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A Temperature-Dependent Timing Mechanism is Involved in the Circadian System that Drives Locomotor Rhythms in the Fruit Fly *Drosophila melanogaster*

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ABSTRACT—The circadian clock of *Drosophila melanogaster* is thought to include rhythmic expression of *period* gene. Recent studies suggested, however, that a *per*-less oscillation is also involved in the regulation of circadian locomotor rhythms. In the present study, we examined the existence and the property of the possible *per*-less oscillation using arrhythmic clock mutant flies carrying *per*⁰¹, *tim*⁰¹, *dClk*^{Jrk} or *cyc*⁰¹, which lack rhythmic *per* expression. When temperature cycles consisting of 25°C and 30°C with various periods (T=8~32 hr) were given, wild-type (*Canton-S*) flies showed locomotor rhythms entrained to temperature cycles over a wide range of period (T=8~32 hr) in constant light (LL) while only to T=24 hr in constant darkness (DD). The mutant flies showed rhythms synchronizing with the given cycle both under LL and DD. In *per*⁰¹ and *tim*⁰¹ flies, the phase of a major peak slightly changed dependent on Ts in DD, while it did not in *dClk*^{Jrk} and *cyc*⁰¹ flies. When they were transferred from a constant temperature to a temperature cycle under DD, several cycles were necessary to establish a clear temperature entrainment in *per*⁰¹ and *tim*⁰¹ flies. These results suggest that *per*⁰¹ and *tim*⁰¹ flies have a temperature-entrainable weak oscillatory mechanism and that the *per*-less oscillatory mechanism may require *dClk* and *cyc*. In addition, *per*⁰¹ and *tim*⁰¹ flies changed from thermoactive in DD to cryoactive in LL, while *dClk*^{Jrk} and *cyc*⁰¹ flies did not. It is thus suggested that *dClk* and *cyc* are also involved in determining the light-associated temperature preference in *per*⁰¹ and *tim*⁰¹ flies.

Key words: *Drosophila melanogaster*, circadian rhythms, temperature cycle, clock mutants, *per*-less oscillation

INTRODUCTION

Many animals have a well-organized daily temporal structure in their behavior and physiology to adapt to daily changes in environment (Aschoff, 1981). The daily temporal structure is generally driven by an endogenous mechanism, so-called circadian system. In general, the major component of the system is a circadian clock that generates about 24 hr rhythms. In *Drosophila melanogaster*, the oscillatory mechanism of this clock has been profoundly studied at behavioral and molecular levels and it is known that the circadian clock consists of periodic expression of so-called clock genes (Williams and Sehgal, 2001). There are four major clock genes found so far. They are *period* (*per*), *timeless* (*tim*), *dClock* (*dClk*) and *cycle* (*cyc*), which encode PER, TIM, dCLK and CYC proteins, respectively (Konopka and Benzer, 1971; Sehgal *et al.*, 1994; Allada *et al.*, 1998;

Rutila *et al.*, 1998). dCLK and CYC form heterodimer and activate transcription of *per* and *tim* (Allada *et al.*, 1998; Darlington *et al.*, 1998; Rutila *et al.*, 1998). PER and TIM also form heterodimers, are transferred into the nucleus and then inactivate the transcriptional activity of dCLK and CYC (Darlington *et al.*, 1998). Because *per* and *tim* finally suppress their own transcription, they are referred to as the negative elements, whereas *dClk* and *cyc* as the positive elements (Dunlap, 1999).

The circadian clock can be entrained to environmental cycles to set physiological events to occur at an appropriate timing using various environmental information. Temperature is one of the important environmental zeitgeber (Zimmerman *et al.*, 1968; Ikeda and Tomioka, 1993; Lankinen and Rihimaa, 1997). From a profound study on photic and temperature entrainment of eclosion rhythms of *Drosophila pseudoobscura*, Pittendrigh *et al.* (1958) proposed that the *Drosophila* circadian system consists of two coupled oscillators; one is a light-sensitive master oscillator and the other is a temperature-sensitive slave oscillator. The hypothesis

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has not been extensively examined in *Drosophila melanogaster* so far. However, studies on arrhythmic *per⁰¹* flies lacking functional PER that is essential for the molecular rhythm generation (Hardin *et al.*, 1990) have yielded lines of evidence suggesting that *D. melanogaster* also has a secondary oscillatory component other than *per*-dependent central oscillation (Weitzel and Rensing, 1981; Helfrich and Engelmann, 1987; Helfrich-Förster, 2001). A circadian incorporation rhythm of positively charged fluorescent dye, probably associated with a circadian change in membrane potential, has been reported in salivary glands of *per⁰¹* flies (Weitzel and Rensing, 1981). When exposed to light cycles with long periods, *per⁰¹* flies clearly anticipated the dark to light transition (Helfrich and Engelmann, 1987). By an experiment using photic entrainment of *per⁰¹* flies with light cycles of various periods, Helfrich-Förster (2001) suggested that the morning peak is driven by an oscillator that can operate independently from the *per*-dependent central oscillator.

To characterize the secondary oscillation, temperature cycle was often used in several insects with the primary circadian clock ablated. In optic-lobeless crickets and cockroaches, the entrainment of the secondary oscillation was reportedly achieved with temperature cycles (Rence and Loher, 1975; Page, 1985). We previously found that the arrhythmic *per⁰¹* mutants of *D. melanogaster* can be entrained to temperature cycles (Tomioka *et al.*, 1998). In this study we examined the possibility of involvement of secondary oscillator other than *per*-dependent central oscillator using arrhythmic clock mutant flies carrying *per⁰¹*, *tim⁰¹*, *dClk^{Jrk}* and *cyc⁰¹*. The *tim⁰¹* flies are known to suppress the circadian oscillation of *per* mRNA (Sehgal *et al.*, 1994), and the *dClk^{Jrk}* and *cyc⁰¹* flies have little or no transcription of the *per* and *tim* genes (Allada *et al.*, 1998; Rutila *et al.*, 1998). Thus all these mutant flies are behaviorally arrhythmic in constant conditions. We entrained the rhythms of the mutant flies to temperature cycles of various periods (*T_s*) ranging from 8 hr to 32 hr. The results show that a temperature-dependent timing mechanism is operating in *per⁰¹* and *tim⁰¹* flies but not in *dClk^{Jrk}* and *cyc⁰¹* flies, suggesting the involvement of *dClk* and *cyc* in the mechanism. We also found that *dClk* and *cyc* are involved in determining the active phase in the thermoperiodic conditions in association with the lighting condition.

MATERIALS AND METHODS

Experimental animals

Adult flies, *Drosophila melanogaster*, were used. They were raised on standard cornmeal-glucose-yeast medium at 25°C under a light cycle with 12hr light to 12hr dark (LD12:12). The strains that we used for behavioral assay were *Canton-S* (wild-type), *period⁰¹* (*per⁰¹*), *timeless⁰¹* (*tim⁰¹*), *dClock^{Jrk}* (*dClk^{Jrk}*) and *cycle⁰¹* (*cyc⁰¹*).

Recording of locomotor activity

Male flies of about five days old were individually housed in transparent acrylic rectangular tubes (3×3×70 mm). The tube was plugged at one end with agar/glucose medium as food and was

sealed with Parafilm, and at the other end with a silicon tube filled with damped absorbent cotton as water source. A moving fly interrupted an infrared beam and the number of interruptions during each 6 min was recorded using a computerized system (Tomioka *et al.*, 1997). The fly tubes and activity sensing system was placed in an incubator (Hitachi, CR32S) in which the light and temperature were controlled. Lighting conditions in the incubator were given by a cool white fluorescent lamp connected to an electronic timer. Light intensity at the animals level was 100 to 300 lx, varying with the proximity to the lamp. Temperature cycles were set by a built-in thermostat driven by an electronic timer. Temperature steps-up and -down were finished within 15 min. Temperature cycles used were composed of an equal duration of thermophase (30°C) and cryophase (25°C) with periods varying from 32 hr (*T_s*=32 hr) to 8 hr (*T_s*=8 hr) at an interval of 4 hr.

Data analysis

The raw activity data were displayed as conventional double-plotted actograms. For judgment of the activity pattern under different *T* values, actograms were drawn at the respective Zeitgeber

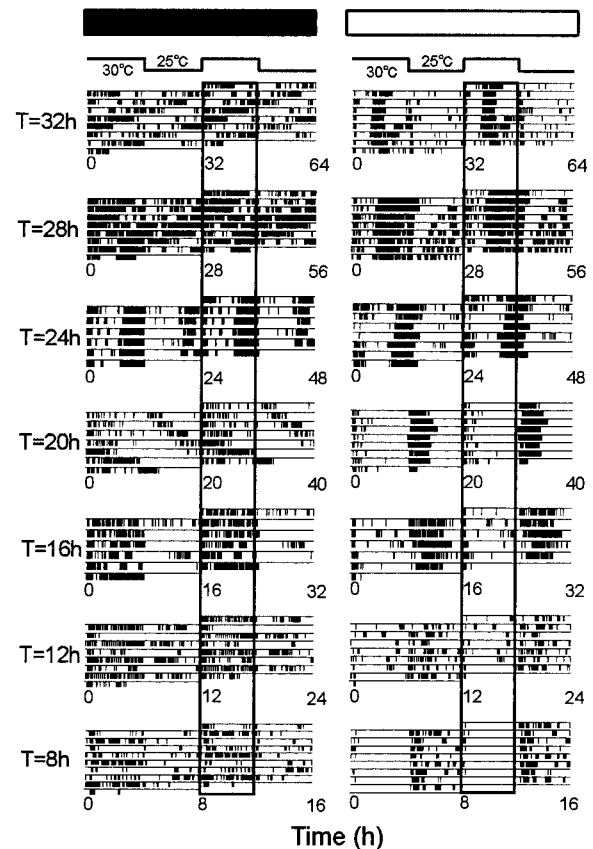


Fig. 1. Double plots of representative actograms of wild-type flies recorded under temperature cycles of various periods, consisting of an equal duration of thermophase (30°C) and cryophase (25°C) in LL (right) and DD (left). Each actogram is double-plotted from top to bottom at the respective Zeitgeber period, which is shown, on the left of each row of the panels. White and black bars on the top of the actograms indicate light and dark, respectively. Boxes on the right half of the actograms indicate the thermophase. In DD, the locomotor rhythm was entrained only to temperature cycle of 24 hr (*T_s*=24hr) and free-ran in other *T_s*. In contrast, the rhythm synchronized with temperature cycles of all *T_s* under LL with the phase of the major peak changing dependent on *T_s*. For further explanations see text.

period. The entrainment of activity of individual animals was objectively examined by the chi-square periodogram (Sokolove and Bushell, 1978) with trial periods around a given T. When the calcu-

lated value at a trial period equal to T exceeded the 0.05 confidence level, the animal was designated as entrained. Average daily activity patterns were calculated for entrained fly groups to compare the

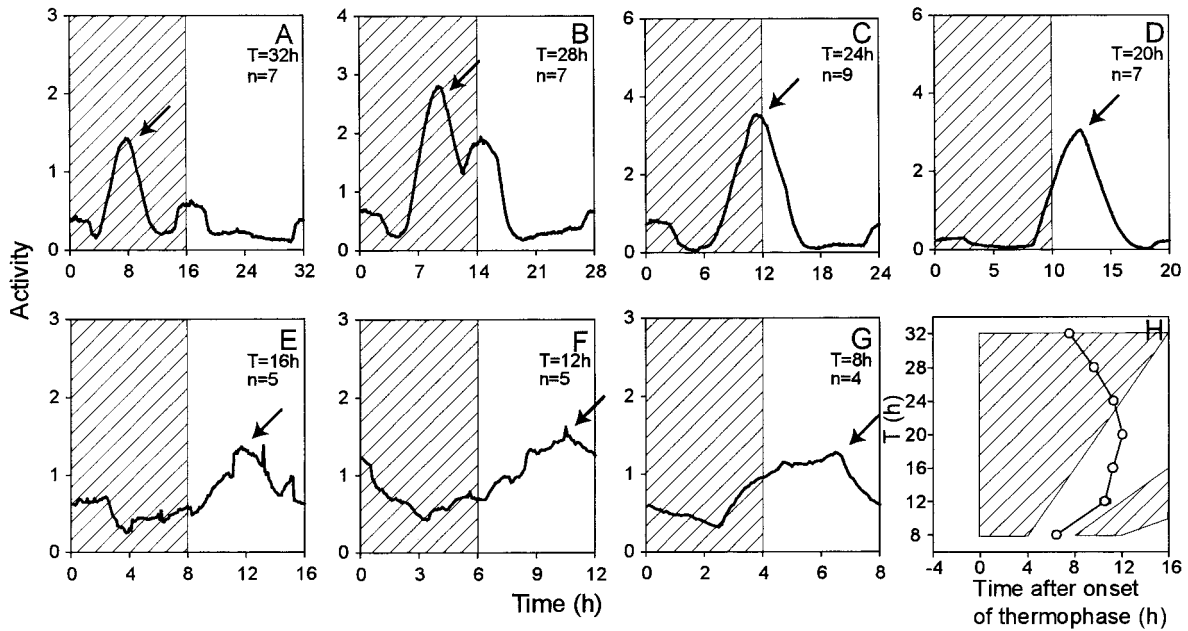


Fig. 2. A–G: Averaged and smoothed daily activity patterns of wild-type flies in LL that were calculated from frequency folded data at a period indicated by T. Shaded area indicates the thermophase. n indicates the number of flies entrained. Note that a primary peak occurred in the middle of the thermophase (arrow) in addition to small peaks at temperature transitions in T=32hr, gradually delayed to the middle to late cryophase as the temperature cycle was shortened. The time difference from the onset of thermophase and the primary peak changed dependent on T. H: Mean phase (\pm SEM) of major peaks in temperature cycles under LL. The phase of the primary peak occurring at the middle of thermophase in T=32hr gradually delayed toward the late cryophase as T was shortened.

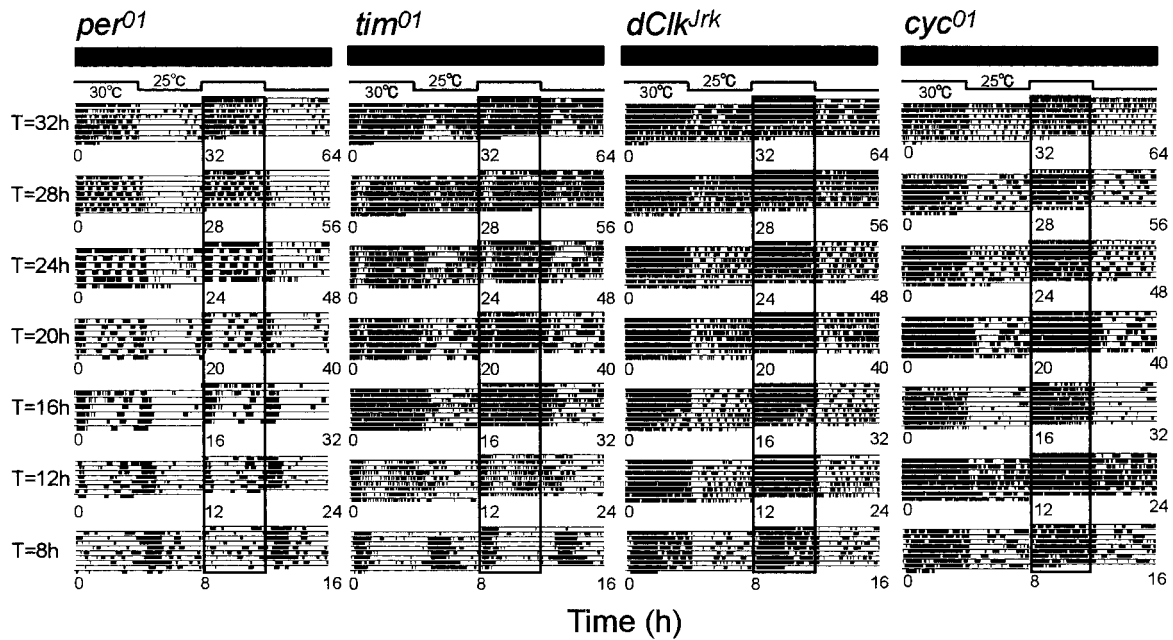


Fig. 3. Double plots of representative actograms of *per⁰¹*, *tim⁰¹*, *dClk^{Jrk}* and *cyc⁰¹* flies recorded under temperature cycles of various periods, consisting of an equal duration of thermophase (30°C) and cryophase (25°C) in DD. Actograms were plotted from top to bottom at the respective Zeitgeber period that was shown on the left of each row of the panels. Black bars on the top of the actograms indicate constant darkness. Boxes on the right half of the actograms indicate the thermophase. In all flies, the locomotor rhythm synchronized with temperature cycles in all Ts. Note that the phase of the activity changed dependent on the period of temperature cycles in *per⁰¹* and *tim⁰¹* flies, while in *dClk^{Jrk}* and *cyc⁰¹* flies the active phase consistently occurred in the thermophase. For further explanations see text.

average waveform entrained to temperature cycles of different T values. To determine the phases of peaks of activity rhythms, moving average of 41 data points was performed. The free-running period was calculated by the chi-square periodogram (Sokolove and Bushell, 1978). Analysis of variance (ANOVA) was used to test whether significant differences among the time intervals between either onset of the thermophase or the cryophase and the activity peak were observed at different T values.

RESULTS

Locomotor rhythm of wild-type flies under temperature cycles

We first examined the entrainment of wild-type (*Canton-*

S) flies to temperature cycles under LL and DD. The locomotor activity rhythm of the flies was recorded under temperature cycles with T=32 hr, 28 hr, 24 hr, 20 hr, 16 hr, 12 hr and 8 hr consisting of an equal duration of thermophase (30°C) and cryophase (25°C). They showed clear entrainment to all temperature cycles under LL without evidence of frequency division or frequency demultiplication (Fig. 1). They showed a large primary peak in addition to small peaks associated with temperature transitions (Fig. 2). The phase of the major peak was apparently dependent on the period of temperature cycles ($P < 0.001$, ANOVA). It occurred in the middle of the thermophase in T=32 hr, gradually delaying toward the cryophase as T was shortened to

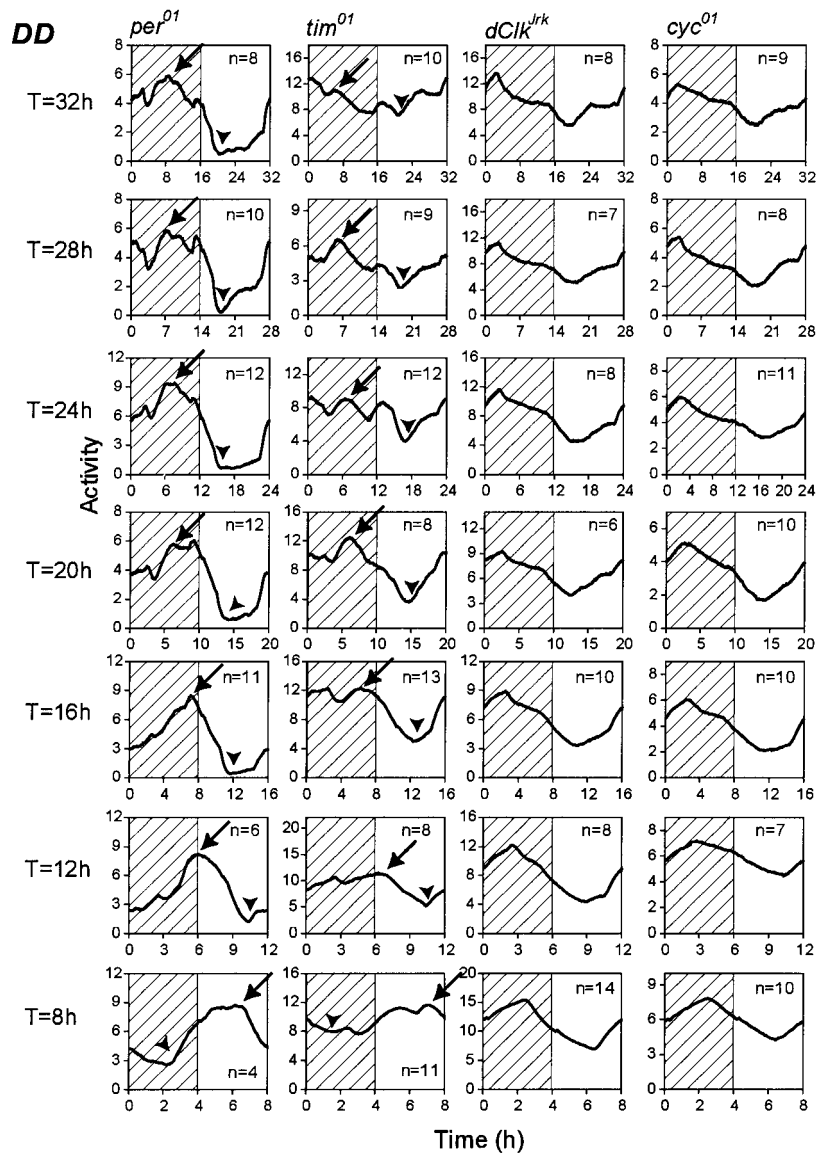


Fig. 4. Averaged and smoothed daily activity patterns of *per*⁰¹, *tim*⁰¹, *dCik*^{Jrk} and *cyc*⁰¹ flies in DD that were calculated from frequency folded data. Shaded areas indicate the thermophase. n indicates the number of flies used. Note that the activity phase was always in the thermophase in *dCik*^{Jrk} and *cyc*⁰¹ flies while, in *per*⁰¹ and *tim*⁰¹ flies, a major peak that occurred in the middle of the thermophase (arrow) in addition to small peaks occurring at temperature transitions in T=32 hr, gradually delayed to the middle to late cryophase as the temperature cycle shortened. The time difference from the onset of thermophase and the major activity peak was 6–8 hr and a discernible trough (arrow head) occurred about 4 hr after the onset of cryophase in *per*⁰¹ and *tim*⁰¹ flies.

20 hr. In $T=20$ hr or shorter, the peak always occurred in the cryophase (Fig. 2H). The results indicate that locomotor rhythms in the wild-type flies are driven by a circadian clock in temperature cycles even under LL. In DD, however, they showed free-running rhythms even under temperature cycles, except $T=24$ hr (Fig. 1). Free-running periods were close to 24 hr but substantially varied dependent on T , ranging from 23.8 ± 0.02 hr ($T=16$ hr, $n=8$) to 24.9 ± 0.2 hr ($T=28$ hr, $n=8$). In $T=24$ hr, they synchronized to the temperature cycle with a primary peak occurring at the late thermophase (11.3 ± 0.2 hr after the onset of thermophase); the daily activity pattern was similar to that under LL.

Locomotor rhythm of clock mutants under temperature cycles in constant darkness

Thermosteriodic entrainability of arrhythmic mutant flies carrying per^{01} , tim^{01} , $dClk^{Jrk}$ or cyc^{01} was examined by recording their locomotor activity in the temperature cycles under DD. Fig. 3 shows representative actograms. In all mutant flies, locomotor activity clearly synchronized with temperature cycles of various T values without evidence of frequency division or frequency demultiplication. $dClk^{Jrk}$ and cyc^{01} flies showed a definite tendency to be more active in the thermophase than the cryophase. Activity increased immediately after the start of thermophase in all T s (Figs. 3 and 4). The phase of activity peak always occurred at about 2.5 hr after the onset of thermophase (Fig. 5B), and there was no systematic dependency of the phase on T except

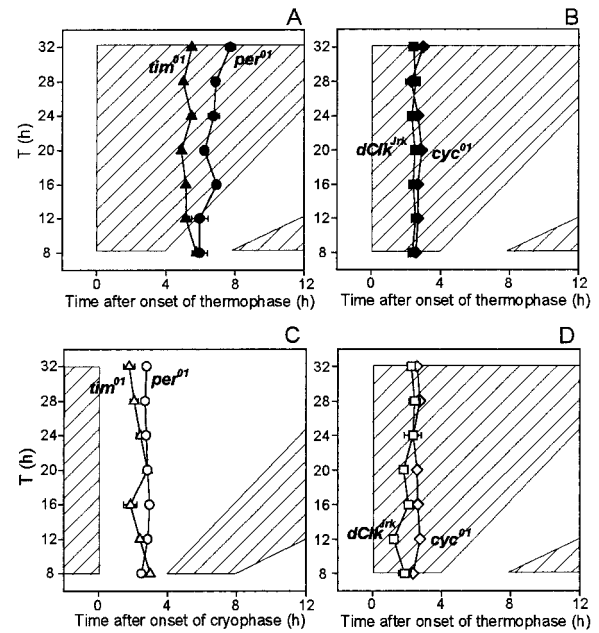


Fig. 5. Mean phase (\pm SEM) of major peaks in temperature cycles under DD (closed symbols) or LL (open symbols). Shaded areas indicate the thermophase. Ordinate indicates periods of temperature cycles and abscissa the time after the onset of thermophase (A, B and D) or cryophase (C). In per^{01} and tim^{01} flies, primary peak occurs about 6–8 hr after the onset of thermophase in DD (A) but about 2–3 h after the onset of cryophase in LL (C). The major peaks of $dClk^{Jrk}$ and cyc^{01} occurred consistently about 2–3 hr after the onset of thermophase both in DD and LL (B and D). For further explanations see text.

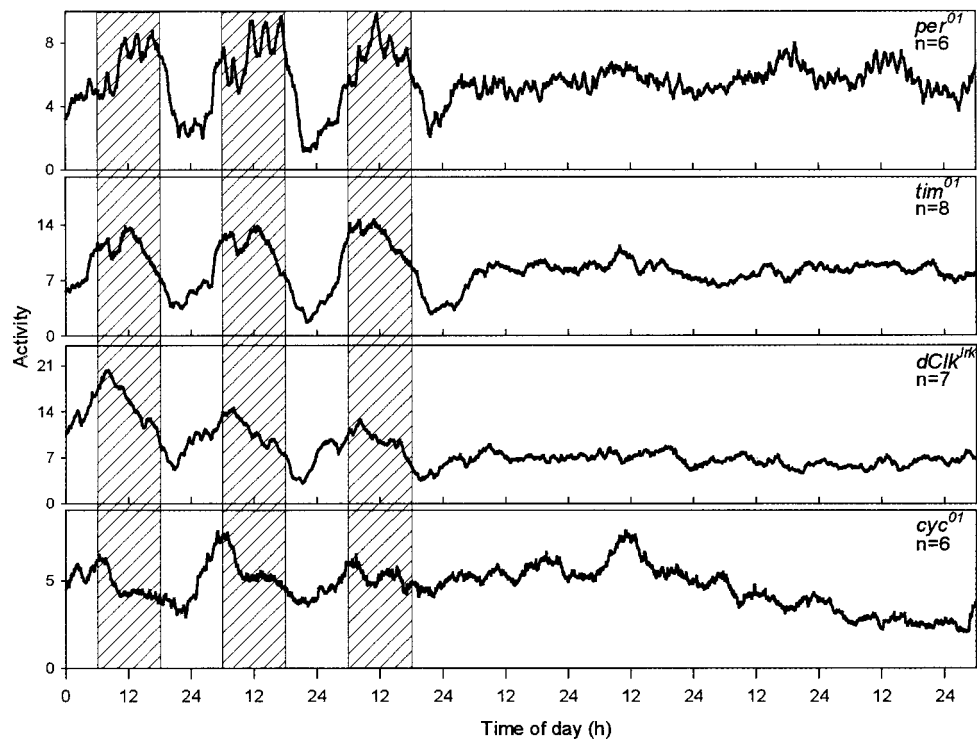


Fig. 6. Averaged longitudinal activity of per^{01} , tim^{01} , $dClk^{Jrk}$ and cyc^{01} flies recorded consecutively in temperature cycle of 30°C 12 hr: 25°C 12 hr and then in constant 25°C under DD. Shaded areas indicate the thermophase. n indicates number of flies used. On transfer to constant 25°C, activity rhythms disappeared after showing a trough associated with a transition from 30°C to 25°C in all flies. For further explanations see text.

T=28 hr in *cyc⁰¹* ($P>0.05$, ANOVA).

per⁰¹ and *tim⁰¹* flies also entrained to the temperature cycles (Figs. 3 and 4); they were also more active during the

thermophase than during the cryophase except for T=8 hr under which activity was concentrated in the cryophase. Average activity curves revealed a major peak that occurred

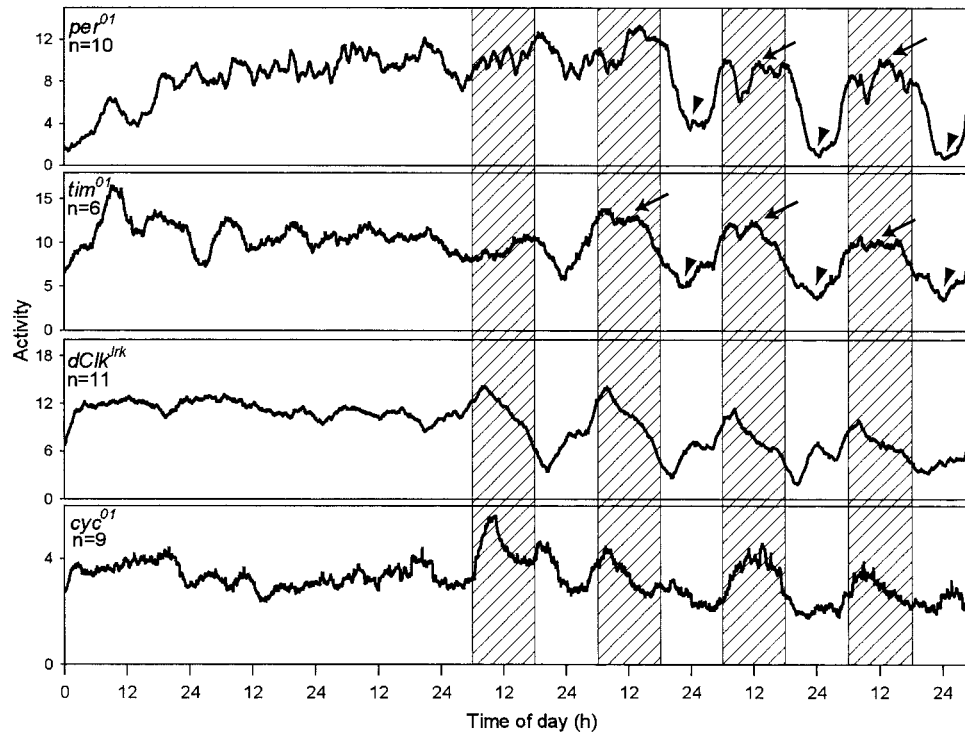


Fig. 7. Averaged longitudinal activity of *per⁰¹*, *tim⁰¹*, *dClk^{Jrk}* and *cyc⁰¹* flies recorded consecutively in constant 25°C and then temperature cycle of 30°C 12 hr: 25°C 12 hr under DD. Shaded areas indicate the thermophase. On transfer to temperature cycle, a robust peak immediately appeared in the first thermophase in *dClk^{Jrk}* and *cyc⁰¹* flies, while at least two or more cycles were necessary for clear entrainment in *per⁰¹* and *tim⁰¹* flies. Arrows and arrow heads indicate a primary peak and a trough, respectively. For further explanations see text.

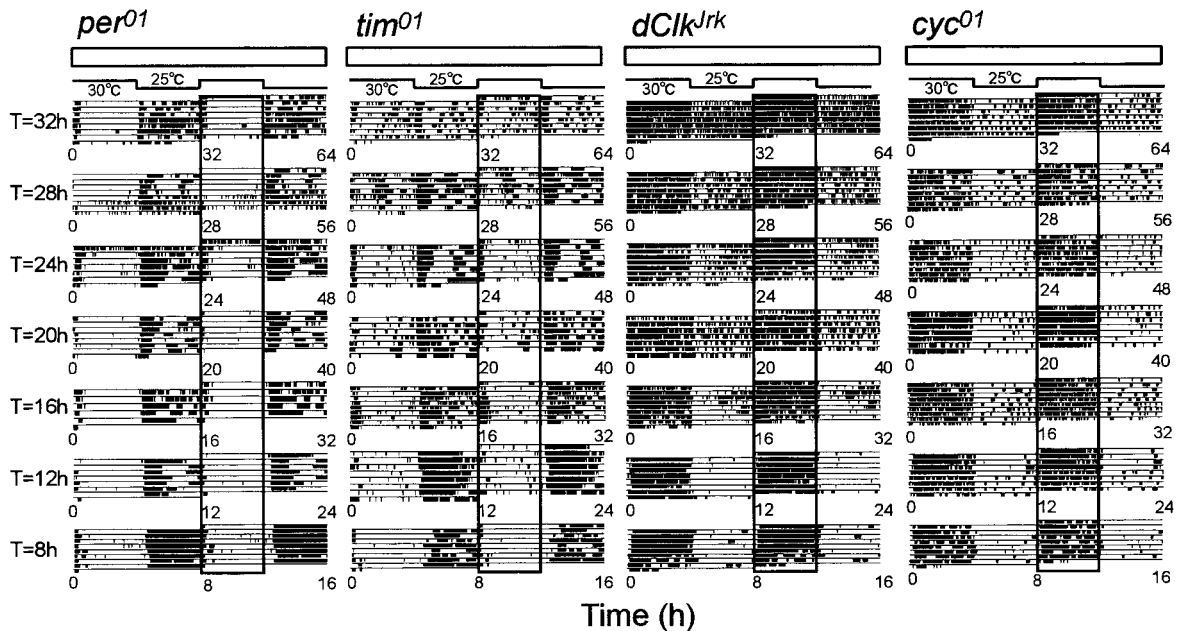


Fig. 8. Double plots of representative actograms of *per⁰¹*, *tim⁰¹*, *dClk^{Jrk}* and *cyc⁰¹* flies recorded under temperature cycles of various periods, consisting of an equal duration of thermophase (30°C) and cryophase (25°C) in LL. Actograms were plotted from top to bottom at the respective Zeitgeber period that was shown on the left of each row of the panels. White bars on the top of the actograms indicate constant light. Boxes on the right half of the actograms indicate the thermophase. In all flies, the locomotor rhythm synchronized with temperature cycles in all Ts. Note that the active phase is antiphase to that in DD (see Fig. 3) in *per⁰¹* and *tim⁰¹* flies. For further explanations see text.

6~8 hr after the onset of thermophase in addition to two rather small peaks associated with temperature transitions. The former gradually became to occur later relative to the temperature cycle as T was shortened, and eventually in the cryophase in T=8 hr. In real time, it occurred slightly but significantly earlier as the T values became smaller in *per*⁰¹ flies (Fig. 5A)(P<0.01, ANOVA), while, in *tim*⁰¹ flies, the peak occurred slightly later at T=32 hr, 24 hr and 8 hr (Fig. 5A)(P<0.01, ANOVA). Another point to be noted is that the *per*⁰¹ and *tim*⁰¹ flies showed a trough at about 4~6 hr after the onset of cryophase in all Ts except T=8 hr (Figs. 3 and 4), where the trough occurred in the thermophase. The trough was more robust in *per*⁰¹ than in *tim*⁰¹ flies.

To clarify whether the rhythmic patterns of the mutant

flies are direct response to temperature cycles, we transferred the flies from temperature cycles to constant temperature and vice versa. Upon transfer from temperature cycles to constant 25°C, all 4 mutant flies became arrhythmic after showing a trough associated with a transition from the thermophase to the cryophase (Fig.6). The activity level at constant temperature was nearly intermediate between the peak and trough in the temperature cycle. When transferred from constant 25°C to temperature cycles of 25°C 12hr : 30°C 12 hr (T=24 hr), *dClk*^{Jrk} and *cyc*⁰¹ exhibited a large activity peak in the first thermophase that persisted thereafter (Fig. 7), whereas *tim*⁰¹ and *per*⁰¹ became to show clear entrainment at 2nd or 3rd cycle, respectively. Interestingly, in *per*⁰¹ a trough occurred around 4~6 hr after the onset of

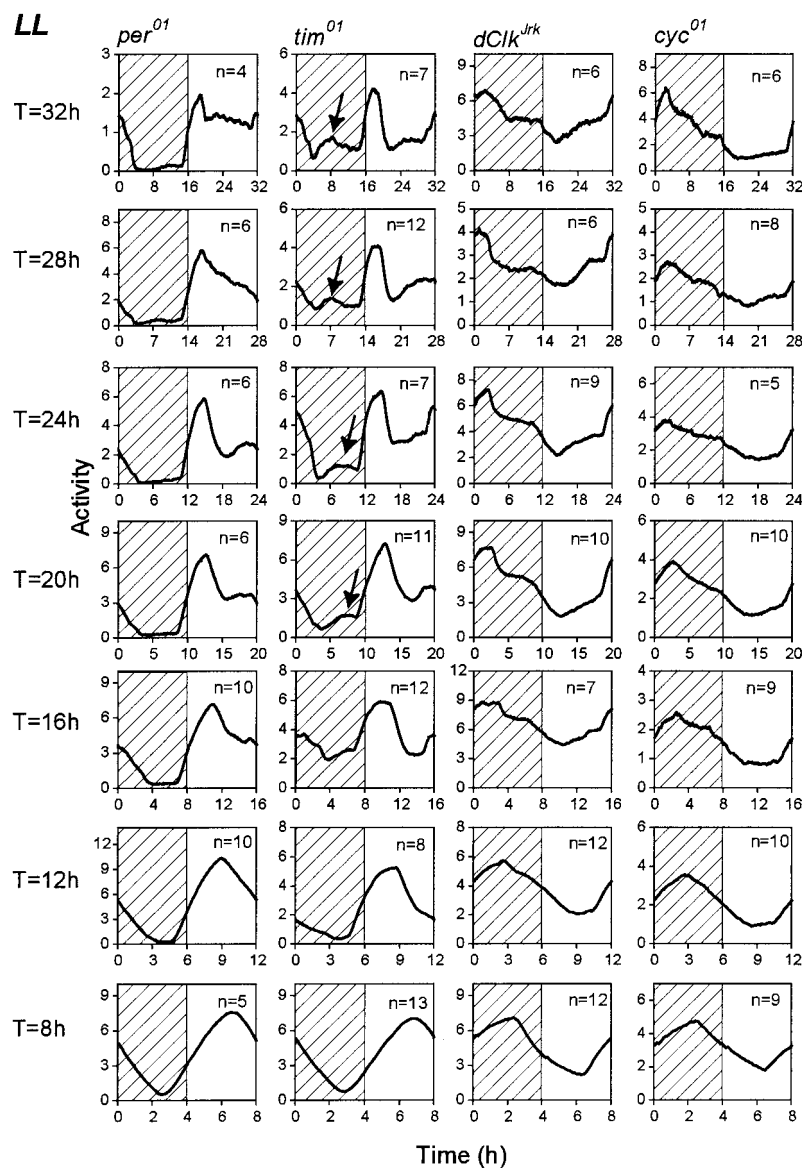


Fig. 9. Averaged and smoothed daily activity patterns of *per*⁰¹, *tim*⁰¹, *dClk*^{Jrk} and *cyc*⁰¹ flies in LL that were calculated from frequency folded data. Shaded areas indicate the thermophase. n indicates the number of flies used. The major peaks consistently occurred in the cryophase in *per*⁰¹ and *tim*⁰¹ flies, and in the thermophase in *dClk*^{Jrk} and *cyc*⁰¹ flies. A small peak (arrow) appeared in the middle to late thermophase in T=20 hr or longer in *tim*⁰¹ flies. For further explanations see text.

cryophase on the first cycle, becoming more prominent day by day to reach at the least level on the third cycle. A similar but less obvious tendency was also observed in *tim⁰¹* flies.

Locomotor rhythm of clock mutants under temperature cycles in constant light

The locomotor activity of arrhythmic mutant flies was recorded in temperature cycles under LL. Fig. 8 shows representative actograms. Again, all mutant flies clearly showed activity rhythms synchronized with temperature cycles without showing evidence of frequency division or frequency demultiplication. In *dClk^{Jrk}* and *cyc⁰¹* flies, the activity patterns were quite similar to those under DD (Fig. 9): the peak occurred about 2~3 hr after the onset of thermophase, and there was no dependency of peak phase on T for both *dClk^{Jrk}* and *cyc⁰¹* flies (Fig. 5D) ($P > 0.05$, ANOVA). However, *per⁰¹* and *tim⁰¹* flies reversed from thermoactive in DD to cryoactive in LL (Figs. 5C and 8), exhibiting a large peak about 2~3 hr after the onset of cryophase. The peak phase was significantly dependent on T in *tim⁰¹* flies (Fig. 5C) ($P < 0.01$, ANOVA). The activity in the thermophase was remarkably reduced especially in *per⁰¹* flies. Interestingly, *tim⁰¹* flies showed a small peak about 6 hr after the onset of thermophase, which seemed identical to the one occurred under DD.

DISCUSSION

Entrainment to temperature cycle in wild-type flies

The results of the present study, not only confirms but also extends the previous reports that wild-type flies of *Drosophila melanogaster* can be entrained to temperature cycles in DD (Wheeler *et al.*, 1993; Tomioka *et al.*, 1998). However, the entrainment was achieved only to the cycle of T=24 hr, and the flies showed free-running rhythm with a period close to 24 hr in Ts shorter or longer than 24 hr. The result indicates that temperature cycle is a much weaker entraining agent than light because light can entrain the rhythm in a wide range of Ts (Helfrich-Förster, 2001). Probably, temperature pulses cause only small phase-shifts in free-running flies. This explanation is also supported by the fact that, in DD, *per^S* and *per^L* flies also entrained to the temperature cycles with periods close to their natural free-running periods (Tomioka *et al.*, 1998).

Under LL, in contrast to under DD, wild-type flies never free-ran but showed activity rhythms clearly synchronized with the given temperature cycles with wide range of Ts, without evidence of frequency division or frequency demultiplication. It has been shown that LL stops the circadian oscillation not only behavioral but also molecular level (Price *et al.*, 1995). The rhythm observed in the present study has characteristics full-filling the empirical rule for the one driven by the circadian oscillation, however: the phase of a primary activity peak changed dependent on the period of temperature cycles. The fact supports our previous hypothesis that temperature cycle can drive the circadian oscillation even in

LL where the clock is stop in constant temperature (Tomioka *et al.*, 1998) and suggests that temperature cycle entrains the rhythm through the pathway different from that for photic entrainment. The locomotor rhythm in *Drosophila* is believed to be driven by a circadian oscillation generated by an autoregulatory feedback loop including rhythmic expression of *per* and *tim* at a period of about 24 hr (Reppert and Weaver, 2000). Their product proteins, PER and TIM, form heterodimer, enter the nucleus and inhibit their own transcription through inactivation of their transcription factors, dCLK and CYC, products of *dClk* and *cyc* (Williams and Sehgal, 2001). Western blot analysis using anti-PER antibody with proteins extracted from fly heads revealed an oscillation in PER abundance at least in T=24 hr and 32 hr under LL (Ibuki, M., *et al.*, unpublished data), suggesting that the temperature induced rhythm in LL involves the rhythmic expression of *per* probably through the autoregulatory feedback loop. An important question that should be addressed in a future study is how temperature drives the oscillation once stopped by constant light. It is also to be answered whether the same molecular oscillation that works in LD or DD (cf. Williams and Segal, 2001) is working in temperature cycle with Ts considerably shorter or longer than 24 hr.

Thermoperiodically induced rhythms in mutants lacking the *per*-feedback loop

The present study revealed that all mutant flies carrying mutation in clock genes, which induces arrhythmicity in constant conditions, exhibited rhythms in thermoperiodic conditions. However, the rhythms were quite different from those of wild-type flies in respect to that they never free-run in DD in Ts longer or shorter than 24 hr and that their peak phase was rather stable over a wide range of Ts, suggesting that the underlying mechanism differs from that for wild-type flies.

The rhythmic pattern was remarkably different between mutant groups lacking so-called negative and positive components. In *dClk^{Jrk}* and *cyc⁰¹* flies lacking either of the positive components, dCLK or CYC (Allada *et al.*, 1998; Rutila *et al.*, 1998), the thermoperiodically induced rhythms seemed to be direct response to temperature cycles. This statement is based on the following facts. Their rhythmic patterns were always quite similar through temperature cycles with various Ts both under DD and LL, with peaks always appearing about 2 hr after the onset of thermophase, and with the active phase always being confined in the thermophase.

In contrast, *per⁰¹* and *tim⁰¹* flies lacking either of the negative components, PER or TIM, changed from thermoactive to cryoactive in shorter Ts in DD. The peak phase was also found to change slightly but statistically significantly dependent on the period of temperature cycles. These facts suggest that their activity rhythms are not a direct response to temperature cycles but driven by an endogenous timing mechanism. The fact that the primary peak consistently occurred around 6 hr after the transition from the cryophase to the thermophase in all Ts suggests that the timing mechanism seems to be reset by the transi-

tion and set the timing of the peak phase. The involvement of circadian oscillation in *per*⁰¹ and *tim*⁰¹ flies is further suggested by the results of transfer of flies from constant temperature to temperature cycles. The activity peak as well as trough never occurred immediately after the transfer but gradually became prominent after several transient cycles. The transients may be explained as the period during which an underlying weak oscillator synchronizes with the given cycle. The oscillator seems to preserve the character of highly damped oscillator since the rhythm almost immediately disappeared when transferred from temperature cycles to constant temperature.

The involvement of *per*-less circadian oscillation in locomotor rhythms has also been demonstrated for the *per*⁰¹ flies entrained photoperiodically (Helfrich and Engelmann, 1987; Helfrich-Förster, 2001). The oscillation demonstrated here occurred when exposed to thermoperiods. It is an interesting question whether these oscillations are based on the same mechanism. Since the thermoperiodically-induced oscillatory components were observed in the flies lacking either PER or TIM but not in flies lacking the positive components, it seems likely that *dClk* and *cyc* are somehow involved in the thermoperiodically-induced oscillation. A similar thermoperiodic entrainment of the circadian oscillation was recently reported for the *frq*⁹ mutant of the bread mold *Neurospora crassa* (Merrow *et al.*, 1999). Although *frq*⁹ mutants are arrhythmic under constant conditions (Aronson *et al.*, 1994), they exhibit clear oscillations in their conidiation under temperature cycles with aspects of typical circadian entrainment. The *frq*-independent oscillation is explained to be driven by a temperature entrainable oscillator called FLO that involves metabolic components (Iwasaki and Dunlap, 2000; Morgan *et al.*, 2001). The relationship between the *Neurospora frq*-independent oscillation and the *Drosophila*-thermoperiodically induced oscillation is an interesting issue to be addressed in future studies.

The thermoperiodically induced oscillation was also reported for cockroaches and crickets (Rence and Loher, 1975; Page, 1985). Their circadian clocks driving the locomotor rhythm reside in the optic lobe. Even after the optic lobes are bilaterally removed, the locomotor activity can be entrained to the temperature cycles with some circadian characteristics. In cockroaches *Leucophaea maderae*, similarly to *D. melanogaster*, the rhythms were driven by cycles with wide range of Ts (12~48 hr) but with predictable T-dependent changes in active phase (Page, 1985). It seems thus a rather general scheme that the circadian system is composed of a temperature entrainable secondary oscillator in addition to the core oscillator involving the autoregulatory feedback loop. Probably the temperature entrainable oscillator is driven by the light entrainable circadian clock in the light of the master-slave organization proposed by Pittendrigh *et al.* (1958) long time ago.

Light-dependent reversal of the active phase in *per*⁰¹ and *tim*⁰¹ flies

The results of the present study demonstrated that the active phase of *per*⁰¹ and *tim*⁰¹ flies showed a reversal from the thermophase under DD to the cryophase under LL in a wide range of Ts. This confirms our previous reports for the light-dependent reversal of active phase in *per*⁰¹ flies in 24 hr temperature cycles (Tomioka *et al.*, 1998). There are multiple photoreceptors known for entrainment of circadian rhythms in *Drosophila*: they are the compound eyes, ocelli, Hofbauer-Buchner's (H-B) eyelet and a deep brain blue-light receptor, cryptochrome (Yasuyama and Meinertzhagen, 1999; Helfrich-Förster *et al.*, 2001). These photoreceptors cooperate to synchronize the circadian locomotor rhythms. Since the photic information for the light-dependent reversal of active phase is abolished in *eya;per*⁰¹ and *so;per*⁰¹ double mutant flies, which lack compound eyes and both compound eyes and ocelli, respectively, but retain the H-B eyelet and cryptochrome, the photic information necessary for this reversal is most likely received by the compound eyes as suggested previously (Tomioka *et al.*, 1998). Interestingly, *dClk*^{Jrk} and *cyc*⁰¹ flies did not show the reversal. Although it is to be answered how *dClk* and *cyc* mediate the light-dependent reversal of temperature preference in *per*⁰¹ and *tim*⁰¹ flies, the photic pathway seems to be disrupted in *dClk*^{Jrk} and *cyc*⁰¹ flies.

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