# Molecular rhythms that regulate rhythm genes in *Drosophila*

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**Almost all living organisms display rhythms in their activities coinciding with the day–night cycles. Our current understanding of the molecular regulation of circadian rhythmicity in *Drosophila* comes from studies integrating genetics and molecular biology, and *Drosophila* is perhaps one of the best understood models in the field of circadian rhythm research. Following the initial discovery of the *per* (*period*) gene some decades ago, several other genes, viz. *timeless*, *dclock*, *cycle*, and *double-time*, that function in the generation of circadian rhythms, have been identified during the past three years: Molecular genetic studies have provided exciting insights into the regulation of the body clocks. Heterodimeric complexes of positive elements (dCLOCK and CYCLE) and their interactions with feedback loops and negative elements of *per* and *tim* genes and their products have been identified and these are providing clues to the general layout of the molecular loops that generate circadian rhythms. The *lark* gene, which encodes an RNA-binding protein, might function as a regulatory element in the circadian clock output pathway controlling pupal eclosion rhythms. However, a clear picture of the output pathways or downstream processes through which the clock regulates the circadian rhythmic events is yet to be understood.**

A large variety of biochemical and physiological functions, and behavioural events in living organisms show periodic or rhythmic fluctuations in parallel with the day–night (light–dark) cycles. However, since even under continuous light or continuous darkness these rhythms persist with a periodicity of approximately 24 h, it demonstrates that the control of these rhythms is by endogenous oscillators or clocks[1]. The spectrum of biological processes controlled by these clocks ranges from the daily sleep/wake cycle and levels of various enzymes/hormones to DNA synthesis and cell division[1]. Extensive studies have shown that these circadian (*L. circa*, about; dies, a day) rhythms indeed have a genetic basis[2].

The circadian organization of any living organism is composed of three broad domains: (i) the input pathways – that transmit the environmental signals (mainly light–dark cycles) to the central oscillator/clock, (ii) the generation of timing signals in the central oscillator/clock, and (iii) the output pathways – that transmit these rhythmic signals with a 24 h periodicity to the various clock-controlled processes which ultimately result in the overt rhythms that can be measured experimentally.

Adult *Drosophila* flies exhibit locomotor activity rhythms, with a periodicity of approximately 24 h, which resembles the mammalian sleep–wake cycles[1,3]. Another well-demonstrated circadian rhythm in fruit fly is the eclosion or emergence of adults from the pupal cases[1,3]. These two rhythms persist under continuous light or continuous darkness with periods of approximately 24 h (refs 1, 3). However, the fruitflies, reared under continuous darkness, when exposed to pulses of light, advance or delay these rhythms in a fashion that is dependent on the time of light administration with respect to the phase of the behavioural cycle[1,3]. In this review article we discuss the molecular basis of circadian rhythmicity in *Drosophila*.

## The master oscillators: PER and TIM

Our current understanding of the molecular regulation of circadian rhythmicity in *Drosophila* comes from the studies that have integrated genetics and molecular biology. Twenty-eight years ago, Konopka and Benzer[4] discovered the X-chromosome-linked *period* (*per*) mutations that altered the daily rhythms of locomotor activity exhibited by *Drosophila*, and its pupal eclosion. The *per* locus was subsequently cloned in the early 1980s, but the amino acid sequence of the 127-kD PER protein[5] gave little clue as to how this gene affected the temporal organization of the above two events. PER protein possesses a protein–protein interaction domain, PAS, through which it binds with the product of the another clock gene, *timeless*. The PAS domain shows similarities with the product of another *Drosophila* gene, *single-minded*, and a mammalian gene, *ARNT*-aryl-hydrocarbon receptor nuclear translocator.

Four years ago, it was reported that along with PER, another product, TIM, of *timeless* (*tim*) gene was also involved in circadian rhythms[6]. In the adult fly head (the presumed anatomical location of the fruit fly circadian pacemaker), the PER and TIM proteins undergo daily fluctuations in their abundance[7–9], phosphorylation state[10],

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sub-cellular distribution[7,11,12], and native size; consistent with an important role complex in the cytoplasm[9,12] that enters the nucleus in a temporally gated manner[11,12]. This sequence of events subsequently results in the inhibition of *per* and *tim* transcription[6,13–15]. This gave an impetus to further studies on these two loci.

Eventually, the *tim* locus was positionally cloned in 1995; but the predicted 165-kD TIM did not show homology/relation to any other proteins known at that time[16]. However, a more recent detailed analysis of the conserved regions of *tim*[17] gene from another species, *D. virilis* have revealed that TIM is better conserved than the PER protein between the two species *D. melanogaster* and *D. virilis* (76% vs 54% overall amino acid identity), and the putative functional domains, such as the PER-interaction domains and the nuclear-localization-signal of the TIM are highly conserved. On the other hand, the acidic domain and the cytoplasmic localization domain, are however, within the least-conserved regions[17].

Hamblen *et al.*[18] characterized four novel *per* mutants of *D. melanogaster* showing altered periodicities. The three mutants: $per^{T}$, $per^{clk}$, and $per^{SLIH}$, arose as a result of a single nucleotide change that led in these mutants the following amino acid substitutions: $per^{T}$-Gly → Asp substitution at position 593; $per^{SLIH}$-Ser → Tyr substitution at 45; $per^{clk}$-Ala → Val substitution at position 969; while the fourth mutant, $per^{04}$, was altered at a splice-acceptor site that resulted in aberrant splicing, and consequently in arrhythmicity[18]. Two of these mutations ($per^{SLIH}$ and $per^{clk}$) lie within regions of PER that have never been mutated before. Both are outside the PER/TIM interaction site and are not within any other PER domains known (whether or not associated with *per* mutants). Thus studies of $per^{SLIH}$ and $per^{clk}$ regions may prove to define functionally important domains within the clock protein.

### *The mode of regulation of per and tim RNA cycling*

Hao *et al.*[23] identified a circadian transcriptional enhancer within a 69-bp DNA fragment upstream of the *per* gene. This enhancer affected high level of *per* mRNA cycling under light–dark or continuous conditions, and this activity was reportedly dependent on the PER protein. An E-box sequence (5′-CACGTG-3′), within this 69-bp fragment, has been reported to be necessary for high-level expression, but not for rhythmic expression, indicating that PER mediated circadian transcription through other sequences within this 69-bp fragment. *tim* gene promoter has also been reported to contain an 18-bp enhancer containing the E-box (5′-GCCGCT**CACGTG**GCGAAC-3′) which functions in a manner similar to the enhancer of the *per* E-box[24].

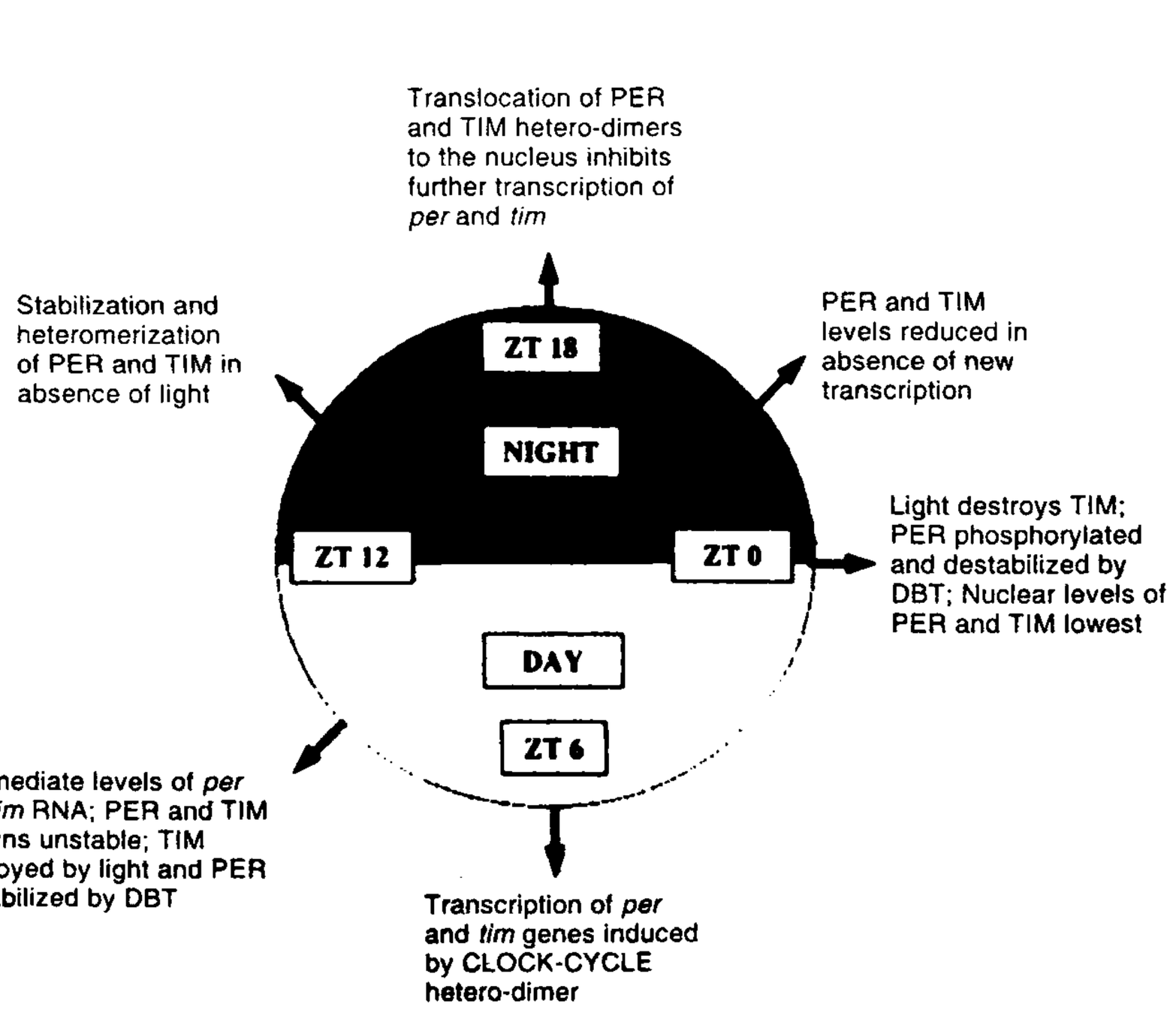


**Figure 1.** Model of circadian rhythm generation in *Drosophila*. The succession of events occurs over a period of 24 h. (ZT = Zeitgeber time = time during a normal LD 12 : 12 cycle; ZT 0 = lights on; ZT 12 = lights off).

### *dCLOCK and CYCLE: The two transcription factors involved in the positive regulation of per and tim activity*

The PAS domain of the PER has been shown to mediate interactions between transcription factors[25]. Most of the PAS-containing transcription factors also contain the well-characterized basic helix-loop helix (bHLH) DNA-binding domains[26]. However, PER lacks the bHLH domain and there is no evidence that PER interacts directly with DNA[27]. It was, therefore, suspected that PER binds with DNA and regulates its own transcription by interacting with other DNA-binding transcription factor(s). An extensive search for such interacting transcription factor(s) by Allada *et al.*[27] through ethyl methane sulphonate mutagenesis, identified the *Jrk* (also called *dclock*) gene which codes for a bHLH-PAS transcription factor, viz. dCLOCK. Rutila *et al.*[28] identified and characterized another bHLH-PAS transcription factor, CYCLE (also named dBMAL, a *Drosophila* homolog of the human BMAL1- brain and muscle ARNT-like factor). Lee *et al.*[29] further demonstrated that the *dclock* transcripts underwent daily oscillations: peaking in the late night/early morning, and reaching trough levels around the light–dark transition. They also reported that the dCLOCK underwent phosphorylation, and interacted with PER and TIM *in vivo*[29]. In addition, dCLOCK levels were constitutively low in the absence of PER and TIM, indicating that PER and TIM can function as transcriptional activators of *dclock* gene[29,30]. Darlington *et al.*[24] have shown that dCLOCK-CYCLE heteromeric complex binds to the E-box sequences to activate transcription of *per* and *tim* genes. Interestingly, co-expression of PER and TIM proteins inhibited transcriptional activation of their own genes, *per* and *tim*, by the dCLOCK-CYCLE complex[24]. Thus, Figure 1 is a model, based on the above-reported findings, of the circadian rhythm generation in *Drosophila*; incorporating the various events (sequentially) that take place over a period of 24 h. However, the precise molecular interactions mediating this activation/inhibition remain unknown.

### *Post-translational regulation of PER and TIM proteins*

Recently, it has been reported that the *double-time* (*dbt*) gene, encoding a kinase, with extensive homology to human casein kinase[31], Iε, is an essential component of *Drosophila* clock. *dbt* gene has been mapped near the tip of the right arm of 3rd chromosome, in between *claret* (*ca*) and *brevis* (*bv*) sites. Furthermore, cloning of the *dbt* gene has also been reported. The conceptual translation of the open reading frame of the *dbt* gene shows that the predicted protein is 440 amino acids in length with a molecular mass of 48 kD, possessing an ATP-binding site between amino acids 15 and 38, and a serine–threonine kinase catalytic domain between amino acids 124 and 136 (ref. 31).

It has also been reported that mutation in the *dbt* gene either shortened ($dbt^S$) or lengthened ($dbt^L$) the period, and a P-element-induced null, or strongly hypomorphic mutation ($dpt^P$), resulted in pupal lethality[32]. Furthermore, $dbt^P$-homozygous mutant embryos were observed to express high levels of stable and unphosphorylated PER protein, independently of circadian time; whereas *tim* RNA and protein rhythms were abolished[32]. Thus, Price *et al.*[32], based on their above-reported findings, hypothesized that the DBT protein destabilizes PER, keeping the levels of PER low, until enough TIM accumulates to pair with PER protein and, consequently, shield it from DBT. Subsequent to these molecular events, PER and TIM proteins enter the nucleus, and turn off the *per* and *tim* genes, leading to waning of the levels of these two proteins, eventually resulting in the dCLOCK and CYCLE – the two transcription factors – turning on the genes once again (see Figure 1): Thus, DBT phosphorylates and destabilizes PER, and thereby contributes to the translational delay-related accumulation of PER, essential for rhythmicity[31,32]. In addition, based on PERIOD-β-galactosidase fusion studies, Dembinska *et al.*[33] suggested that protein instability also was important for PER cycling. A post-translational modification, other than phosphorylation, within the PER amino acids at 637 and 848 residues appeared to regulate cyclic degradation of PER[33].

## Location of circadian clocks in *Drosophila*

Conventional wisdom has it that the circadian clock resides in the anterior hypothalamus of the brain in mammals. In *Drosophila*, it is the lateral neurons which appear to be important for circadian regulation[19]. Intriguingly, however, brain-independent circadian oscillators (cells capable of self-sustained rhythmic output) have been detected in many peripheral tissues of *Drosophila*[20–22]. For example, the Malpighian tubules of the decapitated as well as of the non-decapitated flies carrying the *per-lacZ* reporter transgene, displayed identical circadian rhythms of β-galactosidase expression as well as its localization in the nucleus[20]. Kay and colleagues have elegantly extended this observation, using a real-time Green Fluorescent Protein reporter expression, and have demonstrated that PER appears, disappears and reappears over and over in a cyclic manner in the legs, wings, thorax, head, and abdomen of the fly[22]. Furthermore, even cultured cells and tissues could be entrained by light indicating that nonneuronal *Drosophila* cells are photoreceptive and are capable of supporting their own independent oscillations[22]. Their nature of these photo receptors remains unknown.

## Cryptochrome: the circadian photoreceptor or a component of the clock?

Cryptochrome, a light-absorbing protein sensitive to blue light, was first discovered in the plant *Arabidopsis*

*thaliana*[34], and has been recently shown to be present in fruit flies and mice also. It is reported that, like white light, blue light also shifts the clock by inducing degradation of TIM (ref. 35). However, cryptochrome could not be the fly's only circadian photoreceptor; since the clocks were not cycling properly in most cells of the $cry^b$ mutant flies which lacked cryptochrome, the flies' behaviour followed a normal rhythm and its timing could be reset by new light–dark cycles[36]. Furthermore, it turned out that the clock continued to cycle normally in the brain neurons that control behavioural rhythms[36,37]. However, the possibility cannot be denied that cryptochrome could be more than just a photoreceptor. Since in normal flies it has been reported that the clock functioned perfectly in the dark with the TIM levels oscillating on a usual 24 h schedule; the $cry^b$ mutation resulted in blocking the light response, TIM should have continued to follow its natural circadian cycle as though it were in the dark. But, nevertheless, in most cells of the fly, the $cry^b$ mutation did stop TIM from cycling altogether. This indicates that the mutants were doubly defective. On the basis of the above-reported results, there exists a strong possibility of cryptochrome interaction with some of the other clock factors as well[36,37].

## Output pathways for eclosion rhythms

Although considerable information is available on the molecular elements of the *Drosophila* clock, much less is known about the output pathways that mediate the actual control of rhythmic events. Analysis of the *lark* gene suggested that it is under circadian clock control and might be on the output pathway[38]. Gene dosage experiments showed that decreased or increased levels of LARK protein led to an early or late eclosion phenotype, respectively, indicating that this protein negatively regulates the eclosion process[39,40]. The early eclosion phenotype of *lark* mutants was observed when pupal populations were synchronized to either light/dark, or temperature cycles[39,40], excluding the possibility that the defect resulted from a lesion in the light-input pathway. The *lark* gene encodes an RNA-binding protein (LARK) of the RNA recognition motif (RRM) class. This LARK protein was reported to oscillate rhythmically in abundance over the 24 h period[38], showing peak and trough levels at CT8 and CT20, respectively (CT = circadian time; CT0 = subjective 'sunrise' under continuous conditions) in the presence of a functional *per* gene[38]. In contrast, the *lark* mRNA, however, did not exhibit the above-observed diurnal fluctuations. Therefore, it is not the rhythmic transcription of the gene that contributes to the temporal abundance of the LARK protein. *lark* gene is expressed both in lateral neurons – the proposed site of *Drosophila* master clock – as well as in the eclosion-regulating cells in the ventral nervous system[38]. These results therefore suggest the possibility of a specific output pathway for pupal eclosion, which is controlled by the central circadian oscillator, via the ventral nervous system[38]. Furthermore, it has also been postulated that LARK might mediate the regulation of adult eclosion through a repressor function by post-transcriptionally repressing 'downstream target genes' of the clock output pathway[38–40]. Moreover, although LARK protein is generally localized in the nuclei of neurons[38], it is also known to have a cytoplasmic distribution in a subset (~ 15–20) of neurons that contain the neuropeptide, the crustacean cardioactive peptide (CCAP), which is thought to play a prominent role in the physiological regulation of pupal eclosion[41]. This suggests that a LARK-dependent translational control, by repression, may be involved in regulating the synthesis/release of CCAP in the output pathway[38,42].

### *Involvement of the disco gene and the protein kinase A in the downstream processes: A possibility*

*disconnected* (*disco*) is another gene which was suspected to be a participant in the output pathway. *disco* mutants usually have their eyes disconnected from the optic lobes of the brain, producing optic lobes which exhibit severe anatomical defects, including the missing of lateral neurons, the site of *Drosophila* master clock[43]. These mutants show arrhythmicity of both pupal eclosion events as well as of locomotor activity[44]. Surprisingly, however, circadian oscillations of *per* mRNA in *disco* mutants have been reported as normal[42], thereby suggesting that the defect in *disco* gene was perhaps in the output signals from the oscillator to the effector organs[45,46].

Majercak *et al.*[47] suggested that protein kinase A might be playing a critical role in the flow of temporal information from circadian pacemaker cells to the selective behavioural display. They examined the protein and mRNA, products of *per* gene, in protein kinase A-deficient, *DCO*, mutant flies (*DCO* gene encodes the major catalytic subunit of protein kinase A): while the PER protein and *per* mRNA underwent normal daily cycles in the heads and bodies of the *DCO* mutants, activity rhythms continued to show arrhythmicity. Furthermore, the requirement of protein kinase A in the manifestation of rhythmic activity was preferentially greater under continuous conditions than under the light–dark cycles, indicating thereby that protein kinase A deficiency affected functioning of elements downstream of the *Drosophila*-timekeeping mechanism[47].

## Conclusions

Four years ago, the molecular processes underlying circadian rhythmicity were as unclear as they were 270 years ago, when DeMairan first discovered the circadian

rhythms[48]. Since then, it has been well established that fundamental mechanism underlying the circadian pacemaker is auto-regulatory as well as cross-regulatory in nature. However, how these closed molecular loops are coupled to the output pathways, and in the generation of circadian rhythms, is still unknown.

Significant progress in recent years, in the molecular dissection of the circadian clock, places the circadian field in an exciting era. Future studies need to focus on the functional role of each of these genes in the output pathways of clocks. In addition, further studies are also required to elucidate the signal transduction pathways that link the photoreceptor/s to the clock machinery.


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