Thermosensitive Splicing of a Clock Gene and Seasonal Adaptation

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Similar to many diurnal animals, the daily distribution of activity in *Drosophila* exhibits a bimodal pattern with clock-controlled morning and evening peaks separated by a midday “siesta.” In prior work, we showed that the thermosensitive splicing of a 3′-terminal intron in the RNA product from the *Drosophila period* (*per*) gene (*dper*) is critical for temperature-induced adjustments in the timing of evening activity. Cold temperatures enhance the splicing efficiency of this intron (termed *dmpi8*, *Drosophila melanogaster per* intron 8), an event that stimulates the daily accumulation of *dper* RNA and protein, leading to earlier evening activity. Conversely, warm temperatures attenuate *dmpi8* splicing efficiency contributing to delayed evening activity, likely ensuring that flies avoid activity during the hot midday sun when they are at increased risk of desiccation. Here, we discuss the underlying molecular mechanisms governing the thermosensitive splicing of *dmpi8* and how it contributes to seasonal changes in the daily activity patterns of *Drosophila*. On a broader perspective, RNA–RNA interactions likely have fundamental roles in the thermal adaptation of life forms to the daily and seasonal changes in temperature.

INTRODUCTION

Organisms on this planet have adapted to the daily rotation of the earth on its axis. By means of endogenous circadian (≈ 24 hours) “clocks” or pacemakers that can be synchronized to the daily and seasonal changes in external time cues, most notably, visible light and ambient temperature, life forms anticipate environmental transitions, perform activities at biologically advantageous times during the day, and undergo characteristic seasonal responses (for review, see Hastings et al. 1991; Edery 2000). Light is almost certainly the predominant entraining agent in nature. Under natural conditions the light/dark (LD) cycle aligns the phases of clocks and evokes daily adjustments in the approximately 24-hour endogenous periods of these oscillators such that they precisely match the 24-hour solar day. The duration of day length (photoperiod) can modify the temporal alignment between a circadian rhythm and local time. A physiologically relevant advantage of this inherent flexibility of clocks is that the daily distributions of physiological and behavioral rhythms are not rigidly locked to local time but can be adjusted for seasonal changes in day length. Despite the obvious importance of photoperiod, ambient temperature is also a key environmental modality regulating the timing of circadian rhythms (Sweeney and Hastings 1961; Rensing and Ruoff 2002). This makes intuitive sense because in temperate latitudes, seasonal changes in day length are also accompanied by predictable changes in average daily temperatures. The work presented here is mainly focused on using *Drosophila* as a model organism to address how a circadian system contributes to temperature-dependent changes in the distribution of daily activity.

Before discussing this work, however, it is important to recognize that temperature has multiple effects on circadian-regulated phenomena (Rensing and Ruoff 2002). Perhaps the best known but least understood response of

circadian rhythms to temperature is that period length is relatively constant over a wide range of physiologically relevant temperatures, an active mechanism termed “temperature compensation.” The ability of circadian timing systems to maintain stable periods despite changes in temperature is important because whether it is summer or winter, the solar day does not change in duration. Although period length is rather insensitive to temperature fluctuations, the phases and amplitudes of circadian rhythms can exhibit clear temperature-induced changes. Most notably, circadian rhythms can be phase-shifted by changes in temperature (pulses and step-up or -down) and entrained by daily temperature cycles (see, e.g., Sweeney and Hastings 1961; Wheeler et al. 1993; Liu et al. 1997, 1998; Sidote et al. 1998; and references therein). Other work indicates that the amplitude of a circadian oscillator changes as a function of temperature (usually perceived as increasing with rising temperature), suggested by some as a solution to temperature compensation of period length (Lakin-Thomas et al. 1991; Pittendrigh et al. 1991). 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; Revel et al. 2007; and references therein), which might be an extreme example of amplitude reduction.

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 1961). Many animals display a bimodal distribution of activity, with peaks centered around the morning and evening hours that are separated by a midday “siesta” time of varying lengths. A classic example of how temperature affects daily activity patterns 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 tempera-

tures (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 1961).

If there is such clear adaptive value, why is this effect of temperature on circadian rhythms less studied? There are several reasons, which also highlight some of the challenges in trying to understand the underlying mechanism and the choices of model systems. First, from a historical perspective, the *entre* was based on observing how temperature affects the daily distribution of activity (i.e., movement such as locomotion), which naturally focused the work on animals. Second, it was thought that much of the thermal effect was likely due to the “direct” (or masking) effects of temperature on activity (see, e.g., Matsumoto et al. 1998), minimizing the notion of potential clock involvement. Finally, when it comes to trying to understand “seasonal” adaptation of clocks, much of the focus has been placed on the impact of changing day length or photoperiod. This is quite natural as light is the predominant daily synchronizer in nature, and it is almost certain that circadian rhythms in all organisms, even homeotherms, exhibit phase adjustments in response to changes in day length. Indeed, the dual oscillator model based on coupled morning (M) and evening (E) pacemakers was largely developed over the years as a framework to explain how the M and E peaks track sunrise and sunset, respectively, despite seasonal changes in day length (Pittendrigh and Daan 1976). This photocentric view of seasonal adaptation has for the most part not incorporated the effects of temperature on modulating clock dynamics.

Below, we briefly describe some of our work using *Drosophila* to explore the role of a circadian clock in the adaptation of daily activity patterns to seasonal changes in temperatures. Thermal-sensitive splicing of a clock mRNA has a key role in this adaptation (Majercak et al. 1999). Remarkably, a similar mechanism has also been shown to underlie some temperature responses in the *Neurospora* clock (Colot et al. 2005; Diernfellner et al. 2005; Brunner and Diernfellner 2006). On a broader perspective, the results suggest that naturally selected variations in the strengths of RNA–RNA interactions offer a simple basis for designing an adaptive molecular mechanism displaying highly calibrated thermal responses that can subsequently impact a wide array of physiological and behavioral programs.

DROSOPHILA AS A MODEL SYSTEM TO STUDY THE ROLE OF CIRCADIAN CLOCKS IN REGULATING THE DAILY DISTRIBUTION OF ACTIVITY AS A FUNCTION OF SEASONAL CHANGES IN TEMPERATURE

Several years ago, we used *D. melanogaster* as a model system to understand the role of a clock in regulating the pattern of daily activity as a function of changes in temperature and day length (Majercak et al. 1999). Under stan-

dard daily cycles of 12-hour light followed by 12-hour dark (12:12 L:D) at 25°C, *D. melanogaster* exhibit a bimodal activity pattern with clock-controlled peaks centered around the lights-on (“morning” peak) and lights-off (“evening” peak) transitions that are separated by a midday dip or “siesta” (Fig. 1) (see, e.g., Hamblen-Coyle et al. 1992). For the purposes of this discussion, it is important to note at the outset that despite the clock-controlled bimodal activity pattern, the evening activity component is largely assayed as a bona fide readout of the circadian system in *Drosophila*. This is mainly because the morning peak usually coincides with a direct stimulatory effect of light following dawn, and the evening peak most visibly persists in constant darkness (Wheeler et al. 1993). The photoperiod and ambient temperature have effects on the distribution of activity throughout a daily cycle. Over a wide range of photoperiods, the evening peak occurs around the light-to-dark transition (Qiu and Hardin 1996; Majercak et al. 1999; Rieger et al. 2003; Shafer et al. 2004). As a result, lengthening the photoperiod delays the evening activity relative to the most recent sunrise. Temperature appears to be the main environmental cue determining whether the evening activity will be mostly diurnal or nocturnal (Majercak et al. 1999). With increasing temperature, the evening activity becomes progressively more nocturnal and midday inactivity more pronounced (see Figs. 1 and 3) (Matsumoto et al. 1998; Majercak et al. 1999; Yoshii et al. 2002). This directional response is observed in many diurnal animals, displaying a greater proportion of their activity during the cooler nighttime hours on hot days and conversely the warmer daytime hours during cold days (Sweeney and Hastings 1961).

A Role for Thermal-sensitive Splicing of a Clock Gene in Seasonal Adaptation

We showed that splicing of the 3′-terminal intron in *dper* RNA (termed dmpi8) (Cheng et al. 1998) is an important aspect of how the *Drosophila* circadian clock adapts to seasonal changes in temperature (Majercak et al. 1999). At cold temperatures, the proportion of dmpi8 spliced *dper* mRNA (termed type B′) compared to the unspliced variant (termed type A) is enhanced, leading to more rapid daily increases in *dper* transcript levels and earlier evening activity. Splicing per se, as opposed to retention or removal of the dmpi8 intron, stimulates increases in *dper* mRNA levels. This led to the hypothesis that assembly of active spliceosomes at the 3′-terminal intron somehow produces more mature transcripts, possibly by facilitating 3′-end formation (Fig. 1). Transgenic flies bearing variant *dper* transgenes where splicing of dmpi8 was abrogated manifested nocturnal evening activity even on cold days (Majercak et al. 1999). Long photoperiods partially counteract cold-induced phase advances in the accumulation of *dper* mRNA and protein by delaying the upswing in TIMELESS (TIM) levels. The functional interrelationships between dPER 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.

In more recent findings, we and other investigators

Figure 1. Model for how thermosensitive splicing of the *dper* 3'-terminal intron (dmpi8) regulates seasonal changes in the daily distribution of activity in *Drosophila melanogaster*. Shown at the top is the 3' end of the *dper* precursor mRNA (pre-mRNA); (white box) Sequences from translation stop codon to just upstream of 5' splice site of dmpi8; (horizontal line) dmpi8 intron; (gray box) sequences following 3' splice site of dmpi8 until 3' cleavage and polyadenylation site; (black box) transcribed sequences downstream from 3' cleavage and polyadenylation site. Cold temperatures enhance binding of spliceosome (large oval), which stimulates binding of 3'-end formation factors (small circle). More rapid accumulation of *dper* transcripts leads to advanced evening activity and less prominent midday inactivity. Conversely, on warm days, the inefficient splicing of dmpi8 leads to an extended midday siesta and preferential nocturnal evening activity, events that enhance the ability of *Drosophila* to avoid the deleterious effects associated with the hot midday sun. For *Drosophila* activity profiles: (black bars) dark; (gray bars) light. Locomotor activity was collected in 15 min bins and relative values throughout a daily cycle (example shown, 12 hr light:12 hr dark) shown in arbitrary units. (Left panel) 18°C; (right panel) 29°C (also shown are morning and evening activity components, including midday siesta). (Adapted from Majercak et al. 1999.)

showed that daily fluctuations in the splicing efficiency of dmpi8 are regulated by the clock in a manner that depends on the photoperiod (day length) and temperature (Collins et al. 2004; Majercak et al. 2004). Shortening the photoperiod enhances dmpi8 splicing and advances its cycle, whereas the amplitude of the clock-regulated daytime decline in splicing increases as temperatures rise. This suggests that at elevated temperatures, the clock has a more pronounced role in maintaining low splicing during the day, an adaptive response that for small insects likely minimizes the risks of desiccation during the hot midday hours. Indeed, on entering direct sunlight, a 10-mg insect can heat up by about 10°C in less than 10 seconds (Sayeed and Benzer 1996), a potentially life-threatening event for *Drosophila* that only weigh in the vicinity of 1 mg. In summary, the splicing efficiency of dmpi8 is regulated by the clock, temperature, and day length, ultimately modulating the trajectory of daytime increases in *dper* mRNA levels and hence clock phase. We propose that the multimodal regulation in the splicing efficiency of dmpi8 acts as a “seasonal sensor” conveying *calendar* information to the animal. We and others also identified a novel nonphotic role for NORPA (*no receptor potential A*) in the regulation of dmpi8 splicing (Collins et al. 2004; Majercak et al. 2004). In this chapter, we do not discuss the role of *norpa* further, but we focus on the basis for temperature-dependent changes in dmpi8 splicing efficiency.

Weak 5' and 3' Splice Sites as a Basis for Thermosensitive Splicing

As an initial attempt to understand the basis for the temperature-dependent splicing of dmpi8, we sought to

develop a simplified cell-culture-based assay that recapitulates this thermosensitivity phenotype. *D. melanogaster* (Oregon-R strain [OR]) *dper* genomic sequences encompassing the entire 3'-untranslated region (3'UTR) followed by 90 bp of 3'-flanking nontranscribed region were fused downstream from a *luciferase* (*luc*) reporter gene. Expression of the *luc-dper/3'UTR* hybrid gene was placed under the control of the constitutive actin 5C promoter (pAct) (Fig. 2A) (Angelichio et al. 1991). To enable the simple introduction of different intron and flanking exon sequences, this parent wild-type vector (termed dmpi8) also includes two engineered XhoI and KpnI restriction sites placed 10 bp upstream and downstream from the dmpi8 5' and 3' splice sites (ss), respectively. Following transfection of the popular *Drosophila* Schneider S2 cells, at least two independent lines of stable transformants were isolated for each construct. Subsequently, separate aliquots of cells were incubated for 24 hours at different temperatures in the dark. Total RNA was extracted, and the relative levels of spliced and nonspliced products were determined as previously described using reverse transcriptase-polymerase chain reaction (RT-PCR) whereby values are normalized to an internal RNA standard (Majercak et al. 2004). The dmpi8 control RNA exhibited approximately 2.5–3.2-fold increases ($n > 10$; $P < 0.001$ using Student's *t*-test for values obtained at 12°C and 24°C) in the proportion of spliced-to-unspliced RNA at 12°C compared to 24°C (Fig. 2D). This is similar to the 2–3-fold increase in the ratio of spliced type B' to unspliced type-A *dper* RNA variants observed in wild-type flies at 18°C compared to 29°C (Majercak et al. 1999). Thus, the *dper* 3'UTR is sufficient to manifest thermosensitive splicing of dmpi8 in a

Figure 2. Multiple suboptimal splice sites underlie temperature-dependent splicing of the *dmpi8* intron. (A) A luciferase (*luc*) reporter gene fused to 3' *dper* sequences was used to transfect S2 cells. XhoI and KpnI sites were engineered into the fusion construct such that they flanked the 5' and 3' splice sites of *dmpi8* to facilitate swapping intronic sequences. (B) Schematic of mutations made to increase the predicted strengths of the 5' and/or 3' splice sites of *dmpi8*. (C) Predicted scores for the 5' and 3' splice sites of the different constructs (range, 0–1, where 1 is the highest). (D) Cells were grown at either 12°C or 24°C, and the relative splicing efficiency was determined (values for 24°C were set to 1 and the rest of the values normalized). Note that whereas *dmpi8* exhibits approximately 2.5-fold more efficient splicing at 12°C compared to 24°C, the other transcripts exhibit little (e.g., M3) to no (e.g., M2) temperature-sensitive splicing.

simplified cell culture system, providing a powerful approach to investigate mechanistic issues.

A feature of the *dper* 3'-terminal intron in *D. melanogaster* that caught our attention was that both the 5' and 3' splice sites were predicted to be weak. For example, using a program that predicts splice sites in *D. melanogaster* (www.fruitfly.org/seq_tools/splice.html), *dmpi8* has the lowest scores for both the 5' (score = 0.45) and 3' (score = 0.21) splice sites of all the introns in *per* (the other *per* small introns had scores of 0.88–0.97 for the 5' and 0.42–0.95 for the 3' splice sites, with 1 being highly likely) (Fig. 2C). The presumptive branchpoint (BP) lies in a sequence that is very similar but not identical to the consensus sequence for *D. melanogaster* introns (CUAAU) (Fig. 2A). Multiple suboptimal splice sites for the *dmpi8* intron was eerily reminiscent of prior work from Murphy and co-workers studying the basis for the thermosensitive splicing phenotype of the *ts110* mutant of Moloney murine sarcoma virus (Mo-MSVts110). A series of elegant studies showed that multiple suboptimal splicing signals were the basis for the temperature-sensitive splicing of Mo-MSVts110 (Cizdziel et al. 1988; Touchman et al. 1995; Ainsworth et al. 1996). For example, introducing a consensus branchpoint signal increased splicing efficiency and abrogated the thermodependency (Touchman et al. 1995). A similar result was observed by strengthening the 5' splice site (Ainsworth et al. 1996). Intriguingly, other random mutagenesis strategies to generate temperature-sensitive phenotypes were eventually shown to be a result of introducing mutations that weaken key splicing signals on precursor mRNAs (pre-mRNAs).

Indeed, increasing the predicted strength of either the 5' splice site (e.g., M2) or the 3' splice site (e.g., M1) in *dmpi8* eliminated thermosensitivity, whereas in other cases, the thermal response was diminished (e.g., M3) (Fig. 2B–D). Thus, there is a graded response that correlates with the predicted strengths of the 5' and 3' signals. As with Mo-MSVts110, our findings suggest that temperature regulation of *dmpi8* splicing is not driven by discrete thermosensitive elements but involves an intricate balance of multiple suboptimal splice sites, raising the possibility of intricate coevolution. Studies using transgenic flies whereby we replaced the wild-type *D. melanogaster* 3' UTR (from the standard Oregon-R strain) with the M2 version also exhibited near complete splicing at all test temperatures (data not shown). Moreover, in both S2 cells and flies, versions of the *dper* 3'-terminal intron with higher splicing efficiency also exhibited higher RNA levels (data not shown). Together, these results are highly consistent with our earlier model that the splicing efficiency of the *dper* 3'-terminal intron stimulates the production of mature transcripts.

A Similar Mechanism in the *Neurospora* Clock

Along with *Drosophila*, work in *Neurospora* laid the early foundation for mechanistic insights underlying clock mechanisms (Dunlap 1996). The initial biochemical characterization of the FREQUENCY (FRQ) protein led to a remarkable realization that has grown over the years and can be equally applied to other clock systems. Both dPER and FRQ exhibit dramatic progressive

increases in phosphorylation throughout a daily cycle that are intimately linked to abundance, whereby the most hyperphosphorylated isoforms are associated with sharp decreases in levels (Edery et al. 1994b; Garceau et al. 1997). Today, we know that despite the lack of sequence conservation, there are uncanny similarities between dPER and FRQ proteins with respect to clock function. Importantly, both have been shown to be bona fide “state variables” in their respective clockworks (Aronson et al. 1994; Edery et al. 1994a), indicating that the daily fluctuations in the abundance/activity of these proteins, as opposed to simply their presence, are inextricably linked to the phase of the clock. dPER and FRQ proteins share similar modes of action, whereby they participate in transcriptional autoinhibitory loops crucial to clock function, most likely by acting as conduits for the timely delivery or regulation of *trans*-acting factors that more directly inhibit the key transcription factors which drive their own expression (Schafmeier et al. 2005; He et al. 2006; Yu et al. 2006; Kim et al. 2007). Although we have gotten used to the idea by now, it is still rather intriguing that virtually identical kinases, phosphatases, and F-box proteins regulate the daily life cycles of PER and FRQ proteins (Gallego and Virshup 2007).

Perhaps then, it should not be too unexpected that an aspect of *frq* expression regulated by temperature involves mRNA splicing. Earlier work from Dunlap and co-workers showed that temperature regulates the relative levels of two isoforms of FRQ protein, a short (s-FRQ) and long (l-FRQ) version that arise from alternative use of translation initiation sites (Liu et al. 1997). More recent work from the Brunner and Dunlap labs demonstrated that the ratio of l-FRQ versus s-FRQ is regulated by thermosensitive splicing of an intron that when excised removes the translation initiation site of l-FRQ (Colot et al. 2005; Diernfellner et al. 2005; Brunner and Diernfellner 2006). Moreover, inefficient recognition of nonconsensus splice sites at elevated temperatures was shown to underlie the thermosensitivity (Diernfellner et al. 2005; Brunner and Diernfellner 2006). Additional steps including temperature-dependent effects on the rate of ribosome scanning at the different translation initiation recognition sequences allow for adjustments in FRQ levels that are appropriate for the local ambient temperature (Diernfellner et al. 2005; Brunner and Diernfellner 2006). Thus, in two widely different species, the clockworks adapt to changes in temperature by thermal adjustments in the levels of key state variables via a mechanism involving an initial thermosensitive splicing event that has ramifications for other more downstream aspects of mRNA metabolism or utilization, such as the efficiency of 3'-end formation in the case of *dper* or translation for *frq*.

HOW DO WEAK SPLICING SIGNALS YIELD THERMOSENSITIVE SPLICING?

From the aforementioned examples, such as *dper*, *frq*, and Mo-MSVts110, it is clear that the presence of multiple suboptimal splicing signals can render the splicing efficiency of an intron temperature-dependent. In very general terms, pre-mRNA splicing in eukaryotes occurs via two major steps that differ in the need for ATP consumption

(for review, see Hastings and Krainer 2001). The first step is an ATP-hydrolysis-independent assembly of the core splicing machinery or spliceosome to an intronic sequence. This involves the recognition of *cis*-acting signals on the mRNA such as the 5' and 3' splice sites and 3' polypyrimidine tracts. Subsequently, in the second phase, which requires ATP hydrolysis, the pre-mRNA and splicing machinery undergo structural rearrangements, culminating in cleavage at the 5' intronic site, lariat formation, and finally, removal of the intron with concomitant ligation of the exonic sequences. The initial binding of the spliceosome and the subsequent catalytic events are heavily dependent on dynamic interactions between the substrate transcript and the small nuclear RNAs (snRNAs) that are core constituents of the splicing machinery.

Given the ability of RNA to form intramolecular and intermolecular base-pairing interactions of varying strengths, splicing offers a rich opportunity for generating a molecular mechanism that can elicit calibrated thermal responses. On the basis of the important roles for the 5' and 3' splice sites in spliceosome docking, it is likely that for introns with multiple suboptimal splicing signals, low temperatures stabilize RNA–RNA and/or RNA–protein interactions between the splicing machinery and the RNA substrate, leading to more efficient splicing. For example, low temperatures could enhance the binding of the U1 snRNA to nonconsensus 5' splice sites, modulating a major rate-limiting step in overall splicing efficiency. It is also possible that temperature has effects during the catalytic phase of splicing. For example, the U4 and U6 snRNAs undergo structural rearrangements between them that can be modulated by temperature (Shannon and Guthrie 1991). However, this type of mechanism lacks substrate specificity and would have global effects on splicing. Other possibilities include temperature-dependent changes in (1) RNA secondary structure that modulates the ability of an intron to be recognized and excised and (2) binding of *trans*-acting regulatory splicing factors, such as SR proteins that can enhance or suppress splicing (Bourgeois et al. 2004).

LIGHT-INDUCTION OF *TIM* EXPRESSION AT COLD TEMPERATURES: AN INTEGRATED MECHANISM

Other work from our lab also showed that light stimulates expression of *tim* at low but not warm temperatures (Chen et al. 2006). Intriguingly, although numerous lines of evidence indicate that *dper* and *tim* expression are activated by the same mechanism, light has no measurable acute effect on *dper* mRNA abundance (Chen et al. 2006). The acute effects of light on *tim* expression are temporally gated, essentially restricted to the daily rising phase in *tim* mRNA levels. Because the start of the daily upswing in *tim* expression begins several hours after dawn in long photoperiods (day length), this gating mechanism likely ensures that sunrise does not prematurely stimulate *tim* expression during unseasonally cold spring/summer days. We suggest that photic stimulation of *tim* expression at low temperatures is part of a seasonal adaptive response that helps advance the phase of the clock on cold days, enabling flies to exhibit preferential daytime activity

Figure 3. Integrated effects of photoperiod and temperature on *dper* and *tim* expression. Cold short days stimulate the rapid accumulation of both *dper* and *tim* transcripts, leading to advanced evening activity and little to no midday siesta. Long photoperiods and warm temperatures delay the daily upswings in *dper* and *tim* transcripts, contributing to preferential nocturnal evening activity and pronounced midday inactivity. Thus, temperature and day length have parallel effects on the daily accumulation profiles in *dper* and *tim* transcripts.

despite the (usually) earlier onset of dusk. Thus, we envisage an integrated mechanism whereby during autumn/winter seasons, splicing of *dmpi8* is enhanced, leading to a more rapid rise in *dper* RNA levels, and light stimulates *tim* induction, responses that function in a coordinate manner to advance the timing of when de-novo-synthesized PER and TIM proteins begin to interact, leading to an earlier clock phase (Fig. 3). Otherwise stated, the ability of temperature and photoperiod to adjust trajectories in the rising phases of one or more clock RNAs constitutes a major mechanism contributing to seasonal adaptation of clock function in *Drosophila*.

This raises the role of rhythmic clock gene expression in light of recent findings in cyanobacteria which clearly show that a biochemical oscillator with circadian properties can be reconstituted in vitro (Nakajima et al. 2005). In this case, mixing purified recombinant versions of the key clock proteins KaiA, KaiB, and KaiC in the presence of ATP as an energy source resulted in daily cycles in the phosphorylated state of KaiC. This dramatic result convincingly shows that circadian molecular cycles can occur in the absence of cycling gene expression. KaiC could be considered the equivalent of dPER and FRQ in that these proteins undergo daily cycles in phosphorylation that are likely to be critical signals in period determination and defining the state of the clock. Although it is possible that reconstituted rhythms in dPER or FRQ phosphorylation might be attained, rhythmic clock gene expression is likely to be an important element in optimizing the dynamics of clock systems.

CONCLUSIONS

The work described herein focused on the use of *D. melanogaster* to study the role of a circadian system in

modulating daily activity patterns in response to seasonal changes in temperature. This species has also contributed to other effects of temperature on circadian systems. For example, the elegant work from Kyriacou and colleagues showed that a stretch of Thr-Gly (TG) repeats centrally located in the dPER-coding region modulates temperature compensation of period length (Sawyer et al. 1997). Importantly, these TG repeats are subject to natural selection and exhibit a latitudinal cline in natural populations of *D. melanogaster* (Sawyer et al. 1997). Thus, dPER activity appears to be a focal point for thermal responses in the *Drosophila* clock. Ongoing studies are aimed at determining whether natural selection is also operating at the level of *dmpi8* splicing efficiency and whether there is an association between the thermosensitivity of 3'-terminal splicing and the type of TG repeat present on *dper* (i.e., diurnal phase adjustments can also be attained by changes in period length). More recent work has also identified a factor termed *nocte* that specifically modulates circadian entrainment to daily changes in temperature but not light/dark cycles (Glaser and Stanewsky 2005). Intriguingly, *nocte* appears to be unrelated to *dmpi8* splicing, indicating that multiple pathways convey thermal information to the circadian system in flies.

Among the most exciting recent findings in the study of circadian rhythms was the identification of long-sought “morning” and “evening” pacemakers in *D. melanogaster*. The M cells preferentially regulate the morning peak of activity, whereas the E cells drive the evening component (Grima et al. 2004; Stoleru et al. 2004). Stoleru, Rosbash, and coworkers showed that day length can modulate the relative impact of either M and E cells on the overall circadian system (Stoleru et al. 2007). For example, during long nights, the M cells dominate, whereas during long days, the E cells are predominant. A current challenge is to

integrate the photoperiodic effects on M-E pacemakers with temperature. In this regard, a curious feature is that although the evening activity is delayed during warm days, the morning peak is advanced (e.g., see Figs. 1 and 3). Although this is almost certainly linked to the “desire” of minimizing daytime activity on hot days, how does the clock delay evening activity while advancing morning activity? Recent findings suggest some possibilities, such as differential phase adjustments in M and E clocks (Bachleitner et al. 2007). Moreover, the possibility that some pacemaker cells in the *Drosophila* brain might be preferentially responsive to temperature compared to light (Yoshii et al. 2005; Miyasako et al. 2007) suggests that a complex web of neural networks is involved in integrating photoperiodic and thermal cues to yield seasonably appropriate daily activity patterns. Finally, genome-wide expression studies indicate that information concerning ambient temperature and photoperiod has widespread and coordinate effects on circadian regulation of gene expression in *Drosophila* (Boothroyd et al. 2007). This work also indicated that an intron in *tim* is regulated by temperature, suggesting a broader role for thermosensitive splicing in clock responses to temperature. In addition, the recent demonstration that two different isoforms of *tim* have differential effects on photoperiod-induced ovarian diapause (Sandrelli et al. 2007; Tauber et al. 2007) further indicates that TIM is also a significant clock component in contributing to the seasonal adaptation of *Drosophila* physiology and behavior. Thus, the partnership between dPER and TIM is a key step for the circadian integration of thermal and photic information.

Of course, temperature and day length are highly associated in temperate climates, making it unsurprising that these two modalities are closely intertwined at the level of clock function. Nonetheless, as Pittendrigh noted “although photoperiod is a more reliable indicator of season and organisms have evolved sophisticated methods to measure its length, the physiological value of such a timing device is likely to be mainly directed at anticipating and thus preparing for changes in daily temperatures” (Pittendrigh and Takamura 1987). Recent work in *Drosophila* and *Neurospora* indicates that the thermosensitive splicing of central clock components is a common feature of how circadian clocks respond to temperature. Although other possibilities are likely, weak or nonconsensus splice signals that are more efficiently recognized at lower temperatures appear to be a common theme in generating temperature-dependent splicing of precursor mRNAs. On a broader perspective, RNA–RNA interactions with the ability to form a multitude of short- and long-range base-pair interactions varying in strength appear to be ideal targets for constructing intracellular molecular mechanisms that can be targeted by natural selection to generate highly calibrated thermal responses.

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