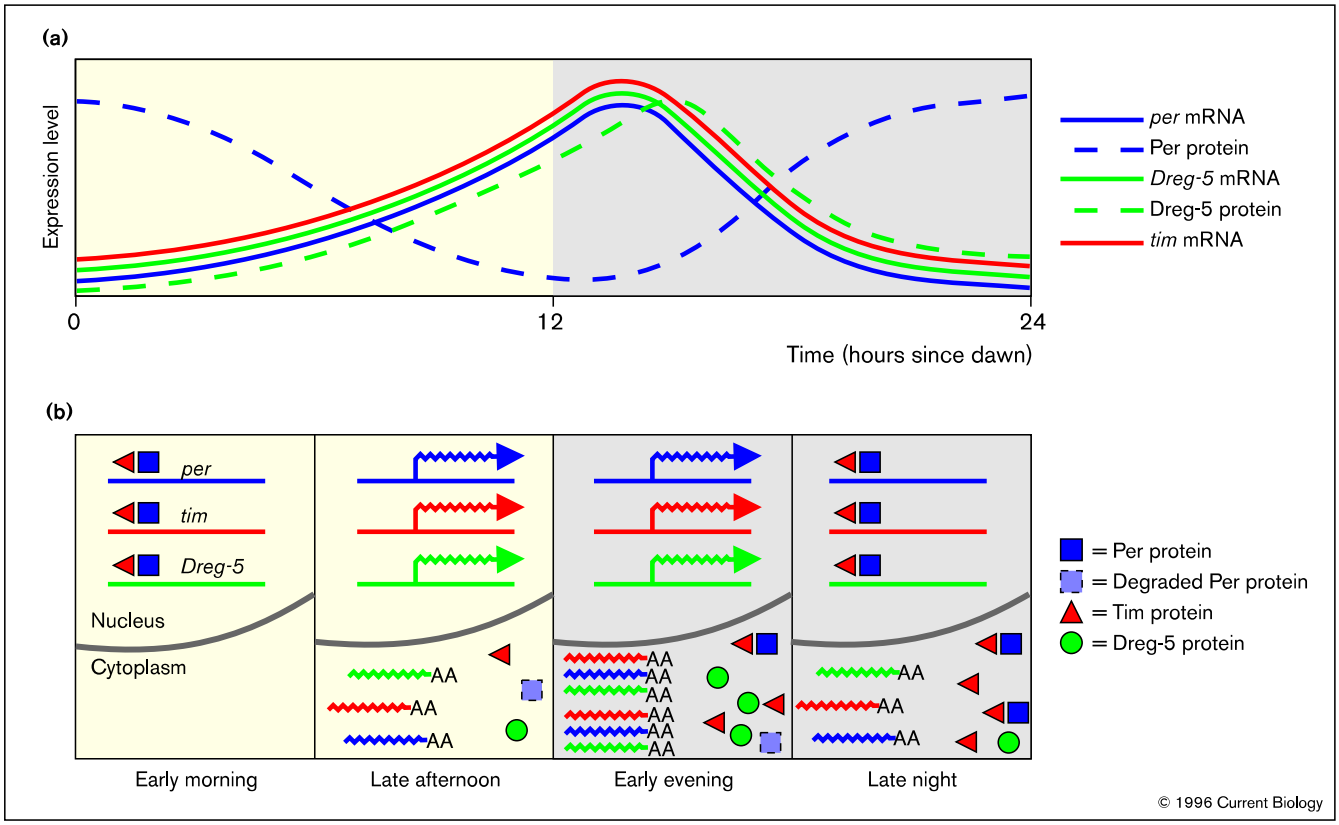
It is 25 years since Konopka and Benzer's pioneering studies identified three *Drosophila* X-chromosome mutants with altered daily rhythms of locomotor activity and pupal eclosion. All three mapped to the same gene, dubbed *period* (*per*). Different *per* missense alleles cause speeding up or slowing down of the circadian cycle, while the null mutant (per^0) is aperiodic. The *per* locus was cloned in the early 1980s, but the sequence of the 127 kD predicted Per protein gave little clue as to how this single gene so greatly affects the temporal organization of behavior. Subsequently, several other proteins — the product of the neurogenesis gene *singleminded* (*sim*) and two subunits of the aromatic hydrocarbon nuclear receptor (ARNT) — were found to show similarity with Per protein in a domain of around 250 amino acids. The other proteins with this so-called PAS domain, (for *per*–ARNT–*sim)*, are all basic helix–loop–helix DNA-binding proteins, although Per itself lacks any recognizable DNA-binding motif. The PAS domain has been shown to function in heterotypic and homotypic protein–protein interactions [2]. Although Per's similarity to DNA-binding proteins and its predominantly nuclear localization in the brain suggest a role in the regulation of gene expression, biochemical functions for the Per protein remained obscure.

Central to the understanding of *per*'s role in the generation of circadian rhythms was the discovery that Per protein and mRNA undergo circadian rhythms of expression in the brain (Fig. 1a) [3,4]. Hardin *et al.* [4] demonstrated that the mRNA rhythm was dependent on the function of wild-type Per protein; the abundance of mRNA from the per^0 allele does not oscillate, but its oscillation is restored by introduction of the wild-type gene. Per protein is thus required to mediate the rhythmic expression of its own mRNA, establishing a genetic feedback loop. This loop is central to the circadian oscillator (see below). Recent work has established that *Neurospora* also has an autoregulatory feedback loop that is central to its circadian oscillator, involving the *frequency* gene [1].

Researchers of circadian rhythms have long sought the 'state variables' of the oscillator: those quantities whose oscillations are essential to the maintenance of the circadian rhythm. The oscillatory feedback of Per protein on its own transcription make *per* mRNA and protein excellent candidates for *bona fide* state variables. This hypothesis predicts that a transient increase in the amount of *per* expression at any one phase of the cycle should shift the clock to a new phase, and in fact such an effect was noted by Edery *et al.* [5]. The hypothesis also predicts that circadian oscillations should not occur in the absence of oscillations in *per* expression. Indeed, Frisch *et al.* [6] have demonstrated a perfect correlation between the oscillation of *per* mRNA and restoration of behavioral rhythmicity in per^0 mutants rescued with *per* transgenes.

Negative feedback of the Per protein on its own transcription, however, is not sufficient to give rise to stable rhythms of expression. Such a feedback system would damp rapidly to a tonic steady state unless there were a time-delay in the feedback process or a secondary oscillator [7]. A time-delay is present in the *per* feedback system: the Per protein rhythm lags that of *per* mRNA by

Figure 1



(a) Idealized graph showing the oscillating levels of *per*, *tim* and *Dreg-5* mRNAs (solid lines), and Per and Dreg-5 protein (dashed lines) in a 24 hour period. The yellow and grey shading indicate ambient light and darkness, respectively. Probes are not yet available for analysis of Tim protein. (b) A cellular model of the *period–timeless* circadian oscillator (see [11]). Each panel shows a brain pacemaker neuron at a different time of day. In the early morning, the Per–Tim protein complex represses the *per* and *tim* genes, as well as the clock-controlled gene, Dreg-5; transcript levels of these genes are at their lowest. In the late afternoon, Per and Tim proteins have been degraded, and transcription of all three genes is coordinately derepressed. In the early evening, transcript levels reach their maximum and Dreg-5, Per, and Tim proteins are all synthesized. Dreg-5 protein accumulates but Per protein does not, perhaps because free Per protein is rapidly degraded. In the night, sufficient Tim has accumulated to bind and stabilize Per protein. The complex enters the nucleus and represses transcription, and mRNA levels from all three loci begin to decline.

approximately 6 hours (Fig. 1a). The biochemical basis for this delay is unknown, but it might involve another state variable of the clock. The existence of additional clock components is also suggested by the observation that, although *per* mRNA is expressed in the fly body as well as the brain, its abundance does not oscillate in the body.

A flurry of recent research has begun to describe the anticipated second component of the circadian clock. The *timeless* (*tim*) gene was identified in a large-scale autosomal screen for rhythm mutants performed in the laboratory of Michael Young [8]. Null *tim* mutant flies behave very much like null *per* mutants: both fail to demonstrate circadian rhythms of eclosion or locomotion, and *per* mRNA does not cycle in either. In *tim* mutants, Per protein is produced at a constant low level, and nuclear localization of a Per–β-galactosidase fusion protein is disrupted [8,9]. There is thus strong evidence that *tim* is essential for the *per* feedback process and the function of the circadian clock.

The recent molecular characterization of *tim* suggests how it may function in this process. The locus was positionally cloned, and the predicted 156 kD Tim protein is not related to any known protein [10]. However, levels of *tim* mRNA were found to oscillate in exact phase with *per* mRNA (Fig. 1a) [11]. Cycling of *tim* mRNA ceases in per^0 mutants, demonstrating that the function of both genes is required to sustain their mutual oscillations, and for behavioral rhythms. The *tim* gene was independently isolated by a second approach. Gekakis *et al.* [12] performed a yeast two-hybrid protein–protein interaction screen using the PAS domain of Per as the 'bait', and they isolated 48 plasmids encoding 'fish' proteins that apparently interact with this domain. Of these, 16 encode large, overlapping

fragments of the Tim protein; experiments with glutathione-S-transferase fusion proteins *in vitro* confirmed a direct biochemical interaction between the Per and Tim proteins. These authors also showed that the per^{L} mutation — a missense mutation affecting the PAS domain that lengthens the circa dian period — may weaken the Per–Tim interaction.

The identification of two interacting clock components has led to the formulation of explicit molecular models of the circadian pacemaker [11]; one is shown in Figure 1b. In this model, transcription of *per* and *tim* is repressed in the early morning by a protein complex containing both Per and Tim. As the concentration of the Per–Tim complex declines in the late day as a result of turnover of the proteins without replacement, *per* and *tim* mRNAs begin to be synthesized. Tim protein begins to accumulate, and after reaching a critical concentration it binds to, and stabilizes, the intrinsically unstable Per protein. The Tim–Per complex then translocates to the nucleus, where it represses both *tim* and *per* gene expression, beginning another cycle. In this way, the accumulation of Tim protein could provide the time delay essential to the function of the circadian oscillator.

A great number of important, addressable questions arise from this model. How do Per and Tim function to modulate gene expression when neither has a recognizable DNA-binding motif? How does ambient light act on the circuit to 'entrain' circadian rhythms to natural light–dark cycles? Light appears not to affect *per* mRNA levels directly; does it affect *tim* mRNA levels, perhaps in a manner analogous to the entrainment mechanism recently described for the *frequency* gene in *Neurospora* [13]? Does light change the phosphorylation state of Per or Tim proteins and so affect their interaction with each other and the phase of the clock? How are *per* and *tim* functions affected by ambient temperature in order to maintain the relatively constant length of the circadian day over a wide range of temperatures [14]?

Finally, there is the q uestion of how information from this feedback oscillator in the brain is transduced into daily timing signals that control such diverse behaviors as locomotor activity and eclosion from the pupal case. Implicit in the genetic feedback model is the existence of other loci whose transcription is also controlled by the Per–Tim protein complex but which function in 'clock-output' pathways. Our recent large screen for clock-controlled genes in *Drosophila* identified 20 mRNAs in the head that have appreciable rhythms of expression, 17 of which are in phase with *per* and *tim* mRNA rhythms [15]. A subset of these oscillating mRNAs were found to be predominantly dependent on the function of Per (and, presumably, Tim). For one such gene, *Dreg-5*, mRNA cycling is phase-locked with *per* and *tim* cycling, although the accumulation of Dreg-5 protein does not show the 6 hour delay seen for Per protein (Fig. 1a) [16]. Dreg-5 is a good candidate for a clock-output signal that is active at dusk and early evening.

Five years ago, the molecular processes underlying circadian rhythmicity were as unclear as they were for DeMarain 267 years ago. It is now well established that a fundamental mechanism underlying the circadian pacemaker is the auto-regulatory and cross-regulatory control of daily expression patterns of critical clock loci, whose functions appear to be dedicated to daily time-keeping. This discovery allows the armamentarium of molecular genetics to be applied to the problem of temporal control of behavior — a problem that at first seemed intractable to molecular genetic analysis, but whose essential elements are now being revealed.

References

1. Loros J: The molecular basis of the ***Neurospora*** clock. *Semin Neurosci* 1995, 7:3–13.
2. Huang ZJ, Edery I, Rosbash M: PAS is a dimerization domain common to ***Drosophila period*** and several transcription factors. *Nature* 1993, 364:259–262.
3. Siwicki K, Eastman C, Petersen G, Rosbash M, Hall J: Antibodies to the ***period*** gene product of ***Drosophila*** reveal diverse tissue distribution and rhythmic changes in the visual system. *Neuron* 1988, 1:141–150.
4. Hardin P, Hall J, Rosbash M: Feedback of the ***Drosophila period*** gene product on circadian cycling of its messenger RNA levels. *Nature* 1990, 343:536–540.
5. Edery I, Rutilla J, Rosbash M: Phase shifting of the circadian clock by induction of the ***Drosophila period*** protein. *Science* 1994, 263:237–240.
6. Frisch B, Hardin P, Hamblen-Coyle M, Rosbash M, Hall J: A promoterless ***period*** gene mediates behavioral rhythmicity and cyclical ***per*** expression in a restricted subset of the ***Drosophila*** nervous system. *Neuron* 1994, 12:555–570.
7. Goodwin B: *Temporal Organization in Cells.* London: Academic Press; 1963.
8. Sehgal A, Price J, Mann B, Young M: Loss of circadian behavioral rhythms and ***per*** RNA oscillations in the ***Drosophila*** mutant ***timeless***. *Science* 1994, 263:1603–1606.
9. Vosshall L, Price J, Sehgal A, Saez L, Young M: Block in nuclear localization of ***period*** protein by a second clock mutation, ***timeless***. *Science* 1994, 263:1606–1609.
10. Myers M, Wager-Smith K, Wesley C, Young M, Sehgal A: Positional cloning and sequence analysis of the ***Drosophila*** clock gene, ***timeless***. *Science* 1995, 270:805–808.
11. Sehgal A, Rothenfluh-Hilfiker A, Hunter-Ensor M, Chen Y, Myers M, Young M: Rhythmic expression of ***timeless***: a basis for promoting circadian cycles in ***period*** gene autoregulation. *Science* 1995, 270:808–810.
12. Gekakis N, Saez L, Delahaye-Brown A-M, Myers M, Sehgal A, Young M, Weitz C: Isolation of ***timeless*** by PER protein interaction: defective interaction between ***timeless*** protein and long-period mutant PER^{L}. *Science* 1995, 270:811–815.
13. Crosthwaite S, Loros J, Dunlap J: Light-induced resetting of a circadian clock is mediated by a rapid increase in ***frequency*** transcript. *Cell* 1995, 81:1003–1012.
14. Huang Z, Curtin K, Rosbash M: PER protein interactions and temperature compensation of a circadian clock in ***Drosophila***. *Science* 1995, 267:1169–1172.
15. Van Gelder R, Bae H, Palazzolo M, Krasnow M: Extent and character of circadian gene expression in ***Drosophila melanogaster***: identification of 20 oscillating mRNAs in the fly head. *Curr Biol* 1995, 5:1424–1436.
16. Van Gelder R, Krasnow M: A novel circadianly expressed gene dependent on the ***period*** gene for its rhythmic expression. *EMBO J* 1996, 15: in press.