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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^b 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^b 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^b flies indicates that photic transduction to the central/peripheral clock (s) is defective in cry^b 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 fruitfly *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-Buchner (H-B) eyelet (Hofbauer and Buchner, 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^b).

MATERIALS AND METHODS

Rearing of flies: Cultures of wild type (Oregon R+), *vg* and cry^b mutants of *D. melanogaster* were reared on a standard medium containing agar, yeast, maize powder, sucrose and the antifungal agent methyl-p-hydroxy

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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 (*Hind* III) 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-dUTP (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 cry^b) 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 protinase K. Hybridization of *per* and *tim* mRNAs with *per* and *tim* cDNA probes (denatured) were carried out at 58-68°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 (nitoblue tetrazolium chloride/bromochloroindolyl 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*, cry^b 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 cry^b 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 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

Time	WT	*vg*	cry^b
08:00	++	++	-
12:00	++	+	-
16:00	+	-	+
20:00	-	-	+
00:00	-	-	-
04:00	-	+	-

+; sign denotes gene expression-; sign represents absence of gene expression

Table 2: Temporal expression patterns of *tim* in WT, *vg* and cry^b mutants in the salivary glands of third instar larvae under constant light (LL) condition

Time	WT	*Vg*	cry^b
08:00	++	+	+
12:00	+	++	-
16:00	+	+	-
20:00	-	-	++
00:00	-	-	++
04:00	+	+	+

+; 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 (Saunder, 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 (Zerr *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.

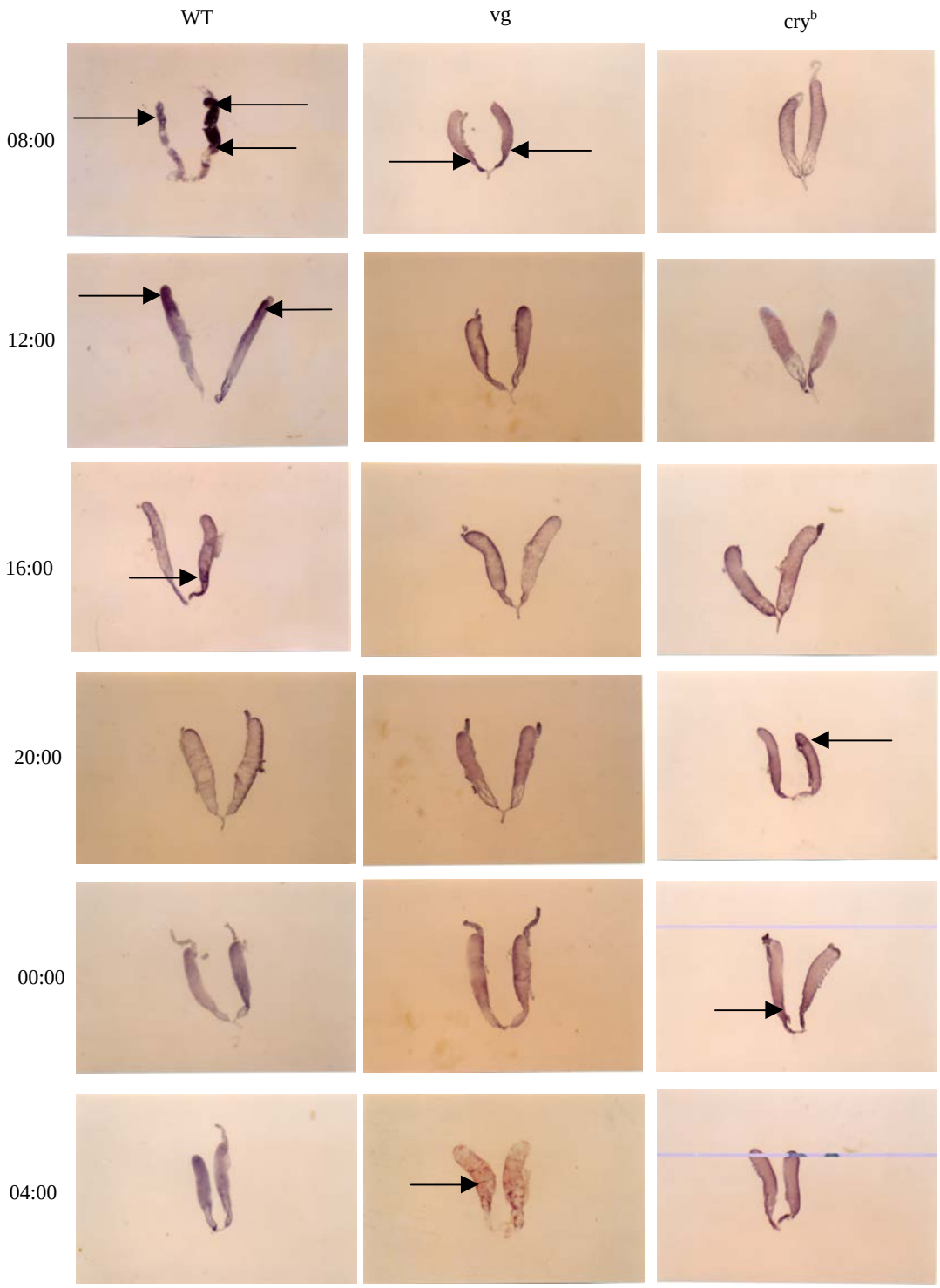

Fig. 1: Temporal expression patterns of *per* in WT, *vg* and *cry^b* mutants in the salivary glands of third instar larvae under constant light (LL) condition

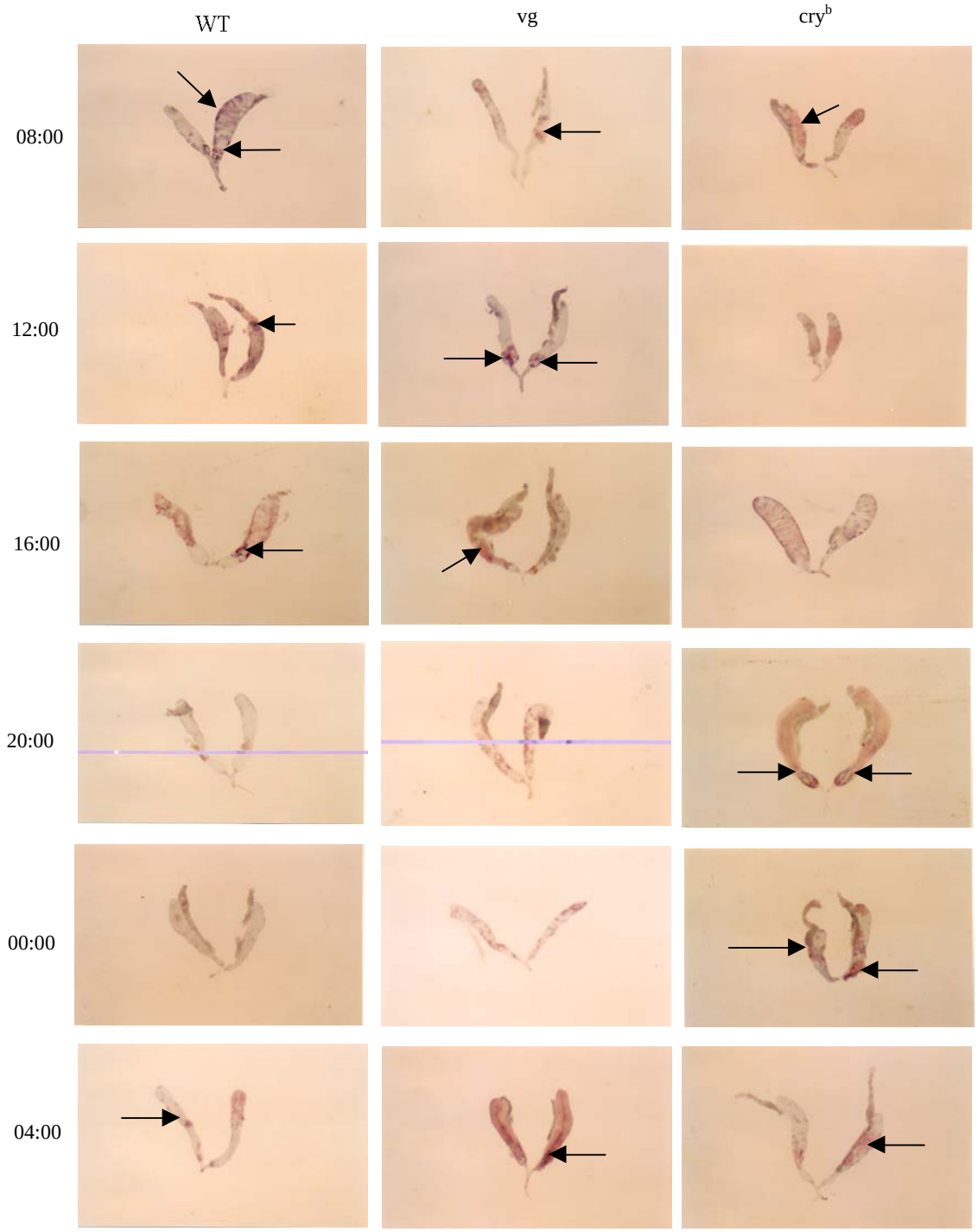

Fig. 2: Temporal expression patterns of *tim* in WT, *vg* and *cry*^{b} mutants in the salivary glands of third instar larvae under constant light (LL) condition

It was reported that CRY is important for entrainment in *Drosophila* and cry^b flies express functionless CRY protein (Emery *et al*., 2000). Under constant light (LL) condition, the expression of *per* and *tim* in cry^b 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^b 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 rhythmcity and low levels of *per* and *tim* gene expression in peripheral tissues like salivary gland in cry^b 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 eyelets: 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.

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