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A CONCISE OVERVIEW OF CIRCADIAN TIMING IN *DROSOPHILA*

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1. ABSTRACT

The molecular aspects of circadian rhyhmicity in *Drosophila melanogaster* are reviewed, with particular regard to the core of the master oscillator and the light signalling input pathway. The core is schematically represented as consisting of two interlocking transcriptional feedback loops based principally on the clock genes *period*, *timeless*, *clock* and *cycle* and their products which, through the interaction with other partners, give rise to a stable 24h endogenous oscillator. Light signalling to the clock is multifaceted and is still the subject of much speculation and research. Here we review data essentially regarding the role of the clock protein Timeless and its interaction with the photopigment Cryptochrome.

2. INTRODUCTION

The molecular analysis of biological clocks is one of the most topical areas of biology and remarkable advances have been made in our understanding of the clock mechanism in a variety of model systems. By now it is well established that the circadian clock of higher eukaryotes is the result of a complex interaction of negative and positive autoregulatory feedback loops (1). In *Drosophila melanogaster*, the genetic and molecular dissection of the components of the endogenous circadian oscillator has so far revealed several 'clock genes' which are *bona fide* components of the clock and are involved in processes connecting it with the external environment, and genes which act downstream of the clock (clock controlled genes, ccg_s). This concise review will be based not only on the published literature, but also on some unpublished results presented recently at the eighth meeting of the Society for Research on Biological Rhythms (Amelia Island, Jacksonville, Florida, May 2002). The organization of the *Drosophila* clock machinery that is proposed below refers almost exclusively to what is known, or inferred, about the "master" circadian oscillator/s located in the central brain (2), although partially autonomous clocks have been identified in peripheral tissues or organs and are involved in the regulation of specific functions (3).

3. A FIRST NEGATIVE LOOP

The first *Drosophila* clock genes to have been identified and cloned were *period* (*per*) and *timeless* (*tim*) (4-8). Both *per* and *tim* encode key components of the circadian clock of *D. melanogaster* (9). They are rhythmically expressed at the RNA and protein levels, with the RNAs peaking early in the evening at Zeitgeber Time (ZT) 13-16, (ZT 0 = light-on, ZT 12 = light-off), whereas the protein products reach a maximum late at night (ZT 18-24) (10, 11). The fact that the RNA levels decline as soon as the protein levels rise, suggests that the expression of both genes is under control of their protein products. In fact, in per^0 and tim^0 mutants RNA cycling is abolished for both genes (8, 12). Figure 1 illustrates the negative feedback loop, which contributes to the core of the clock. The transcription of *per* and *tim* is positively regulated by the heterodimer formed by the products of the *dClock* and *cycle* (aka *dBmal1)* genes, dCLK/CYC (13-16). Mutations in these two latter genes result in loss of rhythmicity in *per* and *tim* expression, which are then constitutively transcribed at low levels. There is a lag of 4-6h between *per* and *tim* mRNA cycles and the corresponding protein peaks. This temporal delay in the molecular oscillations, believed necessary to generate a stable 24h clock (17), is related to posttranslational regulatory mechanisms such as protein phosphorylation and degradation (18, 19). In fact, the cytoplasmic accumulation of PER and TIM proteins is governed by complex dynamics. During the initial process of translation of *per* mRNA, PER is efficiently phosphorylated by the Casein Kinase I epsilon homologue DOUBLE-TIME (DBT). As a consequence, cytoplasmic PER monomers are rapidly degraded until TIM protein, which is light-sensitive (20), reaches a threshold concentration which allows the formation of a

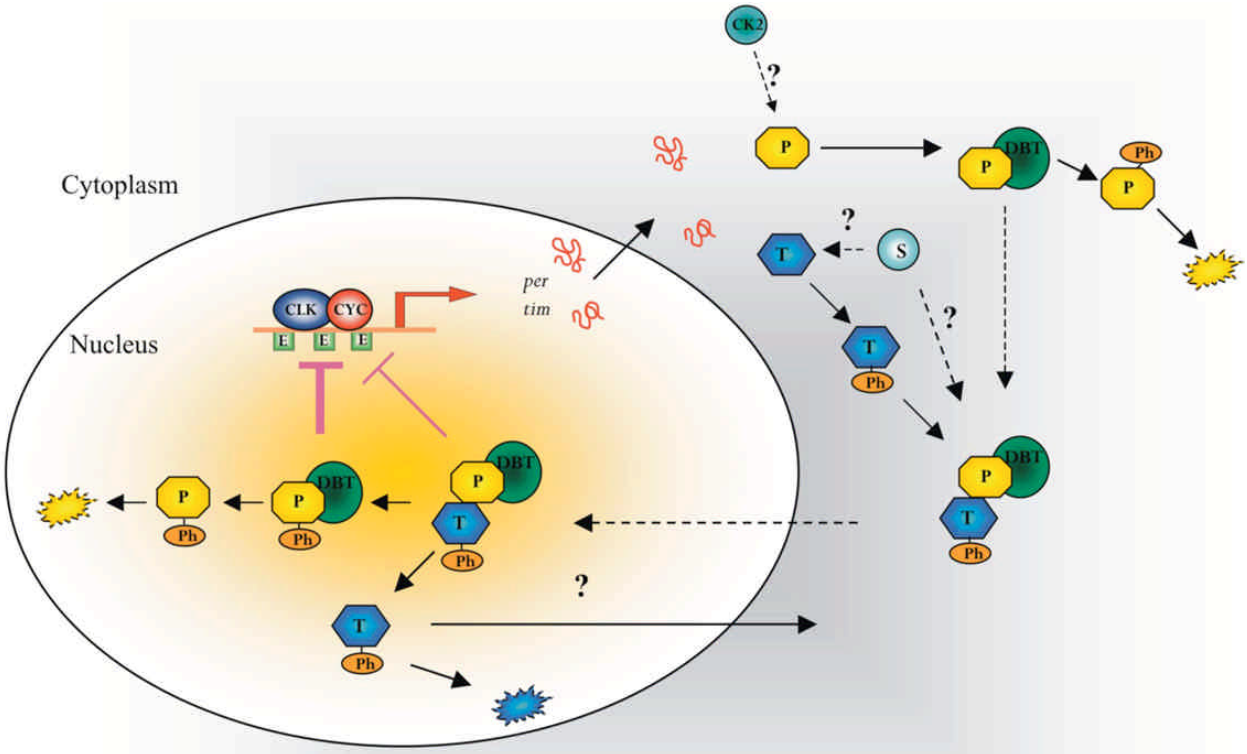

Figure 1. The first negative loop is described. The CLK/CYC heterodimer acts as a positive transcription factor to the *per* and *tim* genes by binding to their E-boxes (E). In the cytoplasm DBT phosphorylates (Ph) PER monomers (P) triggering their degradation until the accumulation of TIM (T) is sufficient to stabilize PER through the formation of a DBT/PER/TIM complex. A possible role for Shaggy (S) in phosphorylating cytoplasmatic TIM is also shown. In addition, a direct role in the phosphorylation of PER has recently been suggested for Casein Kinase 2 (CK2). The latter could be involved in timing the nuclear entry of the DBT/PER/TIM complex. Once in the nucleus the latter complex is able to repress the CLK/CYC function and the loss of TIM enhances this inihibitory effect. Phosphorylated nuclear TIM monomers are either subjected to degradation or perhaps actively exported to the cytoplasm. In the absence of TIM, PER (in the nuclear DBT/PER complex) is phosphorylated by DBT and subsequently degraded. Consequently CLK/CYC repression ceases, and a new cycle of *per* and *tim* transcription can begin. Dashed lines indicate steps at which important delays are known or suggested to be introduced into the feedback loop; question marks indicate putative processes; solid lines ending in a bar indicate a negative effect (negative feedback).

DBT/PER/TIM complex in which PER, thanks to its interaction with TIM, is more stable (17, 19). Once stabilized, the DBT/PER/TIM complex enters the nucleus. *dbt* mRNA and DBT protein are constitutively expressed, nevertheless the subcellular localization of DBT undergoes circadian changes in photoreceptor cells and in the brain's principle oscillator cells, the lateral neurons (19). TIM is necessary to stabilize cytoplasmic PER and the formation of a PER/TIM heterodimer seems to be required to permit nuclear entry of these proteins (21, 22). Once in the nucleus DBT/PER/TIM and DBT/PER complexes, (the latter is formed while TIM is gradually "lost") negatively interact with the "positive element", the heterodimer formed by the transcription factors dCLK and dCYC (19). Rothenfluh et al., (23) have shown that PER monomers, alone, can efficiently inhibit dCLK/CYC transcription activation and suggest that TIM-independent repression by PER normally occurs after dawn. The role of TIM in PER nuclear entry has recently also been questioned by the results of a very precise timing of TIM and PER nuclear accumulation in the large and small ventro-lateral neurons (lvLN and svLN) of *Drosophila melanogaster* (24). These neurons are involved in the onset of robust locomotor rhythms (25-27). In their analysis Shafer et al., (24) find that PER accumulates in the nucleus during the early night in both classes of PDF-expressing neurons at least 3h earlier than TIM, which appears to be restricted to the cytoplasm during this time. These results suggest that PER and TIM might enter the nucleus independently, even if it cannot be completely ruled out that PER and TIM might enter the nucleus as a heterodimer and TIM might then be actively exported back to the cytoplasm. Support for such an idea comes from the realization that TIM possesses a NES domain and so the overall view seems to suggest that TIM might not have a direct role in the inhibition of *per* and *tim* transcription.

The inhibitory action of DBT/PER/TIM and DBT/PER complexes on *per* and *tim* transcription decays with time, due to the nuclear phosphorylation catalyzed by DBT and the subsequent degradation of PER (17, 19). This in turn allows a new cycle of transcription to begin due to the positive action of dCLK/CYC heterodimers on *per* and *tim* transcription. dCLK/CYC heterodimers bind to short sequences (E-box, CACGTG), present in the promoter

regions of both *per* and *tim* genes, which are responsible for the robust rhythmic transcription of *per* (14, 16, 28). Interestingly, both positive (dCLK/CYC) and negative (DBT/PER/TIM or DBT/PER) elements are expressed rhythmically owing to the cycling of dCLK, PER and TIM. Moreover it is worth noting that in tim^0 and per^0 flies, dCLK does not cycle (29). The latter observation strongly suggests the existence of a second interlocked feedback loop.

Another constitutively expressed kinase, SGG, which is encoded by the segment polarity gene *shaggy*/GSK-3 (*sgg*), seems to have a role in TIM phosphorylation and in turn likely regulates the timing of PER and TIM nuclear entry (30). Moreover, overexpression or reduced expression of *sgg* respectively shorthens or lengthens the circadian locomotor activity period of the flies. Finally, a mutant for an additional kinase, CASEIN KINASE 2 (CK2), has been recently identified and characterized by Allada et al., (31). The mutation has significant effects on molecular oscillations of some clock components as well as on circadian behavior. CK2 seems to be co-expressed with pigment dispersing factor (PDF), a neuropeptide present in the ventro-lateral neurons (vLN) which also express *per*, *tim* and *dbt* and are thought to be the major circadian pacemaker neurons in the *Drosophila* brain (26). Based on this information a role for CK2 in the interlocked feedback loops, which are thought to originate the circadian oscillations in the pacemaker neurons of *Drosophila,* has been hypothesized (31).

4. A SECOND INTERLOCKED FEEDBACK LOOP

dClk mRNA and the corresponding dCLK protein show a circadian cycle in abundance and, interestingly, the peak of *dClk* mRNA is in antiphase compared to that of *per* and *tim* mRNAs (13-15). Indeed, d*Clk* mRNA peaks late at night to early in the morning (ZT 23 to ZT 4) at times when the levels of PER/TIM are high (14, 32). The results of recent experiments in transgenic flies with *per* or *tim* promoter regions used to drive *dClk* expression, indicate that the circadian expression of dCLK is not only due to temporal changes in *dClk* mRNA levels, but that postranscriptional mechanisms (such as phosphorylation) significantly affect the circadian fluctuation of dCLK levels (33). Moreover, *dClk* null mutants show constitutively high amounts of *dClk* mRNA, while *per* and *tim* mRNAs fall to constitutive low levels. As *dClk* mRNA transcription is constitutively weak in per^0 and tim^0 mutants it has been suggested that dCLK/CYC, which positively regulates transcription of *per* and *tim*, could act as a repressor of *dClk* transcription. On the other hand, PER and TIM proteins, which repress their own transcription, could serve as positive regulators of *dClk* expression (32, 34). *cyc*, on the other hand, is constitutively expressed (32).

At this point a fundamental question arises: what regulates the *dClk* mRNA oscillation? Are the four proteins mentioned above sufficient to establish two interlocked feedback loops? A very important contribution to this issue comes from the work of M. Young, J. Blau and coworkers. A basic-Zipper transcription factor, encoded by the developmental gene *vrille (vri)* (35), shows circadian oscillations in abundance and might have, in addition to its well established essential role in development, an important function also in the core of the circadian clock, where it is suggested it could act as a repressor of *dClk* (36). In fact, the constitutive expression of *vri* results in arrhythmicity at the behavioral level accompanied by negative effects on *per* and *tim* expression (36, 37). On the basis of results obtained with flies overexpressing *vri*, in which VRILLE (VRI) represses *dClk* independently of PER, Blau et al., (37) suggest that VRI could regulate *dClk* expression directly. Additional support to this hypothesis comes from the identification, by the same authors, of two putative VRI binding sites in the *dClk* promoter.

Another question which Blau et al., (37) have asked is: "what constitutes the *dClk* activator?" They have found that the DNA-binding domain of VRI and that of the protein encoded by the *Par Domain Protein 1* (*Pdp1*) gene are nearly identical, leading to the suggestion that they might bind to the same sites. Therefore they suggest that *Pdp1* could be considered a strong candidate as a direct activator of *dClk* expression. Support for this hypothesis comes from their finding that *Pdp1* is a transcriptional activator capable of activating *dClk* expression *in vitro*. Figure 2 schematically represents this second feedback loop that must be considered putative as far as the identity of some of the components is concerned.

5. THE LIGHT SIGNAL TRANSDUCTION PATHWAY

The capacity of the endogenous circadian clock to synchronize with 24h environmental cycles is one of its most important features. In *Drosophila*, photic entrainment and resetting of the circadian clock within the brain depends on light-mediated degradation of TIM, which in turn significantly affects the stability of another cardinal component of the clock, the protein PER (20, 22). In *Drosophila*, photic stimuli can be transduced to the clock machinery via visual and/or extraocular transduction pathways (38-41). Light triggers proteasome-mediated degradation of TIM (42) through the action of CRY, the product of the gene *cryptochrome* (*cry*) (39, 43). CRY is a blue-light photoreceptor which is thought to physically interact with TIM in a light dependent manner, and consequently may affect PER-TIM interactions. Cryptochromes are pterin/flavin-containing proteins which are thought to be derived from ancestral photolyases. The pterin is proposed to act as an antenna, receiving light energy which is then transferred to a second flavin-based chromophore. This in turn transfers a free excited electron to a redox signalling partner which interacts with the COOH-terminus of the protein. The role of *cry* in the circadian clockwork has not been completely unravelled as yet. *cry* is rhythmically expressed with a mRNA peak early in the morning (ZT 1-ZT 7) (43). CRY protein also cycles, but only in light/dark (L/D) regimes, since light rapidly induces CRY degradation via the proteasome pathway (44). In dark/dark (D/D) regimes CRY tends to accumulate (43). In cry^b mutants (a missense mutation which disrupts a flavin binding site) CRY^b protein is expressed at very low

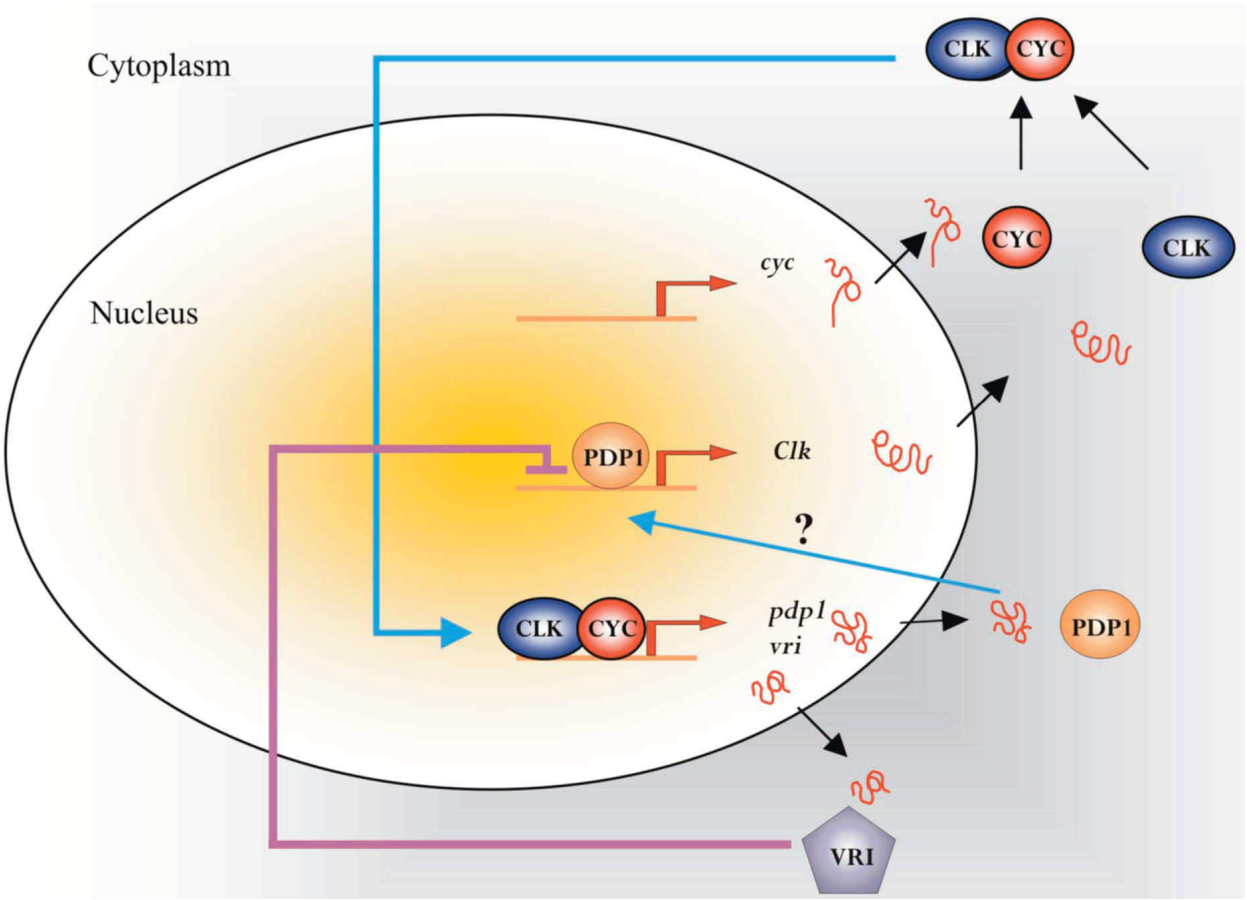

Figure 2. A second interlocked feedback loop is described. The CLK/CYC heterodimer acts as a positive transcription factor to the *pdp1* and *vri* genes. While *cyc* is constitutively transcribed, *Clk* is transcribed cyclically. PDP1 has been hypothesized to be the direct activator of *Clk*, while VRI seems to be directly involved in PER-independent repression of *Clk* transcription. Question marks indicate putative processes; solid lines ending in a bar indicate a negative effect (negative feedback); solid arrowed blue lines indicate a positive transcriptional effect.

levels and this is the most probable cause for the observed defects in some aspects of clock function (39). cry^b flies are defective in light perception but are rhythmic in their behavior under LD and DD regimes, although they are unable to phase shift in response to short light pulses (39, 43), leading to the idea that *cry* should play a cardinal role in the light-signalling process. Other interesting features of the cry^b mutant relate to the finding that this mutant is behaviorally rhythmic also in constant light (LL), a condition under which wild type flies normally behave arrhythmically (45), and that TIM levels do not show the normal degradation in response to light, suggesting that CRY function is also important for TIM light sensitivity (39).

It has been suggested that CRY, in addition to its role in the light input pathway to the clock, might also be directly involved in the clock machinery, at least in peripheral clocks. This hypothesis is based on indirect evidence from the observation of pacemaker abnormalities in cry^b mutants and from experiments with *Drosophila* S2 cells in which, under LL regimes, CRY seems to inhibit the repression of CLK/CYC mediated transcription on the part of PER/TIM (or PER) (39, 46). Moreover, in certain antennal neurons which harbor a circadian clock, CRY seems to be required for the maintenance of molecular rhythms again suggesting that in these cells CRY could also be a component of the clock proper (47). The interaction of CRY with some components of the circadian clock has also been studied in a transcriptional yeast-two-hybrid assay which provided evidence of CRY interacting with TIM and PER under LL conditions (46, 48). A detailed study on the role of the C-terminus of CRY, which has been suggested to be involved in signalling (49), has been conducted in yeast by Rosato et al., (48). The corresponding results show that the removal of the C-terminus allows CRY-TIM and CRY-PER interactions to occur even in DD. This seems to exclude the hypothesis that the interaction of CRY with a signalling partner (TIM, PER) occurs in the same way in which photolyases activate pyrimidine dimers. Therefore, the possibile mechanisms of action of CRY in the light signalling process could be one of the following: (1) the interaction between CRY and the signalling partner is controlled by a light dependent intramolecular redox reaction which changes the conformation of CRY or (2) the interaction between CRY

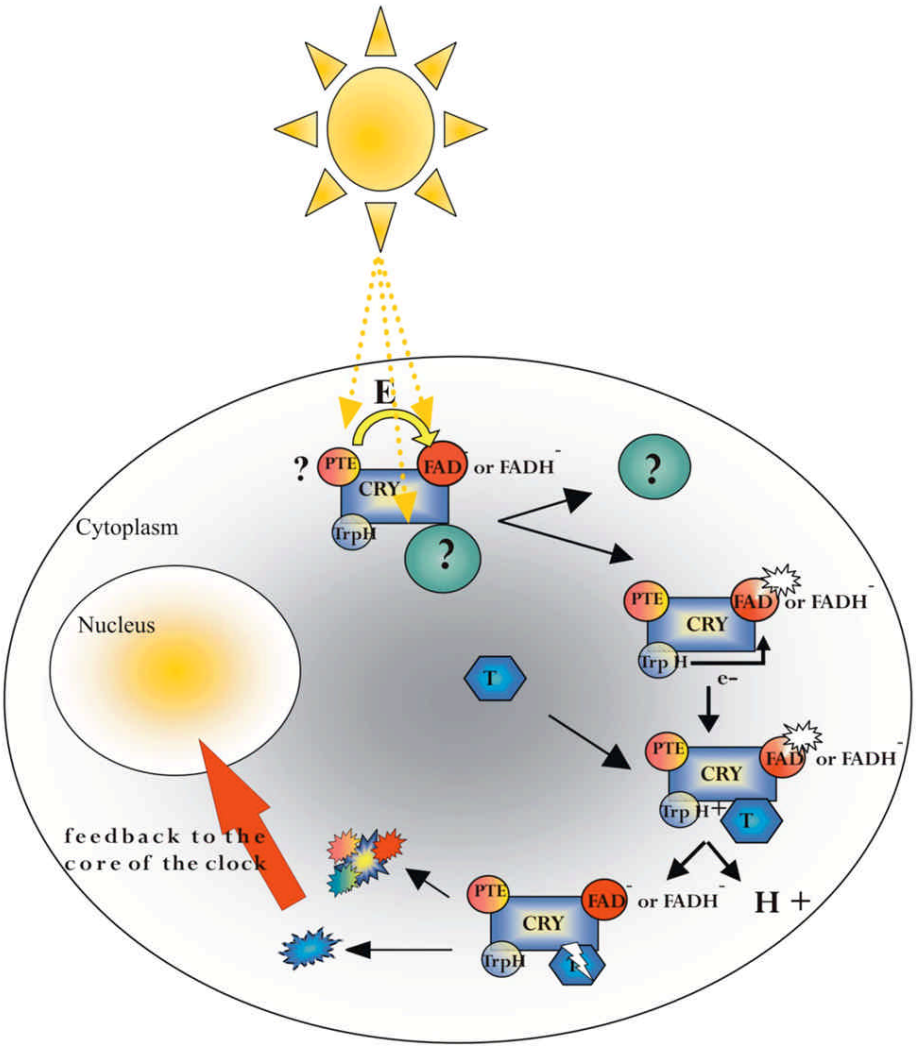

Figure 3. A model for the light signal transduction pathway is proposed. In this model CRY is inactive in the dark due to the inhibitory action of a hypothetical repressor which binds to the C-terminal of CRY (filled green circles with question mark). One possible effect of light could be to either directly induce the removal of the hypothetical repressor or to mediate its removal via an intermolecular redox reaction. Light energy is also absorbed by the pterin (PTE) chromophore, which serves as an antenna, and is transferred to a second flavin chromophore (FAD^- or $FADH^-$). The latter transfer is thought to be mediated by specific tryptophan residues (TrpH). Once CRY has been activated in this way, it is able to bind and transfer an electron to a redox signalling partner i.e. TIM (T). This transfer of energy probably triggers the rapid degradation, via the proteasome, of both TIM and CRY. The degradation of TIM (which negatively affects PER nuclear entry) feeds back to the core of the clock and is interpreted as a resetting stimulus.

and the signalling partner is inhibited in darkness by a repressor bound to the C-terminus of CRY. Light would thus mediate an intermolecular redox reaction that removes the repressor, allowing the signalling partner to interact. The role of redox reactions in the light mediated activation of CRY in *Drosophila* has recently been analysed by Froy et al., (50). They mutagenized flavin binding CRY residues and observed that three out of four are essential for light responses. Moreover two tryptophan residues seem to play a critical role in the intramolecular redox reactions which determine the light dependent activity of CRY. These two tryptophan residues correspond to those present in bacterial photolyases and which are involved in an intramolecular transfer of an electron to flavin as an effect of white light irradiation. The analysis of a flavin-binding site(R381)-tryptophan(W342) double mutant, suggests that flavin excitation triggers an intramolecular redox reaction resulting in dCRY activation (50). Immunoprecipation experiments in S2 cells have suggested that CRY and PER or TIM interactions occur in the cytoplasm even in absence of light (46, 48). Moreover, yeast mutants have been isolated in which the interaction between CRY and PER in a yeast-two-hybrid assay is light independent (48). The overall experimental results available so far suggest that a nuclear protein, conserved in yeast and insects, might be responsible for the light-dependence of CRY interactions in the nucleus. Figure 3 illustrates a putative pathway for light signal transduction to the core of the circadian clock in

Drosophila. In conclusion, the evidence collected so far indicates that CRY in *Drosophila* might have "light" and "dark" functions and may serve as a circadian photoreceptor (in central and peripheral clocks) and as a "clock-gear" (most likely in peripheral clocks).

6. PERSPECTIVE

In this review on the molecular aspects of the *Drosophila* circadian clock any reference to the output of the clock has been deliberately omitted. Work on this very complex issue is relatively recent and it is difficult to identify any general patterns. Moreover the boundaries between what are currently defined as input, core of the clock and output are blurring. Nevertheless there is a good deal of work currently going on in *Drosophila* to identify and characterize the proximal output genes (51) and future research on circadian clocks will most probably dedicate much effort in the attempt to understand how the master clock signals to the peripheral clocks and how the concerted functioning of the former and the latter can lead to highly specific circadian behavior and physiology. Indeed, a great variety of clock-containing cells have been detected in the body of *Drosophila* (reviewed in (3)). These cells can be found in almost any body district: in the gut, malpighian tubules, wings, legs, antennae, brain, and for most of these the ultimate specific meaning for the presence of a clock remains to be established. Nevertheless, the role of the CLK/CYC heterodimer in the regulation of the expression of many genes which are not directly involved in the core of the circadian timing system, is currently being unravelled (3, 18). A consequence of these findings is that many ccg_s seem to be either directly or indirectly regulated by the cycling of clock components. As pointed out by a very recent review on the coordination of circadian timing in mammals (52) "*understanding the mechanisms by which single oscillators interact to form a functional oscillator at the tissue level remains one of the important challenges for future studies*". Once again, *Drosophila* could have a pivotal role in this task, due to the potential for a substantial evolutionary conservation of circadian output processes between insects and vertebrates. The finding of novel genes with rhythmic expression *per se* can only indicate that such genes could operate as components either of the core of the clock and/or as ccg_s working downstream of the clock (and perhaps feeding back to the pacemaker/s). Nevertheless, a contribution to the solution of this conundrum could come from large scale studies of global circadian gene expression in different regions/tissues of the *Drosophila* body. DNA microarray technology has recently opened the way to a new level of investigation in order to rapidly identify high numbers of ccg_s in *Drosophila* tissues (53, 54). A systematic tissue-specific analysis of each transcriptome could then shed some light on many as yet undecifered biochemical processes which are clock controlled and which are involved in determining behavioral and tissue specific physiological phenotypes.

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