Neuron, Vol. 24, 219–230, September, 1999, Copyright ©1999 by Cell Press

How a Circadian Clock Adapts to Seasonal Decreases in Temperature and Day Length

John Majercak,* David Sidote,† Paul E. Hardin,‡ and Isaac Edery*†§

*Graduate Program in Biochemistry
†Department of Molecular Biology and Biochemistry
Center for Advanced Biotechnology and Medicine
Rutgers University
Piscataway, New Jersey 08854
‡Department of Biology and Biochemistry
University of Houston
Houston, Texas 77204

Summary

We show that a thermosensitive splicing event in the 3′ untranslated region of the mRNA from the *period* (*per*) gene plays an important role in how a circadian clock in *Drosophila* adapts to seasonally cold days (low temperatures and short day lengths). The enhanced splicing of this intron at low temperatures advances the steady state phases of the *per* mRNA and protein cycles, events that significantly contribute to the preferential daytime activity of flies on cold days. Because the accumulation of PER is also dependent on the photosensitive TIMELESS (TIM) protein, long photoperiods partially counteract the cold-induced advances in the oscillatory mechanism by delaying the daily increases in the levels of TIM. Our findings also indicate that there is a temperature-dependent switch in the molecular logic governing cycles in *per* mRNA levels.

Introduction

Circadian rhythms are driven by endogenous time-keeping devices known as "clocks" and are an important aspect of the temporal organization observed in a wide range of organisms from bacteria to humans (Dunlap, 1999). An important adaptive feature of circadian clocks is that they are synchronized (entrained) or reset by daily changes in environmental modalities, most notably visible light and ambient temperature. As a result, circadian clocks cannot only track the passage of a daily cycle but also serve as internal indicators of local time, enabling organisms the ability to temporally organize their physiology and behavior such that they occur at biologically advantageous times during a day. Light is almost certainly the predominant entraining agent in nature, (1) evoking daily adjustments in the approximately 24 hr endogenous periods of clocks such that they precisely match the 24 hr solar day and (2) contributing to the synchronization of a clock's phase relative to local time.

The precise role of temperature in the regulation of circadian rhythms in natural environments is not clear. For example, even in poikilotherms, the free-running periods of circadian rhythms are essentially invariant over a wide range of constant temperatures, a phenomenon known as temperature compensation (Pittendrigh, 1954). Yet under certain conditions, circadian rhythms are remarkably thermoresponsive as they can be phase shifted by changes in temperature (pulses and steps) and entrained by daily temperature cycles (e.g., Sweeney and Hastings, 1960; Wheeler et al., 1993; Liu et al., 1997, 1998; Sidote et al., 1998, and references therein). In addition, clocks only function within a restricted temperature range, and outside these limits the oscillatory mechanism appears to be held constant or "stops" (Liu et al., 1997, and references therein). Recent studies using *Drosophila* and *Neurospora* have revealed molecular mechanisms that might underlie (1) temperature compensation, (2) entrainment and phase resetting by changes in temperature, and (3) how temperature limits permissive for rhythmicity are established (Gekakis et al., 1995; Huang et al., 1995; Liu et al., 1997, 1998; Sawyer et al., 1997; Sidote et al., 1998; Stanewsky et al., 1998).

A less studied effect of temperature on clock function is based on the observation that the steady state phases of behavioral rhythms can vary as a function of temperature, even during entrainment by daily light:dark cycles (Sweeney and Hastings, 1960). A classic example is that of garter snakes, which are mainly nocturnal at warm temperatures and diurnal at cold temperatures and display a bimodal distribution of activity at intermediate temperatures (Heckrotte, 1961). In general, diurnal animals respond to colder temperatures by displaying a greater proportion of their activity during daytime hours, whereas nighttime activity predominates at warmer temperatures. This directional response has a clear adaptive value, ensuring that the activity of an organism is maximal at a time of day when the temperature would be expected to be optimal for activity (Sweeney and Hastings, 1960). Importantly, these changes in an organism's daily distribution of activity are not merely driven by exogenous responses to variations in temperature. Under controlled laboratory conditions where animals are exposed to daily light:dark cycles at constant temperatures, the preferential occurrence of activity during the light or dark period is still observed at low and high temperatures, respectively.

In this study, we used *D. melanogaster* as a model system to understand the molecular underpinnings governing how changes in average daily temperatures modulate activity rhythms entrained by daily light:dark cycles. In *D. melanogaster*, the *period* (*per*) and *timeless* (*tim*) genes are essential components of the time-keeping apparatus (for recent reviews, see Hall, 1998; Hardin, 1998; Reppert, 1998; Young, 1998; Dunlap, 1999; Edery, 1999). The *per* and *tim* protein and mRNA products undergo daily fluctuations in abundance, consistent with their roles as state variables in a *Drosophila* circadian clock (a state variable is a clock component whose rhythmic change in abundance or activity, not mere presence in the cell, is a necessary element of the time-keeping mechanism [Crosthwaite et al., 1995]). Two transcription factors of the basic-helix-loop-helix (bHLH)/

§To whom correspondence should be sent (e-mail: edery@cabm.rutgers.edu).

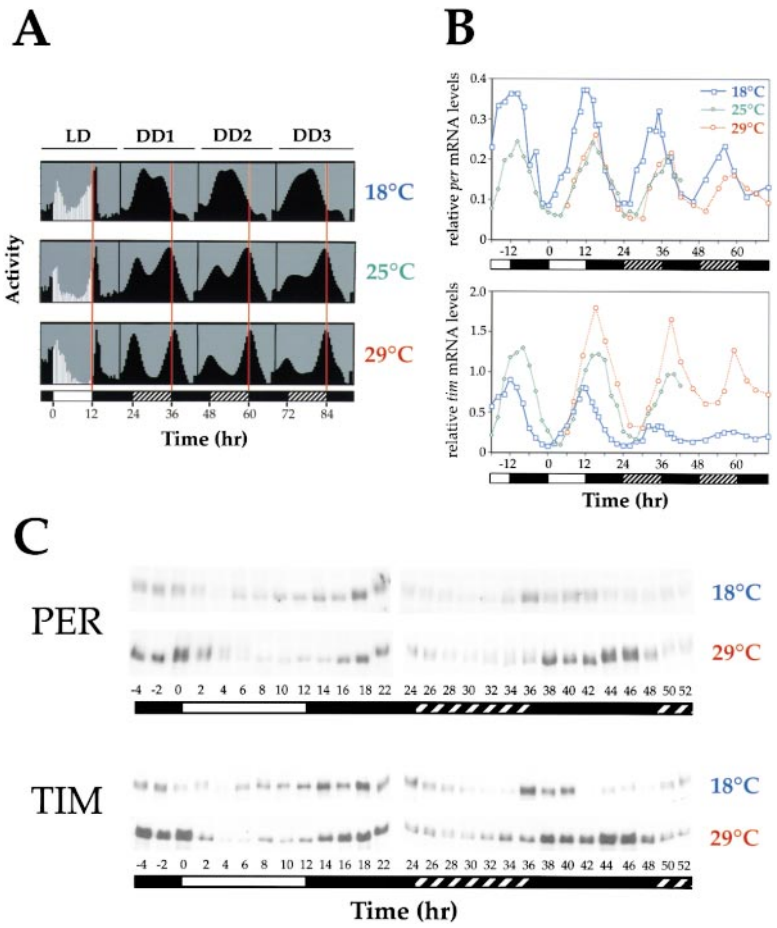

Figure 1. Effect of Temperature on Daily Cycles in Locomotor Activity, and *per* and *tim* mRNA and Protein Levels during LD and DD

Three groups of wild-type CS flies were kept at 18°C (A–C), 25°C (A and B), or 29°C (A–C) and exposed to four cycles of 12 hr light:12 hr dark (12:12 LD) followed by several days in constant dark conditions (DD).

(A) Each panel depicts the average locomotor activity for each group of flies during the last day of LD and the first 3 days of DD (DD1 to DD3) at the different test temperatures. Data collected from at least three independent experiments were pooled. Vertical bars represent activity recorded in 30 min bins during times when the lights were either on (white bars) or off (black bars). To facilitate visual comparison of peak levels in daily activity at the different constant temperatures, a smoothing function was applied to the data collected during DD. The red vertical lines are aligned beginning at time 12 and subsequently every 24 hr.

(B) RNase protection assays were performed on head RNA isolated from flies collected at the indicated times. The data collected from three independent experiments were pooled. Relative mRNA levels refers to either *per*/RP49 (top) or *tim*/RP49 (bottom) values.

(C) Head extracts were prepared from flies collected at the indicated times, and PER (top) and TIM (bottom) proteins were visualized by immunoblotting.

Time refers to hours since the last dark to light transition (defined as time 0). White and black horizontal bars represent times in a daily cycle when the lights were either on or off, respectively. Hatched bars represent subjective day.

PAS (PER-ARNT-SIM) superfamily, termed *Drosophila* CLOCK (dCLOCK) and CYCLE (CYC; also referred to as dBMAL1), interact to form a heterodimer that binds to E box DNA elements found in 5′ upstream regions of *per* and *tim* stimulating their expression (Hao et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998; Lee et al., 1999). PER and TIM physically interact to form a complex that translocates to the nucleus where PER, TIM, or both interact with the dCLOCK–CYC complex (Lee et al., 1998) blocking its activity (Darlington et al., 1998; Lee et al., 1999). In the absence of de novo synthesis, the levels of PER and TIM eventually decline in the nucleus, relieving autoinhibition and enabling the next round of *per* and *tim* transcript accumulation. A remarkably similar mechanism also operates in mammals (Gekakis et al., 1998; Sangoram et al., 1998; Zylka et al., 1998).

Despite the central role of circadian transcription in the *Drosophila* clock, posttranscriptional regulatory pathways also make key contributions to the time-keeping mechanism (reviewed in Edery, 1999). PER and TIM undergo temporal changes in phosphorylation that likely influence their stabilites and hence daily abundance cycles (Edery et al., 1994; Rutila et al., 1996; Zeng et al., 1996; Kloss et al., 1998; Price et al., 1998; Suri et al., 1998). The stability of PER in the cytoplasm is also influenced by TIM (Vosshall et al., 1994; Price et al., 1995, 1998; Kloss et al., 1998). In the absence of binding to TIM, monomeric PER is thought to be phosphorylated by a kinase called DOUBLETIME (DBT) targeting it for rapid proteolysis (Kloss et al., 1998; Price et al., 1998). Once threshold levels of monomeric PER and TIM are attained, formation of a PER–TIM complex is favored, an event that likely overrides *cis*-acting cytoplasmic localization determinants present on each subunit enabling the nuclear translocation of the complex (Saez and Young, 1996). These regulatory events introduce biochemical time constraints that ensure the formation of the PER–TIM complex is slow, thereby delaying its nuclear entry and generating a time window for the accumulation of *per* and *tim* transcripts. Moreover, several lines of evidence suggest that the stability and translational efficiency of *per* mRNA is under circadian regulation (So and Rosbash, 1997; Stanewsky et al., 1997; Chen et al., 1998; Cheng et al., 1998; Suri et al., 1999). The importance of posttranscriptional regulation is further indicated by the observation that light induces rapid decreases in the levels of TIM and consequent disruption of the PER–TIM complex (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996), a response that appears to be the primary clock-specific event during photic entrainment (Suri et al., 1998; Yang et al., 1998).

In this report, we show that splicing of an intron in the 3′ untranslated region (UTR) of *per* RNA (Cheng et al., 1998) is a key aspect of how a circadian clock responds to cold temperatures. At low temperatures, relatively more of the spliced variant (type B′) is present

Table 1. Locomotor Activity Rhythms of Wild-Type (CS) Flies at Different Temperatures[a]

Temperature (°C)	n[b]	Edge[c] (hr ± SEM)	Peak[d] LD (hr ± SEM)	Peak[d] DD1 (hr ± SEM)	Peak[d] DD2 (hr ± SEM)	Peak[d] DD3 (hr ± SEM)	Period (hr ± SEM)	Rhythmicity[e] (%)
18	64	6.9 ± 0.3	11.9 ± 0.1	10.1 ± 0.3	8.0 ± 0.3	8.1 ± 0.4	23.7 ± 0.1	91.2
25	83	10.2 ± 0.2	12.4 ± 0.1	12.6 ± 0.3	12.5 ± 0.4	12.4 ± 0.5	24.2 ± 0.1	90.5
29	29	11.2 ± 0.4	12.9 ± 0.2	12.9 ± 0.2	12.9 ± 0.3	13.0 ± 0.4	23.9 ± 0.2	89.6

[a] Flies were kept at either 18°, 25°, or 29°C and exposed to 4 days of 12 hr light:12 hr dark (12:12 LD) followed by 6 days of constant darkness (DD). Average data from three independent experiments is shown.
[b] Total number of flies that survived until the end of testing period.
[c] Edge is the time at which the upswing in evening activity reached 25% of the peak value and is given in hours from the last lights-on transition where time 0 is lights-on.
[d] Peak is the time at which evening activity of rhythmic flies reached its maximum value. Values for 12:12 LD are given in hours from the last lights-on transition where time 0 is lights-on. DD times are shown in hours relative to the LD cycle. DD1, DD2, and DD3 refer to the first 3 days of DD, respectively.
[e] Percentage of flies with activity rhythms having a power of ≥10 (see the Experimental Procedures).

compared to the unspliced variant (type A). The enhanced ability to splice the *per* RNA 3′ intron at low temperatures is associated with an earlier rise in the levels of PER protein. This cold "adapted" advance in the steady state phase of the PER abundance rhythm enables flies to maintain daytime activity at low temperatures. Long photoperiods partially counteract cold-induced phase advances in the accumulation of *per* mRNA and protein by delaying the upswing in TIM levels. The functional interrelationships between PER and TIM ensure that information concerning ambient temperature and light is properly integrated resulting in activity rhythms that are optimally aligned with the prevailing environmental conditions.

Results

Light Counteracts the Phase-Advancing Effects of Cold Temperatures on the Flies' Evening Activity

The adult head is the anatomical location of the *per-tim*-based circadian clock governing the daily distribution of locomotor activity (Handler and Konopka, 1979; Ewer et al., 1992; Frisch et al., 1994; Vosshall and Young, 1995; Helfrich-Forster, 1998). Thus, the analysis of locomotor activity rhythms coupled with molecular and biochemical studies of the *per-tim*-based oscillator in adult fly heads is likely to reveal a direct causal relationship between clock function and output.

To measure the locomotor activity rhythm as a function of temperature, wild-type (CS) flies were maintained at 18°C, 25°C, or 29°C, exposed to four standard cycles of 12 hr light followed by 12 hr dark (12:12 LD; where zeitgeber time 0 [ZT0] is lights on), and subsequently kept for 6–10 days in constant dark conditions (DD) (Figure 1A). In agreement with previous studies, the endogenous period length of the activity rhythm is nearly constant at the different test temperatures (Ewer et al., 1990) (Table 1), and the activity of *D. melanogaster* during LD at the standard temperature of 25°C is bimodal with one peak centered around ZT0 ("morning" peak) and another around ZT12 ("evening" peak) (Figure 1A, middle and far left) (e.g., Hamblen-Coyle et al., 1992). Under these standard conditions, there is a gradual rise in the evening activity of wild-type flies following a midday nadir that "anticipates" the light to dark transition. This anticipatory increase in activity is tightly controlled by an endogenous circadian clock (e.g., Hamblen-Coyle et al., 1992; Wheeler et al., 1993). However, a significant portion of the flies' morning activity has been attributed to a "startle" effect caused by the lights-on transition (Wheeler et al., 1993). Thus, we will limit our analysis to include only the evening activity as a bona fide indicator of clock function.

In a daily light:dark cycle, the clock-controlled evening activity becomes progressively more day-specific at lower temperatures (Figure 1A, compare far left panels; Table 1). At the two highest test temperatures (i.e., 25°C and 29°C), the relative timing of the evening activity peak observed in LD was maintained during each 24 hr "day" during constant dark conditions (Figure 1A, middle and bottom; Table 1). In sharp contrast, flies kept at 18°C displayed a single broad peak of activity in DD that after two days in constant dark conditions reached a steady state phase occurring approximately 4 hr earlier than that in LD (Figure 1A, top; Table 1). The results indicate that light is partially overriding the phase-advancing effects of cold temperatures on the time-keeping mechanism.

Differential Regulation of *per* and *tim* mRNA Cycles by Temperature

As an initial strategy to investigate the status of the circadian oscillator in flies maintained at different constant temperatures, we measured the *per* and *tim* RNA (Figure 1B) and protein (Figure 1C) cycles. The *per* (Figure 1B, top) and *tim* (Figure 1B, bottom) transcript rhythms are strongly influenced by changes in ambient temperature during LD and DD. Peak levels of *per* mRNA in LD are approximately 30%–35% greater at 18°C compared to 25°C and 29°C. In sharp contrast, during LD the highest levels of *tim* transcripts are observed at 29°C with successive reductions at lower temperatures. A second highly reproducible effect of temperature is that, in general, peak amounts of *per* and *tim* RNA are reached earlier during LD on colder days (i.e., 18°C) compared to those that are warmer (i.e., 25° and 29°C). Nonetheless, these phase advances are essentially restricted to the accumulation phase for *per* mRNA and the declining phase for *tim* mRNA. These temperature-dependent differences in the peak levels and phase of the *per* and *tim* mRNA rhythms continued for at least 2–3 days in

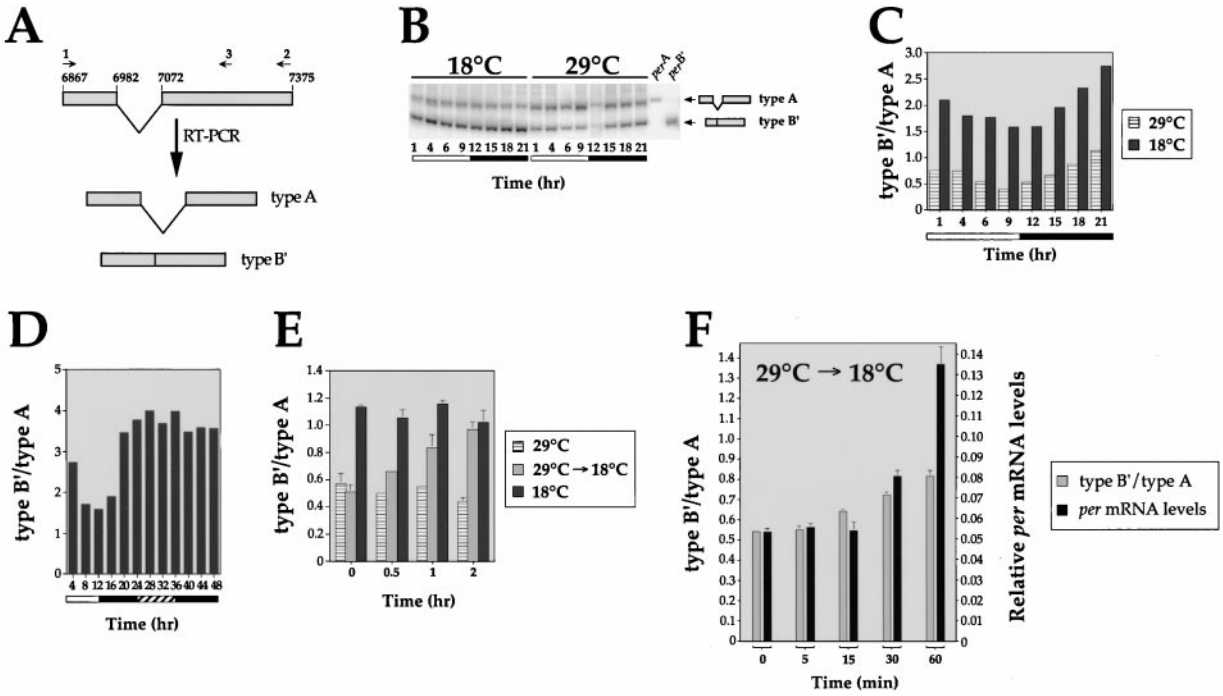

Figure 2. Thermosensitive Splicing of an Intron in the 3′ UTR of *per* mRNA

(A) Schematic representation of assay used to measure the relative levels of two *per* mRNA variants that either contain or are missing an intron in the 3′ UTR. Shown at top is the exon–intron organization of the *per* 3′ UTR and the relative positions including 5′ to 3′ directions of the three oligonucleotide primers used in this study for nested RT–PCR (arrows, top). Following RT–PCR, two *per*-specific products were detected that differ in electrophoretic mobility due to either retention (type A) or removal (type B′) of the 3′-terminal intron (bottom). Exons, horizontal bars; introns, diagonal lines. Numbering of *per* sequences according to Citri et al. (1987).

(B) Two groups of wild-type CS flies were exposed to 4 days of 12:12 LD at either 18°C or 29°C as indicated (top). On the fourth day of LD, flies were collected at the indicated times (hr; time 0 is defined as beginning of light period), and RNA was isolated from heads. Following RT–PCR in the presence of [^{32}P]dATP, the mixture was resolved by gel electrophoresis, and radiolabled products were detected by autoradiography. Control plasmids containing the 3′ UTR of *per* with (per^{A}) or without ($per^{B'}$) the intron were included in the PCR and served as convenient size markers (far right).

(C) Quantitation of results in (B). Shown are the relative levels of type B′ to type A during LD at either 18°C (dark gray vertical bars) or 29°C (lined vertical bars).

(D) Quantitation as in (C) of an independent experiment showing values obtained during LD and DD for flies kept at 18°C.

(E) Two groups of per^{01} flies were exposed to LD for 3 days at 29°C (lined vertical bars and light gray bars), while a third group was exposed to the same light:dark conditions but kept at 18°C (dark gray bars). On the fourth day, one group of flies from 29°C was subjected to an 18°C temperature step-down (light gray bars). Flies were collected at the indicated times (hours after beginning of temperature change). The ratio of type B′ to type A was measured using RT–PCR.

(F) per^{01} flies were kept at 29°C in LD for 3 days. On the fourth day, at time 16 (i.e., 4 hr into the dark period), flies were subjected to a 29°C–18°C temperature step-down and were collected at the indicated times (minutes after beginning of temperature step-down). RNA was isolated from heads, and an aliquot was used to measure the ratio of type B′ to type A (light gray bars), whereas a separate aliquot was used in a RNase protection assay to measure levels of *per* mRNA (black bars). *per* mRNA levels refers to *per*/RP49 values. For each time point, values for the ratio of type B′ to type A and *per* mRNA levels are plotted side by side to facilitate comparisons.

(B–D) White and black horizontal bars (bottom) represent times in a daily cycle when the lights were either on or off, respectively; hatched horizontal bar (bottom) represents subjective day.

constant dark conditions. A dramatic exception, however, is that during constant dark conditions at 18°C, oscillations in the levels of *tim* RNA quickly dampened to trough amounts.

As might be predicted from the RNA profiles, daily oscillations in the levels of PER and TIM proteins were also modulated by temperature (Figure 1C). We noted three main differences between the protein and RNA curves as a function of temperature. (1) During the first day of DD, the timing of peak levels in PER and TIM were significantly advanced at 18°C relative to those obtained at 29°C, whereas temperature had less of an effect on the phase of the PER–TIM temporal program in LD. In the case of *per* RNA, during both LD and the first day of DD, the timing of its daily upswing occurred 3–4 hr earlier at 18°C compared to the two highest test temperatures (Figure 1B). Nonetheless, at 18°C the PER protein cycle is significantly advanced only on the first day of constant dark conditions compared to LD (Figure 1C). This indicates that light is delaying PER accumulation despite the cold-induced phase advance in the *per* RNA cycle. A reasonable explanation that can account for this is that the light-sensitive TIM protein is important for stabilizing PER (Vosshall et al., 1994; Price et al., 1995, 1998; Kloss et al., 1998; Suri et al., 1999) (see Discussion). Light delays the accumulation of TIM and, hence, that of PER. The effects of light on the PER cycle at 18°C nicely account for the cold-specific phase

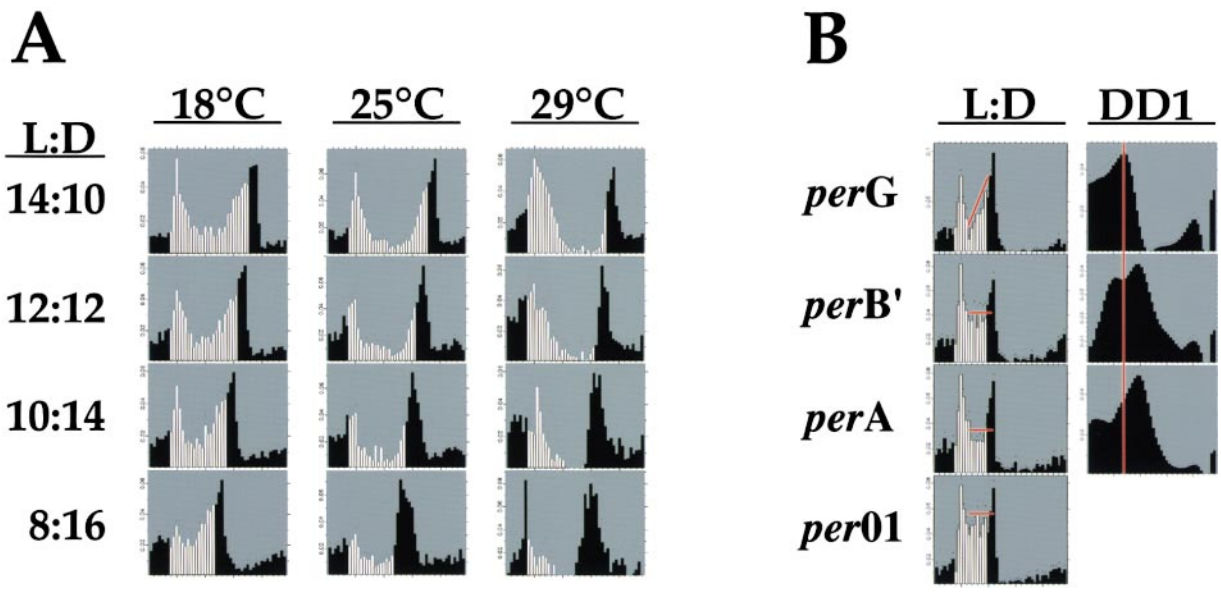

Figure 3. *per*^{A} and *per*$^{B'}$ Mutant Flies Manifest Delayed Activity Rhythms at Cold Temperatures and Short Photoperiods

(A) Locomotor activity was recorded from 12 groups of wild-type CS flies. Each group of flies was exposed for 4 days to a different combination of temperature (18°C, 25°C, or 29°C) and photoperiod (14:10 LD, 12:12 LD, 10:14 LD, or 8:16 LD). Each panel depicts the average locomotor activity during the fourth light:dark cycle for a group of flies exposed to the same temperature and photoperiod regime. Data collected from at least two independent experiments were pooled.

(B) *per*^{G}, *per*$^{B'}$, *per*^{A}, and *per*^{01} flies were kept at 18°C and exposed to four cycles of 6 hr light:18 hr dark (6:18 LD) followed by DD. For each genotype, the average locomotor activity is shown for the last day of LD (left) and first day of DD (right). Note that similar to *per*^{01} flies, but in contrast to control *per*^{G} flies, the daily "anticipatory" upswing in activity is not observed in the *per*^{A} and *per*$^{B'}$ mutants during LD (left, highlighted in red). To facilitate visual comparison of peak levels in daily activity, a smoothing function was applied to the data in DD1 (right). Furthermore, a red vertical line is drawn through the peak of activity in *per*^{G} flies and extended through the data for *per*$^{B'}$ and *per*^{A} flies.

Vertical bars represent activity recorded in 30 min bins during times when the lights were either on (white bars) or off (black bars). White and black horizontal bars (bottom) represent times in a daily cycle when the lights were either on or off, respectively; hatched horizontal bar (bottom) represents subjective day.

advance observed in the flies' locomotor activity during the transition from LD to DD at 18°C (Figure 1A; Table 1). (2) PER accumulated to higher levels at warmer temperatures (Figure 1C, compare overall intensities in top two panels), although *per* RNA abundance was lower under these conditions (Figure 1B). Again, this apparent discrepancy can be explained based on the observation that TIM stabilizes PER. Thus, the low levels of *tim* mRNA and protein at cold temperatures limit the average steady state levels of PER that are attained during a daily cycle (see Discussion). (3) At 18°C, TIM protein continued to undergo robust oscillations in abundance during DD (Figure 1C, lower panels) despite the strongly dampened cycling of its template mRNA (Figure 1B, lower panel). This result strongly suggests that, just as with *per* mRNA, oscillations in the levels of *tim* transcripts are not necessary to generate a time-keeping mechanism that can function in the absence of external time cues and raises the possibility that, at least at cold temperatures, posttranslational mechanisms strongly contribute to generating daily rhythms in TIM abundance.

Temperature Regulates an Alternative Splicing Event in the 3′ Untranslated Region of *per*

Preliminary experiments indicated that shifts in temperature (step-ups or step-downs) induce rapid changes in the levels of *per* transcripts by a posttranscriptional mechanism (see Figure 2; data not shown), whereas the abundance of *tim* mRNA is not acutely sensitive to variations in temperature (data not shown). These results identified *per* RNA as a primary clock-specific target that responds to ambient changes in temperature. Based on these initial observations, we reasoned that understanding the molecular underpinnings governing the thermosensitivity of *per* mRNA abundance should reveal novel regulatory features of the *D. melanogaster* clock and possibly provide important insights into how variations in temperature modulate the timing of locomotor activity in this species. In many cases, *cis*-acting elements that regulate mRNA stability, production, or utilization are found in 3′ untranslated regions (Jackson, 1993). During our investigation of the structure of the 3′ UTR of *per* as a function of temperature, we noticed two different size variants (Figures 2A and 2B; data not shown). The different size variants were due to an alternatively spliced 89 bp intron identical in sequence to results obtained in an earlier report showing that *per* generates two transcripts that differ only by the presence (type A) or absence (type B′) of this intron (Cheng et al., 1998).

In wild-type flies maintained at 18°C, the type B′ variant is present in relatively higher levels at all times in a daily cycle, whereas type A transcripts are proportionally more abundant at 29°C (Figures 2B and 2C). At each time point, the relative abundance of type B′ to type A is approximately 2- to 3-fold higher at 18°C compared to 29°C, strongly suggesting that splicing of this intron

is stimulated at low temperatures. In the *per*^{01} null mutant, the relative levels of type B′ to type A increase after a temperature step decrease from 29°C to 18°C in a qualitatively similar manner to that observed in wild-type flies, indicating that a functional clock is not required for this response (compare Figures 2C–2E and 2F). The relative levels of type B′ to type A in the *per*^{01} and *tim*^{0} null mutants are similar in the light or dark and are not altered by light to dark transitions (Figures 2E and 2F; data not shown), suggesting that the slight decrease in spliced variant observed in wild-type flies during the light phase of a diurnal cycle (Figures 2B and 2C) requires a functional clock. The changes evoked by a temperature step-down on the ratio of type B′ to type A begin to occur just prior to when increases in the overall levels of *per* mRNA are first detected (Figure 2F). For example, between 5 and 15 min after the start of a step decrease in temperature, the ratio of type B′ to type A increases by approximately 17% ($P = 0.01$, two-tailed *t* test), but there is no significant difference in *per* mRNA levels ($P = 0.6$, two-tailed *t* test). The kinetics of this time course is consistent with the notion that at cold temperatures removal of the intron in the 3′ UTR of *per* contributes to increases in the stability and/or synthesis of *per* mRNA (see below; Figure 5).

The Timing of Peak Evening Activity in *Drosophila* Bearing Mutations in the *per* 3′ Intron Is Preferentially Affected at Low Temperatures and Short Photoperiods

To test whether the *per* 3′ intron plays a role in the ability of the clock to respond to variations in temperature, we used three previously generated transgenic lines that produce only type B′ transcripts (*per*$^{B'}$), a variant of type A transcripts having inactivated 5′ and 3′ splice junctions (*per*^{A}), or the control situation where both type A and type B′ transcripts are produced (*per*^{G}) (Cheng et al., 1998). All three *per* transgenes were previously shown to rescue locomotor activity rhythms at the standard temperature of 25°C (Cheng et al., 1998). We measured the locomotor activities of the three different genotypes at two temperatures (18°C and 29°C) and photoperiods (12:12 LD and 6:18 LD) (Figure 3B; Table 2). For each transgene, the results from at least two independent lines were averaged.

The rationale for varying photoperiod is that in natural environments day length usually shortens as average daily temperatures drop. Figure 3A shows examples of how the daily activity rhythms of wild-type flies are influenced by changes in photoperiod as a function of temperature. The results clearly demonstrate that at higher temperatures (e.g., 25°C and 29°C) flies display progressively more nocturnal activity as the photoperiod is shortened (Figure 3A) (Qiu and Hardin, 1996). Flies exposed to colder temperatures (e.g., 18°C), however, continue to display a significant portion of their evening activity during daytime hours even in photoperiods that are only 6–8 hr in length (Figures 3A and 3B; data not shown).

Irrespective of temperature and photoperiod, *per*^{G} flies had a similar distribution of activity to that observed in wild-type flies exposed to similar conditions (Tables 1 and 2, compare peak activity during first day of DD; data not shown), further indicating that *per*^{G} flies have normal clock function (Cheng et al., 1998). Intriguingly, both *per*^{A} and *per*$^{B'}$ flies lack anticipatory rises in evening acitivity under 6:18 LD (Figure 3B, left) and display significant delays in the peak of evening activity (Table 2; Figure 3B, right). The striking similarities in the temperature responses of the *per*^{A} and *per*$^{B'}$ mutants strongly suggest that the splicing process itself, in contrast to retention or removal of the *per* 3′ intron, is critical for the cold-induced phase advance in the activity rhythm (see Discussion). As might be predicted from the ability of light to counteract cold-induced advances in the phase of the clock (Figures 1A and 3A), the daily activity rhythms of *per*^{A} and *per*$^{B'}$ flies were preferentially delayed under the shorter photoperiod (Table 2; data not shown), indicating that splicing at the *per* 3′ intron plays a more significant role in the "cold adaptation" of the clock when day length is short. We speculate that because a long photoperiod is more typical of seasonally warm days, it partially overrides the "contradictory" signal of cold temperatures on the dynamics of the clock (see Figures 4 and 5).

The situation at 29°C is markedly different from that at 18°C. Although the activity peak of *per*^{A} flies was delayed under both photoperiods compared to *per*^{G} flies, the free-running period in this mutant was also lengthened (Table 2). This is consistent with recent findings showing that following entrained to standard conditions of 12:12 LD at 25°C *per*^{A} flies had free-running activity periods that were approximately 1–1.5 hr longer than *per*$^{B'}$ and *per*^{G} flies (Cheng et al., 1998). Lengthening of the period in the *per*^{A} mutant at 29°C likely contributes to the observed delay in reaching peak activity during a diurnal cycle (e.g., Hamblen-Coyle et al., 1992; Hardin et al., 1992a). Thus, the significance of retaining the 3′ intron of *per* in specifically regulating the phase of the clock at higher temperatures (as opposed to its intrinsic period) is not clear. Furthermore, the physiological significance of the delayed activity peak displayed by *per*$^{B'}$ flies exposed to 6:18 LD cycles at 29°C (Table 2) is not clear because seasonally warm days are not usually associated with short photoperiods. In summary, our findings specifically implicate splicing at the *per* 3′ intron in the advanced evening activity during cold days.

Low Amplitude Cycling of *per* RNA in *per*^{A} and *per*$^{B'}$ Flies at Low, but Not High, Temperatures

To gain a better understanding of the molecular underpinnings governing the effects of temperature and photoperiod on the daily activity patterns exhibited by the *per*^{A} and *per*$^{B'}$ mutants, we measured the levels of *per* and *tim* proteins (Figure 4) and mRNAs (Figure 5). Figure 4 shows the PER and TIM abundance cycles in flies kept at 18°C and entrained by two different photoperiods (6:18 LD, Figures 4A and 4C; 12:12 LD, Figures 4B and 4D). Although the average daily levels of PER were similar in *per*^{A}, *per*$^{B'}$, and *per*^{G} flies, the time course of its appearance and disappearance were delayed in both mutants compared to that obtained for the *per*^{G} control (Figures 4A and 4B). For example, in a 6:18 LD cycle, PER is relatively abundant at ZT6 in *per*^{G} flies but barely visible in the two mutants (Figure 4A). Furthermore, PER levels declined earlier in *per*^{G} flies (Figure 4A, compare

Table 2. Locomotor Activity Rhythms of per^A, $per^{B'}$, and per^G Transgenic Flies at Different Temperatures and Day Lengths[a]

Genotype	LD	Temperature (°C)	n[b]	Peak[c] (hr ± SEM)	Period (hr ± SEM)	Rhythmicity[d] (%)
per^G	12:12	18	20	10.5 ± 0.3	23.7 ± 0.2	57.5
$per^{B'}$	12:12	18	19	11.4 ± 0.2[e]	24.2 ± 0.2	40.5
per^A	12:12	18	36	11.2 ± 0.1[e]	24.2 ± 0.1	60.6
per^G	6:18	18	37	4.5 ± 0.2	23.6 ± 0.1	48.7
$per^{B'}$	6:18	18	48	6.0 ± 0.1[e]	23.8 ± 0.1	56.5
per^A	6:18	18	59	6.1 ± 0.1[e]	24.1 ± 0.1	65.4
per^G	12:12	29	24	12.6 ± 0.2	23.5 ± 0.1	62.5
$per^{B'}$	12:12	29	33	12.8 ± 0.1	23.7 ± 0.1	66.7
per^A	12:12	29	27	13.3 ± 0.2[e]	24.9 ± 0.1[e]	80.0
per^G	6:18	29	25	6.1 ± 0.1	23.6 ± 0.1	72.8
$per^{B'}$	6:18	29	20	7.1 ± 0.2[e]	24.2 ± 0.1	64.2
per^A	6:18	29	25	6.8 ± 0.2[e]	24.8 ± 0.1[e]	80.2

[a] Flies were kept at either 18°C or 29°C and exposed to 4 days of either 12 hr light:12 hr dark (12:12 LD) or 6 hr light:18 hr dark (6:18 LD) followed by 6 days of constant darkness (DD). For each genotype, two independent lines were averaged. Average data from three independent experiments is shown.

[b] Total number of flies that gave both significant peak and period values.

[c] Peak is the time at which activity reached its maximum value in the first day of DD following LD. Times are shown in hours relative to a LD cycle where time 0 is lights-on.

[d] Percentage of flies having significant peak values and having activity rhythms with a power of ≥10 and with periods ≥20 hr and ≤28 hr.

[e] Denotes a two-tailed student's t test p value of less than 0.05 when comparing either per^A or $per^{B'}$ flies to the appropriate (tested at same temperature and day length) per^G control group.

panels at ZT18 and ZT21). Under these environmental conditions, we did not observe reproducible differences in the PER abundance rhythm between per^A and $per^{B'}$ flies. The mutant specific effects on the phase of the PER abundance rhythm were more apparent under shorter photoperiods (compare Figures 4A and 4B; data not shown), consistent with the behavioral results (Table 2).

Unlike the situation with PER, for each photoperiod tested, we did not observe reproducible differences in the timing of TIM appearance and disappearance in the three different genotypes (Figures 4C and 4D; data not shown). This is likely due to the predominant effect of light on TIM stability (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). Indeed, irrespective of day length, accumulation of high levels of TIM coincided with the light to dark transition. Thus, the phase alignment between the PER and TIM abundance cycles that normally occurs in per^G flies is altered in the two mutants. As with the situation where flies were kept at 18°C, the temporal distribution of TIM at 29°C was similar for all three genotypes exposed to identical photoperiods (data not shown).

Analysis of the daily fluctuations in the levels of *per* transcripts revealed a striking differential response to

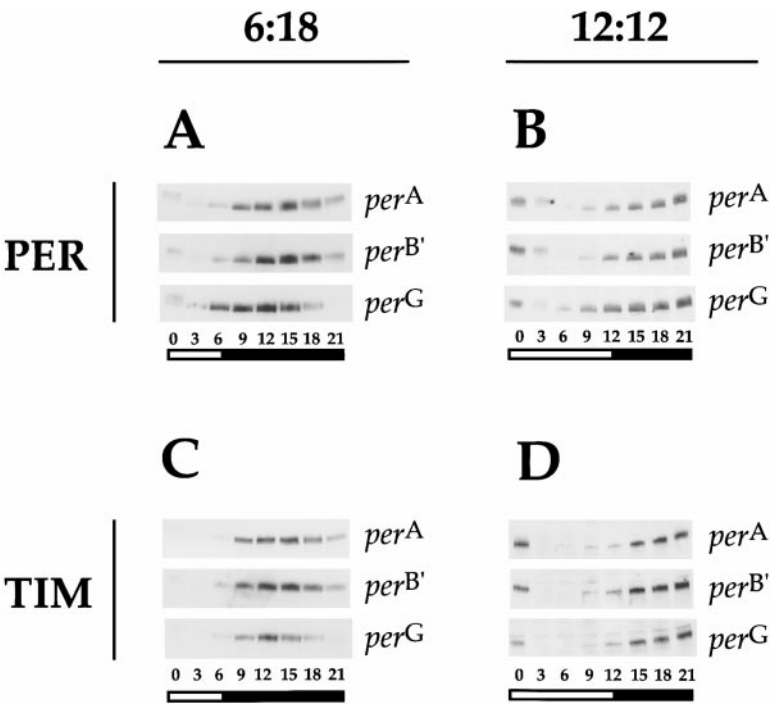

Figure 4. PER Accumulation Is Delayed at Cold Temperatures in the per^A and $per^{B'}$ Mutants

Two groups of per^A, $per^{B'}$, and per^G flies were kept at 18°C, and, for each genotype, one group was exposed for 3 days to 6:18 LD (A and C), whereas the other group was treated in an identical manner except that they were maintained under 12:12 LD (B and D). On the fourth day of entrainment, flies were collected at the times indicated, where time 0 is defined as the last dark to light transition. For each group of flies, immunoblots of head extracts were first probed with antibodies directed against PER (A and B) followed by stripping and reprobing the same blot with antibodies directed against TIM (C and D). Similar results were obtained in three independent experiments, and representative examples are shown.

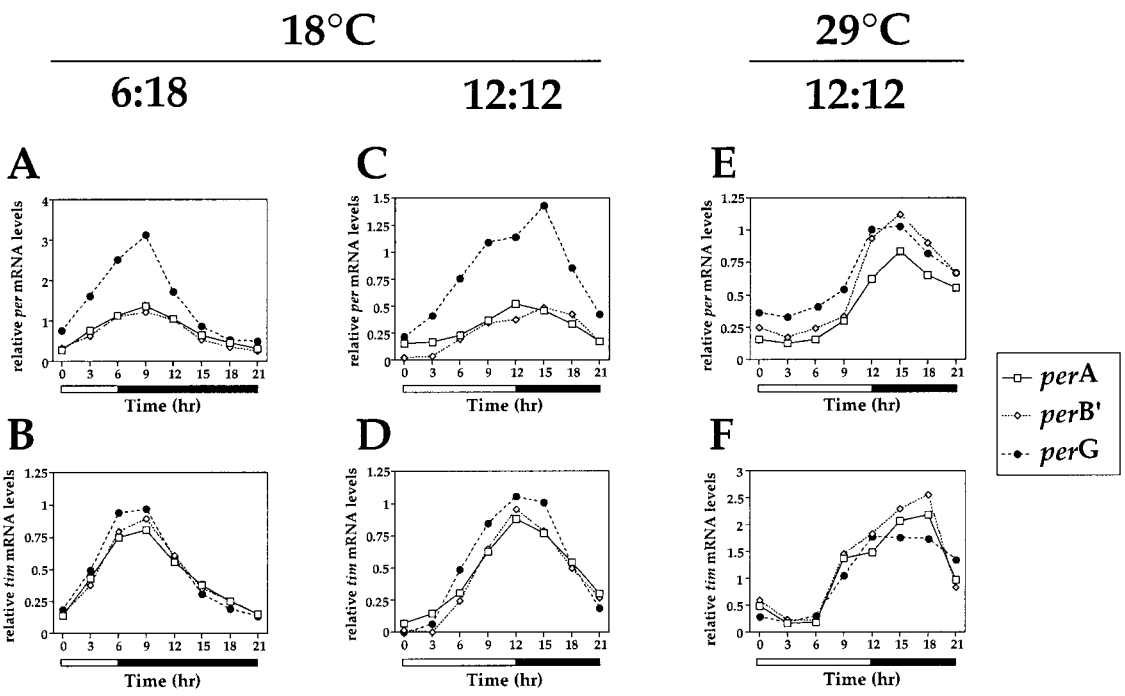

Figure 5. Cold Temperatures Strongly Dampen the Amplitude of *per* mRNA Cycling in per^{A} and $per^{B'}$ Flies

per^{A} (open squares), $per^{B'}$ (gray diamonds), and per^{G} (closed circles) flies were maintained at 18°C (A–D) or 29°C (E and F) and exposed to light:dark cycles of either 6:18 LD (A and B) or 12:12 LD (C, D, E, and F) for 3 days. On the fourth day, flies were collected at the times indicated and head RNA subjected to RNAse protection assays. Relative *per* mRNA levels (A, C, and E) and relative *tim* mRNA levels (B, D, and F) refer to *per*/RP49 and *tim*/RP49 values, respectively.

temperature in the per^{A} and $per^{B'}$ flies that was not observed in control flies (Figure 5, for each genotype, compare 5A and 5C to 5E). Irrespective of photoperiod at cold temperatures, the amplitude in the abundance of the *per* RNA rhythm was severely dampened in both per^{A} and $per^{B'}$ flies (Figures 5A and 5C). In sharp contrast, under warm conditions, the *per* RNA rhythms in all three genotypes are similar (Figure 5E). These cold-induced effects were specific to *per* mRNA because at all temperatures and photoperiods tested daily cycles in *tim* RNA levels were similar for all three genotypes (Figures 5B, 5D, and 5F; data not shown). Thus, the ability to splice the *per* 3′ intron is a major regulatory event that underlies the daily accumulation in the levels of *per* RNA at cold but not warm temperatures. Although cycles in *per* mRNA levels are severely dampened in the per^{A} and $per^{B'}$ mutants at 18°C (Figures 5A and 5C), in both cases PER protein continues to undergo robust rhythms in abundance (Figures 4A and 4B). This is not surprising based on earlier findings demonstrating that daily fluctuations in TIM levels are sufficient to drive a rhythm in the abundance of PER even when *per* mRNA is constitutively expressed (e.g., Suri et al., 1999). However, as results using the mutants clearly show, the timing of PER accumulation at 18°C is strongly modulated by the upswing in *per* mRNA levels. Together, our findings strongly suggest that at low temperatures the PER accumulation rate is controlled by both the advanced increase in *per* mRNA levels and the concentration of TIM (see Discussion).

Discussion

In this study, we used *D. melanogaster* as a model system to understand how changes in average daily temperatures influence the timing of activity rhythms. We show that similar to many other organisms, *Drosophila* are more night active at warmer temperatures, whereas daytime activity is more prevalent at colder temperatures (Figures 1 and 3). Surprisingly, investigation of the underlying time-keeping mechanism revealed that temperature regulates the levels of *per* and *tim* transcripts in opposite directions; for example, at colder temperatures transcript levels of *per* are higher whereas those of *tim* are lower (Figure 1). We show that a thermosensitive splicing event in the 3′ UTR of *per* RNA plays a critical role in regulating daily upswings in the levels of this transcript at low but not high temperatures (Figures 2 and 5). This cold-specific effect of splicing at the 3′ intron on *per* mRNA metabolism contributes to an early accumulation of PER protein (Figure 4) and preferential daytime activity of flies at low temperatures (Table 2; Figure 3). Importantly, the delayed activity rhythms of the per^{A} and $per^{B'}$ mutants at cold temperatures are not accompanied by changes in free-running periods (Table 2 and Figure 3B). We are not aware of any other mutation in a clock gene that specifically affects phase without altering period length. These results further indicate a physiological role for splicing of the *per* 3′ intron in determining the phase of the clock on cold days. Because the stability of PER is highly dependent on

TIM, photoperiod influences the cold-induced advances in the *per* mRNA and protein cycles by regulating the timing of TIM accumulation. Our findings suggest a mechanism for how a circadian clock can adapt to seasonal decreases in temperature and day length.

A Role for the *per* 3′ Intron in the Regulation of the *Drosophila* Clock by Cold Temperatures and Short Photoperiods

Decreases in temperature evoke rapid increases in the ratio of the type B′ to type A RNA variants (Figure 2), suggesting that splicing of the 3′ intron is either enhanced at colder temperatures or repressed at warmer temperatures. By using transgenic flies whose only functional copy of *per* produces either a variant of the type A transcript having inactivated 5′ and 3′ splice junctions (per^{A}) or only the type B′ transcript ($per^{B'}$) (Cheng et al., 1998), we demonstrate that the daily stimulation in the abundance of *per* mRNA at low but not high temperatures is highly dependent on splicing of the *per* 3′ intron (Figure 5). Our data strongly suggest that splicing activity per se at the *per* 3′ intron as opposed to retention or removal of this intron is the important biochemical signal necessary to generate high steady state levels of *per* RNA during cold days. There is a striking temperature-dependent switch in the molecular logic used to control the daily levels of *per* mRNA from a more transcriptional based mechanism at higher temperatures (Hardin et al., 1992b; Hao et al., 1997; So and Rosbash, 1997; Allada et al., 1998; Rutila et al., 1998; Hao et al., 1999) to one that is highly dependent on posttranscriptional regulation at lower temperatures. How the cold-induced splicing of the *per* 3′ intron leads to an increase in *per* mRNA levels is not clear. A possible mechanism is shown in Figure 6 based on evidence from other systems showing that the efficiency of removal of 3′-terminal introns influences the efficiency of pre-mRNA 3′ end formation (i.e., cleavage and polyadenylation) (Nesic and Maquat, 1994; Nesic et al., 1995). In this context, the absence of the 3′ intron in $per^{B'}$ flies presumably eliminates favorable interactions between *trans*-acting factors involved in splicing and 3′ end formation leading to decreases in the rate of *per* mRNA accumulation at cold temperatures and hence delayed activity rhythms. Likewise, although the 3′ intron is present in per^{A} flies, it is flanked by inactivated donor and acceptor splice junctions, which are important *cis*-acting signals necessary in the recruitment of splicesomes to intronic sequences (Green, 1986).

Despite the important role of splicing at the *per* 3′ intron on the ability of the clock to advance the evening activity of flies exposed to low temperatures, these cold-induced effects can be partially counterbalanced by long photoperiods (Figures 1A and 3A; Tables 1 and 2). Due to the photosensitive nature of TIM, the timing of its accumulation is reasonably fixed such that relatively high levels are first observed shortly after the light to dark transition (Figures 1C, 4C, and 4D). Importantly, the cytoplasmic stability of PER is highly dependent on TIM (Vosshall et al., 1994; Price et al., 1995, 1998; Kloss et al., 1998; Suri et al., 1999). Current evidence suggests that monomeric PER in the cytoplasm is phosphorylated by the kinase DBT, an event that targets PER for rapid

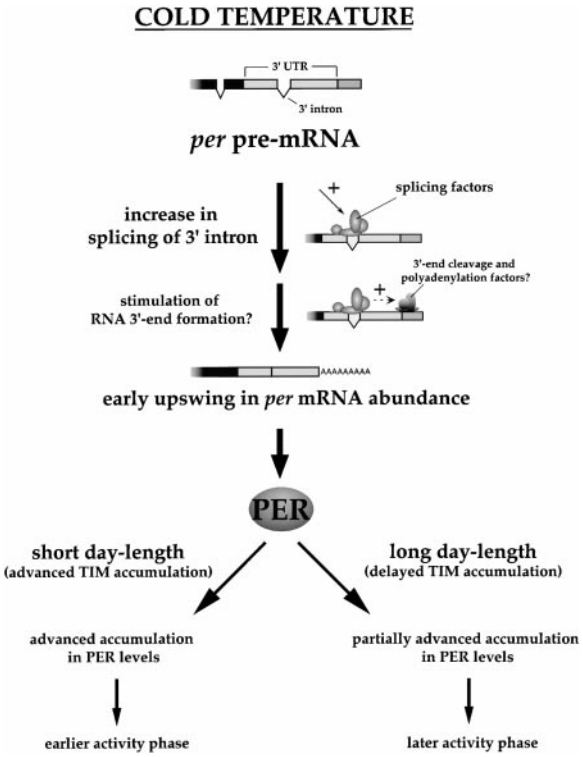

Figure 6. Model for How Decreases in Temperature and Photoperiod Regulate the *D. melanogaster* Circadian Clock

Cold temperatures enhance splicing of the 3′-terminal intron in *per* precursor mRNA. This enhanced splicing activity at the *per* 3′ intron leads to higher levels of *per* mRNA at low temperatures, possibly through the ability of the splicing machinery to stimulate mRNA production by enhancing 3′ end formation. The cold-induced advance in the accumulation of *per* mRNA leads to earlier increases in the abundance of PER protein, contributing to the preferential daytime activity of flies at low temperatures. The accumulation of PER is also dependent on TIM. As a result, long photoperiods partially counteract the cold-induced phase advance in the daily upswing in *per* mRNA abundance because daily increases in the levels of the photosensitive TIM protein are delayed. Thus, PER is a focal point for integrating information regarding ambient temperature and day length. See text for more details.

turnover (Kloss et al., 1998; Price et al., 1998). As *per* and *tim* transcript levels increase during the day, newly synthesized PER and TIM slowly accumulate in the cytoplasm eventually reaching critical concentrations that favor formation of a more stable PER–TIM complex leading to nuclear translocation and continuation of the circadian cycle (e.g., Gekakis et al., 1995; Sehgal et al., 1995).

Although TIM has a strong influence on the accumulation of PER, and light has a predominant effect on timing the daily upswing in TIM levels, results with the per^{A} and $per^{B'}$ mutants clearly show that at 18°C the PER abundance cycles of both mutants are significantly delayed compared to the control situation in per^{G} flies (Figures 4A and 4B). The cold-induced advance in the timing of daily increases in the levels of *per* mRNA likely reduces the time necessary to accumulate a threshold concentration of monomeric PER that favors dimerization with TIM. This interpretation is consistent with the

observations that low temperatures specifically evoke advances in the upswing of *per* mRNA (Figure 1B) and that splicing of the 3′ intron is necessary for the stimulation in *per* mRNA levels at cold temperatures (Figure 5). We propose that the enhanced splicing of the *per* 3′ intron "primes" the clock with the ability to maintain a cold-adapted phase. The advance in the phase of the *per* mRNA cycle that underlies this cold adaptation can be partially neutralized by long photoperiods that are effective in delaying the daily upswing in the abundance of TIM (Figure 6). Similar reasoning was invoked to explain how the per^S mutant, which manifests endogenous rhythms of approximately 19 hr, entrains to a 24 hr light:dark cycle (Marrus et al., 1996). Although *per* mRNA accumulates more rapidly during the day in per^S compared to wild-type flies, light delays increases in the levels of TIM and PER. Our findings implicate PER metabolism as a key event in integrating the "pulls and pushes" imposed by cold-induced advances in *per* mRNA cycles on the one hand and light-induced delays in TIM cycles on the other (Figure 6).

Whether the temperature-induced changes in the levels and phase of the *tim* mRNA cycle (Figure 1B) play a role in the cold-induced advance in the steady state phase of the clock is not clear. The amplitude in *tim* RNA cycling is more sensitive to changes in temperature than that of *per* RNA, which remains relatively constant at higher temperatures (Figure 1B). This raises the intriguing possibility that, whereas splicing at the 3′ intron of *per* is intimately involved in setting the phase of the clock at low temperatures, temperature-dependent changes in the amplitude of the oscillator are mainly driven by changes in the overall levels of *tim* mRNA and protein. Recent evidence in *Neurospora* shows that the overall levels of the key circadian clock protein FREQUENCY (FRQ) increase with temperature, a response that explains several aspects of how the oscillator in this species is regulated by temperature (Liu et al., 1997, 1998). Our findings further reveal that in sharp contrast to results obtained at higher temperatures, during constant dark conditions at low temperatures *tim* RNA is essentially pegged at trough levels. Perhaps the dramatic changes in how the *per* and *tim* RNA cycles are governed in *Drosophila* as a function of temperature are a reflection of the switch from a more night phase clock at higher temperatures to a more day phase clock at lower temperatures. Recent evidence suggests that day phase clocks such as those operating in *Neurospora* and mice use a different molecular logic in responding to photic signals than that used by the night phase clock of *Drosophila* (Shigeyoshi et al., 1997). Future studies are aimed at addressing these possibilities.

Concluding Remarks

In *D. melanogaster*, splicing of a 3′ intron that stimulates *per* mRNA levels appears to be the primary clock-specific response to cold temperatures, whereas the initial clock-specific photoresponsive event is the degradation of TIM. Thus, there is a division of labor among individual components of the time-keeping mechanism in "sensing" different environmental modalities. Nonetheless, the functional interdependence of the PER and TIM biochemical cycles ensures that the effects of temperature and photic signals are coordinated. Different entry points into transcriptional–translational based feedback loops that underlie many circadian oscillators might enable these time-keeping devices to simultaneously sample and integrate multiple environmental modalities resulting in rhythms that are optimally adapted to the prevailing local conditions.

Experimental Procedures

Fly Strains and Collections

The wild-type Canton-S (CS) flies and the mutant per^{01} flies used in this study were descendants of stocks originally maintained in the laboratory of Dr. M. Rosbash (Brandeis University, MA), and were previously described (e.g., Edery et al., 1994). The tim^{01} flies were descendants of stocks originally maintained in the laboratory of Dr. A. Sehgal (University of Pennsylvania Medical School, PA), and were previously described (Sehgal et al., 1994). The transgenic flies per^S, per^A, and $per^{B'}$ were descendants of stocks originally maintained in one of our laboratories (P. E. H.) and were previously described (Cheng et al., 1998). All flies were grown and maintained in vials containing standard agar-cornmeal-sugar-yeast-tegosept media. Vials containing approximately 100 young (2- to 6-day-old) adult flies were placed in incubators (Precision Scientific) at 18°, 25°, and 29°C, exposed to at least three 24 hr photoperiods of alternating light:dark cycles (LD; where ZT0 is defined as lights-on), and in some cases subsequently maintained in the dark (DD) at the indicated temperature. At selected times during LD and DD, flies were collected by freezing. For temperature shift experiments (see Figures 2E and 2F), flies were carefully removed from one incubator and placed in another incubator that was preset to the appropriate temperature.

Locomotor Activity Rhythms

Locomotor activity was monitored by placing individual adult flies in glass tubes and using a Trikinetics (Waltham, MA) system interfaced with an Apple computer as previously described (Hamblen-Coyle et al., 1992). Activity data for individual flies were recorded in 30 min bins and stored until analyzed using a Phase program (Hamblen-Coyle et al., 1992). Using this program, the phase of the locomotor activity rhythm was calculated by measuring the time in each consecutive 24 hr cycle (beginning from the last LD) that the peak of activity occurred. The average time of peak activity was calculated by pooling data from at least three independent experiments. To calculate free-running periods, flies were exposed to four LD cycles before being assayed in constant darkness, and activity periods were determined by Chi-square periodogram analysis as previously described (Hamblen-Coyle et al., 1992).

Immunoblotting

Flies were collected by freezing at the indicated times during LD and DD. Head extracts were prepared and probed for PER and TIM by immunoblotting as previously described (Sidote et al., 1998). Briefly, for each time point, equal amounts of total head protein (~10 μg) were loaded onto 5.7% acrylamide-SDS gels and the mixture resolved by electrophoresis. Following transfer to nitrocellulose paper, PER was visualized using the anti-PER antibody GP73, and TIM was visualized using the anti-TIM antibody GP72 (Sidote et al., 1998).

RNase Protection Assay

For each time point, total RNA was extracted from approximately 10 μl of fly heads using TriReagent (Sigma) and following manufacturer's recommended procedure. The levels of *per* and *tim* transcripts were determined by RNase protection assays (Hardin et al., 1990) performed with the modifications as described in Zeng et al. (1994). *per* and *tim* RNA levels were determined using the *per* 2/3 probe (Hardin et al., 1990) and a *tim* probe (Sidote et al., 1998). As a control for RNA loading in each lane, a ribosomal protein probe (RP49) was included in each protection assay (Hardin et al., 1990). Protected bands were quantified using a Phosphorimager from Molecular Dynamics.

Splicing Assay, Plasmids, and Sequencing

The relative levels of two species of *per* mRNA that arise from an alternative splicing event in the 3′ untranslated region of *per* were measured using a reverse transcriptase–PCR (RT–PCR) based assay. For each time point, approximately 30 μg of total RNA was isolated using TriReagent (see above) and resuspended in 20 μl of water. First strand cDNA synthesis was performed on approximately 5 μg of total RNA using the Superscript Preamplification System (version 2.0) from GIBCO-BRL and an antisense *per*-specific primer (P7373B) that hybridizes immediately 5′ upstream from the putative mRNA cleavage site. The antisense oligonucleotide used for first strand synthesis was 5′-AATTGGATCCGTGGCGTTGGCTTTCG-3′ and includes *per* sequences from 7373 to 7358 bp (numbering according to Citri et al. [1987]; *per*-specific sequences are underlined). Following first strand synthesis, PCR was performed (30 cycles: 93°C [30 s], 60°C [30 s], and 72°C [60 s]) using a sense-specific *per* oligonucleotide that hybridizes immediately 3′ downstream of the stop signal for translation (P6867P2: 5′-AATTCTGCAGCCATCATCG GTGTGG-3′ [includes *per* sequences from 6867 to 6881 bp]) and oligonucleotide P7373B. An aliquot (0.5 μl) of the total RT–PCR mix (50 μl) was used for a second PCR (15 cycles: 93°C [30 s], 60°C [30 s], and 72°C [45 s]) using a nested *per*-specific primer (P7215: 5′-GCTTTGCTTGGCTTGAG-3′ [includes *per* sequences from 7215 to 7199 bp]) and P6867P2. In addition, 0.5 μl of [^{32}P]dATP (3000 Ci/mmol) was added during the second PCR. Amplified products were treated with phenol/chloroform and precipitated with ethanol. Radiolabled samples were separated on 4.5% polyacrylamide (19:1 ratio of polyacrylamide to bis-acrylamide) gels, and the intensity of *per*-specific bands was quantitated using a Phosphorimager from Molecular Dynamics. For each time point tested, similar ratios of type B′ to type A were obtained under conditions, whereby the PCR reaction was either in the linear range or the plateau phase (data not shown).

Two *per*-specific bands that differed in size were detected in total RNA following RT–PCR in the presence of oligonucleotides P6867P2 and P7215. The two *per*-specific amplified products were purified from agarose gels and ligated into the pGEM-T Easy vector (Promega), and JM109 cells were used for transformation. Several independent clones representing each of the two different size classes were sequenced by the DNA synthesis and sequencing laboratory at the University of Medicine and Dentistry of New Jersey using an ABI 377 Sequencer with the enzyme amplitaq polymerase FS. The results confirmed that the two *per*-specific RT–PCR size variants are due to the absence or presence of a 3′ intron identical in sequence to that previously described (Cheng et al., 1998).

Acknowledgments

We thank Dr. S. Peltz for many helpful discussions and critical reading of the manuscript and Dr. S. Hitchcock-DeGregori for the use of her densitometer. This work was partially supported by grants from NIH and NSF (I. E.). J. M. was supported by a Neuroscience training grant from NIH.

Received July 3, 1999; revised August 3, 1999.

References

Allada, R., White, N.E., So, W.V., Hall, J.C., and Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell *93*, 791–804.

Chen, Y., Hunter-Ensor, M., Schotland, P., and Sehgal, A. (1998). Alterations of *per* RNA in noncoding regions affect periodicity of circadian behavioral rhythms. J. Biol. Rhythms *13*, 364–379.

Cheng, Y., Gvakharia, B., and Hardin, P.E. (1998). Two alternatively spliced transcripts from the Drosophila *period* gene rescue rhythms having different molecular and behavioral characteristics. Mol. Cell. Biol. *18*, 6505–6514.

Crosthwaite, S.K., Loros, J.J., and Dunlap, J.C. (1995). Light-induced resetting of a circadian clock is mediated by a rapid increase in *frequency* transcript. Cell *81*, 1003–1012.

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S., and Kay, S.A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. Science *280*, 1599–1603.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. Cell *96*, 271–290.

Edery, I. (1999). Role of posttranscriptional regulation in circadian clocks: lessons from Drosophila. Chronobiol. Int. *16*, 377–414.

Edery, I., Zwiebel, L.J., Dembinska, M.E., and Rosbash, M. (1994). Temporal phosphorylation of the Drosophila *period* protein. Proc. Natl. Acad. Sci. USA *91*, 2260–2264.

Ewer, J., Hamblen-Coyle, M., Rosbash, M., and Hall, J.C. (1990). Requirement for *period* gene expression in the adult and not during development for locomotor activity rhythms of imaginal Drosophila melanogaster. J. Neurogenet. *7*, 31–73.

Ewer, J., Frisch, B., Hamblen-Coyle, M.J., Rosbash, M., and Hall, J.C. (1992). Expression of the *period* clock gene within different cell types in the brain of Drosophila adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. J. Neurosci. *12*, 3321–3349.

Frisch, B., Hardin, P.E., Hamblen-Coyle, M.J., Rosbash, M., and Hall, J.C. (1994). A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the Drosophila nervous system. Neuron *12*, 555–570.

Gekakis, N., Saez, L., Delahaye-Brown, A.M., Myers, M.P., Sehgal, A., Young, M.W., and Weitz, C.J. (1995). Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PERL. Science *270*, 811–815.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

Green, M.R. (1986). Pre-mRNA splicing. Annu. Rev. Genet. *20*, 671–708.

Hall, J.C. (1998). Genetics of biological rhythms in Drosophila. Adv. Genet. *38*, 135–184.

Hamblen-Coyle, M.J., Wheeler, D.A., Rutila, J.E., Rosbash, M., and Hall, J.C. (1992). Behavior of period-altered rhythm mutants of Drosophila in light:dark cycles. J. Insect. Behav. *5*, 417–446.

Handler, A.M., and Konopka, R.J. (1979). Transplantation of a circadian pacemaker in Drosophila. Nature *279*, 236–238.

Hao, H., Allen, D.L., and Hardin, P.E. (1997). A circadian enhancer mediates PER-dependent mRNA cycling in Drosophila melanogaster. Mol. Cell. Biol. *17*, 3687–3693.

Hao, H., Glossop, N.R., Lyons, L., Qiu, J., Morrish, B., Cheng, Y., Helfrich-Forster, C., and Hardin, P. (1999). The 69 bp circadian regulatory sequence (CRS) mediates *per*-like developmental, spatial, and circadian expression and behavioral rescue in Drosophila. J. Neurosci. *19*, 987–994.

Hardin, P.E. (1998). Activating inhibitors and inhibiting activators: a day in the life of a fly. Curr. Opin. Neurobiol. *8*, 642–647.

Hardin, P.E., Hall, J.C., and Rosbash, M. (1990). Feedback of the Drosophila *period* gene product on circadian cycling of its messenger RNA levels. Nature *343*, 536–540.

Hardin, P.E., Hall, J.C., and Rosbash, M. (1992a). Behavioral and molecular analyses suggest that circadian output is disrupted by *disconnected* mutants in D. melanogaster. EMBO J. *11*, 1–6.

Hardin, P.E., Hall, J.C., and Rosbash, M. (1992b). Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. Proc. Natl. Acad. Sci. USA *89*, 11711–11715.

Heckrotte, C. (1961). The effect of the environmental factors in the locomotor activity of the plains garter snake (*Thamnophis radix radix*). Anim. Behav. *10*, 193–207.

Helfrich-Forster, C. (1998). Robust circadian rhythmicity of Drosophila melanogaster requires the presence of lateral neurons: a brain-behavioral study of disconnected mutants. J. Comp. Physiol. [A] *182*, 435–453.

Huang, Z.J., Curtin, K.D., and Rosbash, M. (1995). PER protein interactions and temperature compensation of a circadian clock in Drosophila. Science *267*, 1169–1172.

Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the Drosophila protein *timeless* suggests a mechanism for resetting the circadian clock by light. Cell *84*, 677–685.

Jackson, R.J. (1993). Cytoplasmic regulation of mRNA function: the importance of the 3′ untranslated region. Cell *74*, 9–14.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998). The Drosophila clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell *94*, 97–107.

Lee, C., Bae, K., and Edery, I. (1998). The Drosophila CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER–TIM complex. Neuron *21*, 857–867.

Lee, C., Bae, K., and Edery, I. (1999). PER and TIM inhibit the DNA binding activity of a dCLOCK–CYC/BMAL1 heterodimer without disrupting formation of the heterodimer: a basis for circadian transcription. Mol. Cell. Biol. *19*, 5316–5325.

Lee, C., Parikh, V., Itsukaichi, T., Bae, K., and Edery, I. (1996). Resetting the Drosophila clock by photic regulation of PER and a PER–TIM complex. Science *271*, 1740–1744.

Liu, Y., Garceau, N.Y., Loros, J.J., and Dunlap, J.C. (1997). Thermally regulated translational control of FRQ mediates aspects of temperature responses in the Neurospora circadian clock. Cell *89*, 1–20.

Liu, Y., Merrow, M., Loros, J.J., and Dunlap, J.C. (1998). How temperature changes reset a circadian oscillator. Science *281*, 825–829.

Marrus, S.B., Zeng, H., and Rosbash, M. (1996). Effect of constant light and circadian entrainment of *per*^{S} flies: evidence for light-mediated delay of the negative feedback loop in Drosophila. EMBO J. *15*, 6877–6886.

Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A., and Young, M.W. (1996). Light-induced degradation of TIMELESS and entrainment of the Drosophila circadian clock. Science *271*, 1736–1740.

Nesic, D., and Maquat, L.E. (1994). Upstream introns influence the efficiency of final intron removal and RNA 3′ end formation. Genes Dev. *8*, 363–375.

Nesic, D., Zhang, J., and Maquat, L.E. (1995). Lack of an effect of the efficiency of RNA 3′ end formation on the efficiency of removal of either the final or the penultimate intron in intact cells. Mol. Cell. Biol. *15*, 488–496.

Pittendrigh, C.S. (1954). On tempertaure independence in the clock system controlling emergence time in Drosophila. Proc. Natl. Acad. Sci. USA *40*, 1018–1029.

Price, J.L., Dembinska, M.E., Young, M.W., and Rosbash, M. (1995). Suppression of PERIOD protein abundance and circadian cycling by the Drosophila clock mutation *timeless*. EMBO J. *14*, 4044–4049.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M.W. (1998). *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell *94*, 83–95.

Qiu, J., and Hardin, P.E. (1996). *per* mRNA cycling is locked to lights-off under photoperiodic conditions that support circadian feedback loop function. Mol. Cell. Biol. *16*, 4182–4188.

Reppert, S.M. (1998). A clockwork explosion! Neuron *21*, 1–4.

Rutila, J.E., Zeng, H., Le, M., Curtin, K.D., Hall, J.C., and Rosbash, M. (1996). The *tim*^{SL} mutant of the Drosophila rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron *17*, 921–929.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., and Hall, J.C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of Drosophila *period* and *timeless*. Cell *93*, 805–814.

Saez, L., and Young, M.W. (1996). Regulation of nuclear entry of the Drosophila clock proteins *period* and *timeless*. Neuron *17*, 911–920.

Sangoram, A.M., Saez, L., Antoch, M.P., Gekakis, N., Staknis, D., Whiteley, A., Fruechte, E.M., Vitaterna, M.H., Shimomura, K., King, D.P., et al. (1998). Mammalian circadian autoregulatory loop: a *timeless* ortholog and *mPer1* interact and negatively regulate CLOCK-BMAL1-induced transcription. Neuron *21*, 1101–1113.

Sawyer, L.A., Hennessy, J.M., Peixoto, A.A., Rosato, E., Parkinson, H., Costa, R., and Kyriacou, C.P. (1997). Natural variation in a Drosophila clock gene and temperature compensation. Science *278*, 2117–2120.

Sehgal, A., Price, J.L., Man, B., and Young, M.W. (1994). Loss of circadian behavioral rhythms and *per* RNA oscillations in the Drosophila mutant *timeless*. Science *263*, 1603–1606.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P., and Young, M.W. (1995). Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. Science *270*, 808–810.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J.J., Dunlap, J.C., and Okamura, H. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the m*Per*1 transcript. Cell *91*, 1043–1053.

Sidote, D., Majercak, J., Parikh, V., and Edery, I. (1998). Differential effects of light and heat on the Drosophila circadian clock proteins PER and TIM. Mol. Cell. Biol. *18*, 2004–2013.

So, W.V., and Rosbash, M. (1997). Post-transcriptional regulation contributes to Drosophila clock gene mRNA cycling. EMBO J. *16*, 7146–7155.

Stanewsky, R., Jamison, C.F., Plautz, J.D., Kay, S.A., and Hall, J.C. (1997). Multiple circadian-regulated elements contribute to cycling *period* gene expression in Drosophila. EMBO J. *16*, 5006–5018.

Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A., Rosbash, M., and Hall, J.C. (1998). The *cry*^{b} mutation identifies cryptochrome as a circadian photoreceptor in Drosophila. Cell *95*, 681–692.

Suri, V., Qian, Z., Hall, J.C., and Rosbash, M. (1998). Evidence that the TIM light response is relevant to light-induced phase shifts in *Drosophila melanogaster*. Neuron *21*, 225–234.

Suri, V., Lanjuin, A., and Rosbash, M. (1999). TIMELESS-dependent positive and negative autoregulation in the Drosophila circadian clock. EMBO J. *18*, 675–686.

Sweeney, B.M., and Hastings, J.W. (1960). Effects of temperature upon diurnal rhythms. In Cold Spring Harbor Symposia on Quantitative Biology (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY: Long Island Biological Association), pp. 87–104.

Vosshall, L.B., and Young, M.W. (1995). Circadian rhythms in Drosophila can be driven by *period* expression in a restricted group of central brain cells. Neuron *15*, 345–360.

Vosshall, L.B., Price, J.L., Sehgal, A., Saez, L., and Young, M.W. (1994). Block in nuclear localization of *period* protein by a second clock mutation, *timeless*. Science *263*, 1606–1609.

Wheeler, D.A., Hamblen-Coyle, M.J., Dushay, M.S., and Hall, J.C. (1993). Behavior in light-dark cycles of Drosophila mutants that are arrhythmic, blind, or both. J. Biol. Rhythms *8*, 67–94.

Yang, Z., Emerson, M., Su, H.S., and Sehgal, A. (1998). Response of the Timeless protein to light correlates with behavioral entrainment and suggests a nonvisual pathway for circadian photoreception. Neuron *21*, 215–223.

Young, M.W. (1998). The molecular control of circadian behavioral rhythms and their entrainment in Drosophila. Annu. Rev. Biochem. *67*, 135–152.

Zeng, H., Qian, Z., Myers, M.P., and Rosbash, M. (1996). A light-entrainment mechanism for the Drosophila circadian clock. Nature *380*, 129–135.

Zylka, M.J., Shearman, L.P., Levine, J.D., Jin, X., Weaver, D.R., and Reppert, S.M. (1998). Molecular analysis of mammalian *Timeless*. Neuron *21*, 1115–1122.