Molecular analysis of the circadian clock in the firebrat, *Thermobia domestica*

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Abstract

Circadian clocks are important regulators of daily behavioral and physiological timing of animals. In *Drosophila melanogaster*, the circadian clock is thought to be composed of interlocked autoregulatory transcriptional/translational feedback loops consisting of a set of clock genes such as *period*, *timeless*, *Clock* and *cycle*. Recent studies suggest that in other insect species, the clock mechanism may differ from that of *Drosophila*. To dissect the molecular mechanism of the circadian clock in the firebrat *Thermobia domestica*, cDNAs of the clock genes *Clock* (*Thermobia domestica Clock*, *Td'Clk*), *cycle* (*Td'cyc*), and *timeless* (*Td'tim*) were cloned. *Td'tim* and *Td'cyc* were rhythmically expressed in the firebrat under light-dark cycles with a peak similar to that of *Drosophila* or to that of the mammalian clock, respectively, and the rhythms persisted under constant darkness. *Td'Clk* transcripts did not show any rhythmic change both under light-dark cycles and constant darkness, in contrast to *Drosophila Clk* that shows rhythmic expression with antiphase against *timeless*. Firebrats injected with *Td'Clk*, *Td'cyc* or *Td'tim* dsRNA became arrhythmic under constant darkness and mRNAs of respective genes were reduced to about 50% of the control firebrats, respectively. To examine the interrelation among *Td'Clk*, *Td'cyc* and *Td'tim*, effects of RNAi of each clock gene on the expression levels of other clock genes were analyzed. *Td'cyc* expression was found to be influenced by*Td'tim* and *Td'Clk,* while *Td'tim* seemed to receive regulation involving *Td'cyc* and*Td'Clk.* However, there was no indication that *Td'Clk* received influences from other clock genes.

These results clearly showed that RNA interference is a powerful tool to

dissect the circadian clock in the firebrat and revealed that *Td'Clk*, *Td'cyc* and *Td'tim* are involved in the central machinery of the firebrat circadian clock. More importantly, taken together, these results suggest that the molecular machinery of the firebrat circadian clock has traits both of *Drosophila* type and mammalian type clocks.

List of abbreviations

ANOVA, analysis of variance
bHLH, basic helix-loop-helix
bZip, basic leucine zipper
CLD, cytoplasmic localization domain
CK1, CASEIN KINASE1
CK2, CASEIN KINASE2
Clk, *Clock*
CLK, CLOCK
CODEHOP, consensus-degenerate hybrid oligonucleotide primers
cry, *cryptochrome*
CRY, CRYPTOCHROME
Cry2, *cryptochrome2*
CRY2, CRYPTOCHROME2
CT, circadian time
cwo, *clockwork orange*
cyc, *cycle*
CYC, CYCLE
DBT, DOUBLETIME
DD, constant darkness
DNs, dorsal neurons
dsRNA, double-stranded RNA
LD, light-dark cycle
LL, constant light
l-LNvs, large ventral-lateral neurons
LNds, dorsal-lateral neurons
LPNs, lateral posterior neurons
NLS, nuclear localization signal
pdf, *pigment-dispersing factor*
PDF, pigment-dispersing factor
PDH, pigment-dispersing hormone
Pdp1ε, *PAR domain protein 1 ε*
PDP1ε, PAR DOMAIN PROTEIN 1ε
per, *period*
PER, PERIOD

RORα, retinoic acid receptor-related orphan receptor α
SCN, suprachiasmatic nuleus
SGG, SHAGGY
s-LNvs, small lateral-ventral neurons
tim, *timeless*
TIM, TIMELESS
vri, *vrille*
VRI, VRILLE
UTR, untranslated region
ZT, zeitgeber time

Chapter 1.

General introduction

1.1. Observations on circadian rhythms

The first scientific observation of the existence of a daily rhythm was described in 1729 by the French astronomer de Marian (De Marian, 1729). He showed that the plant "*Mimosa*" maintained its diurnally periodic leaf movements in constant darkness at a relatively constant temperature. This phenomenon later attracted the attention of many botanists in the 19th century, including A. P. De Candolle, J. Sachs, C. R. Darwin, and W. F. Pfeffer. Especially W. F. Pfeffer was primarily concerned with the interaction of the leaf movement rhythm in plants with light and temperature (Pfeffer, 1915). Their results clearly indicated that these rhythms persist in darkness and at a constant temperature, but opinions were divided as to whether such rhythms were internally generated in the plants or externally driven by "X" in the environment which had not yet been detected by the researchers. In 1930, E. Bünning and K. Stern showed that the period of most daily rhythms observed were not precisely 24 h but rather approximately 24 h with a range from 22 to 28 h and thus brought severe difficulty to the "X" theory (Bünning and Stern, 1930). To their date, their observation remained as one of the strong indications to researchers that the observed rhythms are of an endogenous nature (Brown, 1972). Now, we can find the rhythm in most organisms from cyanobacteria to humans. There are lines of evidence for the rhythm to be controlled by an endogenous clock referred to as the circadian clock.

1.2. Essential properties of circadian rhythms

The circadian clock can act as an alarm clock to initiate a physiological process at an appropriate phase of the daily environmental cycle. It can also help an organism prepare in anticipation of actual need. Another important function in some organisms is the accurate measurement of ongoing time throughout the daily cycle. Many criteria of circadian rhythms were defined by C. S. Pittendrigh, E. Bünning, J. Aschoff, and others almost from the begging of circadian research as necessary, formal properties of living oscillator clocks. Three key characteristics are emphasized below.

The first criterion is the persistence of an overt circadian rhythm in constant temperature and constant light or dark conditions with a period of approximately 24 h (Decoursey, 1961; Menaker, 1971). To demonstrate the persistence, the rhythm must be assayed in constant conditions for several consecutive cycles, preferably five or more.

Temperature compensation is the second criterion. This characteristic refers to the observation that free-running period lengths in an organism are very similar when measured at different ambient temperatures (Pittendrich, 1954). Temperature compensation is often considered to be one aspect of a general compensation mechanism that keeps period lengths similar despite differences in factors affecting metabolism (Sweety and Hasting, 1960; Rudy et al., 1999).

The third criterion is that endogenous rhythms of approximately 24 h can be entrained by certain 24 h environmental signals, such as light-dark cycle, temperature cycle, or other stimuli (Bovet and Oertli, 1974; Pittendrigh, 1981).

The controlling system for overt circadian rhythms is called the circadian system. The system consists of three components: a receptor for entrainment to environmental cycles, an oscillator that generates 24 h oscillation, and the output system driving overt activity (Fig1-1A) (Dunlap et al., 2004). As the three components work together, organisms show circadian activity rhythms.

1.3. Circadian rhythms of insects

Most insects show daily rhythms in their various behavior or physiology. These rhythms could potentially serve as an indirect marker for the state of the circadian clock. For example, daily locomotor activity rhythms are commonly observed in many insects and have been studied in holometabolous insects such as flies, beetles and moths as well as in hemimetabolous insects including cockroaches, and crickets (Konopka and Benzer, 1971; Truman, 1972; Page and Barrett, 1989). The cricket *Gryllus bimaculatus* shows locomotor activity at night and this rhythm persists in isolated individuals in constant conditions in the laboratory (Tomioka and Chiba, 1984).

Another behavioral category is gated population rhythms, such as adult emergence or larval hatching rhythms of insects. For example, the hatching of the cricket *Gryllus bimaculatus* occurs rhythmically during the night, persisting in constant conditions (Tomioka et al., 1991). The population rhythms occur only once in the life of an individual and could be observed in a population which includes individuals at various developmental stages.

The sun-compass orientation in birds is another example of the clock output. In this case the clock is used to measure lapse of time to allow for compensation of the sun's movement by 15 degrees per hour across the sky (Hoffman, 1960). The appearance of bees at flowers that open only at specific times of the day is well known as a clock based time memory (Forel, 1910; Beling, 1929; Renner, 1955).

1.4. Insect photoreceptors for circadian entrainment

In most organisms light is an essential zeitgeber to synchronize the circadian clock to environmental 24 h cycles. Thus, the photoreceptors are necessary for the entrainment of the clock. In cockroaches and crickets, the compound eyes are the exclusive photoreceptive organs for the entrainment. Severing the optic nerves between the retina and optic lobe eliminates the entrainment of locomotor activity rhythms to light cycles (Fig1-1B) (Nishiitsutsuji-Uwo and Pittendirgh, 1968; Loher, 1972; Tomoka and Chiba, 1984). In other species, extraretinal photoreceptor may be important. For example, blind or eyeless mutants *Drosophila melanogaster* can synchronize well to light-dark cycles. In this entraiment, CRYPTOCHROME (CRY), which is a blue-light photoreceptor in the family of flavoprotein and is expressed in some cerebral neurons, plays an important role (Emery et al., 1998; Stanewsky et al., 1998; Helfrich-Föster et al., 2001).

1.5. Localization of the circadian pacemaker in insects

The pacemakers regulating the circadian rhythm have been studied and

localized to discrete regions of the brain. The brain of insect can be divided into two major areas, i.e. the optic lobe and the cerebral lobe (central brain). The optic lobes are paired bilateral structures that recieve input from the compound eyes. The visual imformation is then transmitted to the central brain.

The optic lobes generate circadian oscillations in many insect species. For example, removing the two optic lobes in the cockroach *Leucophaea maderae* or in the cricket *Gryllus bimaculatus* resulted in a loss of locomotor activity rhythms (Page,1982; Tomioka and Chiba, 1984, 1989). The results suggested that the optic lobes either contain the circadian clock or are a part of the output pathway of the clock. Unequivocal evidence that the optic lobe contains a circadian clock was provided by a demonstration that the optic lobe continued to generate oscillations even when isolated and kept in vitro in the cricket. When the optic lobes were extracted and maintained in organ culture conditions, they expressed a circadian rhythm in spontaneous neural impulse activity (Tomioka and Chiba, 1992).

Not all insects use the optic lobe as circadian clock, however. In contrast to cockroaches and crickets, it is known that in house flies, the optic lobes are not necessary for the rhythm and the clock appears to reside in the central brain: Locomotor activity rhythms of the house fly *Musca domestica* continued after surgical lesions of the optic lobes but disappeared after lesions of the cerebral lobes (Helfrich et al., 1985). The importance of the cerebral lobe in the rhythm generation has been shown by a transplantation experiment in the fruit fly *D. melanogaster* (Konopka and Benzer, 1971). Circadian rhythms of

locomotor activity in the fruit fly persist in a variety of neuroanatomical mutants with strongly reduced optic lobes.

1.6. The output signals

Output signals have been studied in some insects. The secretion of a variety of insect hormones is under the control of the circadian system. Included are ecdysone, prothoracicotropic hormone, and eclosion hormone. These hormones are involved in the regulation of various developmental events, such as ecdysis.

Pigment-dispersing factor (PDF), a homolog of the crustacean pigment-dispersing hormone (PDH) (Helfrich-Föster and Homberg, 1993), is the most distinguished output neuropeptide of the insect circadian clock. PDF has been immunohistochemically detected in some cerebral neurons in many insects (Pyza et al., 2003; Reischig et al., 2004; Abdelsalam et al., 2008). In *D. melanogaster*, *pdf* mRNA expression exhibits no rhythmic profiles in the cell body, while the dorso-medial termini of PDF-positive axons show daily changes in morphologies, probably controlled by the circadian clock (Park et al., 2000; Fernandez et al., 2008).

1.7. Molecular oscillatory mechanism of the *D. melanogaster* circadian clock

The fruit fly, *D. melanogaster*, has been used for studies on the circadian clock for more than 50 years. The fly shows bimodal locomotor activity rhythms with peaks at around dawn and before dusk. When the flies are transferred to DD, they show free-running rhythms with a period of approximately 24 hr

(Konopka and Benzer, 1971). Under LD cycles, light received by photoreceptors adjusts the phase of the clock, which otherwise ticks autonomously with its own period. The clock sends its time information to the downstream through an output pathway to regulate overt behavioral rhythms.

In *D. melanogaster*, the oscillation of the clock is thought to be generated by a molecular mechanism that composed of transcriptional-translational autoregulatory feedback loops (Dunlap, 1999). In the fly's brain there are about 150 neurons which express the so-called clock genes such as *period* (*per*) and *timeless* (*tim*), and they are classified into seven groups (Fig1-2). Three groups (DN1, DN2 and DN3) are located in the dorsal regions of the brain, and the remaining four groups (s-LNv, l-LNv, LNd and LPN) are located in the lateral region between the optic lobe and the cerebral lobe (Helfrich-Föster, 2005).

The molecular machinery of the circadian clock has been extensively studied (Fig1-3). At least three interdependent feedback loops are thought to constitute the rhythm-generating machinery. One negative feedback loop is formed by *per* and *tim*. dCLOCK (dCLK) and CYCLE (CYC) proteins form a heterodimer and bind to specific enhancer elements (E-boxes), which exist in the upstream regulatory regions of *per* and *tim*, and activate the transcriptions of these genes at early night (Darlington et al., 1998). Translated PER and TIM increase during the night, and their posttranslational regulations are provided by DOUBLETIME (DBT), CASEIN KINASE 2 (CK2) and SHAGGY (SGG), to control the stability and the timing of nuclear transport of PER and TIM by phosphorylation (Akten et al., 2003; Martinek et al., 2001; Price et al., 1998). PER and TIM form a heterodimer and enter the nucleus to inhibit their own

transcription by inactivating dCLK-CYC heterodimers. The second negative feedback loop, *clockwork orange* (*cwo*), plays an important role in another loop. *cwo* is a transcriptional repressor belonging to the basic helix-loop-helix (bHLH) ORANGE family. *cwo* is rhythmically expressed, and its protein represses the dCLK-CYC transcriptional activity (Kadener et al., 2007; Matsumoto et al., 2007). The third positive/negative feedback loop consists of *dClk*, *cyc*, *vrille* (*vri*) and *PAR domain protein 1ε* (*Pdp1ε*), and regulates the rhythmic expression of *dClk*. The dCLK-CYC heterodimer activates the transcription of *Pdp1ε* and *vri* during late day to early night. Translated VRI enters the nucleus and bind to a V/P-box in the promoter region of *dClk* and inhibits its transcription. Thus *dClk* mRNA is reduced during the night. PDP1ε is thought to bind to the V/P-box competitively with VRI and activates transcription of *dClk* (Cyran et al., 2003; Glossop et al., 2003). Newly translated dCLK is responsible for the transcription of *vri* and *Pdp1ε* in addition to *per* and *tim*, and is repressed by PER and TIM. When PER is degraded, dCLK can drive the next cycle.

1.8. Circadian rhythm of vertebrates

A variety of physiological functions are known to show circadian rhythms in mammals. For example, the metabolic activity switches between various nocturnal processes that contribute to growth and repair of the body and diurnal processes that support energetic demand and psychological alertness. The sleep-wake cycle is a prominent circadian phenomenon in mammals (Buysse et al., 2001). In parallel the core body temperature starts to fall in the evening in anticipation of sleep and rises in the early morning in

preparation for the active phase in diurnal mammals (Refinetti and Menaker, 1992). Other important circadian functions are the rhythmic secretions of melatonin that occurs during nighttime and of the adrenal hormone, cortisol, that is secreted primarily during the circadian day to help mobilize energy (Tosini and Menaker, 1998).

1.9. Circadian clock of nonmammalian vertebrates

Most nonmammalian vertebrates have multiple circadian oscillators, and most have multiple photic input pathways. The pacemaker units, i.e., pineal grand, suprachiasmatic nuleus (SCN), and/or retina, involve a coupling of separate sites through neural and humoral pathways to ensure a coherent regulation of the multiple overt rhythms of an individual (Gaston and Menaker, 1968). Environmental signals for photic entrainment of the pacemaker system may come from the retina of the eyes, extraretinal photoreceptors of the brain, or pineal photoreceptors (Gaston and Menaker, 1968). Nonphotic entraining agents, such as temperature or social interaction, may also be important. Output messages may be humoral, such as melatonin and cortisol, or neural (Tosini and Menaker, 1998; Ebihara and Fukada, 1999). Some of these components have been remodeled extensively during the course of evolution from fish to mammals.

1.10. The suprachiasmatic nucleus (SCN) is the primary clock in mammals

For many years after the discovery of the SCN in 1972, the mammalian circadian system appeared to be monolithic in having only one chief central

clock (Moore and Lenn, 1972; Moore and Eichler, 1972; Stephan and Zucker, 1972). A single primary central clock, the SCN, is connected to a single photic sensor, the retina. Multiple modalities mediating output include efferent nerve tracts and humoral signals such as melatonin of the pineal or hormones of the pituitary gland (Arendt, 1995).

Peripheral clocks in mammals and their interaction with the SCN pacemaker are an important topic in current circadian research. Although extra-SCN oscillators have been suspected in mammals for some time, a direct demonstration was first provided by measurement of a circadian rhythm of melatonin synthesis in hamster retinae *in vitro* (Tosini and Menaker, 1996) (See Chapter 1.12).

The best characterized SCN input is a direct neural projection from the retina through the retino-hypothalamic tract (Yamazaki et al., 1999). Because light is the most salient feature of the entrainment affecting circadian rhythms, a direct neuronal projection to the SCN from the eyes is not surprising. The fact that animals deprived of their eyes free-run shows that the eyes are necessary for photic entrainment in the SCN clock (Meijer, 2001).

1.11. Circadian clock of mice

The vertebrate circadian feedback loops are based on transcription and translation similar to those of *D. melanogaster*, and they employ a heterodimer of proteins containing PAS domain, acting as transcriptional activator, as well as a family of PER proteins and mammalian type CRY2 proteins as negative elements (Fig.1-4).

High levels of BMAL1 protein lead to the formation of CLK-BMAL1 heterodimers that in turn promote transcription of the *Per*, *Rev-erbα*, and *Cry* genes at early morning (Lee et al., 2001). Levels of *Per*, *Rev-erbα*, and *Cry* mRNAs are rising, and the corresponding proteins appear at during the day. REV-ERBα moves to the nucleus to repress *Bmal1* expression (Preitner et al., 2002). PER and CRY2 shuttle to and from the nucleus, and they may be stabilized in the nucleus by mutual association. PER proteins are bound and phosphorylated by CASEIN KINASE 1 (CK1), marking them for eventual turnover. Eventually, the PER-CRY blocks BMAL-CLK activity at early night. As a result of BMAL1-CLK inhibition, *Per*, *Rev-erbα*, and *Cry* mRNA levels are declining, and likewise from the turnover of PER, REV-ERBα, and CRY protein levels are decreasing. *Bmal1* mRNA levels are increased by RORα at midnight. Turnover of the PER-CRY-CK1 inhibitory releases the BMAL1-CLK to reactivate transcription of the *Per*, *Rev-erbα*, and *Cry* genes to reinitiate the next cycle (Dunlup, 1999; Reppert and Weaver, 2002).

1.12. Peripheral clocks

For many years after the discovery of the SCN as the clock locus in 1972, the mammalian circadian system appeared to be monolithic in having only one chief central pacemaker. In 1996, a surprising new circadian mammalian clock was announced residing locally in the retina (Tosini and Menaker, 1996). By culturing whole retinas in a flow-through chamber, scientists could follow the melatonin synthesis and secretion rhythm for as long as five days. The properties of this system resembled those of the SCN. Recent molecular studies

have revealed that wide variety of peripheral tissues have their own clock that oscillate independent of the central SCN clock (King et al., 1997; Tei et al., 1997; Balsalobre et al., 1998; Yamazaki et al., 2000; Yoo et al., 2004; Reyes et al., 2008).

1.13. The objective of this study

The machinery of the *D. melanogaster* circadian clock has been understood in details as described above. However, this hypothesis is not fully supported by resent studies using other insect species. For example, in the crickets *Gryllus bimaculatus*, no rhythmic expression of *Clk* can be observed (Moriyama et al., 2012) or *tim* is not essential for the persistence of circadian clock oscillation (Danbara et al., 2010); the honey bee *Apis mellifera* lacks *timeless* gene in its genome and has a mammalian-type *cryptochrome* (*cry2*) gene, leading a conclusion that *A. mellifera* has a mammalian-type circadian clock (Rubin et al., 2006); in the silkmoth *Antheraea pernyi*, PER has an oscillation in the cytoplasm but not in the nucleus (Sauman and Reppert, 1996); in the monarch butterfly *Danaus plexippus*, *cry2* works together with *per* as a transcriptional repressor of negative feedback loop similar to the mammalian clock (Zhu et al., 2008). These results suggest that the molecular mechanism of the *D. melanogaster* circadian clock is rather specific and might not be the general mechanism for insect clocks, and that there might be diverse clock mechanisms among insect species. It is thus necessary to compare the clock mechanism across the diverse insect classes to understand the diversification of insect clock mechanisms. The firebrat, *Thermobia domestica* is an especially suitable insect to study this issue, because

the firebrat is one of the most primitive insects and expected to retain an ancestral clock.

In this study, the clock genes *Clock*, *cycle*, and *timeless* have been cloned from the firebrat *T. domestica* and their functions in circadian rhythm generation were analyzed. Since it is very difficult to isolate mutants in the firebrat, RNA interference mediated gene silencing was used as a major tool to dissect the molecular mechanism.

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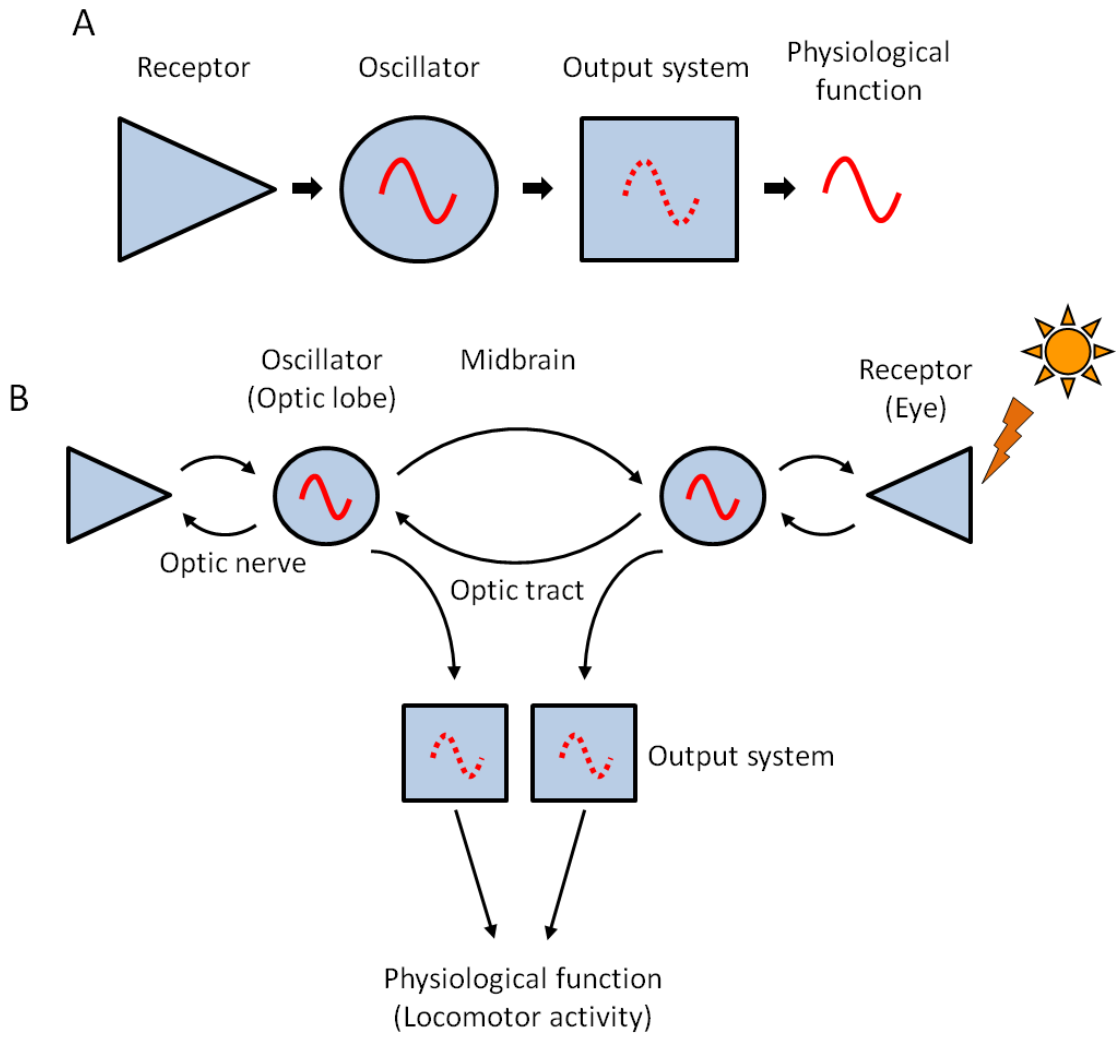

Chapter 1 Figures

Figure 1-1. Schematic diagrams of the circadian system (A) and the pacemaker system of cockroach (B).
(A) The circadian system is composed of three constituents, i.e. a receptor mediating the environmental cues for entrainment, an oscillator generating the rhythm, and an output system that mediates time information from the oscillator to downstream systems. (B) A diagram showing circulation of the circadian nervous system.

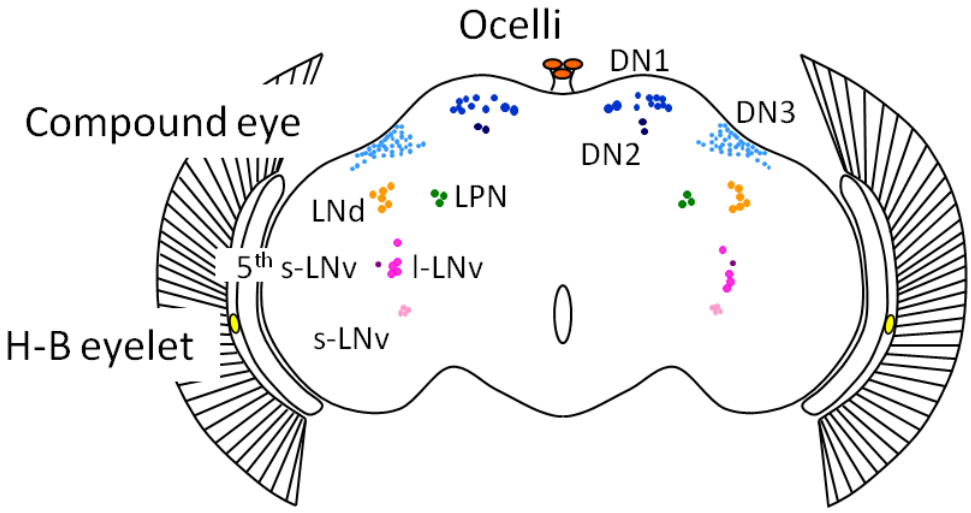

Figure 1-2. Identified clock neurons in the fly's brain.
Clock neurons are roughly classified into seven groups according to the size and the location in the hemisphere; three groups are localized at the dorsal protocerebrum (DN1, DN2, and DN3) and the remaining four groups (s-LNv, l-LNv, LNd, and LPN) are at the lateral side between the cerebral lobe and the optic lobe.

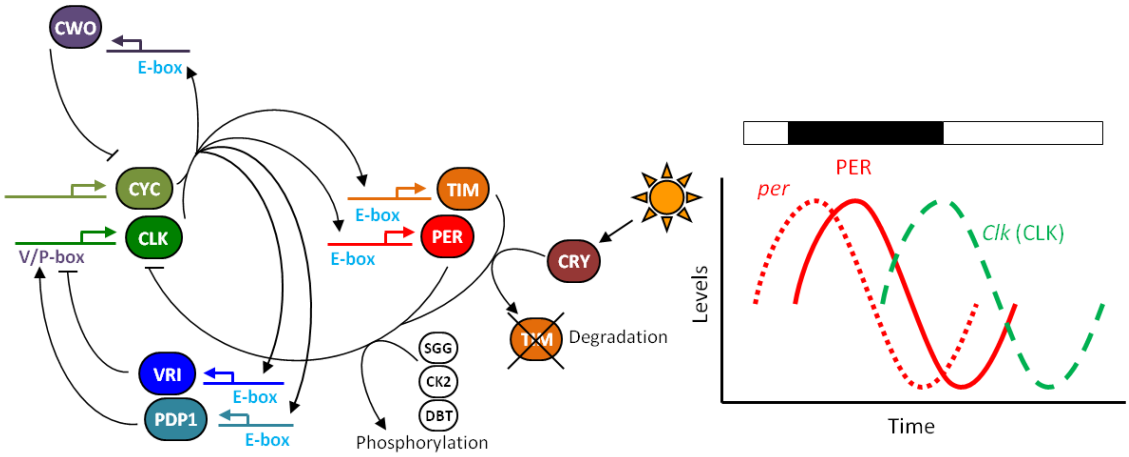

Figure 1-3. The molecular mechanism of the *Drosophila* clock.
CLK and CYC form a CLK-CYC heterodimer that promotes transcription of *per*, *tim*, *vri* through E-box during late day to early night. Thus levels of *per* and *tim* transcripts begin to rise. During the late day, translated TIM proteins are degraded by light-activated CRY: a light-dependent reset mechanism of the clock. As PER and TIM levels increase during midnight, the proteins together form a stable complex that is capable of moving into the nucleus, a process promoted through phosphorylation by the CK2 and SGG. In the nucleus, the PER-TIM-DBT complex represses transcription of *per* and *tim* through inhibitory action to CLK. In the late night, phosphorylated PER is no longer bound by TIM, and TIM is degraded by the proteasome system. The breakdown of PER releases the CLK-CYC heterodimer to reactivate *per* and *tim* transcription, starting the next cycle. Meanwhile, VRI accumulates during early night and represses *Clk* transcription through its binding to VRI/PDP1-box (V/P-box). Later accumulating PDP1ε activates *Clk* transcription by competitive binding to V/P-box with VRI, leading to a rhythmic expression of CLK with a peak at early day. *cwo* is rhythmically expressed to peak under the regulation by dCLK-CYC and forms its own negative feed-back loop. White and black bars indicate light phase and dark phase respectively.

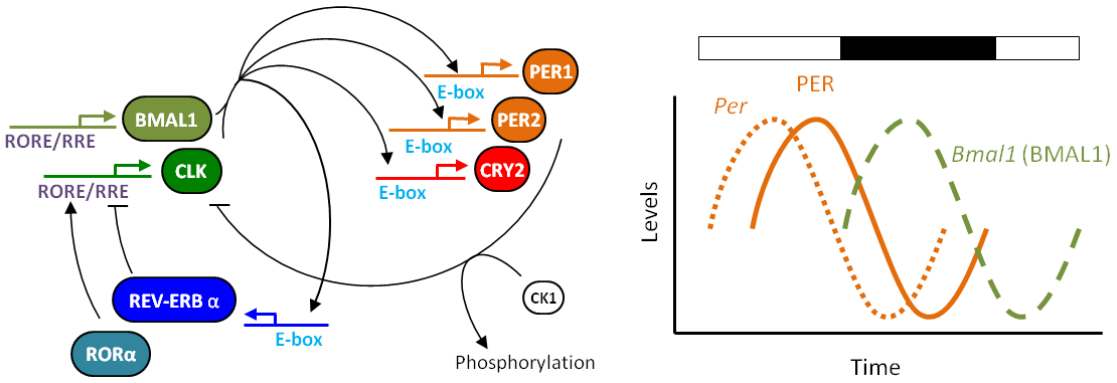

Figure 1-4. The molecular mechanism of the mammalian clock.
BMAL1-CLK binds to E-boxes of *Per1*, *Per2*, *Cry1* and *Cry2* to activate their transcription, and PER1, PER2, CRY1 and CRY2 increase during late day. After they are phosphorylated by CK1, PER1-PER2-CRY1-CRY2 complex enters the nucleus, and restrain their own transcription by inactivation of BMAL1-CLK transcriptional ability during early night. After activation of *Rev-erbα* transcription through E-box by BMAL1-CLK, REV-ERBα immediately accumulates in the nucleus and inhibits *Bmal1* transcription through RRE domain, and RORα accumulates in the nucleus and promotes *Bmal1* transcription through RORE domain during night. Newly produced BMAL1 binds to CLK, and BMAL1-CLK initiates the next cycle.

Chapter 2.

Functional analysis of the clock genes *Clock* and *cycle* in firebrats

2.1. Abstract

Comparative molecular analysis reveals a wide variation of clock mechanisms among insects. In this chapter, the clock gene homologues of *Clock* (*Td'Clk*) and *cycle* (*Td'cyc*) were cloned from an apterygote insect, *Thermobia domestica*. Structural analysis showed that *Td'*CLK includes bHLH, PAS-A, PAS-B domains but lacks a polyglutamine repeat in the C terminal region that is implicated for transcriptional activity in *Drosophila* CLK. *Td'*CYC contains a BCTR domain in its C terminal in addition to the common domains found in *Drosophila* CYC, i.e. bHLH, PAS-A, PAS-B domains. Unlike in *Drosophila*, *Td'Clk* mRNA levels showed no significant daily fluctuation, while *Td'cyc* exhibited rhythmic expression. A single injection of double-stranded RNA (dsRNA) of *Td'Clk* or *Td'cyc* into the abdomen of adult firebrats effectively knocked down respective mRNA levels and abolished the rhythmic expression of *Td'cyc*. Most *Td'Clk* or *Td'cyc* dsRNA-injected firebrats lost their circadian locomotor rhythm in constant darkness up to 30 days after injection, whereas those injected with *DsRed2* dsRNA as a negative control clearly maintained it. From these results, it is likely that *Td'Clk* and *Td'cyc* are involved in the circadian clock machinery in the firebrat. However, the structure and expression profile of *Td'Clk* and *Td'cyc* more closely resemble those of mammals than *Drosophila*.

2.2. Introduction

Circadian rhythms are endogenous oscillations with a period of about 24 h driven by a timing machinery called the circadian clock. The circadian clock is currently believed to be based on feedback loops in gene expression

(Hardin, 2009; Tomioka and Matsumoto, 2010). The molecular machinery of the clock is best studied in the fruit fly *Drosophila melanogaster*. The *Drosophila* clockwork consists of three loops. The first loop is composed of two negative elements, *period* (*per*) and *timeless* (*tim*), and two positive elements *Clock* (*Clk*) and *cycle* (*cyc*). The loop is initiated by transcription of *per* and *tim* by the heterodimerized product protein of *Clk* and *cyc*, CLK and CYC, during late day to early night. The resultant proteins PER and TIM accumulate in the cytoplasm during night, form a complex with DOUBLETIME (DBT) and enter the nucleus at mid to late night to suppress their own transcription by inhibiting CLK-CYC. The second loop consists of *vrille* (*vri*), *PAR domain protein 1ε* (*Pdp1ε*), and *Clk*, producing an oscillatory expression of *Clk* (Cyran et al., 2003; Glossop et al., 2003). The third loop is for the oscillation of *clockwork orange* (*cwo*) (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007).

However, immunohistochemical and molecular studies have yielded considerably different results among insects. For example, in the silkmoth *Antheraea pernyi*, PER is rhythmically expressed in the cytoplasm of presumptive clock neurons but never enters the nucleus, and the rhythm is hypothetically based on rhythmical expression of *per* sense and antisense RNAs (Sauman and Reppert, 1996). In the cricket *Teleogryllus commodus* and *T. oceanius*, no rhythmic expression of PER was observed (Lupien et al., 2003). In the butterfly *Danaus plexippus* and the honeybee *Apis mellifera*, mammalian-type CRYPTOCHROME (CRY2) is thought to form the first loop together with PER (Rubin et al., 2006; Zhu et al., 2008; Gentile et al., 2009). In the honeybee, *tim* is absent from the genome (Rubin et al., 2006). Considerable differences are

known in the molecular structures of CLK and CYC between *Drosophila* and other insects (Chang and Reppert, 2003; Rubin et al., 2006). These differences suggest that insect clocks have diversified during the course of evolution. To understand how this variety of clock mechanisms derived from an ancestral one, further comparative study is necessary. The apterygotes may be suitable for this purpose because they are inferred to retain ancestral clock traits. Previous studies showed that in the firebrat *Thermobia domestica*, PER-like immunoreactivity exhibited daily oscillations in the cytoplasm (Zavodska et al., 2003, 2005), implying that this insect also possesses a circadian clock based on a molecular feedback loop.

In this study, I have cloned cDNAs of the clock genes *Clock* and *cycle* (*Td'Clk* and *Td'cyc*) from the firebrat *T. domestica*, examined their daily expression patterns using quantitative real-time RT-PCR (qPCR), and investigated the role of *Td'Clk* and *Td'cyc* gene in circadian rhythm generation with RNA interference (RNAi). The results showed that the sequences and expression profiles of these clock genes showed surprising similarity to their mammalian orthologs. Knocking-down these genes by RNAi disrupted circadian rhythms, suggesting that both *Td'Clk* and *Td'cyc* play an important role in the circadian system in the firebrat.

2.3. Material and Method

Animals

Adult firebrats, *T. domestica*, an apterygote insect in the order Zygentoma, were used for all the experiments. They were taken from our

culture reared in a light cycle of 12 h light and 12 h darkness (LD12:12) and at a constant temperature of 30°C. They were fed animal food (CA-1, Nihon Crea).

Behavioral analysis

To monitor locomotor activity, adult firebrats were individually housed in transparent acrylic rectangular tubes (6 mm×6mm×70mm). The tubes were plugged at one end with a piece of animal food and were sealed with plastic tape; at the other end, they were sealed with wet cotton connected to a water bottle. Movement of the firebrat was sensed by a photoelectrical detection system: a moving firebrat interrupted an infrared beam, and the number of interruptions during each 6-min interval was recorded using a computerized system. The activity sensing system was placed in an incubator (MIR-153, Sanyo Biomedica, Osaka, Japan), in which temperature was kept at 30°C and lighting conditions were given by a cool white fluorescent lamp connected to an electric timer. The light intensity was 600 to 1000 lux at the animal's level, varying with the proximity to the lamp. The raw data were displayed as conventional double-plotted actograms to judge activity patterns, and free-running periods were calculated by the χ^2 periodogram (Sokolove and Bushell, 1978). If peaks of the periodogram appeared above the 0.05 confidence level, the periods of the peaks were designated as statistically significant. The power of the rhythm was defined as the amplitude from the top of the peak to the confidence level in the χ^2 periodogram (Liu et al., 1991).

cDNA cloning of clock genes *Clock* and *cycle*

Total RNA was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) from 5 heads of adult firebrat collected at zeitgeber time (ZT; ZT0 corresponds to light-on and ZT12 corresponds to light-off) 14. A total of 5 μg of total RNA was used for reverse transcription to obtain cDNA, using SuperScriptIII (Invitrogen, Carlsbad, CA). Using the obtained cDNA as a template, PCR was performed with degenerate primers deduced from the conserved amino acid sequences among vertebrate and insect *Clk* and *cyc* homologues (5′-agaagaagcggcgggaYcaRttYaa-3′ and 5′- ggtctgcagccagatccaYtgYtg-3′ for *Clk* and 5′-caagagatggcgagataagatgaaYacNta- 3′ and 5′-tcgacatcctttgtccatggRttNYKRaa-3′ for *cyc*). PCR conditions were as follows: 30 s for denaturation at 95°C, 30 s for primer annealing at 57°C , and 1 min 30 s for extension at 72°C for 35 cycles with Extaq DNA polymerase (Takara, Otsu, Japan). The purified fragment was cloned into TOPO-pCRII vector (Invitrogen) and sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). 5′ and 3′RACEs were done with GeneRacer™ kit (Invitrogen) and gene-specific primers as follows; fb-Clk-a173 (5′-CCAGTCTTCCTGGATTTCGT-3′) and fb-Clk-a549 (5′-TCTTGCCTTATCCCACTTGC-3′) and fb-cyc-a252 (5′-TCACAGCCCACAACAAAGAG-3′) and fb-cyc-a460 (5′-CTGAGGCACGTCTGTCTTCA-3′) for 5′RACE of *Clk* and *cyc*, respectively, and fb-Clk-s530 (5′-GCAAGTGGGATAAGGCAAGA-3′) and fb-Clk-s913 (5′-TCCTAACGAAAGGCCAACAA-3′) fb-cyc-s519 (5′-GCCTGCAACCTCTTTTGTTC-3′) and fb-cyc-s726 (5′-AAGTCGCTCCTTTCGTCAAA-3′) for 3′RACE of *Clk* and *cyc*, respectively.

RACE fragments were purified, cloned, and sequenced as mentioned above. Sequences were analyzed by Genetyx ver. 6 (Genetic Information Processing Software, Tokyo, Japan) and BioEdit version 7.0.9.0 (Biological Sequence Alignment Editor, Ibis Therapeutic, Carlsbad, CA). Amino acid sequences of CLOCK and CYCLE were analyzed and neighbor-joining trees were inferred with ClustalW (http://clustalw.ddbj.nig.ac.jp/top.html). Sequences of known insects were obtained from GenBank and RefSeq.

Quantitative real-time RT-PCR

mRNA levels were measured with quantitative real-time RT-PCR (qPCR). Total RNA extraction from adult firebrats was performed by TRIzol Reagent (Invitrogen) and the obtained RNA was treated with DNase I to remove contaminated DNA. About 500 ng of total RNA of each sample was reverse transcribed with random 6mers using Exscript RT reagent kit (Takara). Real-time PCR was performed with Mx3000P Real-Time PCR System (Stratagene, Lajolla, CA) using FullVelocity SYBER Green QPCR Master Mix (Roche, Tokyo, Japan) including SYBER Green with fb-Clk-F1 (5′-ATCGCAAGGGTCTGGAAGTG-3′) and fb-Clk-R1 (5′-GGAAAACTCGCCAAGACAGG-3′) for *Clk*, fb-cyc-F1 (5′-CGTGTAATCTGTCGTGTTTGGTG-3′) and fb-cyc-R1 (5′-GAATCGTCCGCCTTTCCTC-3′) for *cyc*, or fb-rp49-F1 (5′-AGTCCGAAGGCGGTTTAAGG-3′) and fb-rp49-R1 (5′-TACAGCGTGTGCGATCTCTG-3′) for *rp49* (GenBank/EMBL/DDBJ Accession no. AB550830). The results were analyzed using software associated

with the instrument. The values were normalized with the values of *rp49*, a housekeeping gene, at each time point. Results of 3 or 4 independent experiments were pooled to calculate the mean ± SEM. Data were analyzed by ANOVA.

RNAi

Double-stranded RNA (dsRNA) of *Td'Clk*, *Td'cyc* and *DsRed2* (Clontech) were synthesized using MEGAscript High Yield Transcription Kit (Ambion). *DsRed2* is a variant of red fluorescent protein gene (*DsRed*), derived from a coral species (*Discosoma* sp.) and is lacked in the firebrat's genome. Primers fused with T7 promoter sequence were designed for synthesis of double-stranded RNAs (dsRNAs) of *Td'Clk* and *Td'cyc* as follows; fb-clk-dsRNA-T7-F1 (5'-TAATACGACTCACTATAGGGACCACCAATCGAAAAATGGA-3') and fb-clk-dsRNA-T7-R1 (3'-TAATACGACTCACTATAGGGCCCAGTTCCCACGAAAACTA-5') for *Td'Clk* and fb-cyc-dsRNA-T7-F1 (5'-TAATACGACTCACTATAGGGAGGGGCTGTTCATTCCTACA-3') and fb-cyc-dsRNA-T7-R1 (5'-TAATACGACTCACTATAGGGCGCCCACGACTTCAAATAAC- 3') for *Td'cyc*. Standard PCR was performed using *Td*'s cDNA as a template. Resulting amplicons, excluding the fused T7 promoters were 600 and 550 bp, respectively, for *Td'Clk* and *Td'cyc*. *DsRed2* was linearized from pDsRed2-N1 (Clontech,Mountain View, CA), amplified with the forward and reverse primers containing T7 promoter. Then RNA was synthesized with T7 RNA

polymerase. Synthesized RNAs were extracted with phenol/chloroform, and suspended in 50 µl of TE solution after isopropanol precipitation. The yield and quality of RNA were assessed by absorbance with a spectral photometer (Genequant Pro, Amersham Biosciences, Piscataway, NJ) and the same amounts of sense and antisense RNA were mixed. The RNAs were denatured for 5 min at 100°C and annealed by a gradual cool-down to room temperature. After ethanol precipitation, the obtained dsRNA was suspended in Ultra Pure Water (Invitrogen) and adjusted to a final concentration of 10 µM. The dsRNA solution was stored at -80°C until use. A total of 70 nl (10 µM) dsRNA solution was injected with a nanoliter injector (WPI, Sarasota, FL) into the abdomen of adult firebrats anesthetized with CO_2.

2.4. Results

Locomotor activity rhythm of firebrat

Locomotor activity rhythms were recorded in LD 12:12, constant darkness (DD) or constant light (LL) conditions with a total of 84 firebrats. Representative actograms are shown in Fig. 2-1. Animals were recorded in LD 12:12 for the first several days then transferred to DD or LL. Nearly 70% of the firebrats (N = 59) showed a clear rhythm with activity mostly confined to the dark portion in the LD (Fig. 2-1). In DD, 45% of the firebrats showed a significant free-running rhythm. The free-running period was slightly longer than 24 h and the power of the rhythmicity (13.32±3.4 [SEM], N = 21) was significantly less than that in LD condition (29.3±3.84, N = 59). The average free-running period was 24.36±0.35 (SEM) h (N = 21). The remaining insects

became arrhythmic but still showed weak rhythmicity, with an increase of activity during night. In LL, all the firebrats (N = 29) became arrhythmic with sporadic activity dispersed over 24 h (Fig. 2-1B). Daily activity levels fluctuated with a period of about 13-25 days, averaging 20.46±4.87 (SD) days in all the conditions; this seems to be associated with molting cycles.

Cloning and sequencing of clock genes *Clock* and *cycle*

To investigate the molecular mechanism of firebrat circadian clock, cDNA clones of the *Clock* and *cycle* homologs of *T. domestica* were first isolated with primers constructed based on the conserved amino acid sequences from vertebrates and insects and 5′ and 3′RACE strategy. The *T. domestica Clk* cDNA (*Td'Clk*, GenBank/EMBL/ DDBJ Accession no. AB550828) consists of 2379 bp of nucleotide with 5′- and 3′- untranslated regions (UTR) of 396 bp and 417 bp, respectively. The *Td'Clk* is predicted to encode a protein of 522 amino acids. The alignments of conserved domains of *Td*'CLK compared with those of other insects are shown in Fig. 2-2A. *Td*'CLK contains a basic helix-loop-helix (bHLH) domain for binding to DNA (Allada et al., 1998; Darlington et al., 1998), PAS-A and PAS-B dimerization regions that are involved in the protein-protein interaction and mediate the binding to the heterodimeric partner CYC (Allada et al., 1998; Darlington et al., 1998). The overall structure of *Td*'CLK is shown in Fig. 2-2B. A BLAST database search indicated that the amino acid sequence has 55-73% identities along the entire length of the protein to those of other known insect species (Table 2-1). Among the putative functional motifs, PAS-A had identity of 53-75% while bHLH and PAS-B had high identity of 77-94% and

82-93%, respectively, with other insect species, suggesting their conserved functional role (Table 2-1). Like *Antheraea pernyi* CLK, it lacks a polyglutamine region in the C terminal region, which is related to transactivation function in *Drosophila* (Allada et al., 1998; Arlington et al., 1998). A phylogenetic tree based on the amino acid sequences of CLK from known insect species (Fig. 2-2C) reveals a close relationship between the firebrat and *Tribolium castaneum*. The *T. domestica cycle* cDNA (*Td'cyc*, GenBank/EMBL/DDBJ Accession no. AB550829) consists of 2579 bp of nucleotide with 5'- and 3'- UTR of 483 bp and 164 bp, respectively. The *Td'cyc* encodes a predicted protein of 644 amino acids. The alignments of its conserved domains compared with those of other insects are shown in Fig. 2-3A and its overall structure in Fig. 2-3B. A BLAST database search indicated that the amino acid sequence has 47-70% identities along the entire length of the protein to those of known insect CYC (Table 2-2). Among the putative functional motifs, bHLH, PAS-A and PAS-B had high identity of 84-94%, 81-96% and 67-92%, respectively, suggesting their conserved functional role (Table 2-2). Importantly, like most known insect CYC, but unlike *Drosophila melanogaster* CYC (*Dm*'CYC), the C terminal of *Td*'CYC has a BMAL1 C-terminal region (BCTR; Fig. 2-3A and B) that shows potent transcriptional activity in vitro (Takahata et al., 2000; Chang and Reppert, 2003). The BCTR region also has high similarity (74-90% identity) to that of known insect CYC (Table 2-2). A phylogenetic tree based on the amino acid sequences of CYC from known insect species reveals a close relationship between the firebrat and *Tribolium castaneum* (Fig. 2-3C).

Expression patterns of *Td'Clk* and *Td'cyc*

To determine whether transcripts of *Td'Clk* and *Td'cyc* oscillated in a circadian manner, levels of *Td'Clk* and *Td'cyc* mRNAs in *T. domestica* were examined under LD12:12 and in the 2nd cycle of DD by performing qPCR. The *Td'Clk* mRNA showed a slight fluctuation in LD but it was not significant (ANOVA, $p > 0.05$, Fig. 2-4A). No circadian rhythmic change was observed also in DD (ANOVA, $p > 0.05$, Fig. 2-4B). In contrast, the expression of *Td'cyc* mRNA appeared to cycle with a peak during the day (ANOVA, $p < 0.01$, Fig. 2-4C); the peak value at ZT10 (2 h before light-off) was about 2 times higher than the trough level and the difference was statistically significant (Tukey-test, $p < 0.01$). The rhythmic *Td'cyc* mRNA expression persisted in DD with a peak at mid to late subjective day (Fig. 2-4D).

dsRNAs suppressed mRNA levels of *Td'Clk* and *Td'cyc*

To examine whether RNAi of the clock genes is effective in firebrats, levels of *Td'Clk* and *Td'cyc* mRNA were measured by qPCR in adult firebrats treated with dsRNA of either *Td'Clk* (ds*Clk*) or *Td'cyc* (ds*cyc*). dsRNA was injected into the abdomen of adult firebrats, samples were collected 7 days after injection, and qPCR was performed. At ZT10, the amount of mRNAs of *Td'Clk* was significantly reduced by ds*Clk* injection, to 57% of the intact level (*t*-test, $p < 0.01$; Fig. 2-5A). The mRNA levels at the other 5 time points were also reduced to a value lower than the basal level of the intact firebrats and no rhythmicity was observed (ANOVA, $p > 0.55$, Fig. 2-5A), suggesting that injected ds*Clk* suppressed expression of *Td'Clk* through RNAi. In contrast, the *Td'cyc* mRNA

levels were greatly increased throughout the day (Fig. 2-5B) with no significant rhythm in *Td'Clk* RNAi insects (ANOVA, $p > 0.39$). In adult firebrats treated with ds*cyc*, the amount of *Td'cyc* mRNA was significantly reduced to 52% of the intact level at ZT10 (*t*-test, $p < 0.05$; Fig. 2-6A). The mRNA levels were also significantly reduced at ZT 2, 6 and 22 and stayed at nearly the basal level of intact firebrats throughout the day with no daily rhythmic profile (ANOVA, $p > 0.43$, Fig. 2-6A), suggesting that injected ds*cyc* also suppressed expression of its mRNAs through RNAi and abolished the rhythmic expression. The *Td'Clk* mRNA levels were almost the same as that of intact firebrats with no daily changes (ANOVA, $p > 0.3$, Fig. 2-6B).

Effects of dsRNAs on locomotor rhythm

Then the effect of ds*Clk* or ds*cyc* on the circadian locomotor rhythm of adult firebrats was examined. ds*Clk* was injected into abdomen of 33 adult firebrat and their locomotor activity was measured in LD for the first 19 days (N = 21) or 3 days (N = 12), then in DD. For LD, the periodogram analysis was performed only in the former 21 firebrats, revealing that more than 80% of the firebrats (N = 20) were designated as arrhythmic (Fig. 2-7A), and the remaining 20% (N = 4) were rhythmic. In DD, more than 90% (N = 30) of the 33 firebrats showed arrhythmic locomotor activity with activity dispersed over the entire day up to 49th day after ds*Clk* injection (example Fig. 2-7A), and a significant rhythm was detected in only 3 animals; the average free-running period was 24.76±1.2 (SEM) h, which was slightly longer than that of intact firebrats but the difference was not statistically significant (*t*-test, $p > 0.2$). When ds*cyc* was

injected (N = 30), however, 80% (N = 24) of the injected firebrats exhibited nocturnal activity rhythm under LD (Fig. 2-7B). The remainings were arrhythmic. In DD, 80% (N = 24) of firebrats injected with ds*cyc* showed arrhythmic activity with a pattern similar to that of those injected with ds*Clk* (Fig. 2-7B). Only 20% of the injected firebrats (N = 6) showed a rhythm free-running with an average period of 25.11±1.15 (SEM) h. The free-running period was slightly longer than that of intact firebrats but the difference was not statistically significant (*t*-test, $p > 0.3$). The arrhythmicity persisted until the end of recording (up to 35 days after ds*cyc* injection). The occurrence of the arrhythmic insects was significantly higher than intact firebrats both for ds*Clk* and ds*cyc* injected insects (χ^2-test, $p < 0.01$). As a negative control, *DsRed2* dsRNA was injected in 55 firebrats and their locomotor activity was measured in LD 12:12 (N = 26) or DD (N = 29). 73% of the firebrats exhibited a nocturnal locomotor rhythm under LD; in DD, the rhythm free-ran in 62% of them with a period of 24.03±0.29 (SEM) h throughout the recording period up to 36 days (Fig. 2-7C). The free-running period and the occurrence of the rhythmic insects were not significantly different from those of intact insects (*t*-test, $p > 0.43$ for free-running period; χ^2-test, $p > 0.1$ for occurrence of rhythmic insects).

2.5. Discussion

Firebrats have a circadian clock

The present study revealed that the firebrat exhibited a nocturnal rhythm synchronizing to the light cycle and free-running in DD with a period slightly longer than 24 h. This result suggests that firebrats possess an

endogenous clock, in accord with the previous reports based on immunohistochemistry (Zavodska et al., 2003, 2005). However, the rhythm was sometimes unclear and interrupted by an inactive period which seemed to be related to molting. Thus a statistically significant rhythm was not detected by the χ^2 periodogram in a certain fraction of the animals. The molting-related reduction of activity has been often reported in hemimetabolous insects, including crickets and cockroaches (Nowosielski and Patton, 1963; Page and Block, 1980; Tomioka and Chiba, 1982). Nymphal hemimetabolous insects often show weak rhythms without a sharp activity onset (Nowosielski and Patton, 1963; Page and Block, 1980; Tomioka and Chiba, 1982). The rather weak activity rhythm and arrhythmicity in a certain fraction of adult firebrats might be related to their still retaining nymphal traits such as molting and might reflect a weak coupling between the circadian clock and the neural network controlling the rhythm as has been suggested in the cockroach (Page, 1990).

Clock and *cycle* genes in firebrat

In the present study, I have examined the structure, expression profile, and functions of orthologs of the *Drosophila* clock genes *Clock* and *cycle* in an apterygote insect, *T. domestica*, which is thought to retain some ancestral traits such as adult molting. A distinctive characteristic of *Td'Clk* is the presence of 3 defined functional domains, bHLH region for DNA binding (Allada et al., 1998; Darlington et al., 1998), and PAS-A and PAS-B regions for protein-protein dimerization (Allada et al., 1998; Darlington et al., 1998), which are commonly seen among known insect *Clk* genes (Fig. 2-2). The *Td'*CLK is much shorter than

*Dm'*CLK, lacking the polyglutamine repeats in its C terminal that is implicated to have transcriptional activity in *Dm'*CLK (Allada et al., 1998; Darlington et al., 1998). This lack of polyglutamine repeats is also commonly seen among known insect CLKs except for *Dm'*CLK. Thus, it seems likely that CLK originally lacked the polyglutamine stretches, which might have been acquired later in *Drosophila*.

The *Td'*CYC protein also has 3 functional domains, i.e. bHLH, PAS-A, and PAS-B regions (Fig. 2-3), that are commonly observed among known insect CYC (Bembenek et al., 2007). Its C terminal includes a BCTR domain with transactivation function, with highly conserved sequence among mouse BMAL1 and other known insect CYC, again except for *Dm'*CYC, which lacks it (Chang and Reppert, 2003). Thus, it is inferred that the BCTR is evolutionarily conserved and likely widespread in animals, while it might have been somehow lost in *Drosophila*.

The firebrats exhibited a daily rhythm in *Td'cyc* mRNA levels in both LD and DD (Fig. 2-4C and D). A similar rhythmic pattern of *cyc* expression has been reported for *Apis mellifera* (Rubin et al., 2006). On the other hand, no significant daily variation was detected in *Td'Clk* mRNA (Fig. 2-4A and B). These expression profiles of *Td'cyc* and *Td'Clk* are in contrast to those of *Drosophila*, where *Dm'Clk* is expressed in a rhythmic manner while *Dm'cyc* in an arrhythmic manner (Rutila et al., 1998), showing a similarity to mammalian clocks. The mammalian clock is constituted by negative transcriptional feedback loops basically similar to the *Drosophila* clock. The main loop consists of *Per*, *Cry*, *Clk* and *Bmal1* (Emery and Reppert, 2004). Like in *Drosophila*, CLK

and BMAL1 activate transcription of *Per* and *Cry* genes; the resultant CRY and PER form complexes that translocate into the nucleus and inhibit CLK/BMAL1-mediated transcription. In contrast to *Drosophila* but similar to firebrats, *Clk* is constitutively expressed (Shearman et al., 1999) and *Bmal1* shows rhythmic expression with a peak at dawn (Honma et al., 1998; Oishi et al., 1998). The similarity between firebrats and mammals may imply that the expression patterns of *Clk* and *cyc* (*Bmal1*) are a trait of the common ancestral clock both for insects and vertebrates. In the present study, however, whole bodies of firebrats were used for the measurement of mRNAs. Expression profiles of these clock genes should also be examined in the clock neurons that control behavioral rhythms.

RNA interference in firebrat

The present study demonstrated for the first time that dsRNA of *Clk* and *cyc* disrupts the expression of mRNAs and the locomotor rhythm in an apterygote insect (Figs 2-5 and 2-7). The specificity of the dsRNA was confirmed by behavioral assays using *DsRed2* dsRNA. The gene-specific effect of dsRNA agrees well with previous reports on the high specificity of RNAi in crickets (Meyering-Vos et al., 2006; Moriyama et al., 2008) and other insects (Yang et al., 2000; Amdam et al., 2003; Maestro and Belles, 2006; Kotwica et al., 2009). In this study, rather long dsRNA of about 600 bp was used for *Td'Clk* and *Td'cyc*, and these dsRNAs turned out to be highly effective for gene silencing and the effect persisted for more than 30 days. Thus, RNAi seems to be a powerful tool to dissect the *Thermobia* circadian clock by silencing gene

expression. The long term effect is comparable to that in crickets (Moriyama et al., 2008) and could be explained by a long-lived, injected dsRNAs or by amplification of dsRNA by RNA dependent RNA polymerase known for *Neurospora crassa*, *Caenorhabditis elegans* and *Arabidopsis thaliana* (Voinnet et al., 1998; Sijen et al., 2001; Ahlquist, 2002; Tijsterman et al., 2002).

The results showed that even after dsRNA treatments, some firebrats (9% for ds*Clk* and 20% for ds*cyc*) continued to show a locomotor rhythm. The free-running period of these rhythmic firebrats tended to be slightly longer than those of intact and *DsRed2* dsRNA-injected, negative controls, suggesting that dsRNA might have some effects even in these rhythmic insects. Similar persistence of circadian rhythms was observed in crickets treated with *per* dsRNA (Moriyama et al., 2008; Moriyama et al., 2009; Sakamoto et al., 2009). The remaining rhythm may be explained by a partial knock-down of mRNA in clock neurons or by variation of effects of dsRNAs among clock neurons. The effects of dsRNAs should be investigated in the clock neurons in future studies.

Interestingly, ds*cyc* injected firebrats showed a rhythm under LD while those injected with ds*Clk* showed no rhythmic profile (Fig. 2-7A and B). Since the rhythm observed in ds*cyc* injected animals immediately disappeared on transfer to DD, the rhythm seems to be a masking effect of light. As ds*Clk* treated firebrats lacked the masking of LD, *Td'Clk* might be involved in the light input pathway inducing direct behavioral responses to light.

The expression of *Td'cyc* seems to be regulated by *Td'Clk*, since knocking-down *Td'Clk* greatly up-regulated *Td'cyc* mRNA (Fig. 2-5B). This finding contrasts to the results in *Clk*/*Clk* mutant mice where *Bmal1* (*cycle*)

mRNA levels were low (Shearman et al., 2000), suggesting a different role of *Td'Clk* from its mammalian ortholog. However, the *Td'Clk* expression seems not to be dependent on *Td'cyc* because no apparent changes were observed in its mRNA levels after ds*cyc* treatment (Fig. 2-6B). The *Td'Clk* dependent *Td'cyc* expression may be explained according to the *Drosophila* clock oscillatory model that CLK-CYC transactivates the *period* and *timeless* genes, and their product proteins PER and TIM negatively act on their transcription factors, including CLK-CYC (Dunlap, 1999; Tomioka and Matsumoto, 2010): knocking-down of *Td'Clk* would lead to a reduced level of PER and TIM, which in turn would release a yet unknown transcription factor for *Td'cyc* from inhibition by PER and TIM. This would eventually increase the *Td'cyc* mRNA level. Cloning and functional analysis of the presumptive transcription factor deserve future studies (See Chapter 4.1).

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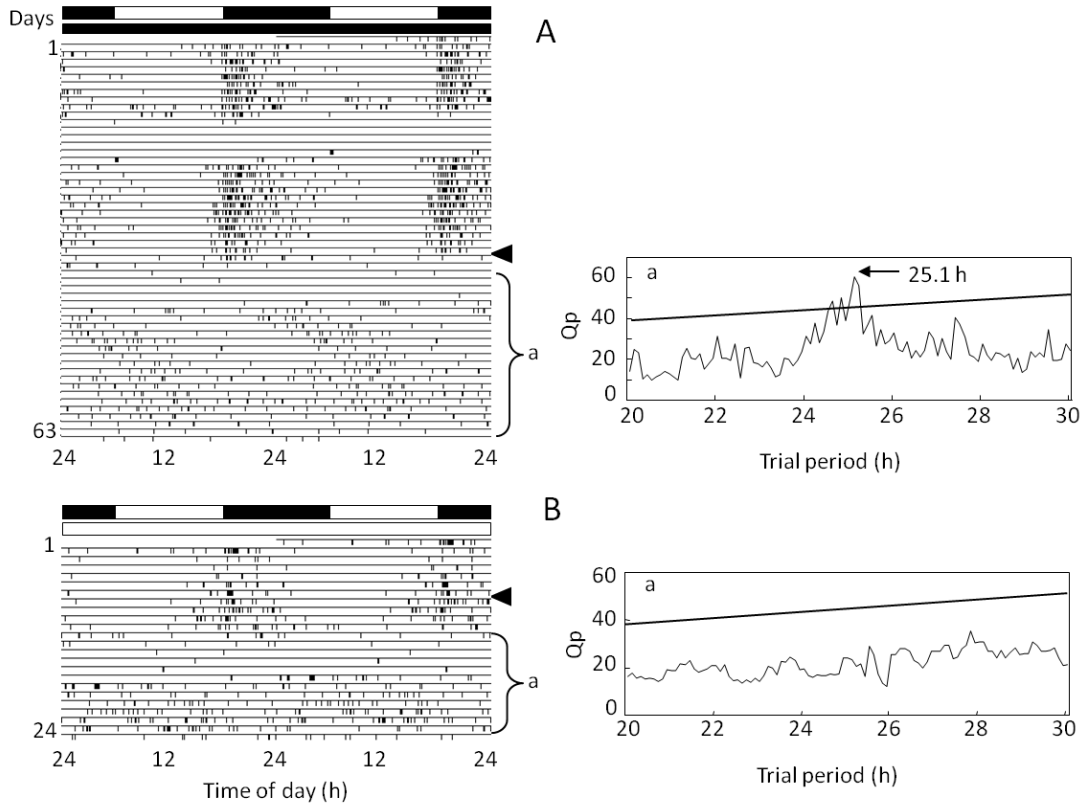

Chapter 2 Figures

Figure 2-1.

Double-plotted actograms (left) and χ^2 periodograms (right) of locomotor rhythms of firebrats *Thermobia domestica* under LD12:12 and DD (A) or LL (B) at a constant temperature of 30 °C. Arrowheads indicate the day when the firebrats were transferred to DD (A) or LL (B). a indicated in the periodogram corresponds to the analyzed time span, a, indicated in the actogram. An oblique line in the periodogram indicates significant level of $p < 0.05$ and the peak value above the line was designated as significant. The firebrats show a nocturnal rhythm in LD that persists in DD (A) but disappeared in LL (B). For further explanations see text.

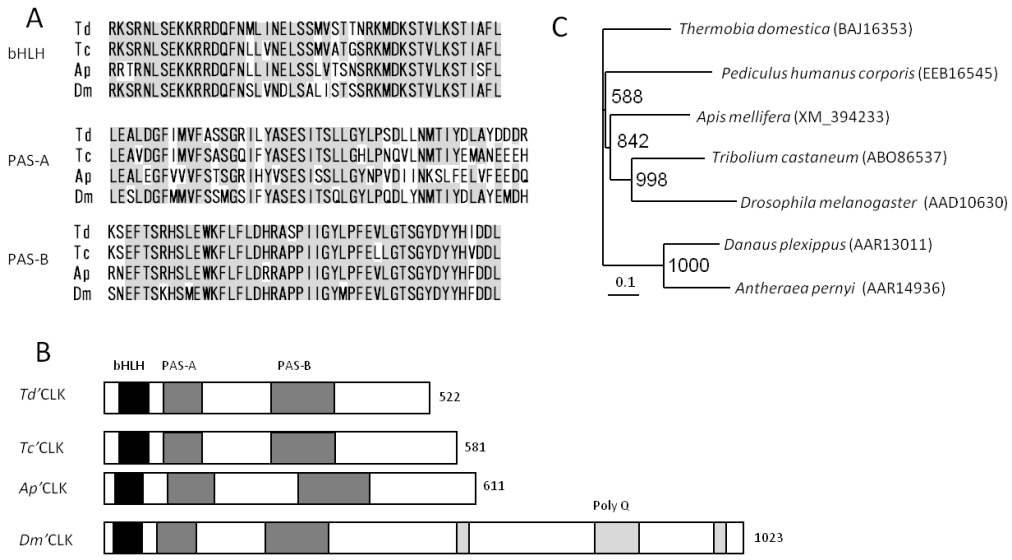

Figure 2-2.

(A) Sequence alignments of conserved domains of *Td'Clk* compared with those orthologous sequences from other insects. bHLH, PAS-A and PAS-B of *Thermobia domestica Clock* (Td) aligned with that of *Tribolium castaneum* (Tc), *Antheraea pernyi* (Ap), and *Drosophila melanogaster* (Dm). The amino acid residues showing more than 60% identity are shaded. (B) Schematic structure of various CLK proteins, comparing the organization of the 3 conserved domains. (C) A phylogenetic neighbor-joining tree of known insect CLK proteins. CLK amino acid sequences were analyzed and neighbor-joining tree was inferred with ClustalW. The GenBank or RefSeq accession numbers are indicated in the bracket. A reference bar indicates distance as number of amino acid substitutions per site.

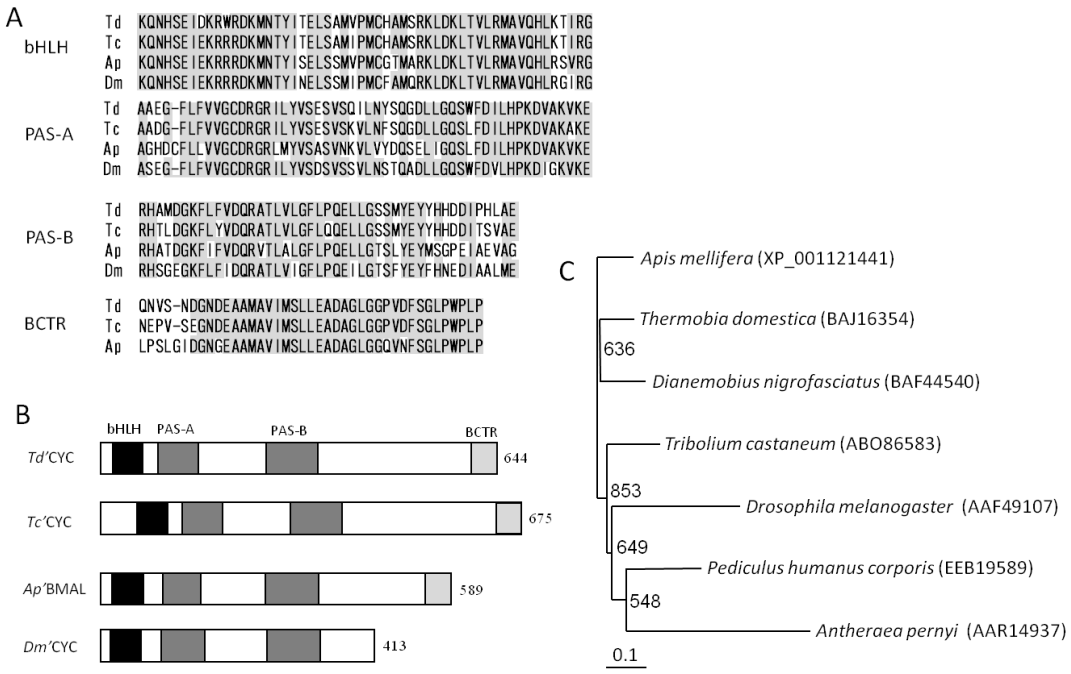

Figure 2-3.

(A) Sequence alignments of conserved domains of *Td'cyc* compared with those orthologous sequences from other insects. bHLH, PAS-A, PAS-B and BCTR of *Thermobia domestica cycle* (Td) aligned with that of *Tribolium castaneum* (Tc), *Antheraea pernyi* (Ap), and *Drosophila melanogaster* (Dm). The amino acid residues showing more than 60% identity are shaded. (B) Schematic structure of various CYC proteins, comparing the organization of the 4 conserved domains. (C) A phylogenetic neighbor-joining tree of known insect CYC proteins. CYC amino acid sequences were analyzed and neighbor-joining tree was inferred with ClustalW. The GenBank or RefSeq accession numbers are indicated in the bracket. A reference bar indicates distance as number of amino acid substitutions per site.

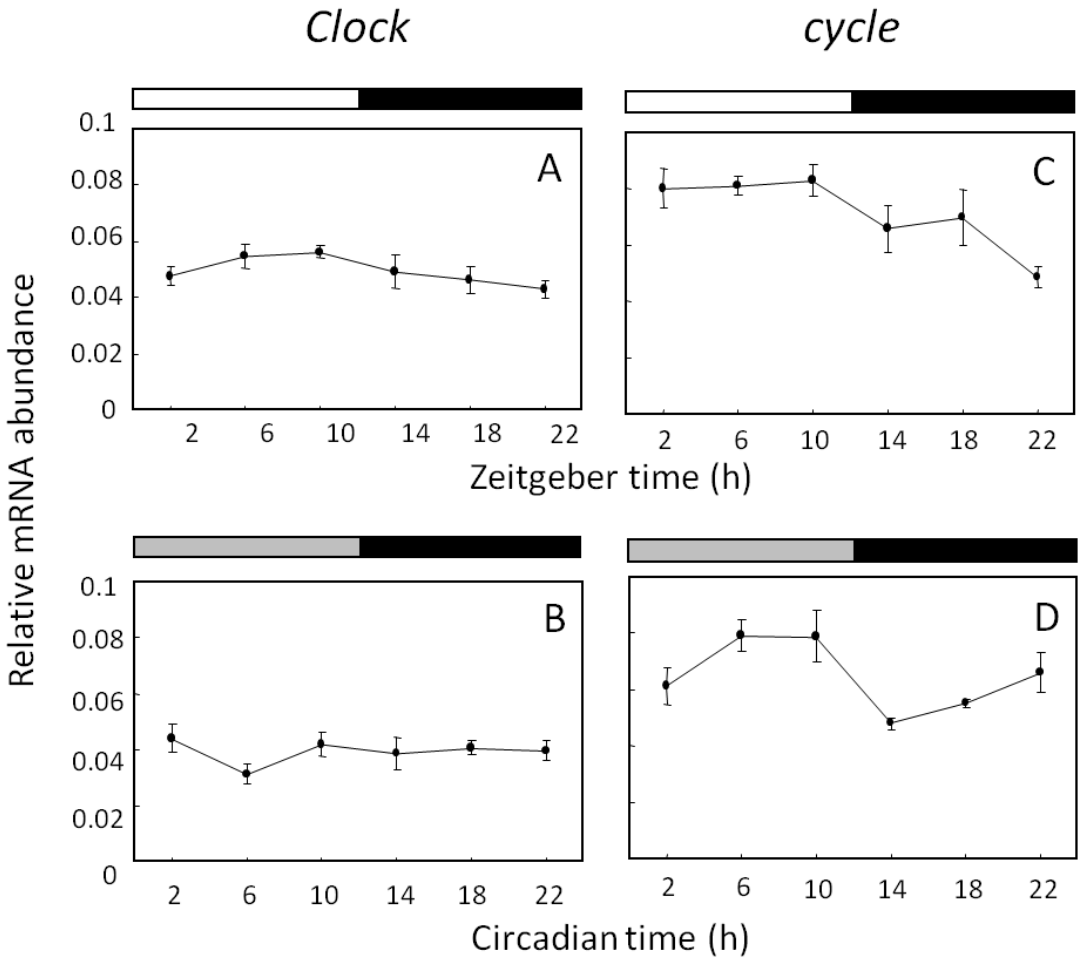

Figure 2-4.

Expression patterns of *Td'Clk* (A, B) and *Td'cyc* mRNA (C, D) in LD12:12 (A, C) and DD (B, D). The abundance of *Td'Clk* and *Td'cyc* mRNA was measured by quantitative real-time RT-PCR. Total RNA was extracted from firebrats collected at 4-h intervals starting at 2 h after lights-on (ZT2). White, black, and gray bars indicate light phase, night/subjective night, and subjective day, respectively. The data collected from 4 independent experiments were averaged and plotted as mean ± SEM values relative to the value of *rp49* mRNA used as reference. For further explanations see text.

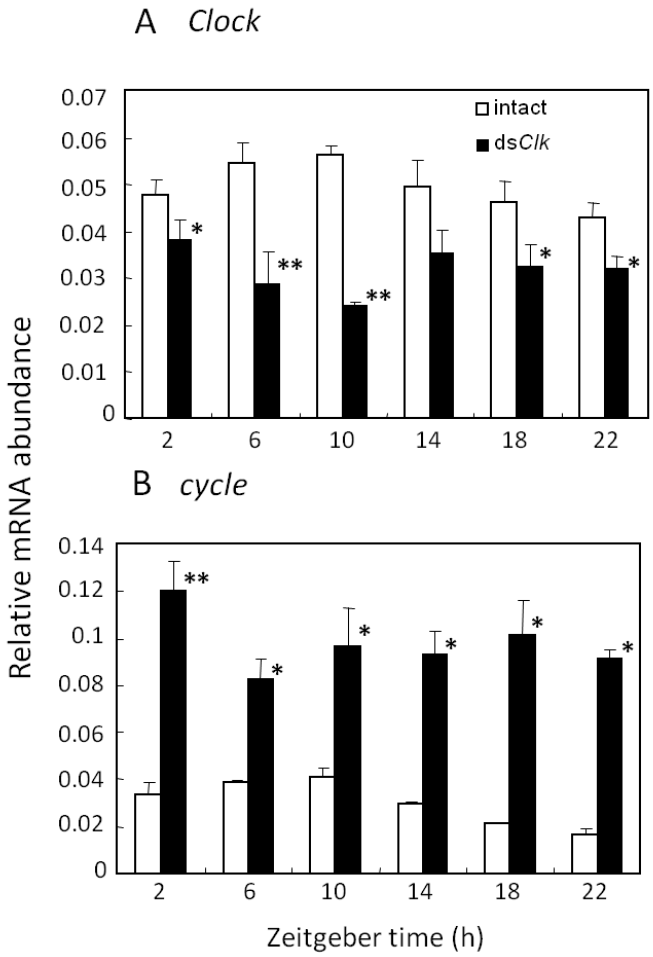

Figure 2-5.

(A) Relative abundance of *Td'Clk* and (B) *Td'cyc* mRNA in intact (white column) or *Td'Clk* double-stranded RNA (ds*Clk*) injected firebrat (black column). Ordinate indicates relative abundance of mRNA. Ds*Clk*-injected firebrats were collected 7 days after the injection at an interval of 4-h starting at 2 h after lights-on (ZT2) at 30ºC. The abundance of *Td'Clk* and *Td'cyc* mRNAs was measured by quantitative real-time RT-PCR with total RNA extracted from the firebrat. For ds*Clk* injected firebrats, the data collected from 3 independent experiments were averaged and plotted as mean ± SEM values relative to the value of *rp49* mRNA used as reference. The data for intact firebrats are those under LD shown in Figure 2-4. *, $p < 0.05$; **, $p < 0.01$, *t*-test. For further explanations see text.

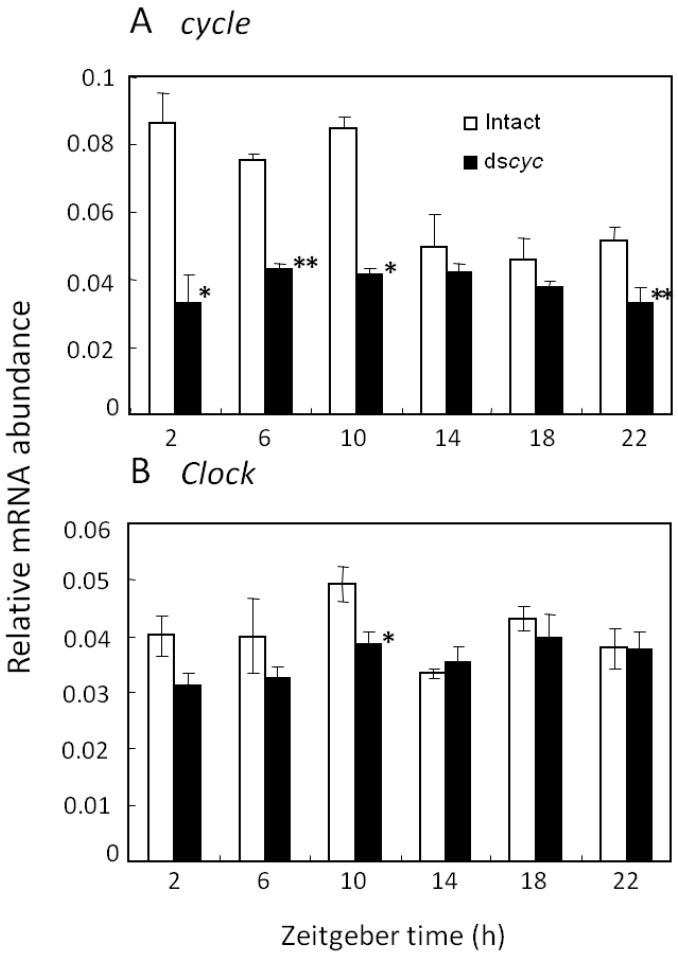

Figure 2-6.

(A) Relative abundance of *Td'cyc* and (B) *Td'Clk* mRNA in intact (white column) or *Td'cyc* double-stranded RNA (ds*cyc*) injected firebrat (black column). Ordinate indicates relative abundance of *Td'cyc* and *Td'Clk* mRNA. Ds*cyc*-injected firebrats were collected 7 days after the injection at an interval of 4-h starting at 2 h after lights-on (ZT2) at 30°C. The abundance of *Td'Clk* and *Td'cyc* mRNAs was measured by quantitative real-time RT-PCR with total RNA extracted from the firebrat. For ds*cyc* injected firebrats, the data collected from 3 independent experiments were averaged and plotted as mean ± SEM values relative to the value of *rp49* mRNA used as reference. The data for intact firebrats are those under LD shown in Figure 2-4. *, $p < 0.05$; **, $p < 0.01$, *t*-test. For further explanations see text.

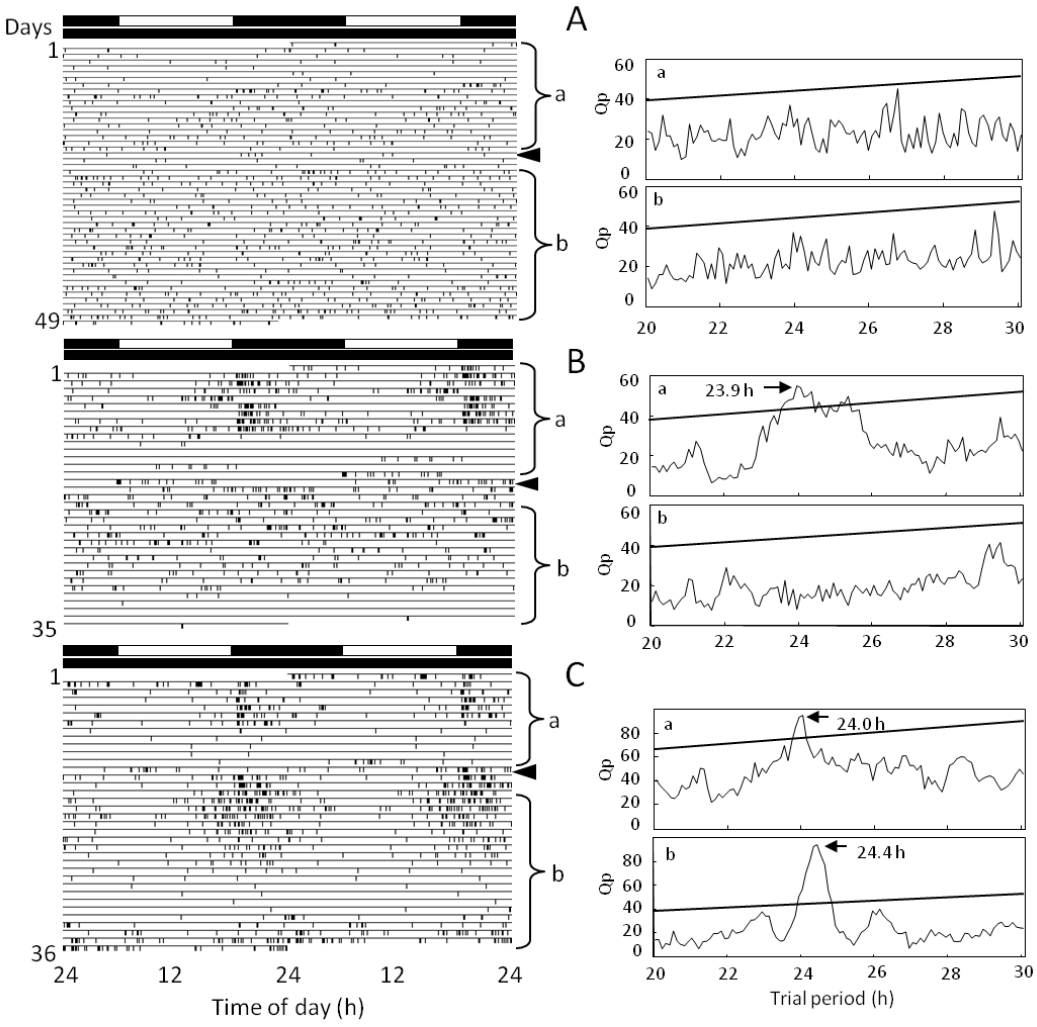

Figure 2-7.

Double-plotted actograms (left) and χ^2 periodograms (right) of locomotor rhythms of firebrats *Thermobia domestica* injected with *Td'Clk* dsRNA (A), *Td'cyc* dsRNA (B), or *DsRed2* dsRNA (C) under LD12:12 and DD at a constant temperature of 30 °C. Arrowheads indicate the day when the firebrats were transferred from LD to DD. a and b indicated in the periodogram correspond to the analyzed time span, a and b, indicated in the actogram. An oblique line in the periodogram indicates significant level of $p < 0.05$ and the peak value above the line was designated as significant. The firebrat showed arrhythmic locomotor activity throughout the recording (A), but the insect in (B) showed a clear rhythm in LD which disappeared on transfer to DD. The control firebrat injected with *DsRed2* dsRNA showed a clear rhythm throughout the recording (C). For further explanations see text.

Chapter 2 Tables

Table2-1. Comparison of amino acid identity and similarity (%) of whole sequence and functional domains among insect CLOCK homologs.

Order	Species	Identity	Similarity	bHLH	PAS-A	PAS-B
Psocodea	*Pediculus humanus corporis*	57	70	94	57	82
Coleoptera	*Tribolium castaneum*	71	83	83	67	93
Diptera	*Drosophila melanogaster*	60	75	85	75	87
Lepidoptera	*Danaus plexippus*	55	67	85	65	93
Lepidoptera	*Antheraea pernyi*	55	70	77	53	91
Hymenoptera	*Apis mellifera*	73	84	88	63	93

Table 2-2. Comparison of amino acid identity and similarity (%) of whole sequence and functional domains among insect CYCLE homologs.

Order	Species	Identity	Similarity	bHLH	PAS-A	PAS-B	BCTR
Orthoptera	*Dianemobius nigrofasciatus*	70	79	84	96	88	90
Psocodea	*Pediculus humanus corporis*	60	72	94	94	69	74
Coleoptera	*Tribolium castaneum*	66	78	94	91	83	85
Diptera	*Drosophila melanogaster*	63	78	85	81	67	-
Lepidoptera	*Antheraea pernyi*	47	61	85	81	69	79
Hymenoptera	*Apis mellifera*	68	78	94	94	92	90

Chapter 3.

Functional analysis of the clock gene *timeless* in firebrats

3.1. Abstract

Recent studies show that the *timeless* (*tim*) gene is not an essential component of the circadian clock in some insects. In this chapter, I have investigated whether the *tim* gene was originally involved in the insect clock or acquired as a clock component later during the course of evolution using an apterygote insect, *Thermobia domestica*. A cDNA of the clock gene *tim* (*Td'tim*) was cloned, and its structural analysis showed that *Td'*TIM includes 4 defined functional domains, that is, 2 regions for dimerization with PERIOD (PER-1, PER-2), nuclear localization signal (NLS), and cytoplasmic localization domain (CLD), like *Drosophila* TIM. *Td'tim* exhibited rhythmic expression in its mRNA levels with a peak during late day to early night in LD, and the rhythm persisted in DD. A single injection of double-stranded RNA (dsRNA) of *Td'tim* (ds*tim*) into the abdomen of adult firebrats effectively knocked down mRNA levels of *Td'tim* and abolished its rhythmic expression. Most dsRNA-injected firebrats lost their circadian locomotor rhythm in DD up to 30 days after injection. DsRNA of *cycle* (*cyc*) and *Clock* genes also abolished the rhythmic expression of *Td'tim* mRNA by knocking down *Td'tim* mRNA to its basal level of intact firebrats, suggesting that the underlying molecular clock of firebrats resembles that of *Drosophila*. Interestingly, however, ds*tim* also reduced *cyc* mRNA to its basal level of intact animals and eliminated its rhythmic expression, suggesting the involvement of *Td'tim* in the regulation of *cyc* expression. These results suggest that *tim* is an essential component of the circadian clock of the primitive insect *T. domestica*; thus, it might have been involved in the clock machinery from a very early stage of insect evolution, but

its role might be different from that in *Drosophila*.

3.2. Introduction

Circadian rhythms are about 24 h oscillations observed in various physiological functions of a wide variety of organisms and are driven by an endogenous mechanism called the circadian clock. The molecular oscillatory mechanism of the circadian clock has been extensively studied in the fruit fly *Drosophila melanogaster* and is currently believed to consist of 3 transcriptional/translational loops (Hardin, 2005; Tomioka and Matsumoto, 2010). In the first loop, a heterodimer of CLOCK (CLK) and CYCLE (CYC) activates the transcription of negative elements, *period* (*per*) and *timeless* (*tim*) (Allada et al., 1998). The product proteins, PER and TIM, increase during the night, peaking at late night, and enter the nucleus by forming a heterodimer to repress their own transcription through inhibitory action on CLK-CYC. The repression of *per* and *tim* transcription results in a reduced level of PER and TIM, which eventually releases CLK-CYC from the inhibition to reactivate the transcription of *per* and *tim*. The CLK-CYC dimer also activates transcription of *vrille* (*vri*) and *PAR domain protein 1ε* (*Pdp1ε*) in the second loop (Cyran et al., 2003; Glossop et al., 2003), and VRI and PDP1ε in turn regulate the transcription of *Clock* (*Clk*) (Zheng and Sehgal, 2008). The third loop consists of cyclic expression of *clockwork orange* (*cwo*), which regulates the amplitude of the *per-tim* oscillation (Kadener et al., 2007; Lim et al., 2007; Matsumoto et al., 2007; Richier et al., 2008).

However, the circadian oscillatory mechanism seems to differ

considerably among insect species. It has been shown that *tim* is not essential for the circadian clock mechanism in the cricket *Gryllus bimaculatus* (Danbara et al., 2010) and even does not exist in the genome of the honeybee, where mammalian-type CRYPTOCHROME (CRY2) is thought to form the first loop together with PER instead of TIM (Rubin et al., 2006). In the monarch butterfly *Danaus plexippus*, it has been suggested that the negative loop consists of PER, TIM, and CRY2, where CRY2 is shuttled into the nucleus, repressing CLK/CYC-mediated transcription, while TIM mostly stays in the cytoplasm and is mainly involved in light-dependent resetting of the clock (Zhu et al., 2008). These facts raise a question whether the *tim* gene was originally involved as an essential component in the insect clock or acquired later during the course of evolution.

As one of the ancestral insects, the firebrat appears to be suitable for searching for the answer to this question. My previous study showed that the firebrat *Thermobia domestica* has a circadian clock and possesses *Clock* (*Td'Clk*) and *cycle* (*Td'cyc*) genes (Kamae et al., 2010). Interestingly, the sequence and expression profile of these clock genes showed similarity to their mammalian orthologs (Kamae et al., 2010). Thus, I infer that primitive insects retain the common traits of *Clk* and *cyc* with vertebrates, but higher insects such as *Drosophila* later obtained new traits. In this chapter, I have cloned cDNA of the clock gene *timeless* (*Td'tim*) from the firebrat, examined its daily expression patterns using quantitative realtime RT-PCR (qPCR), and investigated the role of *Td''tim* in the rhythm generation with RNA interference (RNAi) technology. The results showed that *Td'tim* was structurally similar to its *Drosophila*

homolog. Knockdown of *Td'tim* by RNAi disrupted circadian rhythms, suggesting that *Td'tim* plays an essential role in the circadian system in the firebrat like in *Drosophila* and that *tim* might have been involved in the insect circadian clock from an early stage of the insect's evolution.

3.3. Material and Method

Animals

Adult firebrats, *T. domestica*, raised in the laboratory under a light cycle of 12 h light and 12 h darkness (LD12:12) at a constant temperature of 30 °C, were used for all experiments. They were fed laboratory chow (CA-1, Clea Japan, Tokyo, Japan).

cDNA Cloning of Clock Gene *timeless*

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA) from 5 adult firebrats collected at zeitgeber time (ZT; ZT0 corresponds to light-on and ZT12 to light-off) 14. A total of 5 μg of total RNA was used for reverse transcription to obtain cDNA, using SuperScript III (Invitrogen). Using the obtained cDNA as a template, I performed PCR with degenerate primers deduced from the conserved amino acid sequences among insect *tim* homologues (5'-aaaggagctgaggcggaaRaaRHtNgt-3' and 5'-gtggtgcatcatggtgaagatRcaRtcRtt-3'). The amplified fragments were cloned into TOPO-pCR II vector (Invitrogen) and sequenced with BidDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA). 5' and 3' RACEs were performed with GeneRacer^{tm} kit (Invitrogen) using Blend Taq Plus

(Toyobo, Osaka, Japan) with gene-specific primers as follows; 5′-TAATACGACTCACTATAGGGAGAGGCGTGTGCCTTGTACT-3′ for 5′RACE, and 5′-TGCATTTGGTTGTGACTGCT-3′ and 5′-AAACGACTGCATCTTCACCA-3′ for 3′RACE. RACE fragments were purified, cloned, and sequenced as mentioned above. Sequences were analyzed by Genetyx ver. 6 (Genetic Information Processing Software, Tokyo, Japan) and BioEdit version 7.0.9.0 (Biological Sequence Alignment Editor, Ibis Therapeutic, Carlsbad, CA). Amino acid sequence of TIMELESS (TIM) was analyzed and a neighbor-joining tree was inferred with ClustalW (http://clustalw.ddbj.nig.ac.jp/top.html). Sequences of known insects were obtained from GenBank.

Quantitative Real-Time RT-PCR

Quantitative real-time RT-PCR (qPCR) was used to measure mRNA levels. Both males and females were used for measurement because preliminary experiments revealed no significant gender differences in *tim* mRNA levels. Total RNA extraction from the entire bodies of adult firebrats was performed with TRIzol Reagent (Invitrogen, Carlsbad, CA), and the obtained RNA was treated with DNase I to remove contaminated DNA. About 500 ng of total RNA of each sample was reversely transcribed with random 6mers using PrimeScript RT reagent kit (Takara, Otsu, Japan). Real-time PCR was performed with Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA) using Universal SYBR Green Master (Roche, Tokyo, Japan) including SYBER Green with primers 5′-TACAAGCCAGGTCCATCACA-3′ and

5′-TCAAGCGTCAATTCAGCATC-3′ for *tim*, 5′-ATCGCAAGGGTCTGGAAGTG-3′ and 5′-GGAAAACTCGCCAAGACAGG-3′ for *Clk* (GenBank/EMBL/DDBJ accession no. AB550828), 5′-CGTGTAATCTGTCGTGTTTGGTG-3′ and 5′-GAATCGTCCGCCTTTCCTC-3′ for *cyc* (GenBank/EMBL/DDBJ accession no. AB550829), and 5′-AGTCCGAAGGCGGTTTAAGG-3′ and 5′-TACAGCGTGTGCGATC TCTG-3′ for *rp49* (GenBank/EMBL/DDBJ accession no. AB550830). The results were analyzed using the software associated with the instrument. The values were normalized with the values for *rp49*, a housekeeping gene, at each time point. Results of 3 or 4 independent experiments were pooled to calculate the mean ± SEM. Data were analyzed by a *t* test or ANOVA followed by the Tukey test.

RNA Interference

Double-stranded RNA (dsRNA) for *Td'tim* and *DsRed2*, derived from a coral species (*Discosoma* sp.), were synthesized using MEGAscript High Yield Transcription Kit (Ambion, Austin, TX). Primers fused with T7 promoter sequence were designed for the synthesis of dsRNA of *Td'tim* as follows: 5′-TAATACGACTCACTATAGGGTGCATTTGGTTGTGACTGCT-3′ and 5′-TAATACGACTCACTATAGGGAGAGGCGTGTGCCTTGTACT-3′.

Standard PCR was performed using *Td*'s cDNA as a template. Resulting amplicons excluding the fused T7 promoters were 600 bp for *Td'tim*. *DsRed2* was linearized from pDsRed2-N1 (Clontech, Mountain View, CA) and amplified with the forward and reverse primers. Then, RNAs were synthesized

with T7 and SP6 RNA polymerases. The synthesized RNAs were extracted with phenol/chloroform and suspended in 50 μL of TE solution after isopropanol precipitation. The yield and quality of RNA were assessed by absorbance with a spectrophotometer (Genequant Pro, Amersham Biosciences, Piscataway, NJ), and the same amounts of sense and antisense RNA were mixed. The RNA was denatured for 5 minutes at 100 °C and annealed by a gradual cool down to room temperature. After ethanol precipitation, the obtained dsRNA was suspended in Ultra Pure Water (Invitrogen) and adjusted to a final concentration of 10 μM. The dsRNA solution was stored at -80 °C until use. A total of 70 nL (10 μM) dsRNA solution was injected with a nanoliter injector (WPI, Sarasota, FL) into the abdomen of adult firebrats anesthetized with CO_2.

Recording of Locomotor Activity

To monitor locomotor activity, adult firebrats were individually housed in transparent acrylic rectangular tubes (6 × 6 × 70 mm). The tubes were plugged at one end with a piece of animal food and were sealed with plastic tape; at the other end, they were sealed with wet cotton connected to a water bottle. Movement of the firebrat was sensed by a photoelectrical detection system: A moving firebrat interrupted an infrared beam, and the number of interruptions during each 6-minute interval was recorded using a computerized system. The activity sensing system was placed in an incubator (MIR-153, Sanyo Biomedica, Osaka, Japan), in which the temperature was kept at 30 °C and lighting conditions were given by a cool white fluorescent lamp connected to an electric timer. The light intensity was 600 to 1000 lux at the animal's level,

varying with the proximity to the lamp. The raw data were displayed as conventional double-plotted actograms to judge activity patterns, and existence of the rhythmicity and free-running periods were analyzed by the χ^2 periodogram written by Schmid et al. (2011). If peaks of the periodogram appeared above the 0.05 confidence level, the periods of the peaks were designated as statistically significant.

3.4. Results

Cloning and Sequencing of Clock Gene *timeless*

A *tim* homolog of *T. domestica* was cloned with primers constructed based on the conserved amino acid sequences of known insect *tim* genes and 5′ and 3′ RACE strategy. Obtained was a full length of 5490 bp cDNA of *T. domestica timeless* (*Td'tim*; GenBank/EMBL/DDBJ accession no. AB644410). *Td'tim* has 5′- and 3′-untranslated regions (UTR) of 128 bp and 1735 bp, respectively. Sequences were analyzed by Genetyx version 6 (Genetic Information Processing Software, Tokyo, Japan) and BioEdit version 7.0.9.0 (Biological Sequence Alignment Editor, Ibis Therapeutic, Carlsbad, CA). Amino acid sequence of TIMELESS (TIM) was analyzed with ClustalW (http://clustalw.ddbj.nig.ac.jp/top.html). The putative product protein consisted of 1209 amino acid residues, which is shown in Figure 3-1. It contains PER-1 and PER-2 dimerization regions that are involved in protein-protein interaction and mediate TIM's binding to its heterodimeric partner PER (Gekakis et al., 1995; Myers et al., 1995; Saez and Young, 1996), a cytoplasmic localization domain (CLD) (Saez and Young, 1996), and a nuclear localization

signal (NLS) (Baylies et al., 1993; Vosshall et al., 1994). A BLAST database search indicated that the amino acid sequence has 43% to 57% identities along the entire length of the protein to those of known insect TIMs (Table 3-1). Among the putative functional motifs, NLS had the highest identity of 50% to 83% with other insect species, PER-1 and PER-2 had rather high identities of 34% to 74%, suggesting their conserved functional role, and CLD had a rather low identity of only 9% to 22% (Table 3-1). A phylogenetic tree based on the amino acid sequences of TIMs from known insects, sea urchin, and mice reveals that there are 3 clusters of *tim* and that *Td'tim* forms a cluster with the cricket (*G. bimaculatus*) and the louse (*Pediculus humanus corporis*) *tim* (Fig. 3-2).

Temporal Expression Pattern of *Td'tim* mRNA

To determine whether transcripts of *Td'tim* oscillated in a circadian manner, levels of *Td'tim* mRNA were measured in *T. domestica* under LD12:12 and on the second day of DD by qPCR (Fig. 3-3). Sample collections were performed every 4 h starting at zeitgeber time (ZT) 2 (ZT0 corresponds to lights-on and ZT12 to lights-off) or circadian time (CT) 2 (CT0 corresponds to projected lights-on and CT12 to projected lights-off in DD). In LD, *Td'tim* mRNA showed a rhythmic expression with a peak at late day to early night (ANOVA, $p < 0.01$); the peak value at ZT10 (2 h before lights-off) was about 2 times higher than the trough level, and the difference was statistically significant (Tukey-test, $p < 0.05$). The rhythmic *Td'tim* mRNA expression persisted in DD with a peak at late subjective day (CT10; ANOVA, $p < 0.01$), and the peak was about 3-fold of the trough level.

Td'tim dsRNA Suppressed *Td'tim* mRNA Levels

To examine whether RNAi of the *tim* gene worked effectively in *T. domestica*, *Td'tim* mRNA levels were measured by qPCR in adult firebrats injected with *Td'tim* dsRNA (ds*tim*) into the abdomen. The firebrats were sampled 7 days after the injection. The *Td'tim* mRNA expression was found to be arrhythmic with significantly lower levels than those of intact firebrats (ANOVA, $p > 0.9$) (Fig. 3-4A), suggesting that ds*tim* suppressed *Td'tim* mRNA levels and abolished its rhythmic expression through RNAi.

To examine the effects of *tim* RNAi on the expression of other clock genes, *cycle* (*Td'cyc*) and *Clock* (*Td'Clk*), levels of those mRNAs were measured in firebrats treated with ds*tim*. *Td'cyc* mRNA levels were significantly reduced at most of the examined time points (ZT2-ZT10, ZT22; *t* test, $p < 0.05$) (Fig. 3-4B), with the greatest suppression of 41% at ZT2. The rhythm that was evident in intact firebrats was abolished (ANOVA, $p > 0.5$) (Fig. 3-4B). In contrast, *Td'Clk* mRNA showed both expression levels and patterns similar to those of intact firebrats, and no significant difference was observed at any time point (*t* test, $p > 0.05$) (Fig. 3-4C).

As a control, mRNA levels were measured in ds*DsRed2*-treated firebrats (Fig. 3-4D). Although a significant reduction was observed at ZT2, *Td'tim* mRNA levels showed a clear rhythm similar to that of intact firebrats with a peak at ZT10 (ANOVA, $p < 0.05$). The peak to trough ratio was about 3.

Effects of ds*Clk* and ds*cyc* on *Td'tim*

In *Drosophila*, transcription of *tim* is hypothesized to be activated by transcription factors CLK and CYC. To test this possibility in the firebrat, levels of *Td'tim* mRNA were also measured in firebrats treated with ds*cyc* and ds*Clk*. The knockdown of *Td'cyc* and *Td'Clk* by ds*cyc* and ds*Clk*, respectively, has been confirmed previously (Kamae et al., 2010). In both treatments, *Td'tim* mRNA levels stayed at near basal levels of intact animals throughout the day, and no significant rhythm was observed (ANOVA, $p > 0.7$) (Fig. 3-5): reductions at ZT6 to ZT14 and at ZT10 and ZT14 were statistically significant for ds*Clk* and ds*cyc*, respectively (*t* test, $p < 0.01$ for ds*cyc* at ZT10; $p < 0.05$ for the remainings).

Effects of dsRNA on Locomotor Rhythm

It has been shown that intact firebrats exhibit a nocturnal rhythm synchronizing to LD and free-running in DD with a period slightly longer than 24 h (Kamae et al., 2010). To examine the effect of ds*tim* on the circadian locomotor rhythm, locomotor activity was recorded in 23 adult firebrats injected with ds*tim*. As a control, the locomotor activity of adults injected with ds*DsRed2* was recorded. The results are summarized in Table 3-2. Under LD, the majority (93%) of ds*DsRed2*-injected controls showed a nocturnal locomotor rhythm, which was interrupted by an inactive phase associated with molting (Fig. 3-6C and Table 3-2). The rhythm persisted in the ensuing DD in 84% of them, with a period slightly longer than 24 h (Table 3-2). The ratio of rhythmic animals and free-running periods were very close to those of intact animals (Table 3-2), which were reanalyzed with the χ^2 periodogram (Schmid et al., 2011), suggesting that injection of ds*DsRed2* has no significant effect on the

rhythm.

In the firebrat injected with ds*tim*, 22 animals (95%) exhibited a nocturnal activity rhythm under LD with a peak in the early night (Fig. 3-6A, B). The remaining animal was arrhythmic. In the ensuing DD, 13 (65%) animals almost immediately lost the nocturnal rhythm and became arrhythmic (Fig. 3-6A), suggesting that the rhythm observed in LD was a masking effect of light. Only 7 (35%) of the injected firebrats showed the nocturnal peak free running with an average period of 21.6 ± 2.5 h (Fig. 3-6B). The free-running period was significantly shorter than that of intact and ds*DsRed2*-injected firebrats (*t* test, $p < 0.05$) (Table 3-2).

3.5. Discussion

timeless Gene in the Firebrat

In this study, I obtained the *timeless* (*Td'tim*) gene from the firebrat *T. domestica*. This is the first *tim* homolog in Thysanura insects. Phylogenetic analyses with sequence data from other species revealed that the insect's *tim* consists of 3 clusters. The firebrat lineage belongs to a rather primitive group, including the cricket and the louse, compared with 2 other clusters including dipteran, coleopteran, and lepidopteran species (Fig. 3-2). *Td'*TIM includes 4 defined functional domains, that is, PER-1, PER-2, NLS, and CLD like *Drosophila* TIM (*Dm'*TIM) (Fig. 3-1). The CLD has a very low similarity to other insects' TIM, suggesting its structural and/or functional uniqueness in the firebrat. A comparison between TIMs of the firebrat and fruit fly, *D. melanogaster*, however, revealed that PER-1, PER-2, and NLS domains have high identity (Table 3-1),

suggesting that *Td*''TIM retains a functional ability similar to that of *Dm*'TIM. In the fruit fly, *tim* is one of the major genes constituting the negative feedback loop together with *per* to regulate its own rhythmic expression (Tomioka and Matsumoto, 2010). In fact, the qPCR measurement revealed that *Td'tim* mRNA showed circadian oscillation in both LD and DD conditions with a peak at late day to early night like in *Drosophila* (Fig. 3-2). The fact that *Td'tim* dsRNA abolished the locomotor rhythm in most of the treated firebrats suggests *tim*'s involvement in the rhythm generation (Figs 3-4 and 3-6). The significant reduction and arrhythmic profile of mRNA expression in the *tim* RNAi also indicate *tim*'s importance in the clock machinery of firebrats. Together with the structural similarity to its *Drosophila* homolog, these results suggest that *tim* had been involved as an essential component in the ancestral insect clock.

However, some *tim* RNAi firebrats (35%) maintained a locomotor rhythm in DD, although their free-running periods were significantly shorter than those of intact animals. Persistence of the rhythm after ds*tim* treatment was reported for the cricket *G. bimaculatus*, where *tim* RNAi resulted in only shortening of the free-running period and never abolished the rhythm (Danbara et al., 2010). The role of the cricket's *tim* is thus probably only modificational and not essential in the clock mechanism. In the firebrat, however, the fact that the majority of ds*tim*-treated animals lost the rhythm suggests that the persistence of the rhythm is rather exceptional and probably caused by an insufficient knockdown of *tim* mRNA. The insufficient knockdown may associate with the systemic RNAi strategy because uptake of injected dsRNA may be in a rather passive manner (Huvenne and Smagghe, 2010). The

shortening of the free-running periods in the rhythmic *tim* RNAi firebrats may be caused by faster degradation of the TIM protein due to a less amount of TIM resulting from reduced *tim* mRNA levels. This possibility has also been suggested for the cricket *G. bimaculatus* (Danbara et al., 2010) but needs to be experimentally addressed in future studies.

Possible Role of *tim* in the Firebrat Circadian Clock

The RNAi experiment revealed that knockdown of *Td'tim* had no significant effect on *Td'Clk* mRNA levels but reduced the mRNA levels of a clock gene *Td'cyc* and eliminated its rhythmic expression (Fig. 3-4B and 3-4C). Together with the previous finding that *Td'Clk* is constitutively expressed and *Td'cyc* shows rhythmic expression (Kamae et al., 2010), the fact contradicts what is expected from the *Drosophila*'s clock hypothesis. In *Drosophila*, the transcription of *Clk* is known to be rhythmically controlled by VRI and PDP1ε, of which transcriptions are activated by a CLK-CYC heterodimer, which is repressed by a PER-TIM heterodimer (Cyran et al., 2003). According to this *Drosophila* hypothesis, a knockdown of *Td'tim* would affect *Td'Clk* mRNA levels because of a loss of repression by the PER-TIM complex. However, there was no apparent effect of *Td'tim* RNAi on *Td'Clk* mRNA levels. Regarding the *Td'cyc* gene, there seems to be some regulatory mechanism including *Td'tim* for its rhythmic expression unlike in *Drosophila*, where *cyc* is thought to be expressed constitutively. In vertebrates, a clock gene *Bmal1*, a structural counterpart of *cyc*, is expressed rhythmically by a loop including transcription factors ROR and REV-ERB, which are under the regulation of CLK-BMAL1 (Ko and Takahashi,

2006). In the firebrat, the knockdown of *Td'tim* might affect *Td'cyc* mRNA levels through the yet unknown regulatory pathway for *Td'cyc* by a reduction of inhibitory effects on *Td'*CLK and *Td'*CYC (See Chapter 4.1).

Regulation of *tim* Expression

The present study revealed that *Td'tim* mRNA is rhythmically expressed in both LD and DD to peak late day to early night (Fig. 3-3) like *Drosophila tim* (So and Rosbash, 1997). In *Drosophila*, the transcription of *tim* is activated by a CLK and CYC heterodimer, which binds to a promoter region of *tim*, called E-box (Hardin, 2006). This *Drosophila*'s hypothesis may account for the present results of *Td'Clk* and *Td'cyc* RNAi experiments that a knockdown of these transcription factors resulted in a reduced *Td'tim* mRNA level and eliminated its cyclic expression. I have previously shown that *Td'cyc* has a BMAL C-terminal region (BCTR) domain with a highly conserved sequence to that of mouse BMAL1 with a transactivational function (Kamae et al., 2010). It is thus likely that in the firebrat, *Td'*CLK and *Td'*CYC form a heterodimer and activates the transcription of *Td'tim* like in *Drosophila* and that the rhythmic *Td'tim* expression may be due to the rhythmic expression of *Td'cyc* (Kamae et al., 2010).

The Clock Machinery in the Firebrat

Most interesting is that the firebrat circadian clock possesses both *Drosophila*-type and mammalian-type operation parts. The molecular structure and functional role of *Td'tim* in the clock mechanism appear to be similar to

those in the *Drosophila* clock (Table 3-1 and Fig. 3-4). The rhythmic expression of *Td'tim* is most likely regulated by a negative feedback loop of *Td'tim*, of which transcription is activated by *Td'*CLK/*Td'*CYC. *period* gene probably participates in the loop because I have obtained its cDNA fragment (Kamae and Tomioka, unpublished results) and because a Western blot analysis has revealed a cycling of PER-like antigen (Závodská et al., 2003, 2005). However, the expression profiles of *Td'cyc* and *Td'Clk* seem to resemble those in vertebrate clocks rather than the *Drosophila* clock (Kamae et al., 2010). The mechanism of rhythmic expression of *Td'cyc* is currently unknown. Investigation of the mechanism will reveal whether *vrille* and *Pdp1ε* participate in it like in *Drosophila* or the orthologs of *Ror* and *Rev-erb* are involved like in vertebrates (See Chapter 4.1). Investigation of the role of *per* in the firebrat clock mechanism also needs to be carried out. Elucidation of the firebrat clock machinery will contribute to the understanding of the origin of the insect circadian clocks and how it has been diversified among insects because the firebrat is, to our knowledge, the most primitive insect species, of which the circadian clock has been investigated at the molecular level. In conclusion, it is likely that *tim* is a gene that characterizes the insect's clock machinery because a member of the most primitive insect group incorporates it as a major component of the clock. Those possessing a clock functioning without *tim,* such as honeybees and crickets (Rubin et al., 2006; Danbara et al., 2010), may have lost *tim* or its function during the evolutionary process.

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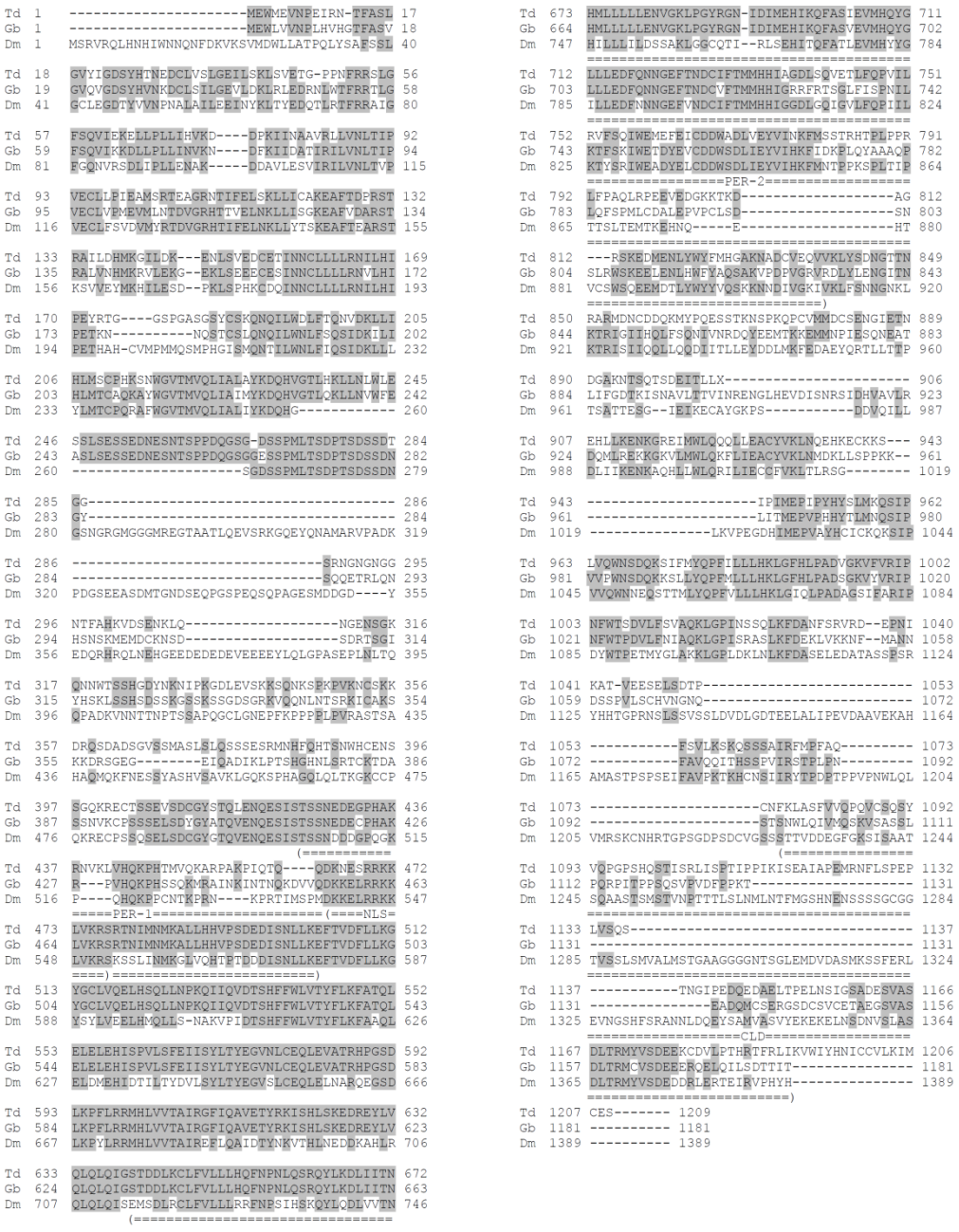

Chapter 3 Figures

Figure 3-1. Alignment of the predicted amino acid sequence of *Thermobia domestica timeless* (AB644410) compared with those of *Gryllus bimaculatus* (BAJ16356) and *Drosophila melanogaster* (AAC46920). Functional domains, NLS, PER-1, PER-2, and CLD are indicated. Gaps are introduced to maximize the homology alignment. Identical amino acid residues are shaded. Td, *Thermobia domestica*; Gb, *Gryllus bimaculatus*; Dm, *Drosophila melanogaster*.

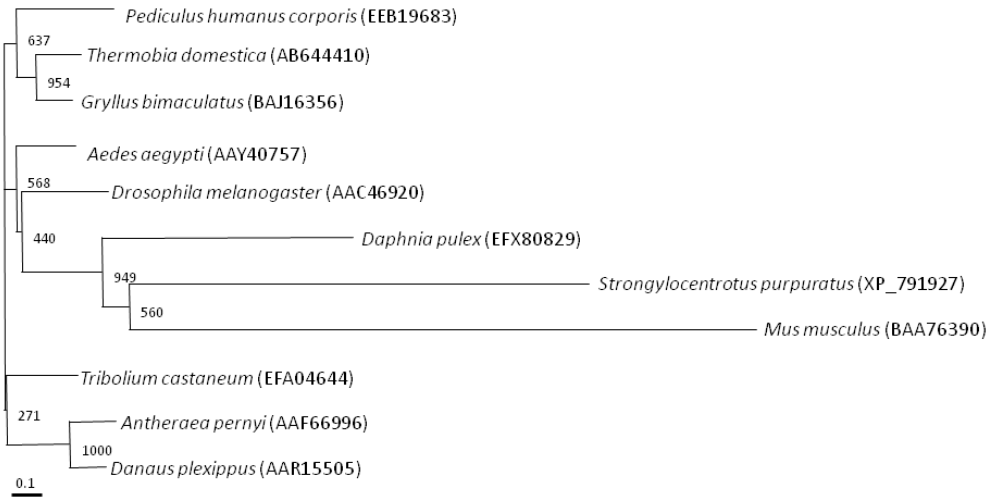

Figure 3-2. Phylogenic neighbor-joining tree of known insect TIM proteins. Neighbor-joining trees were inferred with ClustalW. A reference bar indicates distance as number of amino acid substitutions per site and the numbers indicate bootstrap values. Numbers in parentheses indicate GenBank Accession No.

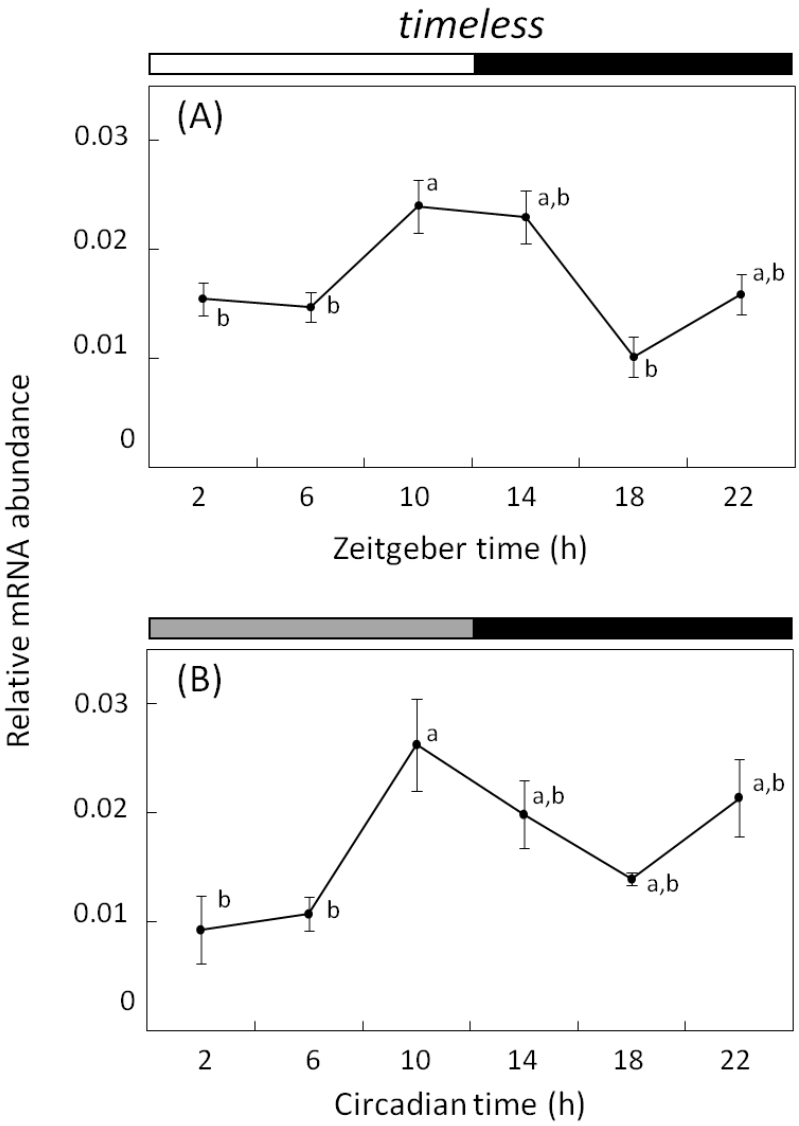

Figure 3-3.

Daily and circadian expression patterns of *Td'tim* mRNA in LD 12:12 (A) and DD (B). The abundance of *Td'tim* mRNA was measured by quantitative real-time RT-PCR. Total RNA was extracted from firebrats collected at 4 h intervals starting at 2 h after lights-on (ZT2) or projected lights-on (CT2). White, black, and gray bars indicate light phase, night/subjective night, and subjective day, respectively. The data collected from 4 independent experiments were averaged and plotted as mean ± SEM values relative to the value of *rp49* mRNA used as reference. Values with different letters significantly differ each other ($p < 0.05$; ANOVA with Tuckey-test).

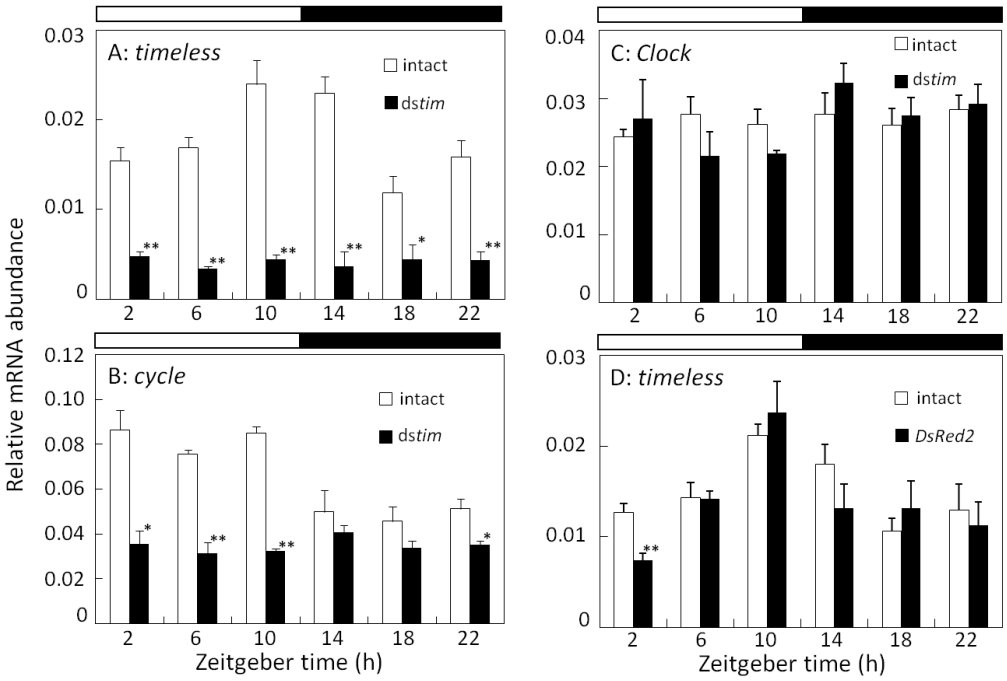

Figure 3-4.

Effects of *tim* dsRNA (ds*tim*) on mRNA levels of *tim* (A), *cyc* (B) and *Clk* (C), and effects of ds*DsRed2* on *tim* mRNA levels (D) in *Thermobia domestica*. (A) Ds*tim* effectively knocked-down *tim* mRNA levels and prevented its rhythmic expression. It also knocked down *cyc* mRNA to abolish its daily rhythm (B) but had no significant effect on *Clk* mRNA (C). Ds*DsRed2* had no significant effect on the *tim* mRNA levels except at ZT2 where a significant reduction was observed (D). The abundance of mRNAs was measured by quantitative real-time RT-PCR. Total RNA was extracted from firebrats collected 7 days after the dsRNA injection at 4 h intervals starting at 2 h after lights-on (ZT2). The data collected from 3 independent experiments were averaged and plotted as mean ± SEM values relative to those of *rp49* mRNA used as reference. ** $p < 0.01$, * $p < 0.05$, *t*-test.

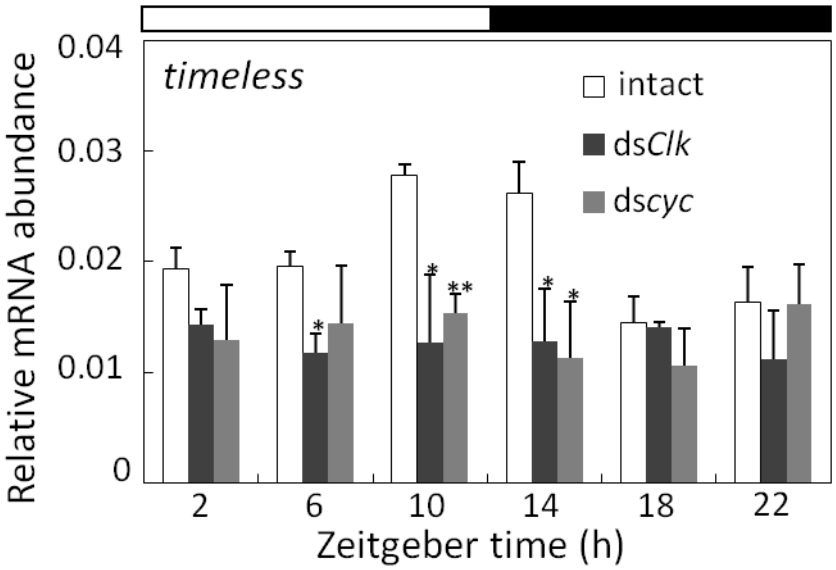

Figure 3-5.

ds*cyc* and ds*Clk* knocked-down *tim* mRNA levels to its basal level of intact animal in *Thermobia domestica*. Total RNAs were extracted from firebrats collected 7 days after dsRNA injection at 4 h intervals starting at 2 h after lights-on (ZT2). The data collected from 3 independent experiments were averaged and plotted as mean ± SEM values relative to those of *rp49* mRNA used as an internal reference. ** $p < 0.01$, * $p < 0.05$, *t*-test.

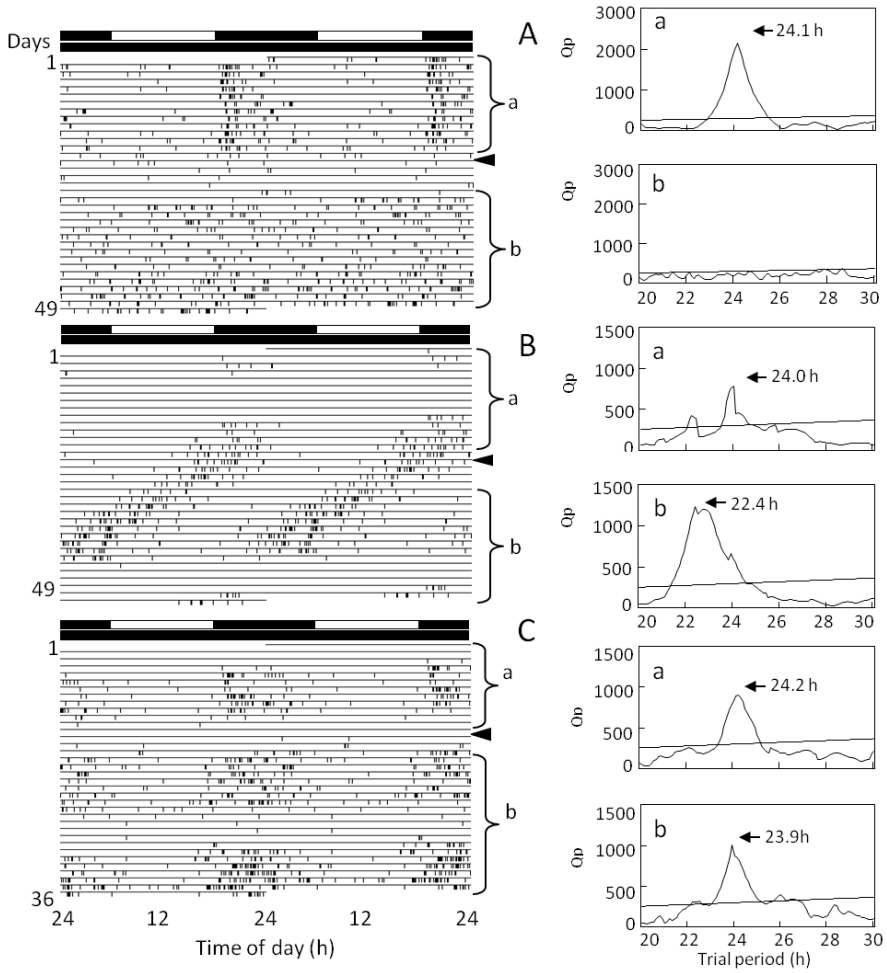

Figure 3-6.

Double-plotted actograms (left) and χ^2 periodograms (right) of locomotor rhythms of firebrats *Thermobia domestica* injected with *Td'tim* dsRNA (A, B) or *DsRed2* dsRNA (C) under LD12:12 and DD at a constant temperature of 30 °C. Arrowheads indicate the day when the firebrats were transferred from LD to DD. a and b indicated in the periodogram correspond to the analyzed time span, a and b, indicated in the actogram, respectively. An oblique line in the periodogram indicates the significance level of $p < 0.05$; peak value above the line was designated as significant. The firebrat in A showed a clear rhythm in LD which disappeared on transfer to DD, but the insect in B showed a rhythm both in LD and DD. The control firebrat injected with *DsRed2* dsRNA showed a clear rhythm throughout the recording period (C). For further explanations see text.

Chapter 3 Table

Table 3-1. Overall amino acid identity and similarity (%) of whole sequence and functional domains among insect TIM homologues.

Species	Identity	Similarity	NLS	PER-1	PER-2	CLD
Gryllus bimaculatus	57	69	83	72	65	19
Periplaneta americana	56	70	83	74	59	-
Drosophila melanogaster	49	67	83	60	53	12
Pediculus humanus corporis	49	64	83	52	47	13
Tribolium castaneum	47	64	83	66	49	19
Aedes aegypti	45	62	83	37	49	9
Antheraea pernyi	44	60	58	34	45	20
Danaus plexippus	43	60	50	36	47	22

Table 3-2. Effects of ds*tim* and ds*DsRed2* on the locomotor rhythm of the firebrat *Thermobia domestica*.

Treatment	n	No of insects		Free-running period (mean±SD) h
		Rhythmic (%)	Arrhythmic (%)	
LD				
intact	49	49 (100)	0 (0)	24.2±1.0
tim dsRNA	23	22 (95)	1 (5)	23.8±0.5
DsRed2 dsRNA	43	40 (93)	3 (7)	24.0±1.8
DD				
intact	35	30 (85)	5 (15)	24.6±2.0
tim dsRNA	20	7 (35)	13 (65)[a]	21.6±2.5[b]
DsRed2 dsRNA	38	32 (84)	6 (16)	24.5±1.6

[a]Chi-square test $p < 0.001$; [b]t-test, $p < 0.05$

Chapter 4.

General discussion

The goal of this study was to characterize the insect ancestral clock using a primitive insect species, the firebrat *Thermobia domesticus*. As reviewed in chapter 1, it has become possible to use various kinds of genetic technique to analyze the circadian rhythm in last two decades. Especially, the behavior geneticist promoted forward genetics of clock genes using the fruit fly in the following three steps: (1) creating mutants of relevant rhythm phenotype; (2) obtaining hints at a behavioral level as to whether the observed mutant phenotype reflects alternation of certain components of the concerned behavior or simply the results of some general pleiotropic effects; and (3) searching for the clock gene responsible for such altered behavior. Unfortunately, however, I could not adopt the forward genetic approach in the firebrat, because it is a non-model insect. Hence, in this work a reverse genetic approach was adopted to analyze the molecular oscillatory mechanism of the firebrat. I have analyzed the role of three circadian clock genes, *Clk*, *cyc*, and *tim* using RNAi technology.

4.1. Possible molecular mechanism of the circadian clock in the firebrat

Circadian rhythms in physiology and behavior are regulated by an endogenous circadian clock. All the molecular oscillatory clocks so far described in multicellular organisms constitute negative feed-back loops in which protein products of clock genes inhibit transcription of their own genes (Young and Kay, 2001).

The analysis of *Td'tim* revealed that it plays a role as a transcriptional repressor like in *D. melanogaster*: it has PER-1 and PER-2 domains for protein-protein interaction and CLD and NLS domains for subcellular

translocation from cytoplasm to nucleus. The oscillation of *Td'tim* mRNA also supports the possibility (Kamae and Tomioka, 2012).

*Td'*CLK-*Td'*CYC heterodimer might also play a role in the core loop as a transcriptional activator like those in *D. melanogaster*. However, *Td'*CLK lacks the transactivational domain (Poly-Q) in C-termini like in *A. pernyi,* and *Td'*CYC contains the other type of transactivational domain (BCTR) in C-termini like its mammalian ortholog, *Bmal1* (Chang and Reppert, 2003). In addition, *Td'Clk* mRNA shows an arrhythmic expression but *Td'cyc* mRNA is expressed in a rhythmic manner like in mammals. Therefore, the CLK-CYC heterodimer plays a role as a transcriptional activator but in a manner different from that in *Drosophila* (Kamae et al., 2010).

A question arising here is how the rhythmic *Td'cyc* expression is regulated under LD and DD. It seems likely that the transcription of *Td'cyc* is regulated in a way similar to that in mammals, because the homologs of *Rev-erb* and *Ror* involved in the rhythmic transcription of *Bmal1* (Preitner et al., 2002; Sato et al., 2004; Cho et al., 2012) have been cloned in the firebrat. A nuclear receptor E75 is called *Rev-erb* and a repressor of the *Bmal1* gene in mammals, and HR3 is a homolog of *Ror*. A preliminary result shows that the E75 mRNA cycles in phase with the *Td'tim* in both LD and DD, and the rhythm was lost in firebrats injected with *tim*, *Clk*, or *cyc* dsRNA in LD cycles, indicating that the E75 gene might be regulated by these clock components. Although it is also a preliminary result, treatment with E75 dsRNA up-regulated *Td'cyc* mRNA and advanced the peak of *Td'tim* mRNA rhythm. These facts suggest that E75 might be an important factor regulating the transcription of *Td'cyc*, and thus affecting

indirectly the transcription of *Td'tim*. In addition, E75 dsRNA treatment prevents moltings, suggesting that E75 is required for ecdysis.

Taken together, these results suggest that the molecular mechanism of the circadian clock of firebrats has traits both of the *Drosophila* type and mammalian type clocks (Fig. 4-1).

4.2. Comparison of the oscillatory mechanism of the circadian clock among firebrats, fruitflies and mammals

The machinery of the *Drosophila* and mammalian circadian clock has been clarified in detail as described in chapter 1-7 and 1-11 (Figs. 1-3 and 1-4). Although the hypothesis of the *Drosophila* clock was believed to explain the clock of various insects, recent studies in other insects yielded results that do not suit the *Drosophila* model, suggesting the divergence of insect clocks (Chang et al., 2003; Lupien et al., 2003; Rubin et al., 2006; Sauman and Reppert, 1996; Zhu et al., 2008). In *Drosophila*, the current cellular clock model comprises a core feed-back loop consisting of repressors, PER and TIM, and activators, CYC and CLK (Fig. 1-3) (Allada, 2003). It seems likely that the firebrat's core loop might be operating in a way similar to that in *Drosophila*. However, unlike *Dm'Clk*, it was shown that cycling of *Td'Clk* mRNA is not necessary for molecular and behavioral rhythms in the firebrat (Kamae et al., 2010; Kamae and Tomioka 2012). For the oscillatory expression of *Dm'Clk*, PDP1ε and VRI, which are directly controlled by the core loop, activate and repress *Dm'Clk* transcription, respectively (Fig. 1-3) (Cyran et al., 2003). However, it is unclear whether PDP1ε and VRI are involved in the firebrat's clock, since the *Td'Clk* is not rhythmically

expressed. To draw a conclusion, molecular analysis of the role of PDP1ε and VRI is required in the future study. On the other hand, the mammalian clock has been shown to contain additional interlocked loops (Fig.1-4). In particular, the loop regulating cyclic expression of BMAL1 (CYC) includes ROR and REV-ERB, members of a subfamily of orphan nuclear receptors, whose expression is directly regulated by the PER/TIM-CLK/BMAL core loop (Giguere, 1999; Preitner et al., 2002; Sato et al., 2004; Emery and Reppert, 2004; Triqueneaux et al., 2004). To drive the rhythmic expression of *Bmal1*, REV-ERB represses *Bmal1* transcription, while ROR acts as a positive driver to activate *Bmal1* expression (Preitner et al., 2002; Guillaumond et al., 2005). Although it is still preliminary, it seems likely that the *Td'cyc* expression might be regulated in a way similar to that in mammals (Fig.4-1).

Taken together, it is likely that the ancestral insect clock had traits of the mammalian type clock but those traits had been gradually replaced by the *Drosophila* type traits during the evolutionary process. In the firebrat, some of the major clock genes, such as *period*, remain still unidentified. To understand the transcriptional/translational feedback loops, it is required to identify those canonical core clock genes and to analyze their functions not only at the transcriptional but also at the posttranslational levels. Other important issue is the identification of the central pacemaker cells in the firebrat's central nervous system, which have been localized in the brain in *Drosophila* (Helfrich-Förster, 2006). Further studies on the firebrat's clock machinery must provide new insights into the evolution of the molecular and cellular clockwork and its functional significance.

4.3. Conclusion

Recent studies on the circadian clockwork in various insects suggest the considerable diversification of the clock mechanism among the insects. The present study has partially revealed the molecular oscillatory mechanism of the circadian clock in the firebrat *Thermobia domestica*, and contributes toward understanding the insect clock mechanism. In the *T. domestica* circadian clock, the two constituents, TIM, acts as a negative element to regulate the expression or activity of a heterodimeric transcriptional activator CLK-CYC, which is formed via their interaction through their PAS domains and regulate the expression of *tim*, like in *Drosophila*. Howver, unlike *Drosophila*, it is not *Clk* but *cyc* that is rhythmically expressed and possesses transactivational domain. These features strongly suggest that the circadian clock in the firebrats possesses properties of both *Drosophila* type *and* mammalian type clocks.

Although the involvement of *Td'Clk*, *Td'cyc*, and *Td'tim* in the central role of the rhythm generation is now apparent, a large body of the molecular mechanism of the firebrat circadian clock is still to be elucidated. The drawbacks of the genetics in firebrats can be circumvented by studies using reverse genetic approach. It is of great advantage for the molecular study of the circadian clock in firebrats that RNAi can be used for functional analysis of the clock genes. Further identification of the clock and clock-regulated genes and their functions using RNAi will uncover the whole mechanism of the insect ancestral clock.

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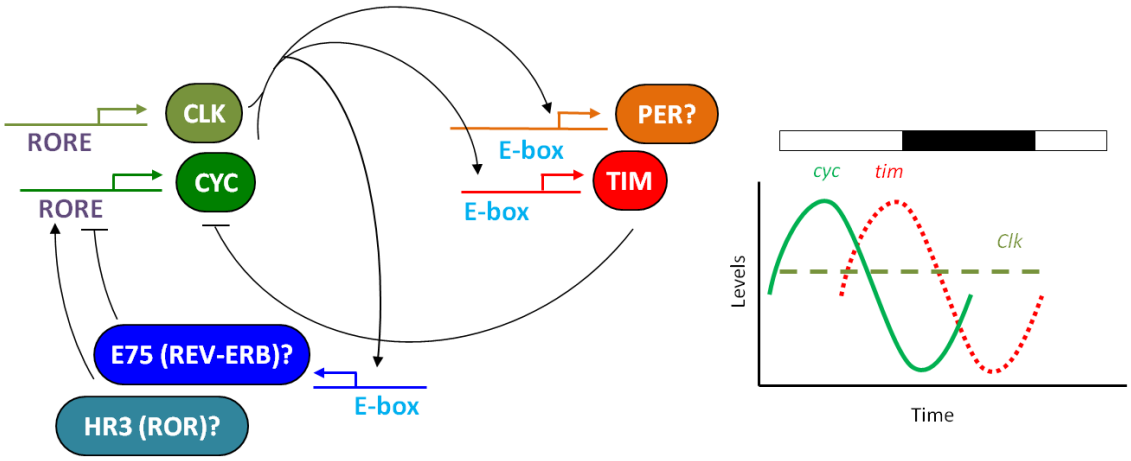

Chapter 4 Figure

Figure 4-1. The molecular mechanism of the *Thermobia* clock.
CYC-CLK probably binds to E-boxes of *tim*, E75, and (*per*) to activate their transcription, and TIM and E75 increase during late day. TIM-(PER) heterodimer enters the nucleus, and restrains their own transcription by inactivation of CYC-CLK transcriptional ability during early night. After activation of E75 transcription through E-box by CYC-CLK, E75 might be immediately accumulates in the nucleus and inhibits *cyc* transcription through RORE domain, and (HR3) might be accumulates in the nucleus and promote transcription through RORE domain during late night.