Expression Patterns of period and timeless Genes in Mutants of Drosophila melanogaster under Constant Light Condition

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Abstract: Genetic and molecular studies indicate that circadian rhythms are generated and regulated by the action of 8-10 genes in Drosophila. Two genes, period (per) and timeless (tim) and their products are found to be essential for the production of well known locomotor rhythms. The rhythmic expression patterns of per and tim in salivary glands of WT, vg and cry\(^2\) mutants at six time points were studied under constant light condition (LL). In wild type, expression of per and tim was noted at subjective day. However, in cry\(^2\) mutants, the expression was found more in night and less in day times. In vg mutants the expression was similar to WT, but less intensive than WT. The similarity of expression in salivary gland in WT and vg suggests that similar kind of feedback mechanism could operate during developmental stages in peripheral tissues/oscillators. The difference in level of expression in cry\(^2\) flies indicates that photic transduction to the central/peripheral clock (s) is defective in cry\(^2\) flies as compared to WT and vg flies.

Key words: Circadian, Drosophila melanogaster, cryptochrome, timeless, vestigial, temporal expression

INTRODUCTION

Several clock genes function together to regulate circadian rhythms of behavior and physiology (Harmer et al., 2001). Most insights into molecular machinery underlying biological clocks has been gained in the fungus Neurospora (Dunlap, 1996) and the fruttiy Drosophila (Hall, 1995). Drosophila’s circadian pacemaker is found to be in lateral brain neurons (Nitabach et al., 2002) and its clock appears to work from the first instar larvae onwards (Price et al., 1998). Complex behaviors such as wake/sleep cycle have come under the control of these clocks and temporal ordering of products of gene and protein expression is required throughout the day and night. Oscillations of period (per) and timeless (tim) are an integral part of the feedback loop that underlines circadian behavioral rhythms in D. melanogaster (Reppert and Weaver, 2001; Scully and Kay, 2000). The clock genes per and tim are circadianly expressed not only in fly’s brain but also in multiple peripheral organs (Hall, 1995) in a tissue autonomous fashion (Emery et al., 2000; Giebultowicz et al., 2000; Suthakar et al., 2005a). Although the molecular basis of feedback loop is relatively well understood, less is known about the molecular basis of regulation on how the clock is entrained by light. D. melanogaster utilizes atleast three photoreceptors (Helfrich-Forster et al., 2001) for entrainment; cryptochrome the blue light photoreceptor (Emery et al., 2000; Krishnan et al., 2001; Klarsfeld et al., 2004), the compound eyes (ocelli) and the Hafbauer-Buechner (H-B) eyelet (Hofbauer and Buechner, 1989). Although, in constant light (LL) and constant darkness (DD) condition several experimental studies have been done, relatively little is known about the expression patterns of per and tim genes in early developmental stages of D. melanogaster particularly in the salivary glands of third instar larvae of the fly. It has been previously reported in our lab, that there is a cyclical change in expression patterns per and tim genes in adult (intestine) and third instar larvae (salivary gland) under different light regimes (Suthakar et al., 2005a,b).

In the present study we address the question, how constant light (LL) affects the expression patterns of per and tim genes in the salivary glands of third instar larvae of WT and mutants (vg and cry\(^2\)).

MATERIALS AND METHODS

Rearing of flies: Cultures of wild type (Oregon R+), vg and cry\(^2\) mutants of D. melanogaster were reared on a standard medium containing agar, yeast, maize powder, sucrose and the antifungal agent methyl-p-hydroxy...
benzoate under constant light (LL) condition in ventilated and light-tight boxes (60×30×30 cm) at 21±1°C. Incandescent bulb (15 W) was used during light phase (300 lux) (Marrus et al., 1996; Zeng et al., 1996). This condition was maintained with a programmable timer (Grasslin, India) for a period of two weeks.

**Probe (per and tim) cDNA preparation:** Clones of per cDNA and tim cDNA were amplified in DH5α E. coli cells and cDNA was separated from the vector by restriction enzyme (HindIII digestion (former) and (Sal I) digestion (latter). The cDNAs of per (2.231 kb) and tim (3.693 kb) were eluted from low melting agarose gel (Sambrook et al., 1989) purified and then labeled with dioxigenin-11-JUTP (Roche Diagnostics, Germany) by random primed DNA labeling method (Feinberg and Vogelstein, 1984; Schmitz et al., 1991). The efficiency of labeling was checked by standard protocols (Suthakar et al., 2005a, b).

**In situ hybridization:** Late third instar larvae of WT and mutants (vg and cryb) were dissected at 6 different time points in phosphate buffered saline (08:00, 12:00, 16:00, 20:00, 00:00, 04:00). The tissues were then subjected to whole mount RNA-DNA in situ hybridization. The tissues were fixed with paraformaldehyde and then treated with diethyl pyrocarbonate (0.1%) and digested with pronase K. Hybridization of per and tim mRNAs with per and tim cDNA probes (denatured) were carried out at 88-68°C for 24 h period. The unhybridized probes were washed off with the hybridization buffer. The expression signals of per and tim mRNA were identified by incubating the tissues with Anti-digoxigenin-AP-Fab fragment (Roche Diagnostics, Germany) and chromogenic mixture (nitroblue tetrazolium chloride/bromo chloroindolyl phosphate) (Suthakar et al., 2005a,b).

**RESULTS**

The temporal expression pattern of per and tim in salivary gland of late third instar larvae of WT, vg, cryb mutants under constant light (LL) are shown (Fig. 1 and 2) and tabulated (Table 1 and 2). More number of (+) indicates higher level of expression; (-) indicates absence of expression. In wild type, the expression of per and tim are noted at subjective day (08:00, 12:00, 16:00, 04:00 h). However, in cryb more expression was seen during subjective night and less expression was observed in subjective day. In vg mutant, the expression was found to be temporally similar to that of WT; but the expression was less intensive than WT.

<table>
<thead>
<tr>
<th>Time</th>
<th>WT</th>
<th>vg</th>
<th>cryb</th>
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<tbody>
<tr>
<td>08:00</td>
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<td>12:00</td>
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<td>16:00</td>
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<td>04:00</td>
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*: sign denotes gene expression; ; sign represents absence of gene expression

<table>
<thead>
<tr>
<th>Time</th>
<th>WT</th>
<th>vg</th>
<th>cryb</th>
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<tr>
<td>08:00</td>
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<td>04:00</td>
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*: sign denotes gene expression; ; sign represents absence of gene expression

**DISCUSSION**

The Drosophila eye is both target of clock control and partly responsible for photic input to the central pacemaker (Young, 1998; Helfrich-Forster et al., 2001). Photoreceptor cells contain peripheral clocks, suggesting that visual function may be regulated by the clocks. The temporal expression patterns of per and tim under constant light in WT and mutants in Drosophila explains the molecular mechanism underlying the biological clock function. In larva, the salivary gland tissue was found to contain per and tim mRNA (Kaneko et al., 2000; Suthakar et al., 2005a). It has long been known that fairly strong constant light induces arrhythmia in insects (Saunders, 1982). In D. melanogaster it was repeatedly demonstrated that adult flies are behaviorally arrhythmic in LL (Helfrich-Forster et al., 2001; Emery et al., 2000). In our studies long term exposure of wild type flies and their larva in constant light reveals that per and tim expression was not rhythmic, which is different from the expression pattern under LD cycle (Suthakar et al., 2005a, b). This is because when wild type flies are exposed to constant and relatively bright light (~ 300 lux in our study): PER and TIM levels are substantially lowered (Zarr et al., 1990; Price et al., 1995); TIM was known to be destroyed by light (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996), providing a possible mechanism for the resetting and of per RNA and protein levels. Under constant light, CRY mediates TIM degradation and inhibits accumulation of TIM and stops the clock, causing arrhythmic behavior in wild type flies and in larvae.
Fig. 1: Temporal expression patterns of per in WT, vg and cry\(^b\) mutants in the salivary glands of third instar larvae under constant light (LL) condition.
Fig. 2: Temporal expression patterns of *tim* in WT, *vg* and *cry* mutants in the salivary glands of third instar larvae under constant light (LL) condition.
It was reported that CRY is important for entrainment in *Drosophila* and cry*<sup>b</sup>* flies express functionless CRY protein (Emery et al., 2000). Under constant light (LL) condition, the expression of *per* and *tim* in cry*<sup>b</sup>* flies are similar to that of LD cycle. Expression was seen in night time and almost nil expression was seen in day time (Emery et al., 2000). cry*<sup>b</sup>* flies were found to be insensitive to LL because they are partially blind to light, conclusively establish CRY as unique circadian photoreceptor and they remain rather robustly rhythmic in that condition (Emery et al., 2000). Present results indicate absence of any conspicuous rhythmicity and low levels of *per* and *tim* gene expression in peripheral tissues like salivary gland in cry*<sup>b</sup>* during developmental stages.

In vg mutants of *Drosophila, per* and *tim* gene expression less intensive than WT, even though vg mutants have all the known photoreceptors (compound eyes; H-B eyeclet: CRY and photopigments in dorsal neuron). The mechanism for low levels of *per* and *tim* expression in vg mutant is not exactly known. However, reduced wing structure caused conspicuous reduction in amount of activity and may be reflected in the expression patterns in vg. Further studies may help to elucidate the functional significance of these expression patterns during developmental stages in the fly.

REFERENCES


