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Photoperiodic Induction of Diapause Requires Regulated Transcription of *timeless* in the Larval Brain of *Chymomyza costata*

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Abstract  Photoperiodic signal stimulates induction of larval diapause in *Chymomyza costata*. Larvae of NPD strain (*npd*-mutants) do not respond to photoperiod. Our previous results indicated that the locus *npd* could code for the *timeless* gene and its product might represent a molecular link between circadian and photoperiodic clock systems. Here we present results of *tim* mRNA (real time-PCR) and TIM protein (immunohistochemistry) analyses in the larval brain. TIM protein was localized in 2 neurons of each brain hemisphere of the 4-d-old 3rd instar wild-type larvae. In a marked contrast, no TIM neurons were detected in the brain of 4-day-old 3rd instar *npd*-mutant larvae and the level of *tim* transcripts was approximately 10-fold lower in the NPD than in the wild-type strain. Daily changes in *tim* expression and TIM presence appeared to be under photoperiodic control in the wild-type larvae. Clear daily oscillations of *tim* transcription were observed during the development of 3rd instars under the short-day conditions. Daily oscillations were less apparent under the long-day conditions, where a gradual increase of *tim* transcript abundance appeared as a prevailing trend. Analysis of the genomic structure of *tim* gene revealed that *npd*-mutants carry a 1855 bp-long deletion in the 5′-UTR region. This deletion removed the start of transcription and promoter regulatory motifs E-box and TER-box. The authors hypothesize that this mutation was responsible for dramatic reduction of *tim* transcription rates, disruption of circadian clock function, and disruption of photoperiodic calendar function in *npd*-mutant larvae of *C. costata*.

Key words: photoperiodism, diapause, seasonal clock, circadian oscillator, *timeless* gene expression

Photoperiodic sensitivity is of primary importance for life-cycle patterning in insects. Seasonal change of the relative proportion of day-length/night-length is perceived by specialized receptors (Shiga and Numata, 2007) and transduced into an appropriate adjustment of developmental mode: change of the rate of development and morphology, migration, entrance into diapause, or various combinations of them all (Tauber
et al., 1986; Danks, 1987). Diapause is a centrally mediated type of dormancy that either represents an obligatory part of ontogeny or is facultatively induced when specific token stimuli (most often photoperiod) signal the onset of less favorable season. Diapause allows the insects to pass through adverse periods, exploit seasonally fluctuating resources, diversify in tropical regions, and colonize temperate and polar regions (Andrewartha, 1952; Danilevskii, 1961; Saunders, 2002; Koštál, 2006).

Physiological mechanisms of photoperiodic timing of diapause remain little understood (Saunders, 2002; Saunders et al., 2004; Danks, 2005; Bradshaw and Holzapfel, 2007). A great deal, however, is known about the molecular basis of the central circadian clock in insects, especially in *Drosophila melanogaster* (Hall, 2003). Several studies tested the hypothesis whether the known molecular elements of insect circadian clocks may serve as functional parts of the photoperiodic “calendar” system. Saunders et al. (1989) found that a double deletion of the *period* (*per*) failed to prevent the ability of female *D. melanogaster* flies to discriminate between long and short days. Despite that negative finding, investigation into the potential involvement of circadian clock genes in photoperiodic response resumed during the last decade. It has been shown that phases, amplitudes, and/or levels of various clock gene expression were affected by photoperiod in fleshflies *Sarcophaga crassipalpis* (Goto and Denlinger, 2002) and *S. bullata* (Goto et al., 2006), linden bug *Pyrrhocoris apterus* (Syróvá et al., 2003), pitcher-plant mosquito *Wyeomyia smithii* (Mathias et al., 2005), and silkmoth *Bombyx mori* (Iwai et al., 2006). Recent studies in *D. melanogaster* and pitcher-plant mosquito indicated that although circadian clocks and photoperiodic calendar appear as genetically distinct mechanisms/processes, the clock gene *timeless* (*tim*) may affect the incidence of diapause directly, independently of its function in the central circadian oscillator (Mathias et al., 2007; Sandrelli et al., 2007; Tauber et al., 2007).

We have contributed to this effort by studying the roles of the clock genes *per* and *tim* in photoperiodism of a drosophilid fly, *Chymomyza costata*. The mature larvae of this fly enter diapause in response to subcritically short days (Riihimaa and Kimura, 1989; Koštál et al., 2000a). Riihimaa and Kimura (1988) selected a mutant strain of flies that did not respond to a photoperiodic signal and named the strain NPD (Non-Photoperiodic-Diapause). Genetic linkage analysis confirmed that the larval nonphotoperiodism and adult eclosion arrhythmicity in the NPD strain were caused by mutation in a single autosomal gene locus *npd* (Riihimaa and Kimura, 1989; Riihimaa, 1996). Formal analysis of the photoperiodic clock’s function revealed that an element with a circadian-oscillatory nature participates in photoperiodic time measurement (Yoshida and Kimura, 1995; Koštál et al., 2000b). Daily and circadian oscillations of *per* mRNA abundance, which were detected in the wild-type strain, were missing in the *npd* mutants (Koštál and Shimada, 2001). The *tim* mRNA transcripts were not detectable by Northern blot analysis in the fly heads of *npd*-mutants, whereas they were detectable and showed typical daily oscillations in the wild-type strain (Pavelka et al., 2003). All these pieces of evidence pointed toward an impaired function of the central circadian clocks in *npd*-mutants and indicated that the locus *npd* could code for the *tim* gene in *C. costata*. Product of *tim* gene may thus represent a molecular link between circadian and photoperiodic clock systems in this fly.

Previous studies used *C. costata* adults. Diapause and photoperiodic sensitivity, however, are expressed only in larval stages. Hence, the main objective of this study was to find if there are any differences in the levels and daily/circadian patterns of *tim* gene expression and TIMELESS (TIM) protein presence in the brains of the wild-type and *npd*-mutant larvae. We also present here the analysis of the genomic structure of *tim* gene in both strains. It revealed that *npd*-mutants carry, in addition to 37 amino acid substitutions, a 1855 bp-long deletion in the 5′-UTR region. This deletion removed the start of transcription and regulatory motifs E-box and TER-box in the promoter. We hypothesize that the deletion was primarily responsible for suppression of *tim* transcription in the brain, malfunctioning of the central circadian clock, and loss of photoperiodic calendar function in *npd*-mutant larvae of the *C. costata*.

**MATERIALS AND METHODS**

**Insects**

Two strains of *Chymomyza costata* (Diptera: Drosophilidae) were used: a wild-type strain originally collected in Sapporo (43°N), Japan, in 1983 and a NPD mutant strain, which was isolated by Riihimaa and Kimura (1988) from wild-type flies collected in Tomakomai (42.3°N; ca. 50 km south of Sapporo, Japan). Larvae, pupae, and adults were cultured on an artificial diet of Lakovaara (1969) under...
a constant temperature of 18 °C. Developmental destiny of larvae was programmed using 2 different photoperiodic regimes (Koštál et al., 2000a): a long-day (LD) regime (16 h light:8 h dark), at which all larvae of both strains continue direct development (pupariate), and a short-day (SD) regime (12 h light:12 h darkness) that induces diapause in 100% larvae of the wild-type strain but no-diapause in larvae of the npd-mutant strain. For mRNA analysis, larval central nervous systems (CNSs) were dissected and stored in RNAlater (Qiagen, Hilden, Germany) at –20 °C until RNA/DNA isolation. Ten CNSs were pooled for each sample. Three to 4 independent replicates (each from different larval generation) were prepared. For immunohistochemistry, the larvae were cut in approximately 1/3 of their length and the front parts containing CNS were immediately fixed in Bouin-Holland solution without acetic acid but supplemented with 0.7% of mercuric chloride (Závodská et al., 2003). Eight specimens were sampled at each larval age and zeitgeber time (ZT) as specified in the Results section.

**DNA/RNA Extractions; cDNA Synthesis; qRT-PCR**

Genomic DNA was extracted from tissues that were dissected from 10 larvae (gut tissues were discarded) using the DNeasy Tissue Kit (Qiagen). Total RNA was extracted using RNA Blue kit (Top-Bio, Prague, Czech Republic). First strand cDNA was synthesized using Reverse Transcription System (Promega, Madison, WI).

Relative abundance of timeless mRNA transcripts in the samples was measured by the quantitative real-time PCR (qRT-PCR) technique using Rotor Gene RG 3000 PCR cycler (Corbett Research, Sydney, Australia) and a Hot Start version of TaKaRa Ex Taq DNA polymerase (Takara, Shiga, Japan). PCR reactions were primed with a pair of gene-specific oligonucleotides (Table 1). One of the nucleotides was designed to span an exon/intron boundary to prevent formation of PCR products from contaminating genomic DNA. In a preliminary experiment, we have verified that no products of expected size are generated from genomic DNA treated with RNase (Top-Bio). Abundance of mRNA transcripts of an endogenous reference gene Rp49 (syn. RpL32) was measured in parallel reactions. Emission of a fluorescent signal resulting from SYBR Green I (Qiagen) binding to double-stranded DNA PCR products was detected with increasing PCR cycle number. Threshold cycle (Cₜ), that is, the cycle at

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5' → 3')</th>
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<tbody>
<tr>
<td>Rp49</td>
<td>upstream</td>
<td>ATGCAAAGCTGTCC/CAAC</td>
</tr>
<tr>
<td>Rp49</td>
<td>downstream</td>
<td>GAAGCCTAGGGCAGCAT</td>
</tr>
<tr>
<td>tim</td>
<td>upstream</td>
<td>CAGATGGCCCAAACCTGGCAC</td>
</tr>
<tr>
<td>tim</td>
<td>downstream</td>
<td>CTCATGATAGTAGGGCACAG</td>
</tr>
</tbody>
</table>

NOTE: ^ indicates position of an exon/intron boundary.

which there is a significant detectable increase in fluorescence signal, was detected for each sample. Each sample was run as a quadruplet of tim and quadruplet of Rp49 reactions. Relative quantification of a target gene to reference gene transcripts was done according to Pfaffl (2001) and Liu and Saint (2002).

**Antibody and Immunohistochemistry**

Recombinant protein corresponding to amino acids 557-663 (EFTVDF...TAIREF) (Swiss-Prot/Q8MML1) produced by a bacterial expression system pET-32 (Merck, Darmstadt, Germany) was used to raise rabbit anti-Chymomyza-TIM antibody. Front parts of larvae were left in the fixative overnight at 4 °C and processed as described earlier (Závodská et al., 2003). Briefly, after dehydration, saturation with chloroform, and embedding in paraplast, fixed tissues were sectioned (sections were 8 μm thick) using 820 Spencer Microtome (American Optical Corp., Southbridge, MA). In deparaffinized and washed sections attached to microscopic slides, nonspecific binding sites were blocked with 10% normal goat serum in PBST. Incubation with the primary antibody was done overnight at 4 °C (preimmunized rabbit serum was used instead of the primary antibody in the control staining), followed by incubation for 1 h at room temperature with the goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch Lab., West Grove, PA). Following washing steps, the HRP enzymatic activity was visualized with hydrogen peroxide and 3,3′-diaminobenzidine tetrahydrochloride. Stained sections were dehydrated, mounted in DPX mounting medium (Fluka, Buchs, Switzerland), and viewed and photographed under a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany) equipped with Nomarski (DIC) optics and CCD camera. The intensity of staining was scored subjectively with a 5-point scale ranging from point 0 (no reaction) to 1 (weak intensity), 2 (low intensity), 3 (high intensity), and 4 (maximal intensity). All preparations were processed simultaneously, and
the entire immunohistochemical procedure, from tissue dissection to staining evaluation, was standardized for all samples in respect to time, temperature, and other conditions.

Cloning and Sequencing

Genomic DNA was analyzed in wild-type and NPD strains. Gene-specific oligonucleotide primers were designed according to a known cDNA sequence of wild-type tim gene (GenBank accession number: AB073724) (Pavelka et al., 2003). Purified PCR products were either inserted into the pGEM-T Easy plasmids and amplified by cloning in JM 109 Competent Cells (both from Promega) or directly sequenced using Big Dye Terminator v1.1 Cycle Sequencing Kit and ABI Prism 377 DNA Sequencer (both from Applied Biosystems, Foster City, CA). Sequences were aligned using Lasergene v5.1 software (DNASTar, Madison, WI).

To obtain tim promoter sequence, genomic DNA was digested by Sau3A1 (Takara) or Hind3 (Amersham, Little Chalfont, UK) and the digests were self-ligated digested by Sau3A1 (Takara) or Hind3 (Amersham, Little Chalfont, UK) and the digests were self-ligated using T4 ligase (Promega). Pair of primers (5′-TTTGACGGATGAAC-3′; 5′-ATTTTGCGCTCACACGACG-3′) were designed based on the known cDNA sequence of 5′ untranslated region (5′-UTR) of wild-type tim gene of C. costata in order to amplify DNA sequence flanking the 5′-UTR using an inverse PCR strategy. Purified inverse PCR product was inserted into pGEM-T Easy vector, cloned, and sequenced as described above. cDNA starts were characterized in both strains by the 5′-RACE technique using 5′-RACE System v2.0 (Invitrogen, Carlsbad, CA).

RESULTS

TIM Protein Is Absent in the Brain of 4-Day-Old npd-Mutant Larvae

The antiserum to C. costata TIM consistently stained 2 neurons in each brain hemisphere of the wild-type larval CNS (Figure 1A) (4-d-old 3rd instar larvae were dissected because they are maximally sensitive to photoperiodic signal). Identical neurons were seen under both photoperiodic regimes, LD and SD. They were located dorsally in the lateral part of the brain as shown in Figure 1B. In contrast to the wild-type strain, no TIM staining was detected in any CNS preparation of similarly aged npd-mutant larvae (Figure 1C). TIM-like immunoreactivity in wild-type larvae was examined in 4 different ZTs under each photoperiodic regime. Relatively high intensity of staining was seen in all 4 ZTs in the LD-reared wild-type larvae; the mean intensity was highest at ZT 2 (4.0 points) and lowest at ZT 20 (2.0 points) (Figure 1D). In contrast, in the SD-reared larvae, the staining was relatively weak at ZT 2 (1.0 point) and increased to 3.0 points at ZT 20 (Figure 1E).

tim mRNA Is Dramatically Suppressed in the Brain of 4-Day-Old npd-Mutant Larvae

Relatively high levels of tim mRNA were detected in the CNSs of wild-type 4-d-old 3rd instars. Daily changes of tim transcript abundance differed between 2 photoperiodic regimes. In the LD-reared larvae, the abundance decreased during the light period (from ZT 0 to ZT 12), reaching a minimum at ZT 12 and later, increased during “evening” and dark period until it reached a maximum at ZT 24. In the SD-reared larvae, a single peak of tim transcript abundance was found at ZT 16 (Figure 2A). Approximately 10-fold lower levels of tim mRNA, and no daily changes, were detected in the brains of npd mutants (Figure 2A).

Daily and Circadian Oscillations in the tim mRNA Level in the Brain of Wild-Type Larvae

CNS samples were taken daily (days 2-8) during the 3rd instar larval development in the wild-type strain. Two characteristic ZTs were examined: ZT 0 and ZT 12 (minimum) in LD-reared larvae and ZT 0 and ZT 16 (maximum) in SD-reared larvae. Clear daily oscillations of tim transcript abundance were observed in the SD-reared larvae. We have confirmed that a similar pattern persists even on days 15 and 30 (data not shown). In the LD-reared larvae, no clear oscillation pattern was detected. On days 4, 5, and 6, however, an incremental increase of tim mRNA level was observed, which indicated that daily oscillations may exist but may be masked by a rapid overall increase of tim mRNA levels in developmentally desynchronizing larvae (Figure 2B). Transferring 4-d-old wild-type larvae from the SD to continuous darkness (DD) regime did not change the larval developmental programming (100% [n = 168] of transferred larvae entered diapause). Endogenously driven (circadian) oscillations of tim transcript abundance were only slightly expressed, and the mRNA level was maintained close to the minimum observed in the SD-reared counterparts (Figure 3A).
An analogous transfer from the LD to DD regime also resulted in no change of developmental programming (92% \( n = 124 \) of transferred larvae pupariated). The mRNA level steadily increased, in a manner similar to the LD-reared counterparts (Figure 3B). Upon Transfer from the LD to SD Regime, the Wild-Type Larvae Change Developmental Destiny and Rapidly Change the \( \text{tim} \) Expression Pattern

When the 4-d-old wild-type larvae were transferred from the LD to SD regime, 93.3% \( n = 239 \) of individuals changed their LD-programmed developmental destiny and entered the diapause state. The developmental pattern of \( \text{tim} \) expression responded very rapidly to LD→SD transfer. Already on day 4, at ZTs 16 and 24 (i.e., 4 h or 12 h, respectively, after the change of regime), no increase of \( \text{tim} \) mRNA was observed as would be characteristic for LD-reared larvae. Instead, the abundance of \( \text{tim} \) transcripts decreased to an apparent minimum at ZT 24, as is characteristic for SD-reared larvae. During the following days (5, 6, 7, and 8), \( \text{tim} \) mRNA levels remained relatively low (at a level characteristic for SD-reared larvae). Although a clear daily oscillation pattern (characteristic for SD conditions) could not be observed in transferred larvae, its development was slightly indicated (Figure 4).

Differences in \( \text{tim} \) Gene Structure between Wild-Type and \( \text{npd} \)-Mutant Flies

Sequences were deposited in NCBI GenBank under accession numbers EU189083 (wild-type) and EU189084 (\( \text{npd} \)-mutant). The \( \text{timeless} \) cDNA coding sequences (CDSs) had equal lengths of 4071 nucleotides in both \( C. \text{costata} \) strains. We found 57 nucleotide substitutions between the wild-type and \( \text{npd} \)-mutant CDSs (98.6% identity). Amino acid (aa) translations of CDSs differed in 37 positions (97.3% identity of 1356 aa in total). Structural homology between the wild-type and \( \text{npd} \)-mutants in individual functional domains was 100% aa identity in 32aa region; 76.2% in acidic domain (21aa); 100% in Nuclear Localization Signal (NLS, 11aa); 94.7% in Per interaction domain 1 (PER-1, 75aa); 97.5% in PER-2 (200aa); 96.4% in Cytoplasmic Localization Domain (CLD). Amino acids differing between 2 strains were highlighted in Figure 5.

Comparison of genomic DNA and cDNA structures of the \( \text{tim} \) gene revealed the presence of 14 exons and 13 introns. The two largest introns (nos. 1 and 12) were sequenced only partially. Positions of all
introns were perfectly conserved in the 2 strains of C. costata. Interstrain differences in the size were found only in 2 introns: no. 7 (550 bp in the wild-type vs. 560 bp in the npd-mutant strain, respectively) and no. 13 (525 vs. 492 bp) (Figure 5).

The most noticeable difference between 2 fly strains was found in the 5′-UTR and promoter regions of the tim gene. In the wild-type cDNA, the putative start of transcription has been found 1409 bp upstream of CDS start. Consensus regulatory motifs, E-box (CACGTG) and TER-box (CACGTTG), were found 234 and 246 bp, respectively, upstream of transcription start. In addition, a lower affinity E-box (CAACGTG) and PERR-like box (GTACGCACGA)
were detected 200 and 560 bp upstream of transcription start, respectively. In npd-mutants, 1855 bp-long deletion was detected, which removed the start of transcription and all regulatory motifs found in the wild-type strain. Bases corresponding to the region from 638 to 2492 bp upstream of CDS start of wild-type tim were removed in npd-mutant tim. No known clock gene-regulatory motifs were found either within the putative promoter fragment (937 bp known) or within the 5′-UTR of cDNA (1693 bp known) that were sequenced in the npd-strain (Figure 5).

**DISCUSSION**

We have previously observed that the tim mRNA was undetectable in the adult heads of npd-mutant flies of C. costata and hypothesized that the lack of tim transcription might be the primary cause of the npd-mutant phenotype, that is, a malfunctioning central oscillator, behavioral arrhythmicity, and nonphotoperiodism (Pavelka et al., 2003). The photoperiodic sensitivity is restricted to the larval stage only (Koštál et al., 2000a). Hence, this article is focused on analysis of tim expression in the CNS of photoperiodically sensitive larvae. We report significant differences in 1) the levels of tim mRNA transcripts, 2) the levels of TIM protein in the brains of wild-type and npd-mutant strains of C. costata, and 3) the structure of the tim gene between 2 strains.

**Figure 5.** Comparison of genomic structures of tim gene in the wild-type (wt) and npd-mutant (npd) strains of Chymomyza costata. Exons are shown as boxes (coding sequence is in black) separated by lines (introns). Some structural features and their positions are highlighted above. All 37 amino acid substitutions and their positions are shown in the center. The position of 1855 bp-long deletion in the 5′-UTR region of tim in npd-mutant strain is indicated by dashed lines. NLS = Nuclear Localization Signal; CLD = Cytoplasmic Localization Domain. For detailed description, see text. Other descriptions as in Figure 1.

**Differences in tim mRNA Levels and Expression Patterns**

Very low levels, and no daily changes, of tim mRNA were seen in the CNS of npd-mutant larvae, which are completely insensitive to photoperiod. In a striking contrast to this situation, not only did we find approximately 10-fold higher levels of tim mRNA in the CNSs of wild-type larvae but we also found that the tim expression is under photoperiodic control in them. The daily profiles of the tim mRNA level differed depending on photoperiodic regime. Although clear daily oscillations (maximum at ZT 16) were seen in the larvae maintained under the short days (SD), the oscillations were less apparent under the long days (LD), where a gradual increase of tim mRNA level appeared as a prevailing trend. Although the larvae were synchronized at their 2nd → 3rd larval moult, individual differences in their developmental rates resulted in an approximately ± 1-day desynchronization by the time of pupariation (Koštál et al., 2000a). Such desynchronization, together with the steady increase of tim mRNA abundance, may partially explain our inability to identify a clear daily rhythm under the LD regime.

Desynchronization at the individual level could also obscure the existence of the endogenous (circadian) component in daily tim mRNA oscillations, as it appeared to be very weak at best (SD) or not apparent at all (LD). Transferring wild-type larvae to DD did not result in any change of their pretransfer developmental programming (pupariation vs. diapause). The basic tim expression pattern was also preserved (i.e., a steady increase after LD → DD transfer vs. relatively low levels and weak daily oscillations after SD → DD transfer). In contrast, transferring wild-type larvae from the LD to SD conditions led to developmental reprogramming (continuous development → diapause), which was preceded with a strikingly rapid adjustment of tim expression pattern from steady increase (LD characteristic) to relatively low levels and daily oscillations (SD characteristic). Perhaps, the aforementioned “weakness” of the endogenous component allowed for such a rapid change. In earlier studies, endogenous behavioral
The circadian gating of eclosion behavior damped within 3 to 4 days under continuous darkness (Lankinen and Riihimaa, 1992, 1997), the rhythm in adult male locomotion activity within 3 days (Koštál, unpublished data), and the oscillations of per mRNA in fly heads within 2 days (Koštál and Shimada, 2001). In nature, Chymomyza sp. larvae live under the bark of fallen forest trees where they feed on decaying phloem tissue (Hackman et al., 1970). Considering microclimate stability of the larval microhabitat on a daily scale, we can assume that there may be a weak selection pressure on developing/maintaining daily rhythmicities in physiology and behavior.

Differences in TIM Protein Levels

No TIM-like antigen was detected in the CNSs of 4-d-old npd-mutant larvae (64 npd-mutant CNSs were analyzed in total). In contrast, a TIM-like antigen has been clearly localized in 2 neurons of each brain hemisphere of 4-d-old wild-type larvae. Under SD, both neurons were stained strongly at night but weakly during the day. Such a pattern conforms well to the situation in D. melanogaster larvae, where several clusters of TIM-expressing neurons were found, and those belonging to lateral group (LNs) showed daily and circadian oscillations of staining intensity with a maximum during night (Kaneko et al., 1997; Kaneko and Hall, 2000). Relatively weak staining during the day indicates that rapid light-induced degradation of TIM protein, as it is known in D. melanogaster (Myers et al., 1996; Ceriani et al., 1999), may take place in C. costata larvae kept under SD. In contrast, under LD, TIM-like staining was relatively strong throughout the 24-h period. This observation suggests that the fate of TIM protein depends on photoperiodic regime. During the early morning time (ZT 2), a minimal intensity of TIM-like staining was observed in SD-reared larvae but there was a maximum in LD-reared larvae. Photoperiodic regulation of daily phase of tim gene expression and TIM protein presence indicates that the C. costata larval clock system may participate in seasonal time measurement. More detailed experimentation, however, is needed for hypothesizing on the precise mechanismic explanation of the TIM role in C. costata photoperiodism. It was shown in adults of D. melanogaster that coupling of brain circadian oscillators changes with photoperiod and may thus serve as a seasonal timer (Grima et al., 2004; Stoleru et al., 2007).

Differences in the Structure of the tim Gene

We found that sequences of TIM proteins differed in 37 amino acids between the wild-type and npd-mutant strains (97.3% identity). Such a relatively large difference probably reflects the fact that the strains were derived from 2 different geographic populations (wild-type, Sapporo; NPD, Tomakomai). There is no major geographic barrier between the 2 populations in nature. The distance of ca. 50 km, however, could be sufficient to prevent mixing. Putative functional domains were relatively well conserved in the 2 strains. In particular, the 32 aa regions and NLSs showed 100% identity. In addition, 4 of the 5 aa substitutions detected within PER-1 domain were for amino acids of the same functional group, as were all 5 aa substitutions found in the PER-2 domain. Reduced conservation of the acidic domain is characteristic for various drosophilid species (Myers et al., 1997; Ousley et al., 1998), and we have observed it in C. costata, too. The number, position, and size of introns appeared conserved between the 2 strains (and closely similar to the situation in D. melanogaster). Thus, we assume that amino acid substitutions had relatively small effect on tim gene transcription. Instead, the upstream events in the regulation of tim gene transcription could be disturbed in npd-mutant larvae. Indeed, we found a large deletion in the 5′-UTR of npd-mutant tim. 5′-flanking regions are known to contain important regulatory sequences, which are necessary for robust and cyclical transcription of tim in D. melanogaster. E-box and TER-box canonical sequences were found in a similar distance from the transcription start in wild-type C. costata tim and D. melanogaster tim (Hall, 2003). The large deletion of 1855 bp, however, removed the E-box and TER-box sequences from the npd-mutant tim promoter region. A more detailed study is ongoing in our laboratory to elucidate what exactly is the effect of the deletion on tim gene transcription and if and how it may influence the function of circadian and photoperiodic timing systems. In D. melanogaster, similar truncation of the promoter region that removes E-box and TER-boxes resulted in a weak and noncyclical expression of tim and a loss of behavioral rhythmicity (Hall, 2003), that is, a phenotype strikingly similar to the C. costata npd-mutant.
TIM and Photoperiodism in Other Organisms

The first hint of a possible role for TIM protein in insect photoperiodism came from the observation of Costa and Kyriacou (1998) that 2 forms of TIM protein of slightly different length (one with 23 aa truncated N-terminus) show a latitudinal cline in their geographical distribution among various populations of *D. melanogaster*. The incidence of diapause was higher in *ls-tim* flies (expressing both short, S-TIM, and long, L-TIM) compared to *s-tim* flies (expressing S-TIM only) (Tauber et al., 2007). In comparison to S-TIM, the L-TIM isoform binds less tightly to the circadian photoreceptor CRYPTOCHROME, resulting in a more stable TIM protein, and consequently, attenuated photosensitivity of the circadian clock and higher incidence of diapause (Sandrelli et al., 2007). Although these results reveal a molecular link between diapause and TIM, the polymorphism of TIM protein N-terminus has been observed in *D. melanogaster* only. In the other drosophilid species, including *C. costata*, the position of single translation initiating codon closely corresponds to the S-TIM form of *D. melanogaster* (Myers et al., 1997; Ousley et al., 1998). Moreover, as no significant interactions were found between the *tim* genotype and photoperiod in the induction of diapause in *D. melanogaster*, it seems that photoperiod and TIM exert their influence on diapause independently. In other words, the role of TIM in diapause induction may be independent of its role in the central circadian oscillator (Bradshaw and Holzapfel, 2007). The pitcher-plant mosquito *W. smithii* is another insect species where potential involvement of TIM in photoperiodism has been studied. Positive and consistent association was observed between the level of *tim* expression and latitude (Mathias et al., 2005). Detailed genetic linkage analysis confirmed that *tim* might play a role in the evolution of photoperiodism in *W. smithii*. It also revealed, however, that such a role was likely independent and incidental to its role in the central circadian pacemaker (Bradshaw et al., 2006; Mathias et al., 2007).

The noncircadian functions of TIM, that is, those that may have played a role in the evolution of insect photoperiodism, were studied in lower eukaryotes and mammals. Orthologs of TIM are bound by Tipin (TIM-interactin protein) (Gotter, 2003), and both proteins participate in cellular processes centered on DNA replication and cell cycle checkpoint signaling (Gotter, 2006). In yeast, both proteins are involved in the cell cycle arrest in response to DNA damage or depletion of dNTP stores (Sommariva et al., 2005). In mammals, *tim*-knockout resulted in developmental arrest and death in early embryonic development (Gotter et al., 2000). In light of such information, it is very salient that insect diapause is, by definition, a regulated developmental arrest (Denlinger, 2002; Koštál, 2006), which involves an arrest of cell proliferation and differentiation in target tissues such as embryonic cells, imaginal discs in larvae, and pupae or gonads in adults (Nakagaki et al., 1991; Tammaro and Denlinger, 1998; Koštál and Shimada, unpublished results). And, to close the circle, it was recently shown that proper orchestration of cell division cycle is influenced by circadian oscillators formed by canonical clock genes (Reddy et al., 2005). *Chironomus costata* may serve as a good model organism, in which relationships between the processes of circadian time control, cell proliferation, and seasonal life-cycle patterning may be tested.

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