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Circadian molecular clockworks in non-model insects

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The recent development of molecular genetic technology is promoting studies on the clock mechanism of various non-model insect species, revealing diversity and commonality of their molecular clock machinery. Like in *Drosophila*, their clocks generally consist of clock genes including *period*, *timeless*, *Clock*, and *cycle*, except for hymenopteran species which lack *timeless* in their genome. Unlike in *Drosophila*, however, some insects show vertebrate-like traits: The clock machinery involves mammalian type *cryptochrome*, *cycle* is rhythmically expressed, and *Clock* is constitutively expressed. Although the oscillatory mechanisms of the clock are still to be investigated in most insects, RNAi and genome editing technology should accelerate the study, leading toward understanding the origin of variable overt behavioral rhythms such as nocturnal, diurnal, and crepuscular activity rhythms.

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Introduction

The insecta is the most prosperous group of animals including nearly a million of species that occur almost everywhere on the earth except for the deep sea. They adapt to various environments and show different daily rhythms, that is, diurnal, nocturnal or crepuscular activity rhythms [1]. The underlying clock mechanism might have been diversified according to the adaptation to those different environmental conditions.

Although the molecular oscillatory mechanism in insect circadian clocks has been extensively studied in *Drosophila*, recent advances in molecular genetic technology allow us to use other non-model insects for the molecular studies. Several different methods have been employed so far to find the molecular components of the circadian clock. At the beginning, antibodies against *Drosophila* PER protein were used in beetles, moths, and crickets [2–4]. Then reverse genetic approach promoted finding of the homologs of *Drosophila* clock genes by molecular cloning (c.f. [5–9]). More recently, microarray, transcriptome analyses, and genome search are used to find clock and clock related genes in various insect species [10,11,12•].

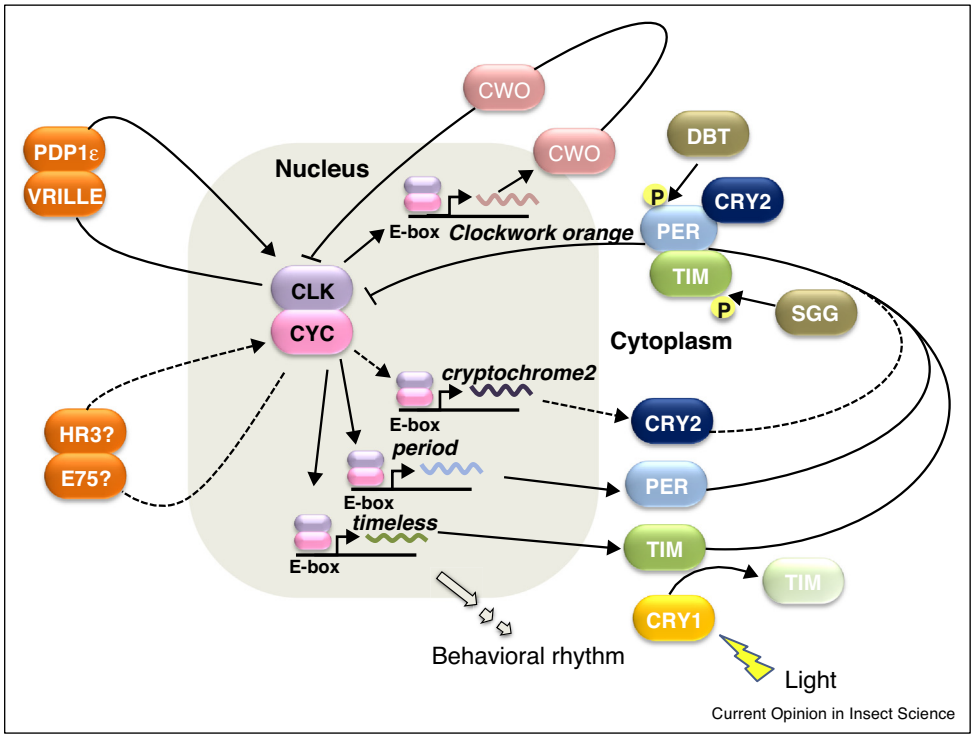
For functional analysis, *in vitro* analysis using cultured cells transfected with a set of clock genes has been employed in some insects such as the monarch butterfly [13], since it was generally difficult to produce mutants of the clock genes in those non-model insects. In other cases, the functional role of a target gene is analyzed by inserting it into the genome of *Drosophila* mutant with mutation of the homologous gene [14,15]. More recently, RNA interference (RNAi) is frequently used to knock-down expression levels of the target gene to examine its role in the clock machinery [16,17]. In this short review, we summarize our current knowledge on the molecular clockworks of non-model insects, focusing on efficacy of recent molecular analyses.

The search for clock genes

Reverse genetic approaches have been performed to search for clock genes in non-model insects, on the bases of the *Drosophila* circadian clock model, in which the clock consists of three loops for oscillatory expression of *period* (*per*) and *timeless* (*tim*), *Clock* (*Clk*), and *clockwork orange* (*cwo*), respectively (Figure 1) (c.f. [18,19]). In the first major loop, the products of *Clk* and *cycle* (*cyc*) genes form heterodimers and activate the transcription of *per* and *tim* during the late day to early night. In the middle of the night, PER and TIM form heterodimers and enter the nucleus to suppress their own transcription by inactivating the transcriptional activity of CLK/CYC. This negative feedback produces the rhythmic expression of *per* and *tim*. CLK/CYC also activates transcription of *vrille* (*vri*) and *Par domain protein 1ε* (*Pdp1ε*). The resultant VRI protein accumulates and represses transcription of *Clk* through a V/P-box in the *Clk* regulatory region. PDP1ε accumulates later than VRI and activates *Clk* transcription during the day so that CLK accumulates during the day. CLK/CYC also activates the transcription of *clockwork orange* (*cwo*) that regulates the amplitude of *per* and *tim* mRNA oscillations [20].

The search for clock genes with molecular cloning, microarray, transcriptome, and genome search revealed that most insects commonly have canonical clock genes

Figure 1



A hypothetical model of an insect circadian molecular clockwork. Solid lines indicate pathways known for *Drosophila* and broken lines for hypothesized for other insects. A core loop consists of transcription factors, CLOCK (CLK) and CYCLE (CYC), and negative regulators PERIOD (PER) and TIMELESS (TIM) in *Drosophila*. In many insects CRYPTOCHROME2 (CRY2) participates as a negative regulator. CRY1 leads TIM degradation in a light-dependent manner to reset the clock's phase. SHAGGY (SGG) and DOUBLETIME (DBT) phosphorylate TIM and PER, respectively, to regulate the timing of nuclear entry of TIM and PER. CLK and CYC are cyclically expressed by other elements, VRILLE (VRI) and PAR DOMAIN PROTEIN 1ε (PDP1ε), and probably HR3 and E75, respectively. CLOCKWORK ORANGE (CWO) is rhythmically expressed by another loop. See text for explanation.

such as *per*, *tim*, *Clk* and *cyc* (Table 1) [11,21•,22–25]. The *per* and *tim* genes are rhythmically expressed in most cases and their structures are similar among insects sharing common functional domains (Figure 2). *per* generally possesses Per-Arnt-Singleminded (PAS)-A and PAS-B domains, a nuclear localization signal (NLS), and a cytoplasmic localization domain (CLD), while *tim* has the first PER dimerization domain (Per-1), NLS, Per-2, and CLD [26]. Their mRNA expressions commonly peak in the early night [27]. The amplitude is generally rather small, often less than two fold [7,27]. Very interestingly, hymenopteran species, including honeybees, do not possess the *tim* gene in their genome (Table 1) [28•]. It has been suggested that *tim* originated as a duplication of the *timeout* gene [29]. *timeout* has been found in many insects (Table 1), and is rhythmically expressed in honeybees, ants and fig wasps [12•,29,30•]. However, its functional role in the clock machinery is not clearly understood.

Recent studies revealed that many insects possess mammalian type *cryptochrome* (*m-cry* or *cry2*), which is different from *Drosophila* type *cry* (*d-cry* or *cry1*) and has lost the ability of photoreception (Table 1) [31••]. It contains several functional domains, including the DNA photolyase domain, a flavin adenine dinucleotide (FAD) binding domain, a CLD, a coiled-coil (C-C) region, and domains necessary for CLK/BMAL interaction (RDs) like in mammalian *cry* (Figure 2) [32,33].

Clk and *cyc* have been detected in many insect species and shown to have common functional domains. The CLK protein generally has five highly conserved regions (Figure 2). They are an amino terminal region that includes a bHLH domain, a PAS-A domain, a PAS-B domain [34], a PAC domain, which has been shown to function as a cytoplasmic localization determinant in *D. melanogaster* PER [26], and polyglutamine repeats (Poly-Q) in C-terminus. The CYC proteins generally have four highly conserved regions that are characteristic of known CYC proteins (Figure 2): a bHLH domain, a PAS-A domain, a PAS-B domain, and a BMAL1 C-terminal region (BCTR). The BCTR, which shows potent transcriptional activity *in vitro* [35,36], has a particularly high similarity among insect CYCs [37•].