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Time Flies for *Drosophila*

Minireview

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Circadian rhythms, based upon a 24 hr cycle, function in both eukaryotes and prokaryotes to keep the organism in tune with its environment. Endogenous cell-autonomous clocks control daily molecular cycles, which manifest themselves as physiological and behavioral oscillations. Environmental stimuli such as light–dark (LD) or temperature cycles entrain, or synchronize, these cellular clocks to the outside world. The molecular basis for circadian clocks in various organisms has been reviewed extensively (Dunlap, 1999). Transcriptional feedback loops have emerged as a common theme in molecular clocks from cyanobacteria to mice. This minireview focuses on the recent progress in the field of clock research in *Drosophila melanogaster*, which has been the most productive for circadian studies to date. The rate of discovery is likely to increase dramatically with the long-awaited release of the fly genome sequence and the availability of genomic tools such as DNA microarrays, which will allow for comprehensive definitions of clock-controlled gene expression. Recent research using *Drosophila* has identified several components of the molecular clock and, of course, raised many questions regarding their mechanism of action. The circadian clock appears to be composed of multiple feedback loops, and the search for proteins that transmit environmental information to the clock, as well as the timing signals from the clock outward to cellular functions, is underway.

Interlocked Feedback Loops

For a few years the clock looked relatively simple: the core transcription/translation feedback loop is dependent upon levels of *period* (*per*) and *timeless* (*tim*) mRNA rising during the day and peaking in the early evening. Transcription of *per* and *tim* is activated by the heterodimer of dCLOCK (dCLK) and CYCLE (CYC), two basic helix-loop-helix-PAS (PER-ARNT-SIM) transcription factors (Darlington et al., 1998; Hogenesch et al., 1998). In the evening, rising levels of PER-TIM heterodimers translocate into the nucleus and downregulate their own activation, thus establishing oscillating levels of PER and TIM (see Dunlap, 1999 and references therein). As PER and TIM are not likely to directly bind DNA, this deactivation presumably occurs by inhibiting the dCLK-CYC heterodimer. Precisely how PER and TIM inhibit their own transcriptional activation by the dCLK-CYC complex has been unclear until recently.

Work by Lee et al. (1999) has begun to fill in the gaps in our understanding of the actual mechanics of the dCLK-CYC/PER-TIM feedback loop. Previous research suggested that dCLK and CYC activate *per* and *tim* by binding an E box (CACGTG) target sequence in their promoters (Hogenesch et al., 1998). Indeed, Lee et al. showed that in vitro dCLK and CYC together, but not separately, can bind to the E box with flanking DNA from the *per* promoter. When the mobility shift assay was repeated using dCLK-CYC premixed with PER and/or TIM, the ability of dCLK-CYC to bind to the E box target sequence of *per* was diminished. Were PER and TIM acting as competitive inhibitors of heterodimerization by sequestering dCLK, or were they preventing the dCLK-CYC heterodimer from binding DNA? Lee et al. found that when dCLK and CYC were mixed together with PER and/or TIM in vitro, the same amount of CYC was bound to dCLK as when PER and TIM were not present. These results suggest that PER and TIM do not disrupt the dCLK-CYC complex, but instead associate with dCLK-CYC to form trimers or tetramers that have a diminished ability to bind DNA. As PER or TIM can interfere with the dCLK-CYC complex individually, it is possible that PER or TIM monomers have a role in regulation of the feedback loop once inside the nucleus. Little is known about the state or fate of these proteins following nuclear translocation. Furthermore, the precise cellular expression pattern of dCLK or CYC is not known, and it would be reassuring to find that they are present in the same cells as PER and TIM, and more importantly in cells known to control rhythmic behavior.

Another interesting question is how PER and TIM contribute to the antiphasic cycling of *dClk*. Recent progress toward answering this has come from examining *dClk* mRNA levels in various clock mutant backgrounds, as *dClk* cycling may also be regulated by an interplay of PER, TIM, CYC, and dCLK. *dClk* mRNA is upregulated in *dClk* or *Cyc* single mutants, downregulated in *per* null flies, and upregulated again in *per* and *dClk* or *Cyc* double mutants (Glossop et al., 1999). This suggests that *dClk* may act as its own repressor. However, it is not known whether *dClk* represses its own transcription directly, or whether the repressor is an unidentified factor whose expression is dependent upon the dCLK-CYC heterodimer (Glossop et al., 1999). There must also be a separate *dClk* activator(s) that is independent of PER, dCLK, and CYC (see Figure 1); otherwise, high levels of *dClk* mRNA would not occur in the absence of PER and dCLK or CYC. Intriguingly, expression of *cryptochrome* (*cry*), which is involved in entrainment of behavioral rhythms by light, cycles in phase with *dClk*, and *cry* (Emery et al., 1998) and *dClk* (Glossop et al., 1999) mRNA levels are affected the same way in various clock mutant combinations. Therefore, *cry* is likely to be regulated by the same mechanism as *dClk*.

New Spoke in the Wheel

Now that your head is spinning thinking about the interlocked feedback loops within the circadian oscillator, it is time to add a new player: *vrille* (*vri*). Most of the fly clock components have been identified in forward genetic screens, but genomics approaches are now contributing important results. In a differential display screen using adult fly heads, Blau and Young identified *vri* as a gene oscillating in phase with *per* and *tim* (Blau

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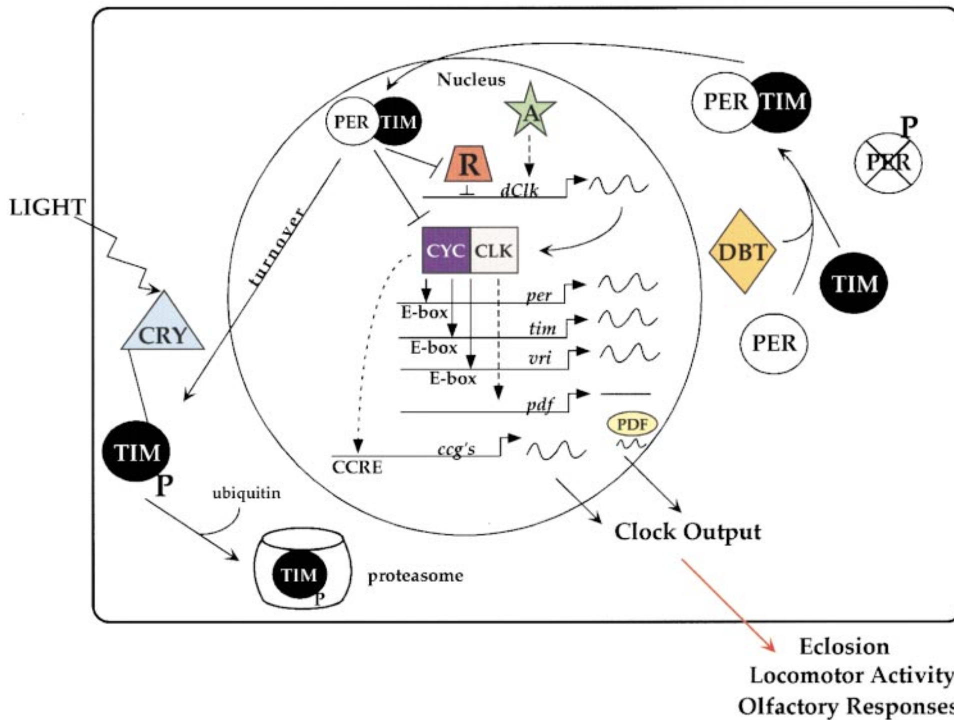

Figure 1. The Circadian Oscillator in *Drosophila*

Depiction of the interlocked feedback loops of the central pacemaker and the clock's response to light. Arrows indicate positive regulation and lines ending in bars denote negative regulation. R, repressor; A, activator; CCRE, circadian clock regulatory element; and ccg, clock controlled gene.

and Young, 1999). Like *tim*, *vri* mRNA is expressed in the ventral and dorsal lateral neurons (LNs) and photoreceptor cells (Blau and Young, 1999), which have functional clocks. Since *vri* cycles in phase with *per*, the dCLK-CYC-dependent mechanism for *per* activation may apply to *vri* as well. These studies demonstrated that *vri* does indeed have an E box sequence, similar to that of the *per* promoter, from which dCLK activates transcription in cultured S2 *Drosophila* cells. Furthermore, when *vri* dosage is reduced by half, locomotor activity period length is shortened by about half an hour, as with a heterozygous deletion of *per* (see Blau and Young, 1999 and references therein). However, placing *vri* within the clockworks is difficult, as it is becoming harder to distinguish between cyclic input factors and "central" pacemaker components. Nevertheless, constitutively expressing *vri* in pacemaker cells gives some evidence for *vri* being a central clock protein. These experiments resulted in *per* and *tim* mRNAs being suppressed in the pacemaker cells, and long period and arrhythmic phenotypes in the behavioral rhythms (Blau and Young, 1999). Disturbing *vri* oscillation disrupts normal cycling of clock gene products, but determining if *vri* is a component of the core feedback loop awaits more elaborate manipulations of its expression pattern.

Connection of TIM to Light

The feedback loop is dependent upon cyclic expression of *per* and *tim*, and the entrainment of the clock relies upon the degradation of TIM in response to light. Exactly how light is relayed to signal TIM's degradation still remains ambiguous, but another piece of the puzzle has recently been put into place by connecting TIM to *cry* and its perception of light. The entrainment of the behavioral rhythms by light uses both rhodopsin and *cry*, which is related to flavin-mediated blue light photolyases (for review see Devlin and Kay, 1999). Yeast two-hybrid assays showed that CRY interacts with TIM and PER-TIM in the presence of light but not the dark (Ceriani et al., 1999). This interaction was specific to functional CRY since CRY^b, a mutation that leaves CRY at least partially inactive (Stanewsky et al., 1998), is unable to interact with TIM or PER-TIM. The potential for CRY-TIM physical interactions in flies is supported by the immunoprecipitation (IP) of CRY-GFP from S2 cells stably transfected with TIM (Ceriani et al., 1999).

The same authors investigated the influence of various combinations of clock genes on dCLK-based transcriptional activation using transient transfection in S2 cells and a luciferase reporter under the control of the *tim* promoter. In this assay, the dCLK-CYC heterodimer activates the *tim* promoter, and the addition of PER-TIM reduces this activation. Adding CRY to these clock proteins allows for almost full activation of the *tim* promoter when the S2 cells are maintained in the light, but not the dark (Ceriani et al., 1999). This appears to be the result of CRY interacting with TIM, presumably signaling its degradation, as CRY does not have this effect in the absence of PER-TIM, while CRY^b does not have any effect on this assay (Ceriani et al., 1999). In vivo, TIM is degraded in larval lateral neurons in response to light (Naidoo et al., 1999), but more experiments (such as co-IPs from fly tissues) will be needed in order to

demonstrate that CRY and TIM interactions are functionally relevant in the animal.

How TIM is degraded was a mystery until the recent demonstration that proteasomes are involved in TIM degradation (Naidoo et al., 1999). TIM protein was shown to be degraded in the presence of head extracts from LD-entrained flies light-pulsed in the dark. Unpulsed head extracts did not affect TIM levels, PER levels remained unaffected, and proteasome inhibitors blocked the degradation of TIM (Naidoo et al., 1999). In addition, S2 cell culture assays demonstrated that TIM, but not PER, is ubiquitinated in cells following a 2 hr light treatment (Naidoo et al., 1999). The ubiquitination of TIM supports the idea that TIM is degraded by the proteasome, as most cellular proteins that are degraded by the proteasome are ubiquitinated before proteasomal targeting.

PER is rendered unstable by phosphorylation by DBT (Price et al., 1998), which led Naidoo et al. to examine whether TIM is phosphorylated in response to light as part of its degradation pathway. Although *per* and *tim* mRNA levels peak in the early evening, PER and TIM protein levels do not peak until several hours later. This delay is due to *double-time* (*dbt*) (Kloss et al., 1998; Price et al., 1998). PER monomers are unstable once they are phosphorylated by DBT; however, when bound to TIM, PER remains stable and the feedback loop is maintained. TIM contains sequences that are putative degradation/phosphorylation signals. Light-induced TIM degradation was blocked by the presence of tyrosine kinase inhibitors (Naidoo et al., 1999). In addition, TIM immunoprecipitated from fly head extracts showed phosphorylation when the flies received a light-pulse prior to collection (Naidoo et al., 1999). Based upon these observations, TIM appears to be phosphorylated, ubiquitinated, and then targeted to the proteasome for degradation in response to light. The questions remain as to which tyrosine kinase is phosphorylating TIM to signal its degradation, and which kinase (if it is not DBT) causes cyclic phosphorylation of TIM in constant darkness (DD).

Flies on Their Best Behavior

Perhaps the greatest current challenge in *Drosophila* circadian research is linking the central pacemaker to the control of behavior, metabolism, or development. In an effort to understand how the pacemaker actually converts its molecular cycles into rhythmic behavioral output, recent research has focused on the neuropeptide pigment-dispersing factor gene (*pdf*) (Park and Hall, 1998). Renn et al. (1999) examined the *pdf* gene for its potential as a circadian transmitter, and asked whether the ventral cluster of lateral neurons (LN_vs), which are candidate pacemaker cells, is the location in which the function of the clock interfaces with output factors. Most LN_v cells that express *per* also express neuropeptides related to the crustacean pigment dispersing hormone (β-PDH) which has pharmacological properties consistent with a circadian role in insects.

Analysis of the null mutation for *pdf*, *pdf*^{01}, demonstrates that *pdf* is indeed involved in the circadian output regulating rhythmic locomotor behavior in flies. Renn et al. report that flies lacking PDF are well entrained during LD conditions, although the evening peak in activity occurs ~1 hr earlier than wild-type, and mutant animals do not anticipate the lights-on signal as well (Renn et al., 1999). In free-running/DD conditions, 50%–98% of the flies become arrhythmic depending upon the genetic background (Renn et al., 1999). Most of the flies maintain rhythmicity for the first 2–3 days of DD, which is lost with continued free-running conditions. Although PDF is expressed in LN_vs, these neurons are normal in number and morphology in *pdf*^{01} mutant animals and the mutant phenotypes in *pdf*^{01} flies can be rescued with wild-type *pdf* (Renn et al., 1999).

What happens to circadian rhythms when these LN_vs are missing from the animal? In flies lacking many or all PDF-expressing lateral neurons, locomotor activity phenotypes closely mimic the *pdf*^{01} mutant effects (Renn et al., 1999). Intriguingly, both the ablated as well as *pdf*^{01} mutant animals retain some aspects of rhythmicity. This suggests that additional timekeeping mechanisms are present in neurons other than the LN_vs.

Recent research has shown that some of the clock genes affect *pdf* expression. Mutations in *dClk* or *Cyc* reduce PDF staining in the large LN_vs (l-LN_v), and disruption of *vri* oscillations also results in reduced PDF staining (Blau and Young, 1999). However, the level of *pdf* mRNA in l-LN_vs is not affected in larvae continuously expressing *vri*, but it is absent in *dClk*^{Jrk} mutants (Blau and Young, 1999). Although these three clock proteins affect *pdf* expression, *per* and *tim* null mutations do not affect PDF levels (Blau and Young, 1999). How dCLK, CYC, and VRI regulate *pdf* expression remains to be elucidated. However, as *pdf* mRNAs levels do not appear to cycle (Park and Hall, 1998), PDF protein activity is likely to be cycling at some level in order for PDF to affect regulation of rhythmic locomotor activity.

As we begin to unravel how the molecular clock signals to output relays, we must determine what are the physiological outputs of the circadian clock that might provide an adaptive advantage for the organism. Circadian rhythms in locomotor activity and emergence from the pupal case, eclosion, have been known for quite some time. However, identifying other biological processes the clock controls in flies has only just begun. Previous studies have shown that autonomous rhythms in *per* expression occur in *Drosophila* antennae as well as other tissues (Hege et al., 1997; Plautz et al., 1997b) and that almost every body part has a clock (Plautz et al., 1997a). However, until recently there was no evidence that these peripheral oscillators are necessary for any behavioral or physiological response in flies. One group has now identified circadian rhythms in olfactory responses in flies, and has shown that this novel circadian response fails to occur when the antennae lack a functional clock. Krishnan et al. (1999) measured electroantennogram (EAG) responses to odorants in flies and found that LD-entrained wild-type flies gave EAG responses to ethyl acetate which were elevated at night. In *per* or *tim* null mutant flies, the EAG responses remained steady state. Similar EAG patterns were seen in DD and LD. Furthermore, this rhythm is not restricted to food odors since benzaldehyde, an avoidance odor, produced similar results (Krishnan et al., 1999). In *per* transgenic flies that have rhythmic *per* expression solely in the LNs, EAG responses were robust in DD but did not have a circadian rhythm. This implies that peripheral

oscillators are necessary for circadian rhythms in olfactory responses. Olfaction is essential for acquiring food, predator avoidance, and social interactions including mating in many organisms. Therefore, by maintaining olfactory responses, peripheral clocks could have a profound effect on the ability of an animal to survive in the wild.

The ubiquity of circadian clocks from cyanobacteria to humans implies that they have some intrinsic adaptive value. The discovery of homologs of *Drosophila* clock genes in humans provides one of the richest opportunities to explore the genetics of the behavior in man using a candidate gene approach. Understanding the molecular underpinnings of the clock from input to output in the amenable system of *Drosophila* will shed light on the workings of the clock and its influence on the behavior and physiology of more complex organisms.

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