ADAPTATION OF THE *DROSOPHILA* CIRCADIAN CLOCK TO SEASONAL CHANGES IN TEMPERATURE AND PHOTOPERIOD

by

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ABSTRACT OF THE DISSERTATION

Adaptation of the *Drosophila* circadian clock to seasonal changes in temperature and photoperiod

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Dissertation Director: Dr. Isaac Edery

In *Drosophila melanogaster*, splicing of an intron in the 3' untranslated region of the *period* (*per*) mRNA (herein referred to as dmpi8) is enhanced at cold temperatures, leading to more rapid daily increases in *per* transcript levels and earlier "evening" activity. We show that shortening the photoperiod (day length) enhances daily fluctuations in the splicing of dmpi8 and advances its cycle, whereas the amplitude of the clock-regulated daytime decline in splicing increases as temperatures rise. Our results identify a novel non-photic role for NORPA in the temperature regulated repression of dmpi8 splicing.

In related work I found that two haplotypes (termed VT1.1 and VT1.2) in the *per* 3' UTR with six natural polymorphisms showed differential splicing efficiency in inbred flies, transgenic flies, and S2 cells. The VT1.1 haplotype leads to better dmpi8 splicing and earlier evening activity. Splicing assays in S2 cells also suggest that efficient dmpi8 splicing promotes *per* mRNA export. Our results suggest that splicing of dmpi8 plays a major role for *Drosophila* in adapting to local climates.

This dissertation also shows that light acutely stimulates the expression of *tim* at cold but not warm temperatures and that this is regulated at the transcriptional level.

Cold-specific stimulation of *tim* RNA abundance requires CLK or CYC but not PER, TIM, CRY, or the canonical phototransduction pathway. The photoinduction of *tim* is temporally restricted to the daily rising phase in *tim* mRNA levels, likely ensuring that sunrise does not prematurely stimulate *tim* expression during unseasonably cold long days.

My work suggests a model whereby temperature and day length are integrated to set the phase of the clock in a seasonably appropriate manner. Namely, during cold short days, stimulation of *per* dmpi8 splicing and photoinduction of *tim* mRNA transcription ensures that both *per* and *tim* transcripts accumulate more rapidly, leading to *Drosophila* exhibiting preferential activity during the warmer daytime hours despite the earlier onset of dusk. Conversely, during warm long days, the inefficient splicing of dmpi8 and lack of light stimulated *tim* transcription delay evening activity such that flies avoid the deleterious effects of the hot midday hours.

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DEDICATION

To my wife, Huihui Pan

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Chapter 1. Introduction

Circadian Clocks

The revolution of the Earth around the Sun generates a day-night cycle of 24 hours with rhythmic changes in both temperature and day light. As an adaptation to these daily changes of environmental timing signals (i.e., Zeitgeber), diverse organisms, from bacteria to humans, evolved a crucial mechanism in order to temporally coordinate internal biological processes with the daily rhythmic environmental cues. Central to this mechanism is a cell-autonomous clock that regulates the rhythmic expression of many clock-controlled genes (CCGs), as illustrated by microarray data (20, 82, 90, 107, 144), and thus affects downstream biological processes such as daily rhythms in locomotor activity of the fruit fly and the metabolism of cholesterol to bile acids in mice (74) (Figure 1.1). The synchronization of this time-keeping circadian clock allows an organism to anticipate daily changes in the physical environment, enhancing survival and well-being of the organism. Indeed, a lack of clock synchrony with the environment leads to health problems, such as impaired cognitive function associated with jetlag and shift work (18, 118).

Despite diverse phylogenetic origins of species that show circadian rhythmicity, core clock mechanisms generally include negative and positive elements that form transcriptional-translational autoregulatory feedback loops that generate molecular oscillations in clock gene expression with a period of about 24 hours (8) (Figure 1.1).

Circadian rhythms are described as having three major characteristics. First, 24

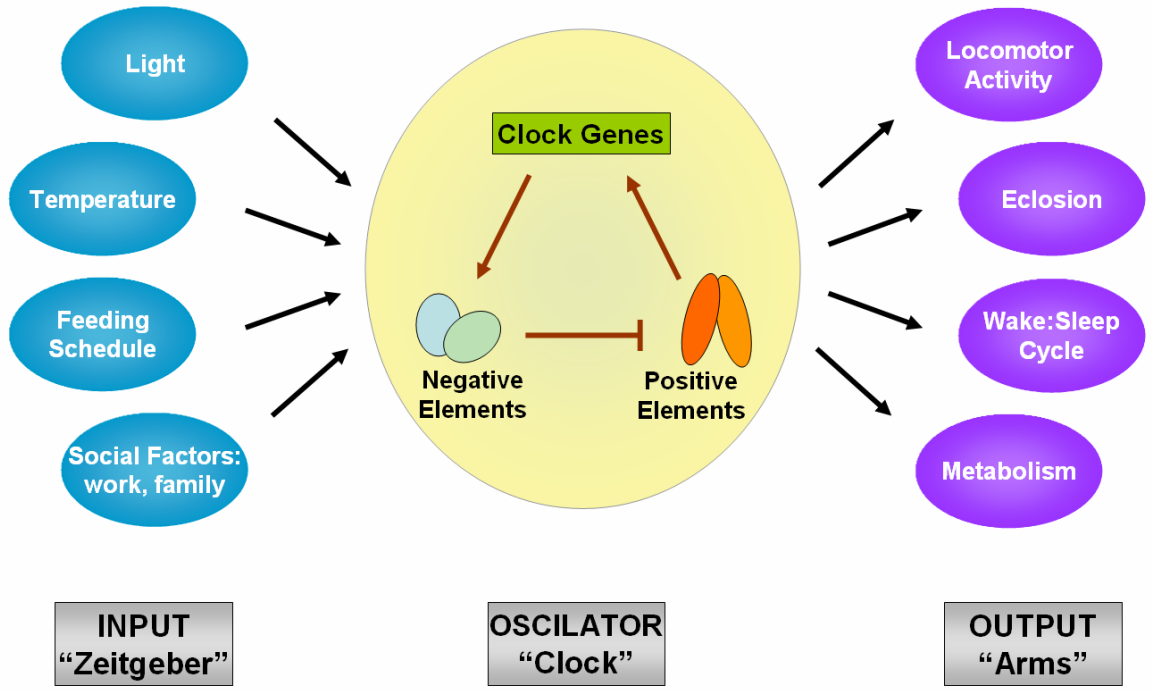

Figure 1.1. Three interconnected parts of the circadian system: input, core clock and output. Light, temperature and other environmental stimuli function as input signals synchronize (entrain) the intrinsic circadian clock. The circadian clock consists of a series of interconnected negative and positive feedback loops marked with many clock genes oscillating in their expression with a period of c.a. 24 hrs. By controlling the expression of diverse clock-controlled genes (CCGs), the clock is then able to regulate many behavioral and physiological processes, such as the wake:sleep cycle.

hours under laboratory conditions lacking any external time-giving (Zeitgeber) cues. This free-running feature has been observed in both unicells (96) and dissociated cells from mammals (153), indicating that the circadian period is generated cell-autonomously. Second, the period lengths of circadian rhythms are compensated with regard to environmental temperature changes such that the periods are essentially invariant (i.e., temperature compensation) (109). Although the period length of the clock is relatively constant, both temperature and day length can profoundly affect amplitude and phase of the clock, which can lead to modulation of daily activity patterns of many organisms, as I discuss in this dissertation regarding the *Drosophila* circadian clock. Third, circadian rhythms can be resynchronized, or entrained, by external time cues such as the daily light:dark cycle and temperature. If a shift in external cues happens (e.g., traveling across time zones), the phase of the clock will be reset to maintain synchrony with the new environmental program. This re-alignment to a new phase is called entrainment, which literally means "to get aboard a train".

The *Drosophila* Circadian Clock

Modern chronobiology research was founded by Colin Pittendrigh with the fruit fly, and Jürgen Aschoff with mice, as models in the early 1950s. Today, the *Drosophila melanogaster* circadian clock is one of the most extensively studied (6, 45, 49, 140). Genetic analysis has revealed over a dozen clock genes in the central oscillator circuitry (Figure 1.2), which includes 3 transcription activators [*clock* (*dClk*), *cycle* (*cyc*), and *PAR domain protein 1ε* (*pdp1ε*)], 3 transcriptional repressors [(*period* (*per*), *timeless* (*tim*), and *vrille* (*vri*)], one mediator of light input [*cryptochrome* (*cry*)], 4 proteins that regulate protein stability and subcellular localization [*doubletime* (*dbt*), *casein kinase 2* (*CK2*),

shaggy (*sgg*), and *protein phosphatase 2a* (*PP2a*)], and 2 F-box proteins that regulate protein degradation [*Drosophila slimb*, and *jetlag* (*jet*)]. The *Drosophila* circadian clock is composed of two interconnected transcriptional-translational feedback loops: a *per*/*tim* loop and a *dClk* loop (41, 52). Essentially, the *per*/*tim* loop is activated by a dCLK-CYC heterodimer and repressed by PER-TIM heterodimer and the *dClk* loop is activated by PDP1ε and repressed by VRI, while both PDP1ε and VRI are activated by a dCLK-CYC heterodimer (49).

Around dusk, PER and TIM proteins begin to accumulate in the cytoplasm. The levels of both proteins are modulated by post-translational events. PER is progressively destabilized by phosphorylation by the kinases DBT (111) and CK2 (1, 81), and stabilized by the phosphatase PP2A through dephosphorylation (120). On the other hand, phosphorylation by SGG destabilizes TIM (88). However, in recent report by Stoleru *et al.*, SGG binds directly to CRY and stabilizes TIM (133). Furthermore, upon receiving light through the blue light photoreceptor CRY, TIM is hyperphosphorylated and targeted for degradation. The degradation of both PER and TIM occurs through the proteasome pathway mediated by F-box proteins, SLIMB and JETLAG, respectively. In the absence of light, TIM accumulates rapidly in the early night time and forms a PER/TIM complex (78, 163), which stabilizes PER protein from degradation (70, 111, 112, 148). The PER/TIM complex enters the nucleus in the middle of the night (26, 95). Nuclear PER-TIM heterodimer binds to the dCLK-CYC dimer and facilitates the phosphorylation of dCLK by the kinase DBT, thereby destabilizing the binding of the dCLK-CYC to E-box elements and deactivating dCLK-CYC dependent transcription of *per* and *tim* transcripts (7, 29, 68, 76, 77, 162). The steady state levels of PER and TIM decline following

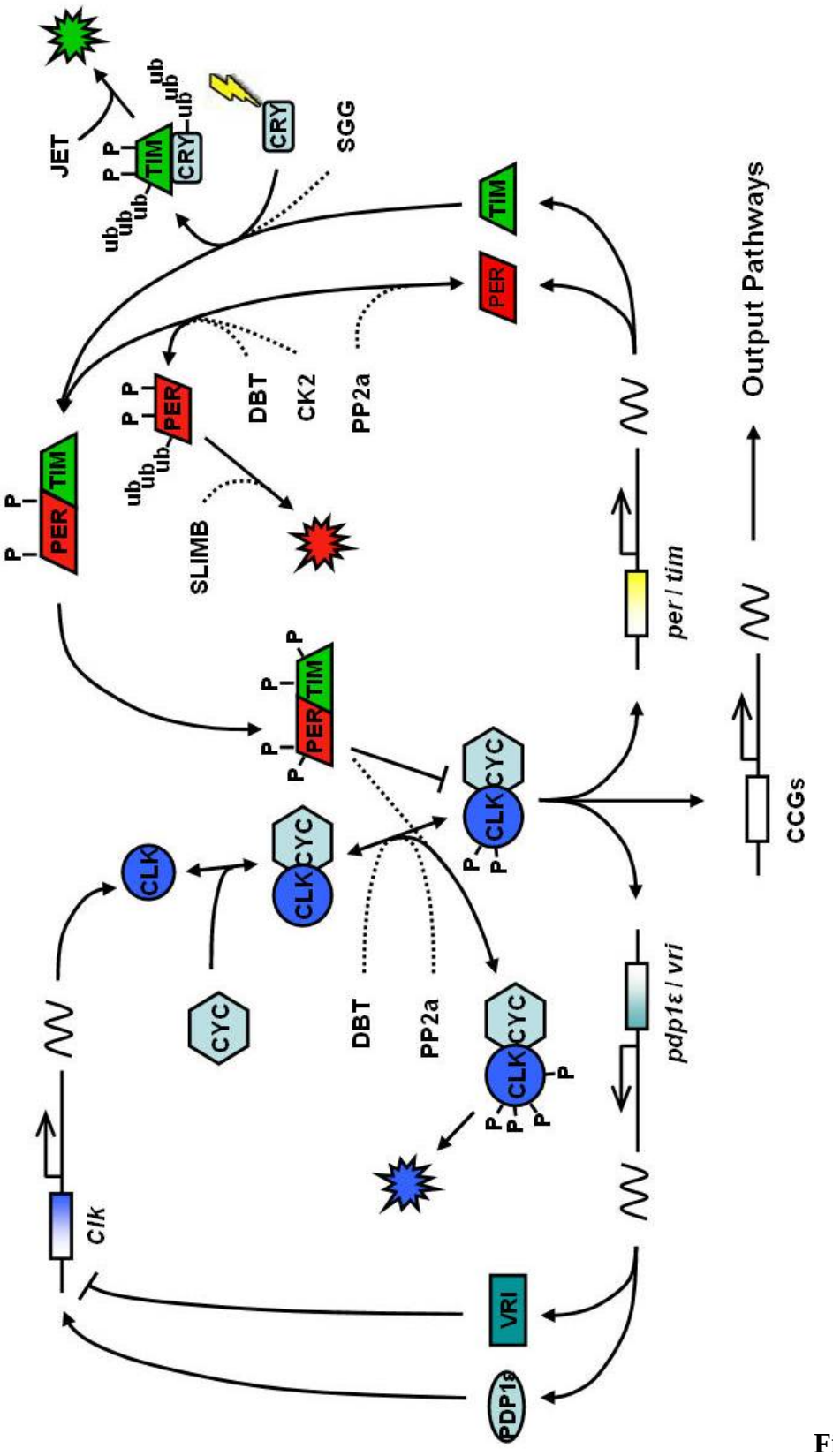
JET
CRY
SGG
TIM
PER
DBT
CK2
PP2a
SLIMB
CLK
CYC
Clk
VRI
PDP1ε
per / tim
pdp1ε / vri
CCGs
Output Pathways

Figure 1.2.

Figure Legend 1.2

Figure 1.2. Model for the circadian clock in *Drosophila melanogaster*. dCLK-CYC drives the transcription of *per*, *tim*, *vri*, and *pdp1ε*, which peak in the late evening/early night, when PER and TIM accumulate and form a heterodimer, an event that stabilizes both proteins and stimulates the nuclear-entry of the PER-TIM complex in the mid-night. PER is destabilized by DBT and CK2 phosphorylation, whereas TIM is destabilized following light-mediated activation of CRY. Destabilization of both PER and TIM in the cytoplasm during the day contributes to a delay in their nuclear accumulation. In the nucleus, PER and TIM interact with dCLK-CYC, and DBT acts in the same complex to hyperphosphorylate both dCLK and PER, inhibiting the complex from binding to E-Box elements, thereby blocking dCLK-CYC from stimulating transcription of *per*, *tim*, *vri*, *pdp1ε*, and other clock controlled genes (CCGs). On the other hand, both *vri* and VRI accumulate 3-6 hours earlier than *pdp1ε* and PDP1ε through an unknown regulatory mechanism, enabling the repression of *dClk* expression by VRI before PDP1ε out-competes VRI on the *dClk* promoter to drive *dClk* expression and dCLK accumulation. Line, pathways of the clock circuitry; dashed line, up-regulatory effects to the circuitry; Small P, phosphorylation; ub, ubiquitin.  , light.

hyperphosphorylation, a situation that contributes to the subsequent rise of *per* and *tim* mRNA levels around dawn, driving the next round of cytoplasmic PER and TIM accumulation.

In the interconnected dCLK loop, dCLK-CYC drives expression of *vri* and *pdp1ε*. VRI protein accumulates first and represses dClk expression. PDP1ε protein then peaks and out-competes VRI on the *dClk* promoter to activate *dClk* transcription after VRI-mediated repression ends in the middle of the night. *dClk* RNA peaks in the late night (27, 40). Post-translational regulation of dCLK is modulated by DBT and PP2A together with PER in such a way that the dCLK-CYC driven transcription of *per*, *tim*, *vri*, and *pdp1ε* does not occur until the dawn of the next day (68, 162).

Roles of Temperature and Day length (Photoperiod)

The axis on which the Earth turns has a tilt of about 23°27' relative to the earth's orbit plane around the sun. As a result of this tilt, there are seasonal changes in temperature and day length (photoperiod). Seasonal changes in temperature and day length profoundly affect the behaviors of many organisms. For example, seasonal affective disorder (SAD), a disease resulting from failing to adapt to short winter days, affects 2-5% of the general population in temperate climates (129) and can be treated with extra hours of exposure to broad- spectrum light (33). In *Arabidopsis thaliana*, the most important environmental condition that affects the timing of flowering is changes in day length (64).

As for *Drosophila melanogaster*, wild type flies show morning and evening peaks of activity separated in the day by a mid-day siesta time (see Figure 1.3). Under conditions of 12:12LD (daily cycles of 12 hours of light and 12 hours of darkness, with Zeitgeber Time 0 (ZT0) as light-on and ZT12 as light-off) at moderate temperatures, wildtype *D.*

melanogaster essentially exhibit bimodal daily activity patterns with one peak centered around ZT0 (‘morning’ peak) and another around ZT12 (‘evening’ peak) (85). Several years ago our lab showed that a thermosensitive intron in the *Drosophila per* mRNA 3’ UTR (17), termed the *Drosophila melanogaster per* intron 8 (dmpi8), affects the length of siesta time and the timing of evening activity onset (Figure 1.3). These flies exhibit delayed evening activity and thus a longer siesta time, when the ambient temperature rises. At low temperatures, the dmpi8 intron splices at enhanced efficiency to produce more of the spliced variant (type B’) of *per* mRNA, which leads to an earlier rise in *per* mRNA and PER protein. In transgenic flies that express only either the spiced variant or the unspliced variant (type A) of *per* mRNA, the cycling amplitudes of both *per* mRNA and protein are strongly dampened at low temperature compared with that of the wildtype. This leads to a slow accumulation of PER and delayed evening activity. Thus, the splicing efficiency of dmpi8 regulate the timing of the phase of the evening activity. Long photoperiods partially offsets cold-induced phase advances in the accumulation of *per* mRNA and protein by delaying the increase in TIM levels. The integration of PER and TIM ensure that signals regarding ambient temperature and light are properly interpreted and transduced to activity rhythms that are optimally associated with the prevailing environmental conditions. Namely, on seasonably cold short winter days, the clock-controlled evening behavior of fruit flies becomes increasingly more advanced and day-specific, while under warm long summer day conditions, their evening activity becomes more delayed and nocturnal with longer siesta time. The delayed activity under warm temperatures is likely adaptive, ensuring that flies avoid the deleterious effects of the hot mid-day hours.

Drosophila circadian oscillators show different light sensitivity to short light pulses

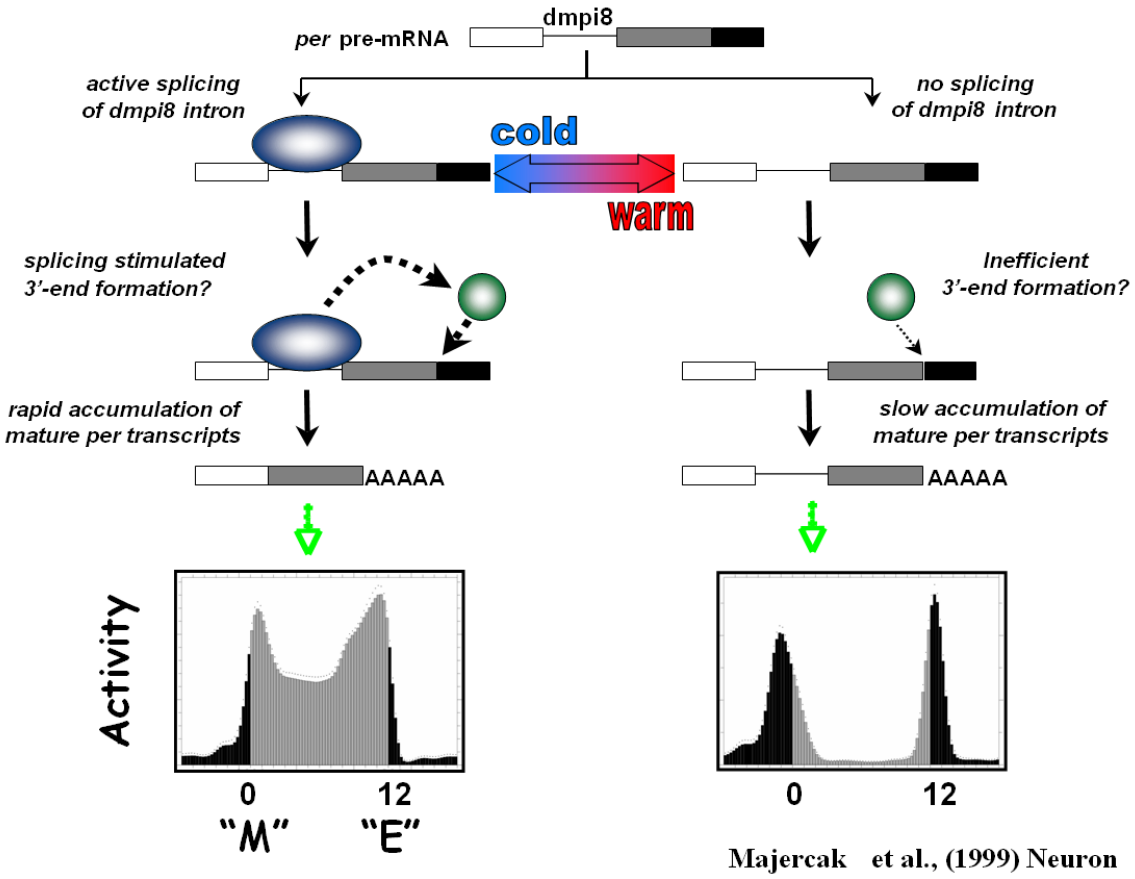

Figure 1.3. Model for how the thermosensitive splicing of dmpi8 contributes to seasonal adaptation of daily activity patterns in *Drosophila melanogaster*. Splicing of the 3' UTR intron of *per* can be stimulated at cold temperature, which presumably promotes 3'-end formation,leading to rapid *per* mRNA accumulation, an event that advances fly evening behavior ("E") onset and also reduces the siesta time between the morning ("M") and evening activity peaks. In the histogram, the black bars indicates night time activities and the gray bars indicates the day time activities.

depending on when the light pulses are given (5, 66). A phase response curve (PRC) is a plot of phase shifts as a function of circadian time when a stimulus such as light is administered (Figure 1.4). Typically PRCs of *Drosophila* circadian oscillators for light pulse show delay phase shifts in the early subjective night ("delay zone") and advance phase shifts in the late subjective night ("advance zone"), with little phase response occurring during the subjective day ("dead zone") (Figure 1.4).

Work described in this thesis provides further insights into how the temperature dependent splicing of dmpi8 regulates the daily activity patters of *D. melanogaster*. In addition, work described in this thesis shows that light stimulates *tim* transcription during cold days. Together, the work suggests a model whereby the *Drosophila* clock integrates temperature and photoperiod signals enabling flies to exhibit seasonably appropriate daily activity patterns.

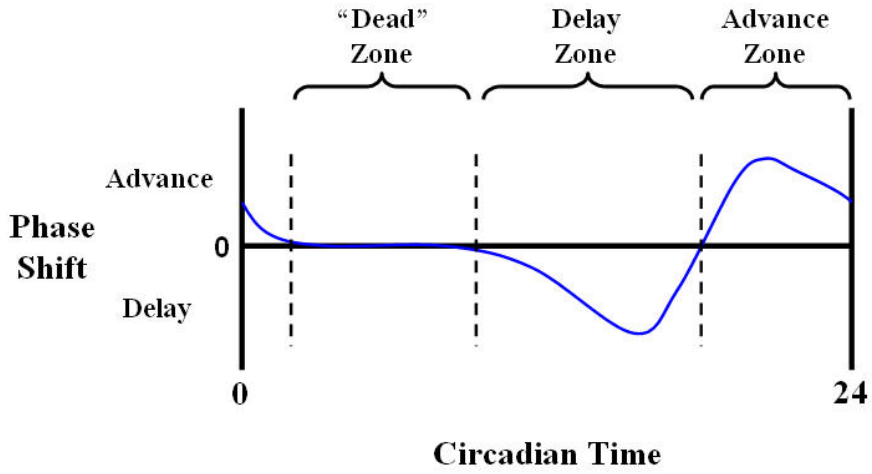

Figure 1.4. Schematic diagram of a typical light-induced phase response curve. In this example is shown a hypothetical phase response curve for the phase of *Drosophila* daily activity as a function of when light-pulses were administered. Groups of flies in darkness were given a short period of light exposure at different times during a day (circadian time from 0 to 24) with an untreated group of flies as phase control. The phase values for the treated flies were subtracted from those of the control flies and the magnitude of the shift [delay (negative) or advance (positive), Y-axis] was plotted relative to the time the pulse was administered (X-axis) to generate a phase response curve (blue line). Typically, phase response curves of *Drosophila* circadian oscillators for light pulse show delay phase shifts in the early subjective night ("delay zone") and advance phase shifts in the late subjective night ("advance zone"), with little phase response occurring during the subjective day ("dead zone").

Chapter 2. Splicing of *period* gene 3' terminal intron is regulated by light, circadian clock factors and phospholipase C

Introduction

Circadian rhythms are driven by cellular oscillators known as clocks or pacemakers and are an important aspect of the temporal organization observed in a wide range of organisms from bacteria to humans (32, 34). These clocks exhibit "free-running" (self-sustaining) periods of ≅24 hr in the absence of environmental cues. Nonetheless, an important adaptive feature of circadian oscillators is that they are synchronized (entrained) by daily changes in environmental modalities, most notably visible light and ambient temperature.

Light is almost certainly the predominant entraining agent in nature. In natural conditions the light-dark (LD) cycle aligns the phases of clocks and evokes daily adjustments in the approximately 24-hr endogenous periods of these oscillators such that they precisely match the 24-hr solar day. The duration of day length (photoperiod) can modify the temporal alignment between a circadian rhythm and local time (54). 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 (139). This makes intuitive sense because in temperate latitudes seasonal changes in day length are also accompanied by predictable changes in average daily temperatures. Thus, circadian clocks

play an important role in endowing organisms with the ability to anticipate daily and seasonal changes in environmental conditions, resulting in physiological and behavioral rhythms that occur at biologically advantageous times throughout the year. However, it is not clear how these pacemakers integrate environmental cues concerning seasonal changes in photoperiod and temperature.

Work using *D. melanogaster* has provided numerous insights into our understanding of the timekeeping mechanisms governing circadian rhythms (recently reviewed in (2, 45, 130). In *Drosophila melanogaster* four circadian clock proteins termed PERIOD (PER), TIMELESS (TIM), dCLOCK (dCLK) and CYCLE (CYC/dBMAL1) function in a transcriptional-translational feedback loop that is a core element of the oscillator mechanism in this species. dCLK and CYC are members of the basic helix-loop-helix (bHLH)/PAS (PER-ARNT-SIM) superfamily of transcription factors that heterodimerize to activate *per* and *tim* expression (3, 29, 119). Transcription of *per* and *tim* begins in the early-to-mid day resulting in peak mRNA levels being reached in the early night. However, the daytime accumulation in the levels of PER and TIM proteins is slow. This is partly because the DOUBLETIME (DBT) kinase promotes the phosphorylation and degradation of PER (70, 111), and light stimulates the turnover of TIM (63, 101, 163). Eventually, as the levels of *per* and *tim* transcripts increase during the late-day TIM and PER reach threshold concentrations that enable them to interact, a process that protects PER against DBT-induced degradation (70, 111) and presumably facilitates the nuclear accumulation of PER and TIM (however see, (125). Once in the nucleus PER/TIM interact with the dCLK-CYC transcription factor blocking its activity (7, 29, 76, 77). Reductions in the concentrations of PER/TIM in the nucleus relieves autoinhibition of dCLK-CYC,

beginning another round of *per*/*tim* expression and in an interconnected loop repression of *dClk* transcription (27, 40, 41).

We recently used *D. melanogaster* as a model system to understand the molecular underpinnings governing how changes in daily temperatures modulate activity rhythms. The 'evening' activity peak of *D. melanogaster* exposed to daily light-dark cycles progressively advances from mainly nocturnal to predominately late-day as ambient temperature is decreased (85). We showed that splicing of an intron in the 3' untranslated region (UTR) of *per* RNA (dmpi8; *D. melanogaster per* intron 8) is a key aspect of how the *Drosophila* circadian clock adapts to changes in temperature. At low temperatures relatively more of the spliced variant (type B') is present compared to the unspliced variant (type A). The enhanced removal of dmpi8 at low temperatures leads to an advance in the timing of the *per* mRNA and protein accumulation phases during the day. Because the interaction of PER with TIM is a key event in the progression of the clock, a more rapid increase in the abundance of PER shortens the time necessary to attain threshold concentrations that favor interactions with TIM, contributing to advanced molecular cycles and the preferential daytime activity of flies on cold days. At cold temperatures, mutations that either abrogate splicing (per^{A} mutant) or remove dmpi8 ($per^{B'}$ mutant) both cause low amplitude cycles in *per* mRNA levels and delayed activity rhythms. These results suggested that active splicing of dmpi8 *per se* as opposed to the presence or absence of the intron is the relevant molecular event mediating the cold-enhanced daily increases in the abundance of *per* mRNA.

How light regulates the clock and the daily distribution of activity in *Drosophila* is not fully understood and is likely to be complex, involving various ocular and extraocular

photoreceptors (37, 61, 115, 145). Early work clearly established an important role for the light-induced degradation of TIM as a key primary clock-specific photoresponse in the entrainment of *Drosophila* clocks to daily light-dark cycles (63, 78, 101, 138, 158, 163). The putative blue-light photoreceptor CRYPTOCHROME (CRY) enhances the light-induced degradation of TIM in most, if not all, clock cells (44, 61, 65, 67, 131, 145). CRY functions as a deep-brain photoreceptor (37) that apparently directly interacts with TIM (13), presumably leading to the rapid ubiquitination and destruction of TIM by the proteasome (102). Circadian photosensitivity is altered in the cry^{b} mutant, which has severely reduced levels/activity of CRY (13, 35-37, 61, 131). Besides a role in transducing photic signals, CRY also functions as a *bona fide* clock element in some peripheral circadian pacemakers (65, 73, 131). Although physiologically or anatomically blind flies manifest robust activity rhythms (e.g., (59, 145, 149, 154), more recent work has supported a role for classic visual signal transduction pathways in the light regulation of daily activity rhythms (e.g., (115) and refns. therein). For example, a mutation in *no-receptor-potential-A* (*norpA*), which encodes phospholipase C (PLC/NORPA), is associated with variant patterns in the clock-controlled evening activity (46, 154). As mentioned above, temperature (85) and in addition mutations in *per* that shorten or lengthen the periodicities of free-running behavioral rhythms, also manifest alterations in the timing of evening activity during entrainment to light-dark cycles (46).

In this report we show that temperature, light and the clock interact to regulate dmpi8 splicing. In short photoperiods the daily proportion of the spliced type B' variant is enhanced and accumulates earlier, contributing to increases in the daily upswing and peak levels of *per* mRNA. Thus, both short day lengths and low temperatures, which are

normally associated in natural conditions, stimulate splicing of dmpi8. Increases in temperature lead to lager amplitude declines in daytime splicing, a response that is regulated by the clock. This more pronounced clock dependent inhibition of splicing as temperatures rise likely contributes to the manifestation of mainly nocturnal activity on warm days, enabling flies to avoid the deleterious effects associated with daytime heat. The proportion of the type B' variant is abnormally high in the *norpA* mutant, and exhibits little daytime decreases even on warm days. Thus, it appears that irrespective of temperature the splicing behavior of dmpi8 in *norpA* flies exhibits characteristics that are normally observed only at low temperatures, consistent with the mutants advanced evening activity even on warm days. The findings suggest a novel non-photic role for PLC in transducing the effects of temperature on the splicing behavior of the dmpi8 intron. Our findings suggest that the dual thermal and photoperiodic regulation of dmpi8 splicing acts as "seasonal sensor" encoding *calendar* information.

Materials and Methods

Fly strains and collections

The wildtype (CS), *per*^{01}, *tim*^{0}, *cry*^{b}, and *norpA* flies used in this study were previously described (3, 72, 119, 123, 131). All the flies used in this study were wildtype for eye and body pigmentation. All flies were reared at room temperature (22-25°C) and maintained in vials containing standard agar-cornmeal-sugar-yeast-tegosept media. Vials containing ~100 young (2-6 day old) adult flies were placed in controlled environmental chambers (Percival) at the indicated temperature (18° or 29°C) and exposed to at least four 24 hr photoperiods of alternating light:dark cycles [LD; where zeitgeber time 0 (ZT0) is defined as lights-on], and in some cases subsequently maintained in constant darkness (DD). Cool white fluorescent light (~2000 lux) was used during LD and the temperature did not vary by more than 0.5°C between the light and dark periods. At selected times during LD and DD flies were collected by freezing.

Locomotor activity

Locomotor activity was continuously monitored and recorded in 30-minute bins by placing individual adult flies (three to seven day-old males) in glass tubes and using a Trikinetics (Waltham, MA) system interfaced with an Apple computer as previously described (46). Fly activity data was analyzed using the Periodogram and Phase analysis software available from the web-based Brandeis Rhythm Package (http://hawk.bcm.tmc.edu/brp/brp.cgi). The Phase program was used to calculate the time in a day (in hr relative to ZT0) for the onset (50% of peak) and peak of the "evening"

activity for each individual fly (Table 2.1). Values for individual flies were pooled to obtain an average value for each genotype and temperature. Pair-wise comparisons were performed using student's *t*-test.

RNase protection assay

For each time point, total RNA was extracted from ~30 μl of fly heads using TriReagent (Sigma) and following manufacturer's recommended procedure. The total (type A and type B') levels of *per* transcripts were determined by RNase protection assays using the *per* 2/3 probe (52). In addition, we also used RNase protection assays to measure the relative levels of type A and type B' *per* RNA variants using a novel probe called *per*3'UTR. *per*3'UTR was generated by using PCR to amplify a genomic region of *per* that contains nucleotides 7009 to 7308 [numbering according to (19)]. The oligonucleotides used in the PCR were P7009 (5' ACCGAGCACCAGCCAGT 3') and P7308 (5' TTAGGGCTGAGAAGGGTGC 3'). The PCR product was subcloned into the pGEM-T easy vector (Promega) and linearized with Spe1, and antisense probe produced *in vitro* by using T7 RNA polymerase in the presence of [α-^{32}P]UTP as previously described (85). The antisense probe protects a *per* RNA region containing the 3' most 62 nt of dmpi8 followed at the 3' end of dmpi8 by the next 238 nt of flanking exon. Thus, in the presence and absence of dmpi8, the protected radiolabeled band is 300 and 238 nt long, respectively. As a control for RNA loading in each lane, a ribosomal protein probe (RP49) was included in each protection assay (52). Protected bands were quantified using a Phosphorimager from Molecular Dynamics, and values were normalized relative to those of RP49 and for uridine content.

RT-PCR splicing assay

The relative levels of the type B' and type A *per* RNA variants were measured using a reverse transcriptase-PCR (RT-PCR) assay based on a method previously described (85). For each time point, total RNA was extracted from ~30 μl of fly heads using TriReagent (Sigma) and following manufacturer's recommended procedure. Approximately 2 μg of total RNA was incubated in a final volume of 20 μl and reverse transcription performed using oligo$(dT)_{20}$ as a primer and the ThermoScript RT-PCR Kit from Invitrogen, according to the manufacturer's recommended procedure. A 2 μl aliquot of the reaction was further processed by PCR in a final volume of 50 μl using the *per*-specific primers P7197 (5' TCTACATTATCCTCGGCTTGC 3') and P6869 (5' TAGTAGCCACACCCGCAGT 3'). This amplified a region of the 3' UTR of *per* from 6869 bp to 7197 bp (numbering according to refn. (19) (Figure 2.1A). To control for sample-to-sample differences in total RNA we also included primers for the non-cycling mRNA encoding for Cap Binding Protein 20 (CBP20). Prior work using DNA microarrays has shown that the mRNA levels for CBP20 are constant throughout a daily light-dark cycle (14); and see, http://expression.gnf.org/cgi-bin/circadian/index.cgi) (see also Figure 2.1A). We further chose *cbp20* RNA as an internal control because the levels of *per* and *cbp20* mRNAs are in a similar range in total head extracts, as inferred by staining intensities following RT-PCR done in the exponential phase (see Figure 2.1A). The primers used in the RT-PCR for amplification of *cbp20* sequences were CBP495R (5' CAACAGTTTGCCATAACCCC 3') and CBP362F (5' GTCTGATTCGTGTGGACTGG 3'). This amplified a region of *cbp20* from 362 bp to 495 bp (numbering according to GeneBank Acc. No. NM079672). PCR products were separated and visualized by

electrophoresis on 2% agarose gels followed by staining with Gelstar^{TM} (Cambrex Co.), and the bands quantified using a Typhoon 9400 Imager. The two *per* band intensities were normalized for product length differences and relative to *cbp20* levels. Two *per*-specific bands of the expected sizes for PCR products that either contain dmpi8 (329-bp) or where the dmpi8 intron is removed (240-bp) were detected in total RNA following RT-PCR (see Figure 2.1A). In addition, one *cbp20*-specific band of the expected size (134-bp) that had relatively constant intensity irrespective of time-of-day was detected (see Figure 2.1A).

Numerous control experiments were performed to ensure the accuracy of the results, including the following: (1) To verify that our assays were performed in the exponential phase of PCR for *per* amplicons, we collected wildtype flies at different times during a daily light-dark cycle, and subjected aliquots from the reverse transcription reaction to PCR ranging in cycle length from 24 to 28. Under the conditions used, all these cycle lengths resulted in curves for *per* RNA levels that have indistinguishable amplitudes, peak times and overall shapes as a function of time in a daily cycle (data not shown). Importantly, the results we obtained using RT-PCR are similar to those using RNase protection assays (e.g., compare Figure 2.1B and E). Furthermore, as expected from the linear dose-response for *per* RNA staining intensities using these different PCR cycle lengths, the different reactions yielded splicing ratios (i.e., relative molar ratio of type B' to type A) that were very similar, and similar to those using RNase protection assays. Control experiments using wildtype flies collected throughout a daily cycle and varying PCR cycle length were performed at least 3 independent times with very similar results (data not shown). In addition, for each independent experiment, several of the first-strand synthesis reactions (usually expected trough and peak values for wildtype flies) were subjected to at

least two different PCR cycle lengths (usually 24 and 26) to verify that *per* amplicon levels were in the linear range. We also routinely included as part of our experiments separate reactions with serial dilutions ranging from 10 to 50-fold differences in *per* RNA concentration to ensure linear band intensities. (2) We included *cbp20* mRNA as an internal non-cycling RNA control during the RT-PCR (see above). Between 24 and 28 PCR cycle lengths, both the *per* and *cbp20* amplicons are in the exponential phase (see Figure 2.1A and data not shown). (3) Similar ratios of type B' to type A were obtained when reverse transcription was performed with either oligo$(dT)_{20}$ or P7308 (see above) as primers. (4) No *per*-specific or *cbp20*-specific amplicons were detected when the RT-PCR was performed in the absence of reverse transcriptase (see Figure 2.1A) or gene-specific primers. (5) The two *per*-specific RT-PCR bands were extracted from gels and sequenced to verify that they were produced from type A and type B' *per* variants (data not shown). (6) We always included wildtype control flies whenever mutant flies were analyzed, and RNA samples were processed contemporaneously. (7) Finally, we optimized conditions by using for example, several different primers, incubation temperatures, reverse transcriptases, and amounts of total RNA or cDNA. For example, more consistent results were obtained using ThermoScript instead of SuperScript reverse transcriptase for cDNA synthesis, possibly because the higher temperature during the incubation (55° versus 42°C) minimizes RNA secondary structure.

Results

Photoperiodic and clock regulation of dmpi8 splicing

We previously showed that in wildtype flies entrained by standard 12h light:12h dark cycles [12:12LD; where zeitgeber time 0 (ZT0) is lights-on] cold temperatures stimulate the relative splicing of dmpi8 (i.e., relative molar ratio of type B' to type A *per* variants), which underlies the earlier daily upswing in total *per* mRNA abundance and higher peak values (85) (see also Figure 2.7A and B). Importantly, it is the more rapid accumulation phase of *per* mRNA that contributes to the advanced evening activity of *Drosophila* on cold days (85). In temperate regions low daily temperatures are normally associated with short day lengths.

To determine whether changes in photoperiod also regulate dmpi8 splicing, wildtype Canton-S (CS) flies were kept at 18°C and exposed for several days at two different photoperiods (6:18LD and 12:12LD) (Figure 2.1). First, we assayed the effects of variations in photoperiod on total *per* mRNA levels. RNA levels were mainly determined by semi-quantitative RT-PCR (Figure 2.1A) but similar results were obtained by RNase protection assays (e.g., compare Figure 2.1B and E; and see Material and Methods). To control for sample-to-sample variations in the RT-PCR we co-amplified the non-cycling *cbp20* mRNA and normalized *per* values (Figure 2.1A; see Materials and Methods). Consistent with our previous findings (85), total *per* mRNA levels accumulated earlier during the day and reached higher peak values as a function of shortening the duration of the photoperiod (Figure 2.1B and E). An earlier accumulation phase for *per* mRNA levels under shorter photoperiods was also previously observed at the standard temperature of

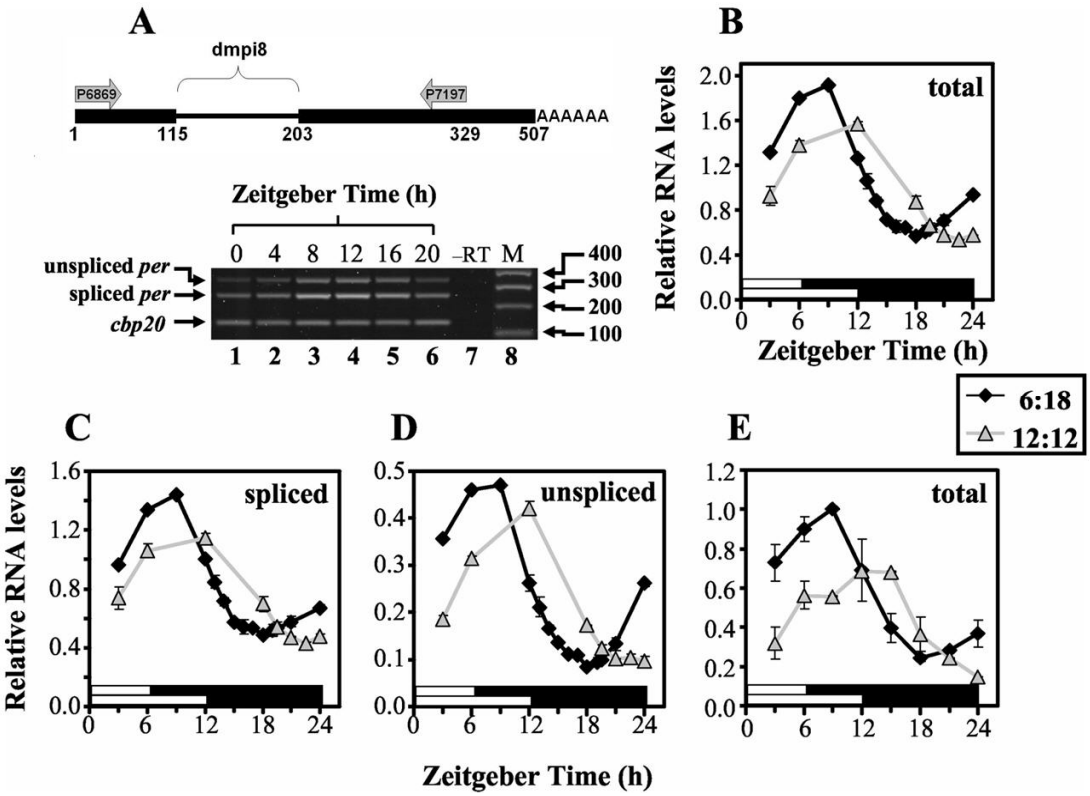

Figure 2.1. Photoperiod affects the waveform of the *per* mRNA abundance profile. Wild-type (CS) flies were kept at 18°C, exposed to 4 days of different photoperiods (6:18LD or 12:12LD as indicated), and collected during the last day of LD. RT-PCR (A to D) or RNase protection assays (E) were used to measure the relative levels of total (i.e., type A and type B' *per* variants together) (B and E), dmpi8-spliced type B' (C), and dmpi8-unspliced type A (D) *per* transcripts in head extracts. For each time point, values shown are from at least two independent experiments. (A) Schematic diagram of RT-PCR dmpi8 splicing assay using two *per* primers (P6869 and P7197) (top of panel; numbers indicate distance from start of *per* 3' UTR). Also shown (bottom panel) is a representative agarose gel showing the *per* (dmpi8 unspliced and spliced) and *cbp20* RT-PCR products from flies collected at different times during 12:12LD (lanes 1 to 6); lane 7, control sample in the absence of RT; lane 8, DNA size markers (M). (B to E) White horizontal bar, lights on; black horizontal bar, lights off.

25°C (113).

We next analyzed the spliced type B' (Figure 2.1C) and unspliced type A (Figure 2.1D) *per* RNA variants individually. Irrespective of day length, at 18°C the majority of *per* mRNA transcripts are type B' (Figure 2.2) (85). The relative abundance of type B' to type A (type B'/type A) at 18°C ranges from ~2- to 4.5-fold higher at the different photoperiods analyzed (Figure 2.2-6). In more than 10 independent experiments we observed that in wildtype flies the proportion of type B' transcripts changes as a function of time in LD cycles (e.g., see Figure 2.2-6). At all photoperiods analyzed (i.e., 6:18LD, 9:15LD and 12:12LD), daily trough and peak values in the ratio of type B' to type A were significantly different ($P<0.0001$ by student's *t*-test; $n = 4$) (Figure 2.2 and data not shown). The most consistent trends in dmpi8 splicing oscillation are that the proportion of the spliced type B' variant are overall lower during the day, and as photoperiod is shortened it accumulates earlier and reaches higher peak values in the night (Figure 2.2). These results suggest that the daily mean splicing of dmpi8 is more efficient under short photoperiods, consistent with the earlier accumulation phase and higher levels of total *per* mRNA (Figure 2.1B and E).

At low temperatures oscillations in the daily proportion of type B' to type A *per* transcripts are observed in constant dark conditions, albeit with dampened amplitudes (Figure 2.3A and C). The dampening in this molecular cycle is mainly attributable to a lowering of peak values in the type B'/type A ratio during constant dark conditions, whereas trough values remain quite constant (Figure 2.3C). Furthermore, trough values remain lower in flies entrained to longer photoperiods even after two days in DD (Figure 2.3C; $P<0.0001$ by student *t*-test for comparison of average trough values during last day

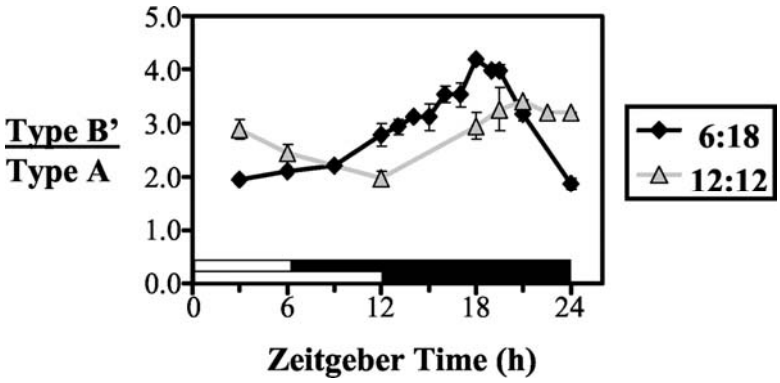

Figure 2.2. Effects of photoperiod on the relative splicing efficiency of dmpi8. Shown are the relative levels of type B' to type A *per* variants as a function of changes in day length. The data are derived from the results shown in Figure 2.1B to D. A significant difference was found on comparison of peak to trough values for either 6:18LD or 12:12LD ($P<0.0001$ by Student's *t* test). White horizontal bar, lights on; black horizontal bar, lights off.

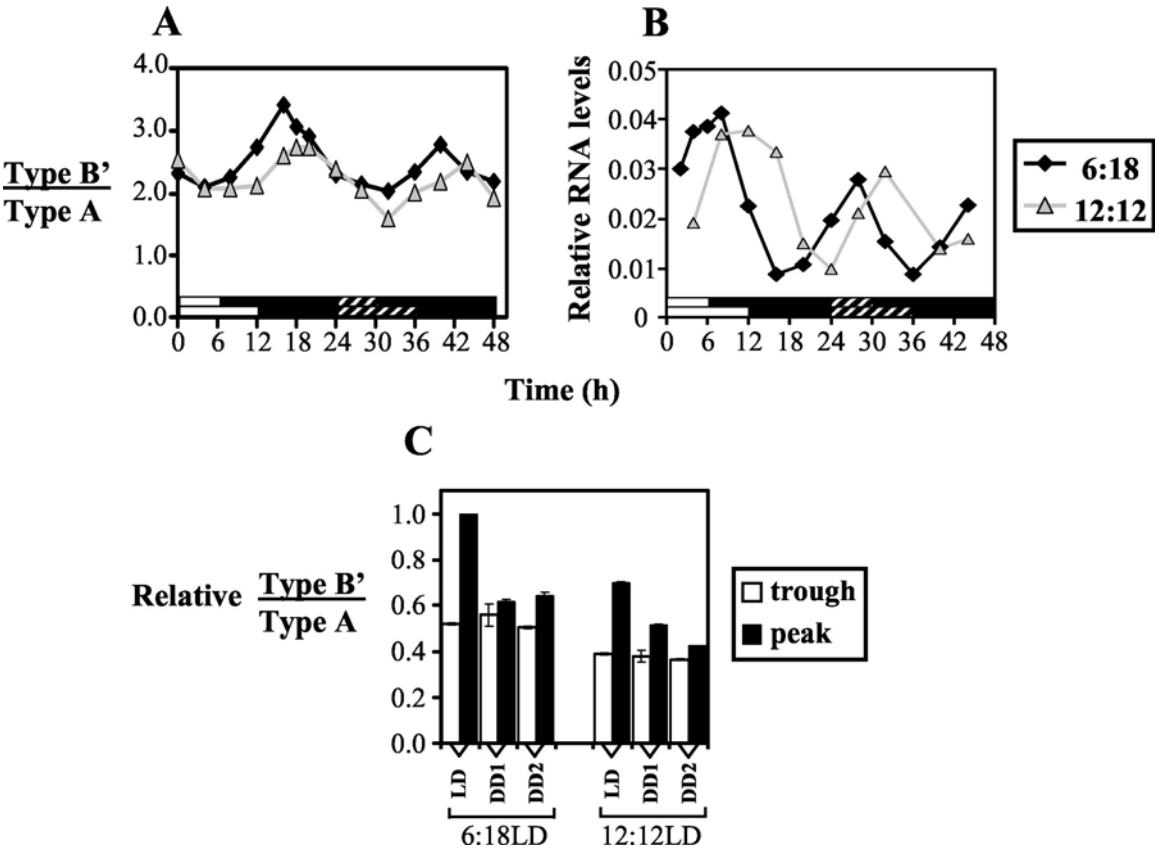

Figure 2.3. Dampening of cycles in dmpi8 splicing levels under DD conditions at cold temperatures. Wild-type (CS) flies were exposed to 4 days of either 6:18LD or 12:12LD at 18°C followed by DD conditions. Flies were collected at different times during the last LD cycle and DD. (A and C) RT-PCR was used to determine the relative levels of spliced (type B') and unspliced (type A) *per* RNA. (B) Total *per* mRNA levels (type A and type B') were measured using RNase protection assays. The data in panels A and B are representative of results obtained in at least two independent experiments. (C) Peak levels in the type B'/type A ratio for 6:18LD were set to 1, and the rest of the values were normalized to show relative peak and trough amounts during either 6:18LD or 12:12LD (indicated at bottom of the panel) followed by the next 2 days of DD (DD1 and DD2). The data are an average of two independent experiments. A P value of <0.0001 was determined by Student's *t* test on comparison of average trough values during the last day of LD and first 2 days of DD between 12:12LD- and 6:18LD-entrained flies.

of LD and first two days of DD between 12:12D and 6:18LD entrained flies), suggesting that day length has long-range indirect effects on setting the basal splicing efficiency of dmpi8. A likely mechanism for how the duration of day (or night) is 'remembered' during constant dark conditions is via stable changes in the dynamics of the clock as a function of changes in photoperiod length. In any event, the results demonstrate circadian regulation of the daily changes in the splicing of dmpi8. Indeed, irrespective of temperature and photoperiod the overall daily proportion of the spliced type B' variant in several arrhythmic circadian clock mutants tested (i.e., per^{01} and tim^{0}) was at or below trough values observed for wildtype control flies (Figure 2.4A and B; and data not shown). It is possible that the decreased splicing of dmpi8 in the clock impaired flies contributes to the low-to-intermediate levels of *per* mRNA previously observed in these mutants (3, 52, 119, 123); and data not shown). The results also suggest that the clock (or clock components) has a significant influence on the mean splicing efficiency of dmpi8 throughout a daily cycle.

In wildtype flies, higher amplitude rhythms in the proportion of type B' spliced transcripts occur at warmer temperatures, despite the overall lower splicing levels (compare Figure 2.4A to B and C). At 29°C the dmpi8 daily splicing profile is characterized by a relatively steep daytime decline followed by a nighttime upswing that peaks toward the end of the dark phase. There are two notable differences in the daily splicing rhythm of dmpi8 when comparing cold and warm days. First, there is a larger magnitude decline in daytime splicing at warm temperatures. Second, at the higher temperature the splicing rhythm continues with a robust amplitude in the first day of constant darkness, in sharp contrast to the rapid dampening observed at the colder

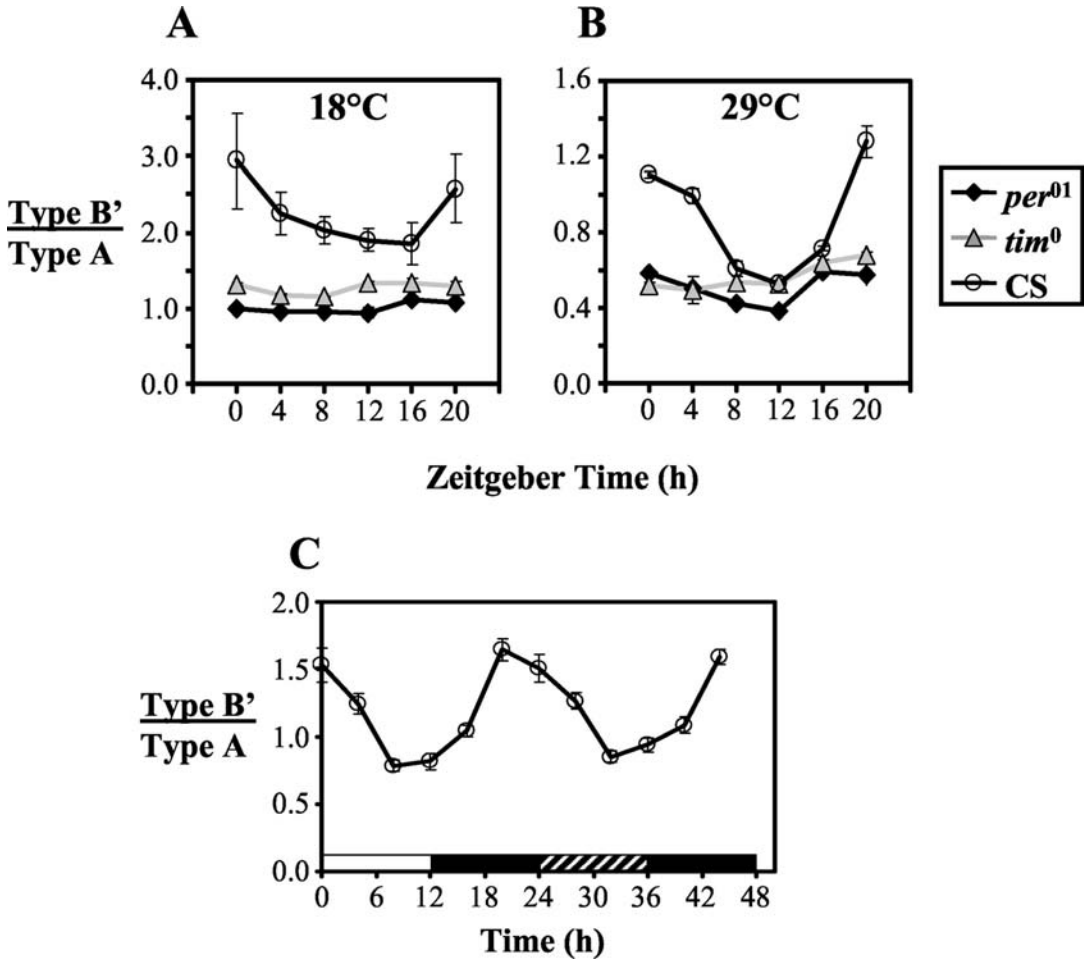

Figure 2.4. Lower splicing of dmpi8 in arrhythmic clock mutants. (A and B) Wild-type (CS) and clock mutant flies (*per*^{0} and *tim*^{0}) were exposed to 4 days of 12:12LD at either 18°C (A) or 29°C (B). Comparisons of peak to trough values resulted in the following P values: *per*^{01} at 18 and 29°C, P<0.01; *tim*^{0} at 18 and 29°C, P<0.03. (C) In separate experiments, CS flies were kept at 29°C and collected on the last day of 12:12LD and the first day of DD. The data are derived from an average of two independent RT-PCR experiments.

temperature (compare Figure 2.3A to Figure 2.4C). The results suggest a more pronounced role for the clock in repressing splicing during daytime on warm days.

Despite the low daily mean splicing of dmpi8 in the *per*^{01} and *tim*^{0} mutants, overall daytime levels in the proportion of type B' transcripts were lower compared to nighttime values during light-dark cycles, especially at 29°C (Figure 2.4A and B; see figure legend for P values showing significant peak-to-trough differences). We did not observe any significant changes in the type B'/type A ratio when the clock mutants were analyzed in constant dark conditions (data not shown), suggesting that light decreases the splicing efficiency of dmpi8 in a manner that does not require a functional clock and that might be enhanced by increases in temperature (see Figure 2.5).

Light acutely inhibits the splicing of dmpi8

To further examine whether light has acute effects on the splicing efficiency of dmpi8, wildtype and clock mutant flies were entrained to LD cycles and exposed to light-pulses administered at different times during the dark phase (Figure 2.5). We noted small but reproducible decreases in the proportion of the spliced type B' variant by nocturnal light-pulses, a response that was observed at all temperatures (i.e., 18°, 25° and 29°C) and photoperiods (6:18LD and 12:12LD) (Figure 2.5; see figure legend for P values; and data not shown). Brief light-pulses did not reduce dmpi8 splicing below the daytime trough levels observed during the preceding light-dark cycle (data not shown and see Figure 2.5B). Thus, it appears that the prevailing conditions of photoperiod and temperature set a basal amount of dmpi8 splicing efficiency that is refractory to acute inhibition by light. This likely explains why we observed reproducible time-of-day differences in the acute photoinhibition of dmpi8 splicing, whereby light-pulses given

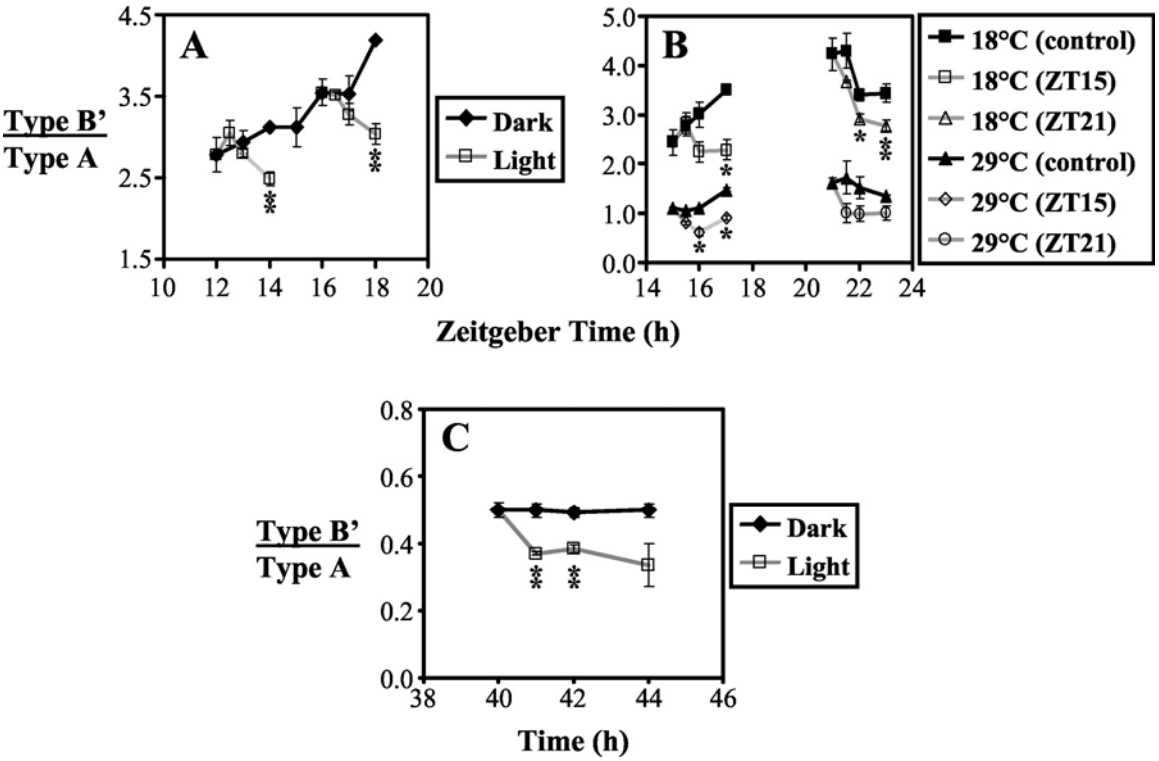

Figure 2.5. Light acutely inhibits the splicing of dmpi8 in wild-type and clock mutant flies. (A and B) Wild-type (CS) flies were kept at either 18°C (A and B) or 29°C (B only) and exposed to 4 days of either 6:18LD, with light beginning at ZT12 or ZT16 (A) or 12:12LD, with light beginning at either ZT15 or ZT21 as indicated (B). On the dark phase of the last LD, groups of flies were exposed to light beginning at different times; other groups served as nontreated controls. (C) *per*^{01} flies were exposed to 12:12LD cycles at 29°C, and on the first day of DD groups of flies were exposed to light beginning at 40 h (time from the last dark-to-light transition at ZT0); other groups served as nontreated controls. The data shown are derived from an average of three independent RT-PCR experiments. Symbols: *, P<0.01; **, <0.05 using Student's *t* test for comparison between light-pulsed and control values. Similar photoinhibition of dmpi8 splicing was also obtained in wild-type flies exposed to 12:12LD at 25°C (data not shown).

during the first half of the night when the proportion of type B' is increasing generally evoked larger magnitude decreases (Figure 2.5B).

During the late-night the proportion of dmpi8 spliced transcripts are already decreasing in wildtype flies and therefore during this phase differences between untreated and light-treated samples are minimized. As a result the clock modulates the magnitude of the light inhibition in a time-of-day specific manner. The ability of light to evoke decreases in the proportion of type B' *per* transcripts is in agreement with the overall lower dmpi8 splicing during the day and higher peak amounts during short photoperiods (Figure 2.2 and Figure 2.4). Importantly, nocturnal light-pulses evoked rapid decreases in dmpi8 splicing in per^0 and tim^0 flies where possible influences from circadian control are eliminated (Figure 2.5C and data not shown).

In summary, the roles of the clock and light in regulating dmpi8 splicing are complex and vary as a function of photoperiod and temperature. Short photoperiod advance the daily upswing in the proportion of the spliced type B' variant and higher peak values are obtained, responses that are likely largely mediated via the effects of day length on the dynamics (phase and amplitude) of the clock. Light also has acute effects, inhibiting the splicing efficiency of dmpi8. The shape of the daily dmpi8 splicing rhythm is strongly modulated by temperature. The 'cold splicing phenotype' is characterized by high overall splicing with relatively low amplitude cycling that dampens quickly in constant dark conditions. Conversely, the 'warm splicing phenotype' is characterized by low overall splicing and a very pronounced clock-mediated daytime decline, a pattern that can persist in the absence of light-dark cycles.

Distinct roles for phospholipase C and CRY in the regulation of dmpi8 splicing

To investigate a possible role for CRY in the photoregulation of dmpi8 splicing we used the *cry*^{b} mutant, which is thought to either severely inhibit or completely inactivate CRY activity (35, 37, 131). The low daily splicing efficiency of dmpi8 in *cry*^{b} flies (Figure 2.6A and B) implies that a different photoreceptor(s) is mediating the inhibitory effects of light. Indeed, daytime decreases in the relative proportion of type B' transcripts still occur in the *cry*^{b} mutant ($P<0.03$ by student *t*-test for comparison of peak to trough values at either 18°C or 29°C). However, completely ruling out any photoreceptor function for CRY in the regulation of dmpi8 is complicated by virtue of the fact that the *cry*^{b} mutation also inactivates circadian regulation in some peripheral clocks (65, 73, 131). Also, because the majority of *per*-expressing clock cells in total head extracts are derived from ocular photoreceptors, this would mask a possible photoreceptor role for CRY in the small number of key brain pacemaker neurons that control activity rhythms. Although at present we cannot attribute a causal relationship, the levels of *per* mRNA are constantly low in head extracts from *cry*^{b} flies (131); and data not shown), consistent with the lower relative splicing efficiency of dmpi8.

The *norpA*^{P41} mutant has been instrumental in revealing novel roles for visual phototransduction pathways in different aspects of how light regulates daily activity patterns in *Drosophila* (e.g., (37, 61, 65, 67, 131). In this regard it is interesting to note that the splicing ratio of dmpi8 is essentially constantly high in the *norpA*^{P41} mutant (Figure 2.6A and B). Although maximal dmpi8 splicing is higher in *norpA* flies compared to wildtype flies, the most dramatic alteration is that daytime values remain high ($P>0.3$ for peak to trough comparisons at both temperatures). The overall high splicing with

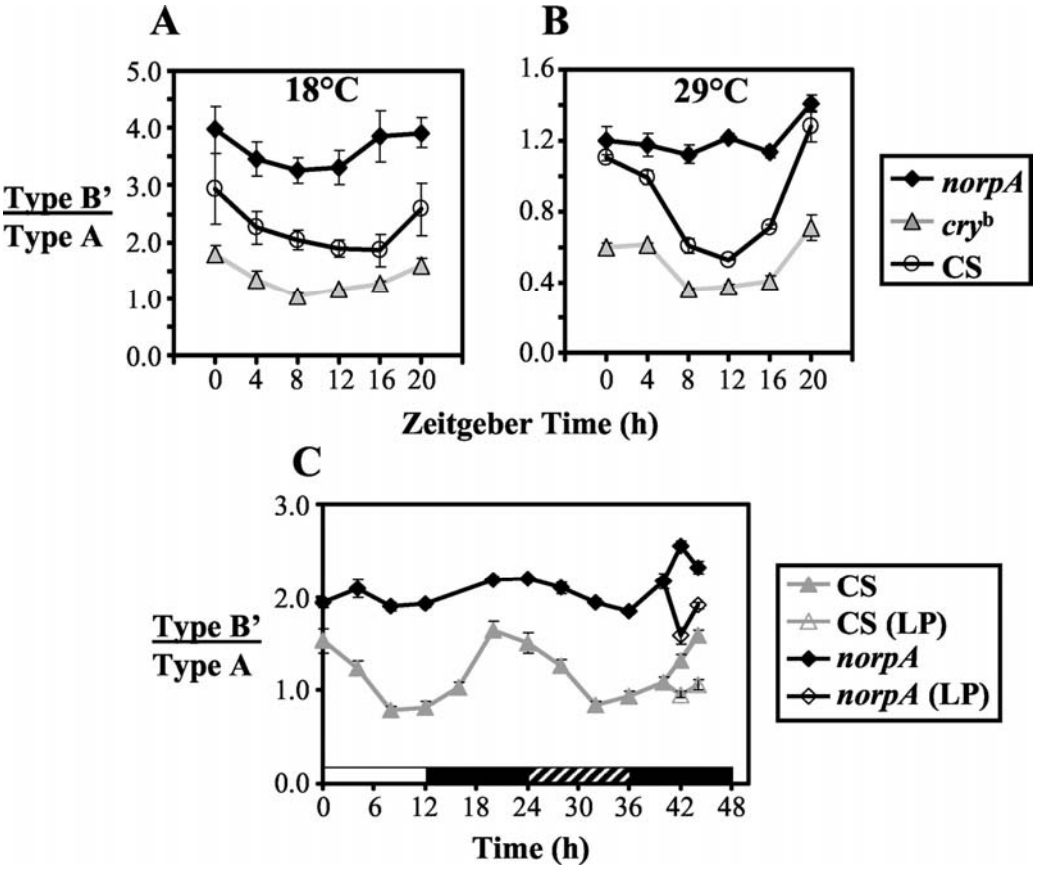

Figure 2.6. Effects of *cry*^{b} and *norpA* mutants on splicing of dmpi8. (A and B) Wild-type (CS) and mutant flies (*cry*^{b} and *norpA*^{P41}) were exposed to 4 days of 12:12LD at either 18°C (A) or 29°C (B). The RNA samples were from fly collections done at the same time as those shown in Figure 2.4. Comparison of peak to trough values in *norpA* flies at both temperatures did not show a significant difference (P>0.3 by Student's *t* test). Similar results were obtained in at least four other experiments (data not shown). (C) In separate experiments, CS and *norpA* flies were kept at 29°C and collected on the last day of 12:12LD and the first day of DD (the CS splicing profile is the same as that shown in Figure 2.4B). In addition, on the first day of DD, groups of CS and *norpA* flies were exposed to light beginning at 40 h (time from the last dark-to-light transition at ZT0); other groups served as nontreated controls. For each genotype, comparison of light-treated (open symbols) and control samples (closed symbols) showed significant differences (P<0.05; Student's *t* test). LP, light pulse-treated samples.

low-amplitude cycling is maintained in constant dark conditions even at 29°C (Figure 2.6C; and data not shown), indicating that the *norpA* mutation plays a role in the regulation of dmpi8 splicing that is independent of the acute effects of light. Indeed, light-pulses can still evoke rapid inhibition of dmpi8 splicing in *norpA* flies (Figure 2.6C), revealing that PLC is not a critical or sole component of the photic-transduction pathway leading to the light inhibition of dmpi8 splicing. Rather, the findings strongly suggest that the *norpA* mutation strongly attenuates the robust clock-regulated daytime decrease in the proportion of the spliced type B' variant normally observed at high temperatures. Otherwise stated, it appears that inactivation of PLC 'locks' the splicing behavior of dmpi8 into a pattern characteristic of that observed on cold days; e.g., high overall splicing with low amplitude rhythm (see Figure 2.9). A greater proportion of the type B' variant is still observed at 18°C compared to 29°C (Figure 2.6A and B), indicating that although the *norpA* mutation has strong effects on minimizing clock-regulated daytime repression of dmpi8 splicing, it does not completely abolish the splicing sensitivity to temperature.

Consistent with the enhanced dmpi8 splicing, the levels of total *per* mRNA during its accumulation phase are greater in *norpA* flies at both temperatures compared to the control situations (Figure 2.7A and B). Nonetheless, in *norpA* flies peak levels of total *per* mRNA are higher and reached earlier at 18°C compared to 29°C (Figure 2.7A and B), as previously shown for wildtype flies (85) (Figure 2.7A and B). Thus, as with the daily average proportion of type B' to type A variants (Figure 2.6), the total *per* mRNA levels in *norpA* flies still responds to temperature. Intriguingly, the daily levels of the unspliced type A *per* variant are in a similar range at 18° and 29°C, for both wildtype and *norpA* flies (Figure 2.7C). It is mainly the abundance of the spliced type B' *per* variant that increases

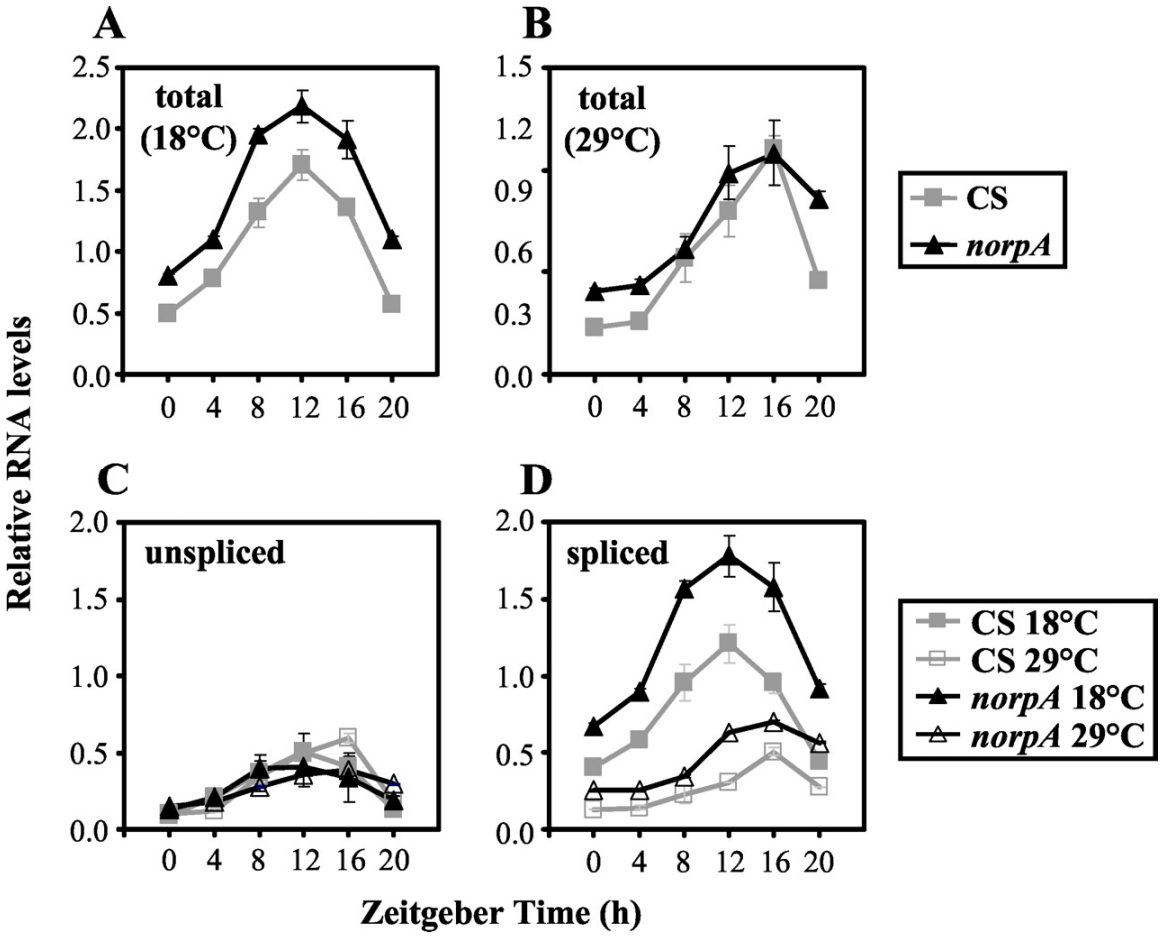

Figure 2.7. Higher overall levels of *per* mRNA were found in *norpA* flies. Wild-type (CS) and *norpA*^{P41} flies were exposed to 4 days of 12:12LD at either 18 or 29°C. RT-PCR was used to measure the relative levels of total (i.e., type A and type B' *per* variants together) (A and B), unspliced type A (C), and spliced type B' (D) *per* transcripts in head extracts. The RNA samples were from the fly collections shown in Figure 2.6. Higher overall levels of total *per* mRNA in *norpA*^{P41} flies were also obtained using RNase protection assays (data not shown).

in response to colder temperatures or the *norpA*^{P41} mutation (Figure 2.7D). These results support our prior findings that cold temperatures lead to advanced and higher amplitude cycles in total *per* mRNA levels mainly via a posttranscriptional mechanism that increases the splicing efficiency of dmpi8 (85), and suggest that the *norpA* mutation essentially phenocopies cold temperatures.

Advanced evening activity in *norpA* mutants

Under standard conditions of 12:12LD at 25°C, wildtype *D. melanogaster* exhibit bimodal daily activity patterns with one peak centered around ZT0 ('morning' peak) and another around ZT12 ('evening' peak) (e.g., Figure 2.8A) (46, 154). The timing of evening activity is clearly regulated by *per*, whereas the morning peak of activity appears to be comprised of (1) a startle response to lights-on characterized by a rapid and transient increase in activity (46, 154), and (2) a circadian component that might be independent of *per* activity (57). Increases in temperature and or photoperiod progressively delay the timing of evening activity and are associated with more pronounced "siesta times" (i.e., mid-day inactivity) (85), likely reflecting adaptive responses that ensure flies avoid desiccation during the hot mid-day hours. We previously showed that the onset and peak of the evening activity is delayed in transgenic lines that produce only type B' transcripts (*per*$^{B'}$) or a variant of type A transcripts having inactivated 5' and 3' splice sites (*per*^{A}) (85). To extend these prior findings, we sought to examine the timing of evening activity in *norpA* and *cry*^{b} flies, which have alterations in overall splicing ratio of dmpi8 (Figure 2.6) but still manifest robust circadian activity rhythms that can be entrained by daily light-dark cycles (37, 131, 154).

As predicted by its effect on the splicing of dmpi8 (Figure 2.6) and the

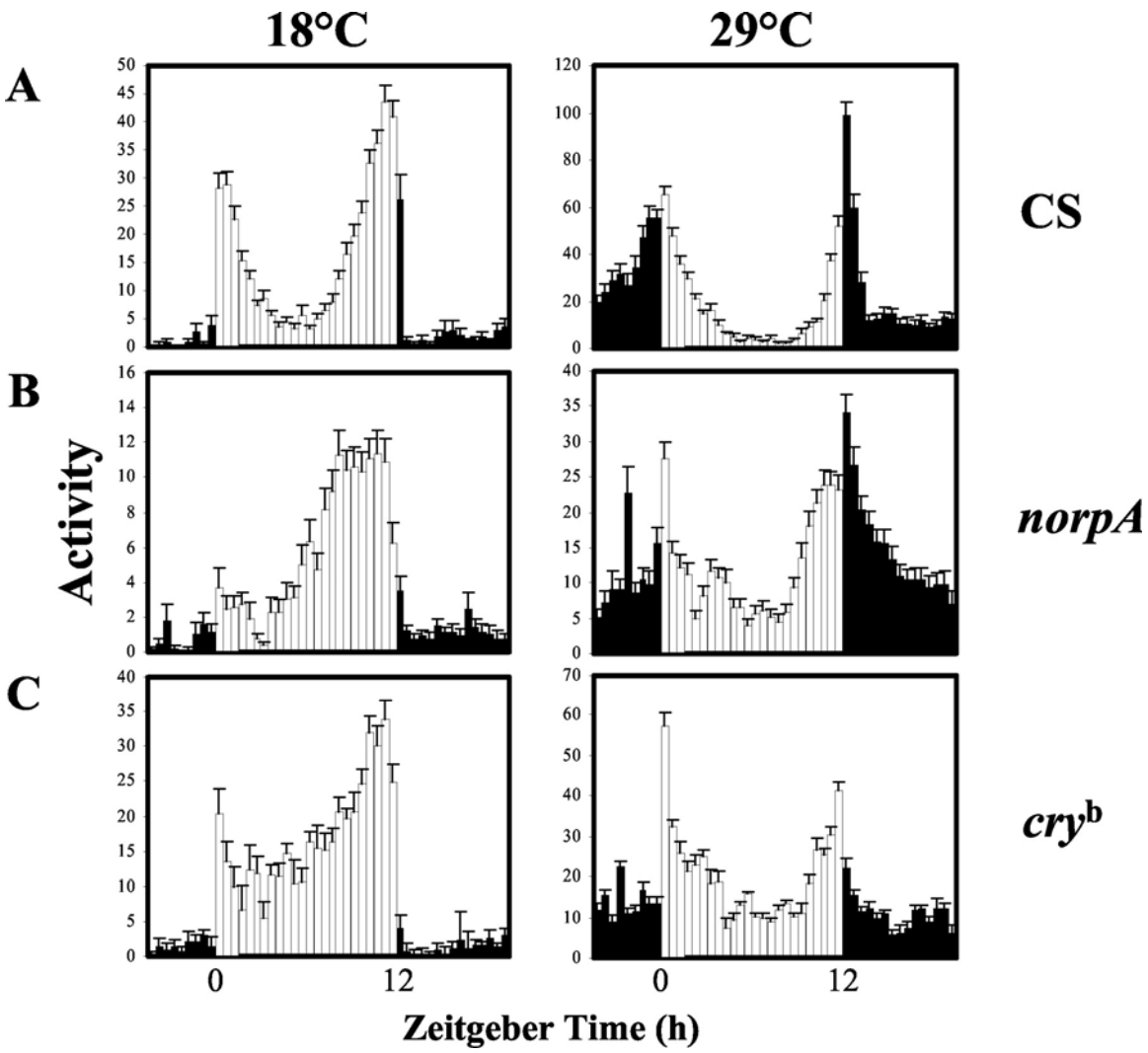

Figure 2.8. Daily locomotor activity rhythms of CS, *norpA*^{P41}, and *cry*^{b} mutants at different temperatures. Shown are the daily distributions of locomotor activity averaged over the fourth and fifth days of LD for wild-type (CS), *norpA*^{P41}, and *cry*^{b} male flies entrained to 12:12LD at either 18°C (left panels) or 29°C (right panels). Vertical bars represent the activity recorded in 30-min bins during times when the lights were on (white bars) or off (black bars). The evening activity occurred around ZT12.

Table 2.1. Locomotor Activity Rhythms of Canton-S, *norpA*^{P41} and *cry*^{b} Flies at Different Temperatures. [a]

Genotype	Temperature (°C)	n [b]	50% onset [c] (hr ± SEM)	Peak (hr ± SEM)
Canton-S	18	32	8.9 ± 0.2	10.4 ± 0.1
norpA^{P41}	18	30	7.2 ± 0.3*	9.1 ± 0.2*
cry^{b}	18	28	7.4 ± 0.2*	9.2 ± 0.2*
Canton-S	29	31	10.6 ± 0.2	12.1 ± 0.2
norpA^{P41}	29	30	8.5 ± 0.2*	11.6 ± 0.2**
cry^{b}	29	27	9.3 ± 0.4*	11.5 ± 0.4

[a] Flies were kept at either 18°C or 29°C and exposed to 4 days of 12 hr light:12 hr dark (12:12LD). Average data from two independent experiments is shown.
[b] Total number of flies that survived until the end of testing period.
[c] Onset is the time at which the upswing in evening activity reached 50% of the peak value and is given in hours from the last lights-on transition where ZT0 is lights-on.
* Significant difference between Canton-S and *norpA* or *cry*^{b} at $P<0.001$ using student's *t*-test.
** Significant difference between Canton-S and *norpA* at $P<0.05$.
No significant differences were observed between *norpA* and *cry*^{b} for either onset or peak at both temperatures.

accumulation of *per* mRNA levels (Figure 2.7), the onset of evening activity peak in *norpA* flies is phase advanced at both temperatures compared to wildtype flies (Figure 2.8 and Table 2.1). This is consistent with earlier findings showing that another allele of *norpA* (*norpA*^{P24}) has an advanced evening activity peak under standard conditions of 12:12LD at 25°C (46, 154). The endogenous activity periods of *norpA* flies are approximately 30 min shorter then wildtype flies (46, 154); and data not shown), whereas the *per*^{A} and *per*$^{B'}$ transgenic flies generally have slightly longer periods compared to control flies (17, 85). While changes in the splicing efficiency of dmpi8 might affect the clock's endogenous period, it is unlikely to fully account for the phase differences in the timing of evening activity. As previously noted for the *per*^{A} and *per*$^{B'}$ transgenics, *norpA* flies manifest larger differences from wildtype flies in the timing of the onset of evening activity compared to when peak values are attained (Table 2.1). Although in *cry*^{b} mutants the low dmpi8 spliced levels are consistent with the decreased amounts of total *per* transcripts (131); and data not shown), these flies also manifest earlier evening peaks compared to wildtype controls, although not as early as *norpA* mutants (Figure 2.8 and Table 2.1). The reason(s) for this apparent discrepancy is not clear but might be related to the fact that in *cry*^{b} flies TIM is less photosensitive, which could override the effects of decreased *per* mRNA and protein levels observed in this mutant (see Discussion).

Although we certainly cannot claim that the effects of *norpA* on the timing of evening activity are solely due to alterations in dmpi8 splicing efficiency and further work will be required to better understand how *cry*^{b} ultimately alters the timing of the evening activity, the main conclusion with regards to dmpi8 splicing is that a *norpA*-dependent pathway likely mediates its temperature regulation, whereas neither *norpA* or *cry* appear to

play major roles in the acute photoinhibition of dmpi8 splicing.

Discussion

In prior work we showed that cold-induced increases in the splicing of the *per* dmpi8 intron contribute to the preferential manifestation of daytime evening activity in flies as temperatures drop (85). Findings in this study expand on this model by revealing that the clock and photoperiod also influence the splicing efficiency of dmpi8. Furthermore, our findings suggest a physiological role for phospholipase C (PLC) in downregulating the production of type B' transcripts. The results indicate multi-pathway integration of photic and temperature signals by the *Drosophila* clock at the level of dmpi8 intron removal. We propose that whereas the light-induced degradation of TIM is critical for the synchronization of *Drosophila* clocks to local time, the dual thermal and photic regulation of dmpi8 splicing acts as a seasonal sensor encoding calendar information. In this model TIM is a major photoresponsive clock component setting the timing of the evening peak to around the light-dark transition by regulating the posttranslational accumulation rate of PER. The splicing efficiency of dmpi8 responds to changes in temperature and photoperiod, contributing to seasonal adjustments in the timing of evening activity by influencing the *per* mRNA accumulation phase, and hence the time-window necessary to attain threshold concentrations of PER that favor interactions with TIM.

Regulation of dmpi8 splicing by temperature, light and the clock

A network of interacting molecular circuits that respond to the clock, temperature, duration of photoperiod and acute effects of light control the daily regulation of dmpi8 splicing efficiency. The effects of temperature and light on dmpi8 splicing are mediated through clock and clock-independent mechanisms. In general, the clock helps maintain

lower dmpi8 splicing levels during the day and stimulates it during the night reaching peak values around midnight to late night, although the shape of the dmpi8 splicing profile is strongly influenced by photoperiod and temperature.

Temperature has a major effect on the overall daily splicing efficiency of dmpi8, probably establishing a dynamic range of upper and lower limits. Under standard 12:12LD conditions the daily proportion of type B' in wildtype flies ranges from ~65 to 85% at 18°C and ~35 to 50% at 29°C. In regards to setting the mean daily splicing efficiency, there is almost certainly a significant clock independent mechanism that responds to temperature changes. For example, our prior findings demonstrated that in clock impaired per^0 flies, decreases in temperature rapidly lead to concomitant increases in the proportion of type B' to type A transcripts (85). This is also supported by the lower average daily splicing values in per^0 and tim^0 flies at 29°C compared to 18°C (Figure 2.4). How changes in temperature directly modulate the splicing efficiency of dmpi8 is not clear.

Temperature also has a strong influence on the clock regulation of dmpi8 splicing. The amplitude of the splicing rhythm is smaller on cold days and dampens more quickly in constant dark conditions (Figure 2.3 and Figure 2.4). A notable difference is that there is a relatively more pronounced effect of the clock in driving daytime repression of dmpi8 splicing on warm compared to cold days, a feature that persists in constant dark conditions. Thus, there is a 'cold splicing phenotype' characterized by high overall splicing of dmpi8 and low amplitude cycling that rapidly dampens in DD. Conversely, the 'warm splicing phenotype' is characterized by low overall splicing of dmpi8 and relatively strong clock-regulated daytime decline in the proportion of type B' transcripts. Because the abundance of *de novo* synthesized *per* transcripts begin to steadily increase during the day

at all temperatures (e.g., Figure 2.7), higher daytime values of dmpi8 splicing on cold days accelerates the *per* mRNA accumulation phase, whereas repression of dmpi8 splicing during the day on warm days delays the upswing in *per* mRNA levels. It is possible that the nighttime increases in the splicing efficiency of dmpi8 at warm temperatures (Figure 2.6) also participate in delaying the *per* mRNA cycle by slowing down the declining phase in *per* transcript levels (Figure 2.7). As previously shown the higher splicing efficiency of dmpi8 at low temperatures is a seasonal adaptation that enables flies to exhibit daytime evening activity during short cold (85). In contrast, a more prominent influence of clock regulation on the daily inhibition of dmpi8 splicing during warm days might ensure that flies avoid the deleterious effects of daytime heat by favoring nocturnal evening activity. In summary, the effects of temperature on dmpi8 splicing by regulating the overall daily efficiency and the amplitude of clock-controlled daytime decreases, influence the accumulation rates of the *per* mRNA and protein products, which adjusts the timing of the evening activity such that it mainly occurs during the day at low temperatures and during the night at high temperatures (85) (Figure 2.9A).

Photoperiod adds another control point in the regulation of dmpi8 splicing by further adjusting the daily profile. A major effect of photoperiod is indirectly mediated via its synchronization of the phase of the clock, which regulates the timing of the dmpi8 splicing rhythm. As day length is shortened the proportion of type B' transcripts increases earlier and reaches higher peak values. The increased efficiency in the mean splicing of dmpi8 during short photoperiods likely underlies the earlier and more robust rhythm in total *per* mRNA abundance (Figure 2.1). Advances in the accumulation phase of the *per* mRNA cycle contribute to earlier evening activity (85) (Figure 2.9B). Thus, short day

lengths and low temperatures, which are normally associated in natural conditions, both increase the overall splicing efficiency of dmpi8. Conversely, long photoperiods likely produce synergistic effects with warm temperatures in decreasing the splicing efficiency of dmpi8, contributing to the manifestation of mainly nocturnal evening activity (85).

Changes in photoperiod also adjust the timing of the nighttime decline in the proportion of type B' *per* transcripts such that it occurs earlier under shorter photoperiods (Figure 2.2). Thus, the start of clock-regulated nighttime repression essentially anticipates the beginning of the next day when values of dmpi8 splicing are on average lower compared to nighttime levels. A similar scenario is also observed for the light-sensitive TIM protein that begins its clock-controlled declining phase in the late night prior to the onset of morning (101, 163). Because photoperiods adjust the dynamics (phase and amplitude) of the clock, the duration of the light phase is "remembered" during the night by the timing mechanism, generating different nighttime profiles for dmpi8 splicing as a function of day length. It also appears that the duration of the photoperiod has long-range indirect effects on the basal splicing efficiency of dmpi8 that are maintained for at least two days in constant dark conditions (Figure 2.3C). Differential "aftereffects" on circadian properties measured during constant dark conditions by variations in the length of the photoperiod are a well established phenomena in *Drosophila* (e.g., (115)). How the clock ultimately regulates the splicing efficiency of dmpi8 is not clear and could be rather indirect.

In addition to a role of photoperiod in the clock regulation of dmpi8 splicing, light acutely inhibits the proportion of the type B' variant (Figure 2.4 and Figure 2.5). This likely helps maintain lower daytime levels in the efficiency of dmpi8 intron removal,

although the "relative strength" of photoinhibition appears modulated by temperature and the clock (Figure 2.4 and Figure 2.5). Certainly, even though light-pulses evoke rapid decreases in dmpi8 splicing (Figure 2.5), wildtype and clock mutant flies exposed to LD cycles of 12 hr light still exhibit a significant amount of dmpi8 splicing at low and high temperatures (Figure 2.4). Thus, a certain basal level of dmpi8 splicing efficiency, that varies as a function of temperature, photoperiod and clock regulation, appears refractory to light-mediated inhibition. The acute photoinhibition in the proportion of the type B' variant might enable the dmpi8 splicing efficiency to rapidly respond to seasonal changes in day length. For example, as the sun sets later, the prolonged acute inhibition by light could delay the rise in dmpi8 splicing efficiency (Figure 2.5), whose timing would reflect the internal clock dynamics set by the previous days slightly shorter photoperiod. This might also explain why the acute photoinhibition of dmpi8 splicing is at least partially clock-independent.

Given the highly integrated molecular circuitry underlying the *Drosophila* clock it is certain that temperature and photoperiod regulate a wide range of diverse steps in the oscillatory mechanism, likely ranging from transcriptional rates to clock protein turnover. Our data identify the temperature and photoperiodic control of dmpi8 splicing as one important control center that regulates the dynamics of the *per* RNA cycle and ultimately seasonal adjustments in the duration of time necessary to reach levels of PER that favor formation of the PER-TIM complex, a critical event in the progression of the clock and the timing of evening activity.

A novel non-photic role for phospholipase C in the temperature regulation of dmpi8 splicing

CRY does not appear to be an important photoreceptor in mediating the

photoinhibition of dmpi8 splicing but it does play a role in generating daily cycles in the proportion of the type B' *per* variant. This is suggested by the observations that in cry^b mutants the mean daily levels of dmpi8 splicing are low and daytime values are lower than during the night (Figure 2.6). Because arrhythmic clock mutants (such as per^0 and tim^0) also result in low daily average levels of dmpi8 splicing (Figure 2.4) the effect of CRY is more likely to be indirectly related to its core clock function (65, 73, 131) and not in its capacity as a circadian-relevant blue-light photoreceptor. Why do cry^b flies have advanced evening activity peaks if they have low dmpi8 splicing and *per* mRNA levels? Here the answer might lie with its photoreceptor function in mediating the rapid light-induced degradation of TIM (13, 35, 36, 131). This raises the possibility that a more robust accumulation of TIM in certain key pacemaker cells of cry^b flies might override the lower levels of *per* mRNA contributing to the observed advanced evening phase. However, the situation is complex because the cry^b mutation has differential effects on PER/TIM dynamics in different groups of brain pacemaker neurons (e.g., (61, 67, 131, 145). In any case, because the photosensitivity of TIM is regulated by CRY in at least some pacemaker cells and TIM influences the stability of PER, it is unclear at present how the low splicing of dmpi8 in the cry^b flies might contribute to the altered activity rhythms observed in this mutant. This also raises a potential limitation of our study as we used whole head extracts to analyze the splicing levels of the dmpi8 intron. Because most of the clocks in the head are present in ocular photoreceptors, we cannot rule out the possibility that the splicing behavior of dmpi8 is different in the small number of key brain pacemaker neurons required for locomotor activity rhythms. In any event, while future studies will be required to address how cry^b alters the timing of evening activity, our data strongly suggest that the

circadian relevant CRY photoreceptor is not a major factor in mediating the light-induced inhibition of dmpi8 splicing.

In contrast, the constitutively high splicing efficiency in *norpA* flies suggests that PLC has a role in downregulating dmpi8 splicing (similar results were obtained by others; C. Kyriacou, personal communication; (22). However, the *norpA* mutation does not appear to have a major role in blocking the ability of light-pulses to inhibit the proportion of type B' transcripts (Figure 2.6C). Rather, the findings suggest a novel non-photic function in mediating increases in splicing repression as temperatures rise. The most dramatic difference between *norpA* and wildtype flies is that splicing does not decline during the daytime in *norpA* flies, especially at higher temperatures where in wildtype flies the daytime decline is relatively greater compared to cold temperatures (compare Figure 2.3A to Figure 2.6C). Thus, even at warm temperatures, *norpA* flies exhibit the 'cold splicing phenotype' characterized by high overall dmpi8 splicing with relatively little clock-controlled daytime decline, a pattern that persists into the first day of constant darkness. These results suggest that in the absence of PLC, the 'default' splicing behavior is that normally observed on cold days.

While there are different models that can be formulated to explain the effects of the *norpA* mutation on the daily splicing behavior of dmpi8, we favor a simple model whereby PLC/NORPA participates in the temperature sensing pathway (Figure 2.9) (a similar model was also recently proposed; (22). We posit that there is a heat signal whose strength increases as temperatures rise. PLC is required to transduce the intensity of this heat signal into a proportional splicing repression response. More heat signal, more PLC-dependent splicing repression. The splicing inhibition mechanism regulates the overall dmpi8

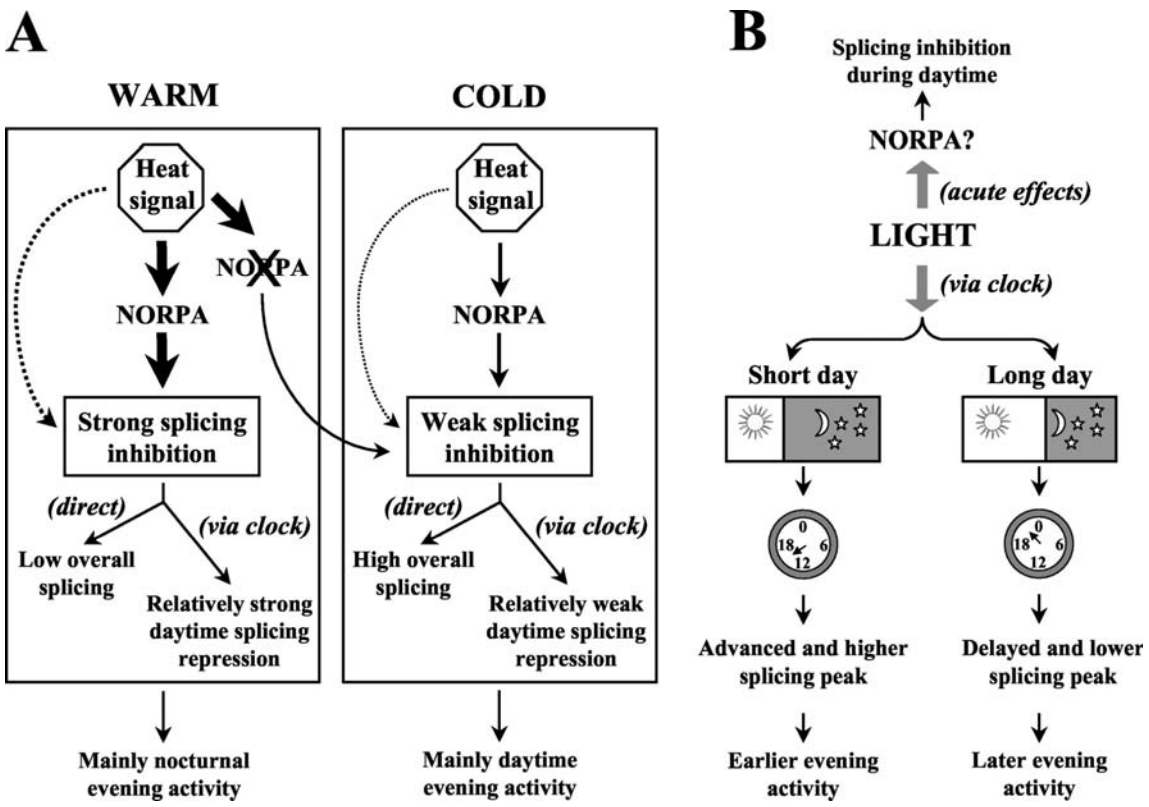

Figure 2.9. Model for how temperature, photoperiod, clock, and PLC regulate splicing of dmpi8. (A) Schematic representation of how temperature regulates the splicing efficiency of dmpi8. The intensity of a hypothetical heat signal is proportionally transduced via PLC to a splicing repressor that inhibits a more direct pathway, modulating the mean daily splicing efficiency (which has a clock-independent component), and a clock-regulated pathway that decreases daytime splicing. In the *norpA* mutant, there is less heat signal transduced to the splicing repressor, resulting in a cold splicing phenotype even on warm days. On cold days the cold splicing phenotype is augmented in *norpA* flies. The broken arrow indicates a possible *norpA*-independent inhibition of dmpi8 splicing by heat. (B) Schematic representation of how light regulates the splicing efficiency of dmpi8. The photoperiod adjusts the dynamics of the clock, which in turn modulates the timing of increases in the dmpi8 splicing rhythm and peak values (bottom). Light also has acute photoinhibitory effects that are at least partially independent of clock function (top). See the text for more details.

splicing efficiency and the relative strength of the clock-regulated daytime decline (Figure 2.9A). Such a model can explain why overall splicing is high and there is little clock-induced daytime inhibition on cold days (little heat signal, low splicing repression) and how inactivation of *norpA* results in the cold splicing phenotype and early activity even on warm days (strong heat signal but no transduction to splicing repressor). Because there is still less splicing in *norpA* flies at warm compared to cold temperatures (Figure 2.6), we also suggest that either the *norpA* mutation is not totally inactive with regards to dmpi8 splicing, and/or there is at least some *norpA*-independent temperature sensing for dmpi8 splicing regulation. Another possibility is that the temperature sensor is intact in the *norpA* mutant but PLC is required for correct temperature calibration. High amplitude cycling of other clock-controlled events in *norpA* flies, such as *per* mRNA rhythms (Figure 2.7), strongly argue that *norpA* does not directly affect core clock dynamics but plays a role between the daytime temporal signal and splicing inhibition (Figure 2.9A). The data strongly suggest that the main role of PLC in regulating dmpi8 splicing is related to sensing temperature and not light. It will be of interest to determine whether PLC affects the splicing efficiency of other introns.

At present it is not known how PLC might downregulate the production of type B' transcripts in a temperature dependent manner. The loss-of-function mutation in $norpA^{P41}$ flies causes the compound eyes and ocelli to be completely unresponsive to light (108). While some of the effects of *norpA* on fly activity are likely due to its role in ocular photic signal transduction (e.g., startle response) (154), as noted above this is not the case for dmpi8 splicing efficiency. Further support for this contention is based on findings showing that several physiologically blind mutants that affect visual photic signal transduction (e.g.,

ninaA[1], *ninaE*[5], *ninaE*[7] and *ninaE*[8]) do not exhibit an increase in the splicing efficiency of dmpi8 (data not shown). Moreover, in contrast to *norpA* mutants, several eyeless mutants manifest longer periods and/or delayed evening activity peaks compared to wildtype controls (59, 115, 149). In this context it noteworthy that *norpA* products are expressed in the eyes and brain, possibly including brain pacemaker neurons critical for rhythmic activity (67, 87, 164). Also, different isoforms of *norpA* gene products arising from alternative splicing are differentially expressed in the eyes and brain, although the function of extra-retinal PLC is not known (69). Future work will be required to determine the extent to which the advanced evening peak in *norpA* flies is due to its effects on dmpi8 splicing, identify the putative pacemaker cells where *norpA*-dependent changes in dmpi8 splicing efficiency presumably influence the timing of evening activity, and understand how temperature might modulate the ability of PLC to inhibit this splicing reaction. Furthermore, the identity of the photic transduction pathway mediating the acute light-mediated inhibition of dmpi8 splicing remains elusive but does not seem to involve major participation from either PLC or CRY.

In summary, our findings suggest that the thermal and photoperiodic regulation of dmpi8 splicing acts as a "seasonal sensor" conveying *calendar* information to *Drosophila*, endowing them with the ability to optimize their daily distribution of activity in accordance with the prevailing environmental conditions. The results also identify a novel non-photic role for *norpA* in mediating direct and clock-mediated effects of temperature on the splicing efficiency of dmpi8, and further illustrate the complexity of how day length, temperature and clocks interface to regulate seasonal adaptation in animals and plants.

Chapter 3. Natural polymorphisms in the *Drosophila melanogaster period* gene 3' UTR affect splicing of the dmpi8 intron and daily activity patterns

Introduction

In adapting to daily light-dark cycle on the earth, a wide range of organisms from bacteria to humans evolved cellular oscillators known as clocks or pacemakers to accommodate their temporal behavior and physiology to the 24-hr environmental modalities (32, 34). Work using the *D. melanogaster* has provided insights for how the clock functions (2, 45, 50, 130).

We recently used *D. melanogaster* to understand how a circadian clock adapts to seasonal changes in daily temperatures and day length, which modulates the daily activity patterns in the species. Prior work from our lab showed that the clock-regulated 'evening' activity peak of *D. melanogaster* progressively delays from mainly late-day to mostly nocturnal as ambient temperature is increased (85). The thermosensitive splicing of dmpi8 in the 3' UTR of *period* (*per*) mRNA has an important role in regulating the daily distribution of *Drosophila* activity as a function of temperature and day length. For example, the enhanced splicing of dmpi8 at low temperatures and/or short day length leads to a quicker accumulation in the timing of the *per* mRNA and PER protein (84, 85). This rapid accumulation of PER shortens the time necessary to attain threshold concentrations that favor interactions with TIM, contributing to advanced molecular cycles and evening activity on cold days. Because the active splicing of dmpi8 *per se* rather than the presence or absence of the dmpi8 intron is the relevant molecular event mediating the cold-enhanced

daily increases in the abundance of *per* mRNA (85), we reasoned that any natural polymorphisms that can affect the splicing efficiency of dmpi8 would affect the accumulation of *per* mRNA abundance and thus regulate the timing of evening locomotor activity in *Drosophila*.

Drosophila melanogaster originated in tropical Africa, migrated into Eurasia during early history and arrived in North America with human movement migration a few hundred years ago (30). Eanes and collegues (121) collected *D. melanogaster* single females to establish isofemale lines in the fall of 1996 and 1997 from the Eastern Coast of United States in a variety of latitudes ranging from Homestead, FL (HFL97) to Whiting, VT (VT97). They examined polymorphisms in the *methuselah* (*mth*) gene, which is thought to have major effects on fly stress response and longevity phenotype. A five-SNP haplotype in the *mth* gene showed a strong latitudinal cline in frequency across populations, indicating adaptive evolution of the loci (121). Intrigued, we set to find out if the *per* 3' UTRs of *D. melanogaster* exhibit natural polymorphisms that could affect dmpi8 splicing efficiency, and hence the distribution of daily activity. In this chapter we show that two polymorphisms found in an isofemale line captured from the wild in Whiting, Vermont (VT97.1) regulates dmpi8 splicing, *per* mRNA levels and behavior. We were able to recapitulate the differences in splicing efficiency using a simple assay in cultured S2 cells. The results suggest intricate co-evolution of polymorphisms that collaborate to affect the overall splicing efficiency of dmpi8. Our results further suggest that splicing of dmpi8 is a control point under natural selection allowing flies to adapt to local seasonal conditions.

Materials and Methods

Fly strains and collections

D. melanogaster isofemale lines were collected in the fall of 1997 from Homestead, FL (HFL97) and Whiting, VT (VT97) (121). Single-pair crosses were performed to generate inbred strains homozygous of *per* 3' UTRs from the VT97.1. Four strains were recovered from each of the two kinds of *per* 3' UTRs, as confirmed by sequencing. Inbred strains of the same *per* 3' UTRs were then pooled to form two inbred lines, termed VT1.1 and VT1.2 respectively, in order to reduce differences in genetic background caused by inbred crossing. To generate transgenic flies that produce the *per* transcripts with different 3' UTRs we used a CaSpeR-4 based transformation vector containing a 13.2 kb genomic *per* insert (19) that was modified with sequences for KpnI and ApaI sites upstream of the *per* translation stop signal, termed CaSpeR13.2-KA. Genomic DNAs from VT1.1 or VT1.2 inbred flies were used as templates to amplify the *per* 3' UTRs with the primers KpnI_P6869 (5'-TAA***GGTACC***TAGTAGCCACACCCGCAGT-3') and P7373 (5'-GTGGGCGTTGGCTTTTCG-3') (the KpnI site is in bold italic, immediately upstream of the two stop codons. Genomic DNA sequences are underlined.). The PCR products were then ligated to the pGEM-T easy vector (Promega). *per* 3' UTRs with different mutations were generated from the above pGEM-T easy derivative constructs with the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA), and reconstructed into the CaSpeR13.2-KA vector by swapping the DNA fragment from KpnI site to a unique Bsu36I site in the *per* 3' UTR to yield final transgenic constructs, which were then confirmed by DNA sequencing. Transgenic flies were generated by Genetic Services Inc (Sudbury, MA, USA) in a w^{1118} background and subsequently crossed into a $per^{0}w$ background with a

double balancer line (*per*^{0}*w*;*Sco*/*Cyo*;*MKRS*/*TM6B*). Standard handling of all flies was as described in Majercak *et al* (85).

Plasmid and dmpi8 splicing assay in S2 cells

To construct the pAct-Luc-UTR reporters for splicing analysis in the S2 cells, the pUChsneo-act(RI) vector was used (141). A luciferase open reading frame (ORF) was amplified flanked with EcoRI and StuI sites at the 5' and 3' ends, respectively. A 598bp *per* 3' UTR, *per* genomic DNA starting from the first stop codon to 100 bp after the presumed poly(A) cleavage site (6869 to 7466, numbering according to refn. (19)) was amplified flanked with StuI and SalI sites at the 5' and 3' ends, respectively. A plasmid backbone fragment was recovered from the pUChsneo-act(RI) vector digested with EcoRI and SalI. A three-way ligation was performed with the above mentioned luciferase ORF, the *per* 3' UTR, and the plasmid backbone to generate the pAct-Luc-UTR vector. To generate pAct-Luc-VT1.1 and pAct-Luc-VT1.2 constructs, genomic DNAs from VT1.1 or VT1.2 inbred flies were used as templates to amplify the *per* 3' UTRs with the primers StuI_P6869 (5'-TA***AGGCC***TAGTAGCCACACCCGCAGT-3') and P7373 (5'-GTGGGCGTTGGCTTTTCG-3') (The StuI site is in bold italic, immediately upstream of the two stop codons. Genomic DNA sequences are underlined.). The PCR products were then ligated to the pGEM-T easy vector (Promega). *per* 3' UTRs with different mutations were generated from the above pGEM-T easy derivative constructs with the Quick Change site-directed mutagenesis kit (Stratagene, CA, USA), and reconstructed into the pAct-Luc-UTR vector by swapping the DNA fragment from StuI site to Bsu36I site to yield final transgenic constructs, which were then confirmed by DNA sequencing.

S2 cells were obtained from Invitrogen and transfected using Effectene reagent

(Qiagen) according to the user manual with optimization by Kwang Huei Low in the lab (unpublished data). 1.5×10^6 S2 cells were placed in 6-well plates and transfected with 125 ng of pAct-Luc-UTR vectors. After the transfection, cells were allowed to recover for 2 days before harvesting. Cytoplasmic and nuclear RNAs of the cells were fractionated according to Graham et al. (42). The RNAs were then extracted with TRI Reagent LS (Sigma) according to the technical bulletin. About 1 μg of each RNA sample was used in RT-PCR for standard dmpi8 splicing assay (84).

Locomotor activity

Unless otherwise stated, locomotor activity data were essentially collected in 15 or 30-minute bins and analyzed as previously described (46, 84). The daily behavior profiles of flies were plotted either on the Faas program kindly provided by Michel Boudinot in the lab of Dr. Rouyer or on Excel spread sheet. The phase of daily activity was determined on a Unix command line version of the Brandeis Rhythm Package (BRP) Phase module. The times when flies exhibit evening peak or 50% of peak-onset were measured for each individual fly and subsequently averaged as activity phases for each genotype. For phase shift experiment, flies were entrained at 12:12LD, 18°C for at least four days. During the last dark period of a group of flies were exposed to 5 minutes of light (~2000lux) at ZT18, ZT19, or ZT20. An untreated group served as controls. After the light pulses, flies were kept in complete darkness for 7 more days. BRP Phase module was used to determine the phase of the evening peak. The values for the treated flies were subtracted from those of the control flies and the magnitude of the shift (delay or advance) was plotted relative to the time the pulse was administered to generate a phase response curve.

RT-PCR splicing assay from fly heads

The relative levels of the dmpi8 spliced type B' and unspliced type A *per* RNA variants in wild type fly heads and S2 cells were measured using a reverse transcriptase-PCR (RT-PCR) assay based on a method previously described (84). In order to differentiate the transgenic *per* mRNA transcripts from the endogenous per^0 transcripts we used the forward primer P6851mF (5'-ACACAGCACGGGGATGGGGGTA-3') with its 3' end base-pairing on the KpnI site which does not exist in the endogenous transcripts. The P6851mF primer, together with the P7197 reverse primer will generate amplicons from 6851 to 7197 bp (numbering according to refn. (19)), with a KpnI restriction site upstream of the two stop codons.

Results

Natural polymorphisms in the *per* 3' UTR modulate the splicing efficiency of the dmpi8 intron

We previously showed that in wildtype flies, the splicing efficiency of dmpi8 is stimulated in cold temperature and/or short day length conditions, which accelerate the earlier daily upswing in total *per* mRNA abundance and higher peak values (84, 85). The advanced *per* mRNA phase and level, in turn, contributes to the advanced evening activity of *Drosophila* on cold short days (84, 85). We took advantage of the flies that Eanes and colleagues (121) collected from the Eastern Coast of the United States to test whether there are natural variations in the *per* 3' UTR and if yes, would they affect the splicing of dmpi8 and consequently the distribution of daily activity.

Two haplotypes of *per* 3' UTRs were isolated from the VT97.1 isofemale line in the process of DNA sequencing (Figure 3.1). These two haplotypes, termed VT1.1 and VT1.2, consist of 6 polymorphisms including two deletions (Del1, 15 nt deletion and Del2, 3 nt deletion) and 4 SNPs (SNP1-4) (Figure 3.1). Since Del1 and SNP1, 3, and 4 are more than 43 bp away from the intron and SNP2 is 13 bp downstream of the 5' splice site (5' SS), they are unlikely to directly affect the strength of these splice sites as suggested by the splice site consensus of *Drosophila* (99). On the other hand, the Del2 polymorphism is less than 10 bp upstream of the branch point and is part of a putative polypyrimidine tract "TTCCCCCT" (the Del2 is underlined) (Figure 3.1). A polypyrimidine tract could bind to many kinds of protein factors such as U2AF, SF1 and polypyrimidine tract binding protein (PTB) to regulate branch point or exon definition (10, 150).

The VT97.1 flies were used to perform single-pair crosses to screen for inbred lines

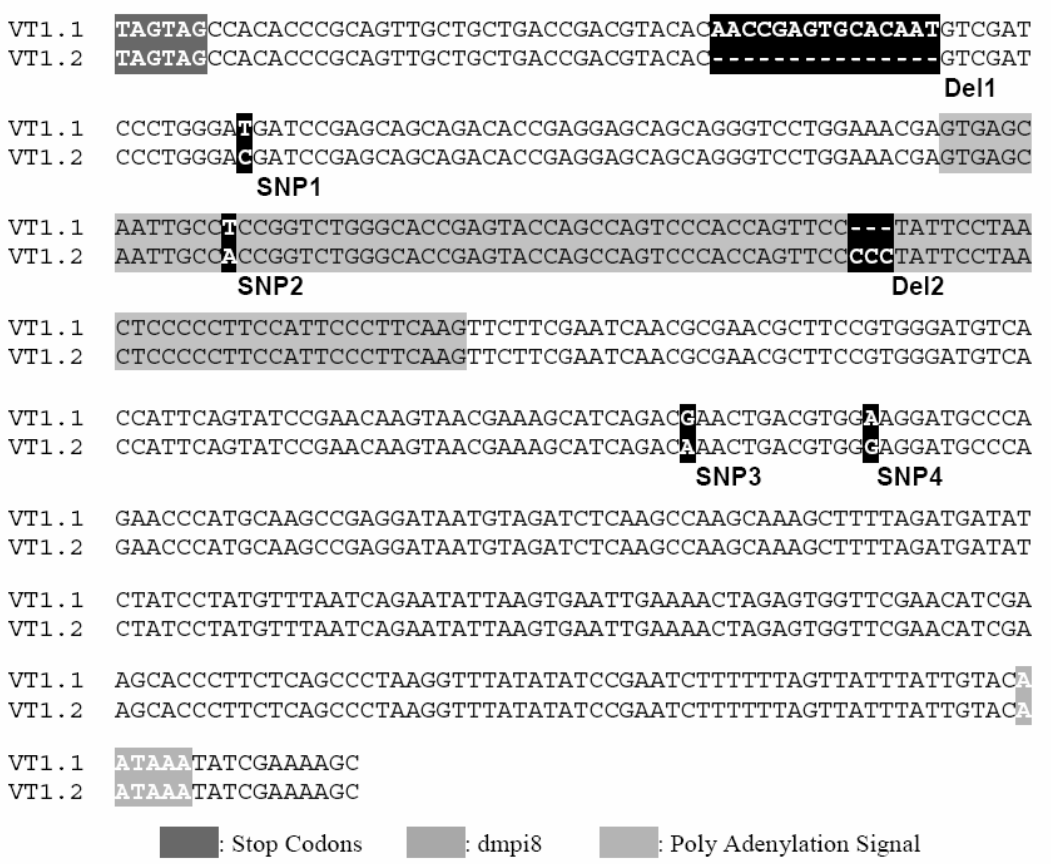

Figure 3.1. Two haplotypes in the *per* 3' UTR were isolated from the VT97.1 isofemale line. Individual flies from VT97.1 were used for genotyping as described by Goor et al (39). For sequencing analysis, the *per* 3' UTRs were amplified with the two primers P6812 (5'-CGAGAGCAAGATCATGGAGC-3') and P7505 (5'-TTCTTCGAAAAGGAATGAACG-3'). Shown above are the VT1.1 and VT1.2 3' UTRs ranging from the stop codons to the presumed poly(A) cleavage site. Stop codons, dmpi8 intron and the Poly(A) signal are in different gray background as shown at the bottom of the figure. Polymorphisms are shown in black ground with their names below them.

that are homozygous for VT1.1 and VT1.2 *per* 3' UTRs, respectively. Four of VT1.1 or VT1.2 inbred lines were then each pooled together to form two lines (termed VT1.1 and VT1.2) to minimize the genetic differences among the lines resulted from the inbred crosses. To determine whether the polymorphisms in these two haplotypes of *per* 3' UTRs affect the splicing of dmpi8, VT1.1 and VT1.2 flies were kept at 29°C and exposed to several days of 12:12LD (Figure 3.2). VT1.1 flies showed significantly higher splicing efficiency ($P<0.01$ for all six time points) than VT1.2 flies (Figure 3.2A). Consistent with previous findings (84), earlier *per* mRNA accumulation and higher peak levels were also observed for VT1.1 flies. VT1.1 flies also showed similar higher dmpi8 splicing ratio and faster mRNA accumulation than VT1.2 when they were tested in shorter photoperiods (6:18LD) and low temperatures (18°C) (data not shown).

VT1.1 flies exhibit advanced evening activity and are less sensitive to the phase shifting effects of light pulses in the late night

Consistent with the difference in the splicing efficiency of dmpi8 and *per* mRNA levels (Figure 3.2), the evening activity onset in VT1.1 flies is phase advanced at both 18°C and 29°C compared to VT1.2 flies (compare dashed lines for VT1.1 and VT1.2 profiles at 18°C or 29°C) (Figure 3.3A) (Table 3.1). No significant period difference is found between the VT1.1 and VT1.2 flies at either temperature tested (Figure 3.3A), suggesting that the observed variation of evening activity onset between VT1.1 and VT1.2 are due to differences in clock phase rather than that of clock period.

The rapid and transient increases in activity caused by the light-on and light-off transitions, termed startle responses, are close in proximity to the morning and evening peaks respectively under 12:12LD conditions (46, 154). This apparently affects the

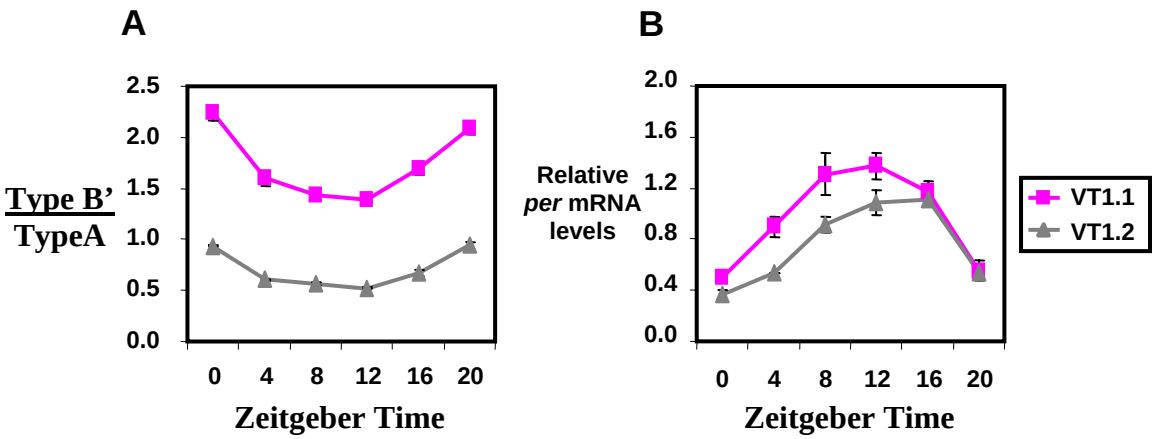

Figure 3.2. VT1.1 flies manifest higher splicing efficiency of dmpi8 and advanced *per* mRNA accumulation compared to VT1.2 flies. VT1.1 and VT1.2 flies were exposed to 4 days of 12:12LD at 29°C and collected at the indicated time during the last LD. The splicing efficiency (A) and relative *per* mRNA levels (B) were measured using RT-PCR as described previously (84).

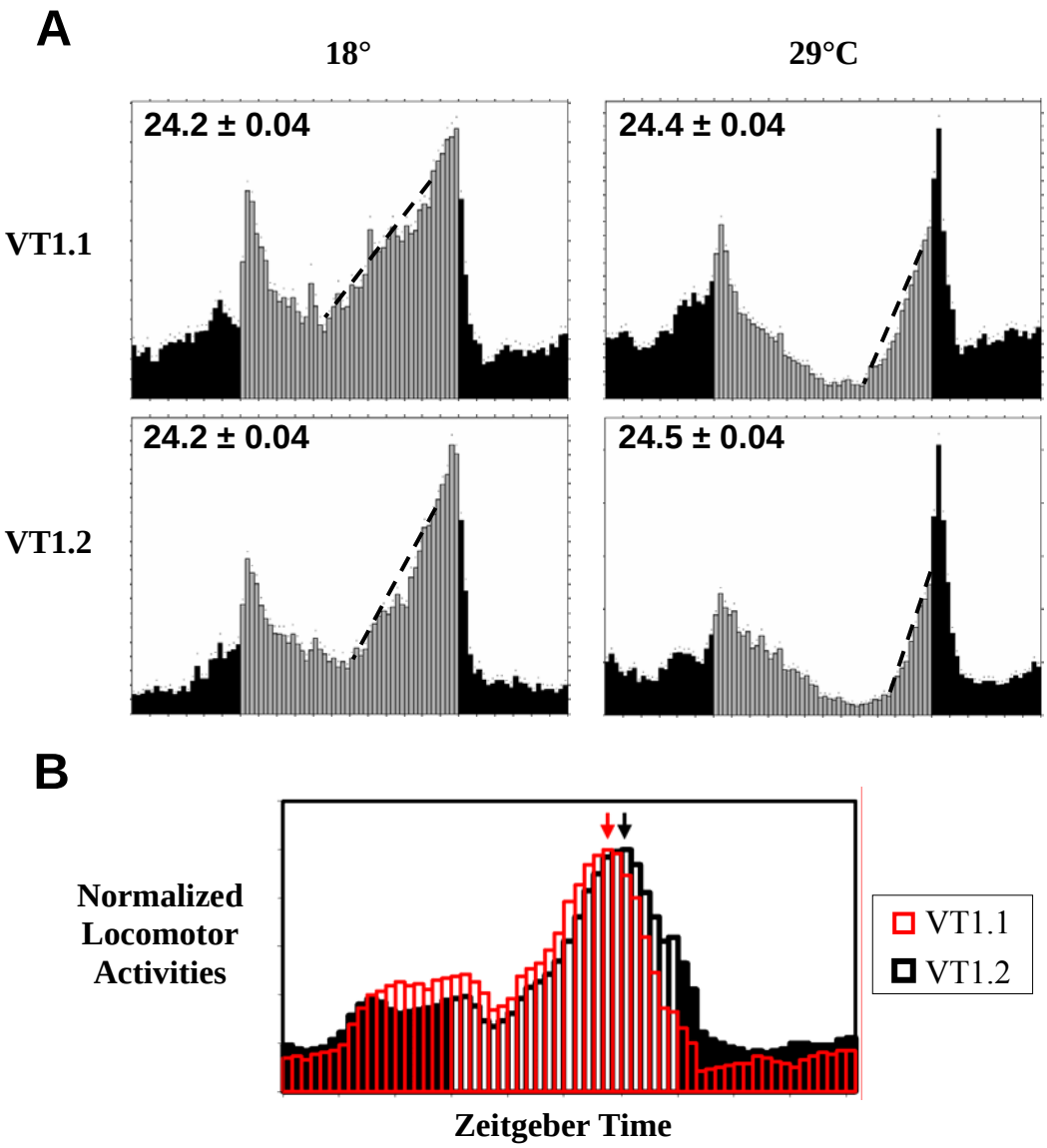

Figure 3.3. Daily Locomotor activity rhythms of VT1.1 and VT1.2 flies at different temperatures and photoperiods. Shown are the daily distribution of locomotor activity averaged over the third and fourth days of LDs for VT1.1 and VT1.2 male flies. (A). The flies were entrained to 12:12LD at either 18°C (left column) or 29°C (right column). Shown at the upper left corner are the free-running periods (± SEM) for the behavior of the same groups of flies kept in complete darkness after the LD cycles. No significant difference in period was found between VT1.1 and VT1.2 flies at 18°C (P=0.94) or

Figure Legend 3.3 Continued

29°C(P=0.13). The dashed lines indicate the approximate rising phase of evening activity of the flies. (B). The VT1.1 and VT1.2 flies were entrained to 12:18LD at 18°C. Behavior data were averaged for each genotype and normalized against the evening activity peaks before the profiles were plotted using a 3D bar chart in Excel. The behavior profile of VT1.1 flies is plotted in red open bars to facilitate comparison of the profiles. The arrow heads denote the times of evening behavior peaks for the flies. Red arrow head, VT1.1; black arrow head, VT1.2. Vertical bars represent the activity recorded in 15-min (panel A) or 30-min (panel B) bins during times when the lights were on (white or gray bars) or off (black bars).

Table 3.1. VT1.1 flies exhibit earlier evening activity onsets and peaks. [a]

		VT1.1	VT1.2	
18°C	Peak ± sem (N)	10.6 ± 0.09 (126)	10.7 ± 0.06 (125)	
	50% onset ± sem (N)	7.1 ± 0.10 (126)	7.9 ± 0.08 (125)	**[b]
29°C	Peak ± sem (N)	12.3 ± 0.10 (127)	12.4 ± 0.08 (126)	
	50% onset ± sem (N)	9.8 ± 0.10 (127)	10.0 ± 0.08 (126)	*

[a] Shown in the table are phase data extracted from the same experiment as in Figure 3.3A.

[b] ** and * indicate that the P-values are <0.01 and <0.05, respectively for student's *t*-test on the 50% evening behavior onset between the VT1.1 and VT1.2 flies.

evening activity peak measurement for the VT1.1 and VT1.2 files entrained in 12:12LD at 18°C and 29°C (Figure 3.3A). To separate the startle responses from the clock regulated bimodal behavior peaks, the VT1.1 and VT1.2 flies were entrained to a non-native 12:18LD cycles at 18°C. Under this 30-hr entrainment regime, flies manifest their "morning" and "evening" behavior peaks many hours earlier than the light-on and light-off transition times (Figure 3.3B) and have to reset their circadian clocks every day. In 12:18LD, 18°C, VT1.1 flies manifested about a 1-hr earlier evening activity peak timing than VT1.2, as indicated by the arrow heads (Figure 3.3B). Together, these results establish that between the two inbred lines derived from VT97.1 isofemale line, the VT1.1 flies exhibit better splicing in the dmpi8 intron of the *per* gene and advanced evening activity phase, consistent with our model.

Light resets the *Drosophila* clock by degrading TIM and consequently destabilizing PER proteins (78, 155, 163). Light pulses given in the early part of the night delay the clock phase, while in the later part of the night advance the phase. Responses of *tim* dosages and analysis of spectral sensitivity of CRY have demonstrated a correlation between the strength of TIM response to light and behavior responses to light (138, 158). Takahashi and colleagues have shown that a *Clock* mutant in the mouse that generates reduced cycling amplitude of mouse *per* mRNAs enhances the amplitude of phase-response by resetting stimuli (146). We reasoned that differences in *per* mRNA levels observed between VT1.1 and VT1.2 flies might affect their ability to undergo phase resetting by light pulses. To test this hypothesis, after entrainment to 12:12LD, 18°C, groups of VT1.1 and VT1.2 flies were given five minutes of light at ZT18, ZT19, and ZT20 on the last dark phase of LD cycle; one group of each genotype without the light pulse

served as untreated controls (Figure 3.4). Interestingly, a light pulse at ZT19 rendered both genotypes arrhythmic, suggesting that the light hit the critical unstable equilibrium point between the "delay zone" and "advance zone" of phase response curve termed "singularity" (157), a phenomena that has been molecularly described recently in *Neurospora* (62) (Figure 1.4 and Figure 3.4). A light pulse at ZT18 delays the clocks for about 5 and 5.4 hours for VT1.1 and VT1.2 flies, respectively. However, five minutes of light at ZT20 advances the phase of VT1.1 flies by only 2.9 hrs, compared with 7.6 hrs for VT1.2 flies, a variation that is statistically different ($P<0.05$) (Figure 3.4).

Transgenic flies carrying the VT1.1 and VT1.2 haplotypes recapitulate the differences in dmpi8 splicing and the daily locomotor activity profiles

To eliminate the possibility that the genetic background difference between VT1.1 and VT1.2 inbred lines leads to their phenotypic differences in dmpi8 splicing assay and locomotor activity, we generated transgenic flies with a 13.2kb *per* genomic DNA carrying the VT1.1 or VT1.2 3' UTRs, as well as VT1.2 3' UTRs with each of the 6 polymorphisms mutated into the counterpart found in the VT1.1 haplotype (Figure 3.5A) (19). In 12:12LD, 29°C, the per^0w;p{VT1.1} transgenic flies, which carry the *per* genomic DNA with the VT1.1 haplotype in the 3' UTR, manifest significantly higher dmpi8 splicing efficiency ($P<0.01$) compared with the per^0w;p{VT1.2} or other transgenic flies in VT1.2 background with single polymorphism mutation (Figure 3.5 and data not shown). On the other hand, no difference is found between the per^0w;p{VT1.2} and other transgenic genotypes in VT1.2 background with single polymorphism mutation ($P>0.1$). These results confirm that the polymorphisms in the VT1.1 and VT1.2 haplotypes indeed lead to the higher splicing efficiency in the inbred lines and that changing any single one of the six

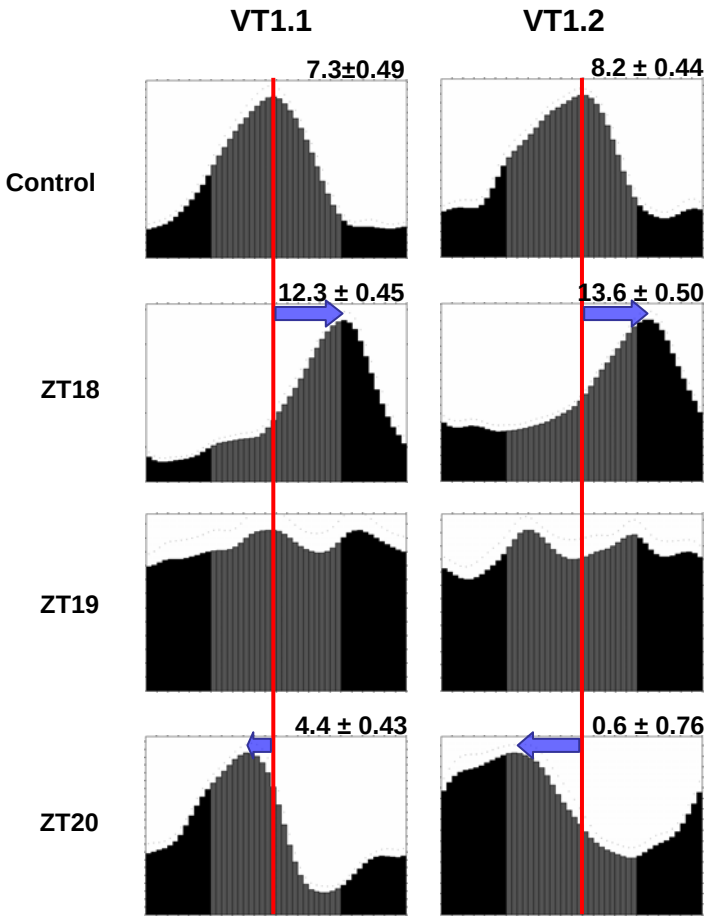

Figure 3.4. VT1.1 flies show less phase advance in behavior than VT1.2 after a light pulse at ZT20. Shown are the daily distributions of locomotor activity averaged over the second and third days of DD for VT1.1 and VT1.2 male flies after the indicated 5 minutes of light given at ZT18, 19, or 20 of the dark period on the last LD. One group of each genotype was kept in darkness without light pulses as phase controls. Before the light pulses, all flies had been entrained to 12:12LD at 18°C. Vertical red lines are the phase reference of behavior peaks for VT1.1 and VT1.2 control flies. Blue arrows show the direction and extent of phase shift in the behavior peaks. The numbers at the upper right corner of the behavior profiles (except for ZT19-light pulsed) show the phases of the behavior peaks. The flies light-pulsed at ZT19 became arrhythmic.

polymorphisms in the VT1.2 haplotype into the counterpart from the VT1.1 haplotype is not enough to enhance the splicing of dmpi8.

To investigate whether the *per*^{0}*w*;p{VT1.1} transgenic flies show a predicted earlier evening locomotor activity onset than the *per*^{0}*w*;p{VT1.1}, we tested male flies of both genotypes in 12:12LD at 18°C and 25°C. The transgenic flies showed very similar periods to the inbred flies with >80% rhythmicity (compare Figure 3.3A**,** Figure 3.6, and data not shown), indicating a full rescue of the *per*^{0}*w* genetic background. At both 18°C and 25°C, *per*^{0}*w*;p{VT1.1} flies exhibit earlier evening behavior onset ($P<0.01$, both evening peak and 50% evening activity onset at either 18°C or 25°C) (Table 2.1), which again are due to the advanced clock phase *per se* in the VT1.1 because there is no significant period difference found between the *per*^{0}*w*;p{VT1.1} and the *per*^{0}*w*;p{VT1.2} flies (Figure 3.6).

Splicing assay in S2 cells reveals that SNP3 and SNP4 are important in regulating dmpi8 splicing

Because single mutations in polymorphisms of the VT1.2 haplotype do not improve dmpi8 splicing (Figure 3.5B), it is likely that multiple polymorphisms work cooperatively to regulate this event. Our lab developed a dmpi8 splicing assay system in S2 cells that recapitulates the thermosensitive splicing of the *per* 3' intron observed in fly heads. To pinpoint which polymorphisms are important, we generated pAct-Luc-UTR constructs for VT1.1 and VT1.2 haplotypes as well as other 3' UTRs in either a VT1.1 or VT1.2 background with one or more polymorphisms swapped with the counterparts from the other haplotype, expecting that mutations of some combinations of polymorphisms would exhibit different dmpi8 splicing efficiency from that of their genetic background,

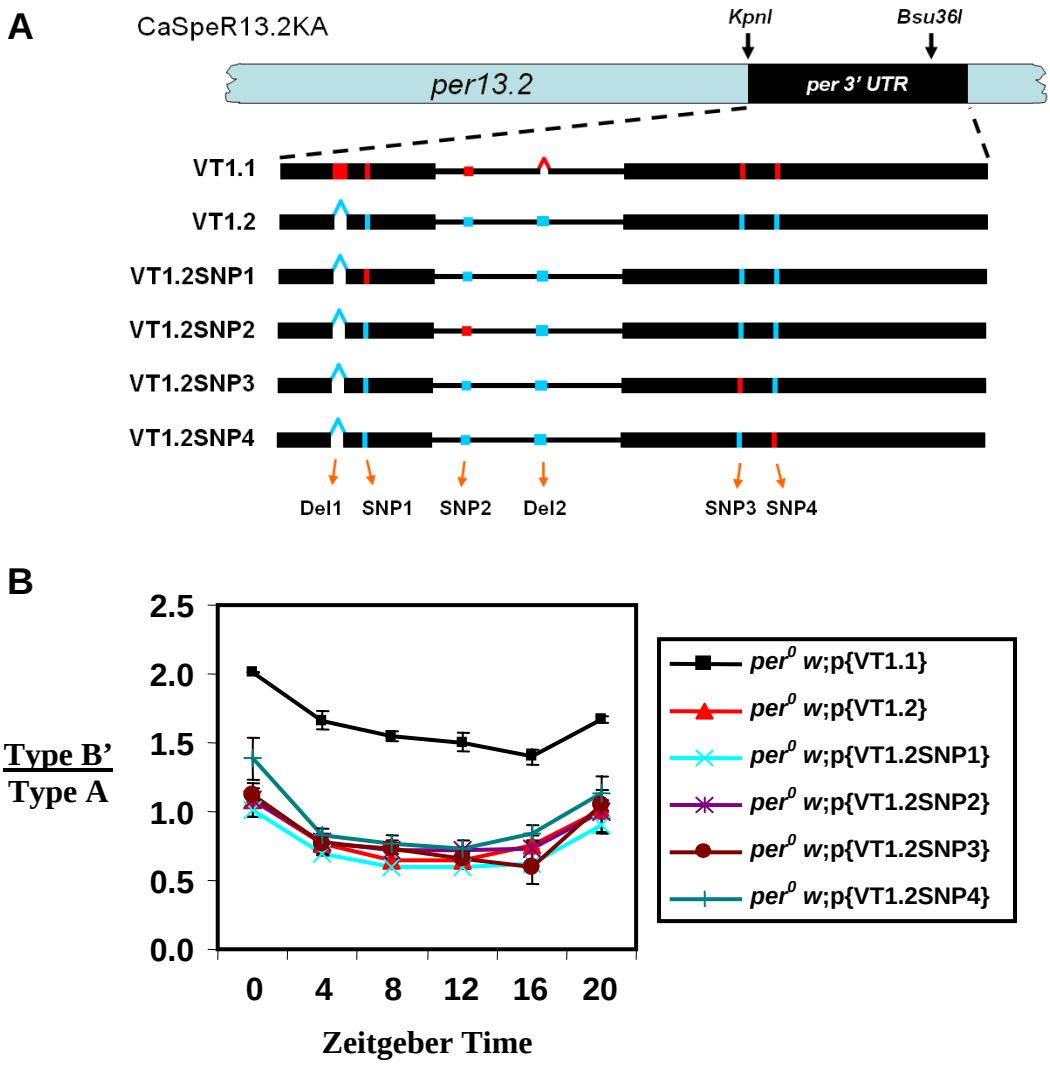

Figure 3.5. Change of individual SNPs from VT1.2 to VT1.1 does not enhance the splicing of dmpi8. (A). VT1.1 and VT1.2 transgenic flies were generated with CaSpeR13.2-KA as backbone by swapping the KpnI and Bsu36I fragments of VT1.1 and VT1.2 3' UTRs into the transgenic construct. For other transgenic constructs with a single SNP mutation, VT1.2 3' UTR was used as template (polymorphisms shown in blue) to mutate one of the VT1.2 SNPs to the corresponding one found in VT1.1 (shown in Red). (B). Flies were exposed to 4 days of 12:12LD at 29°C, and the flies are collected once every 4 hrs on the last LD. Shown are results for the averages of two transformants for

Figure Legend 3.5 Continued

each genotype. Significant differences were found between *per*^{0}*w*;p{VT1.1} compared to *per*^{0} *w*;p{VT1.2} and the other transgenic flies containing single SNP changes ($P<0.01$). No significant differences were found between *per*^{0}*w*;p{VT1.2} and the transgenic flies containing single SNP changes ($P>0.1$).

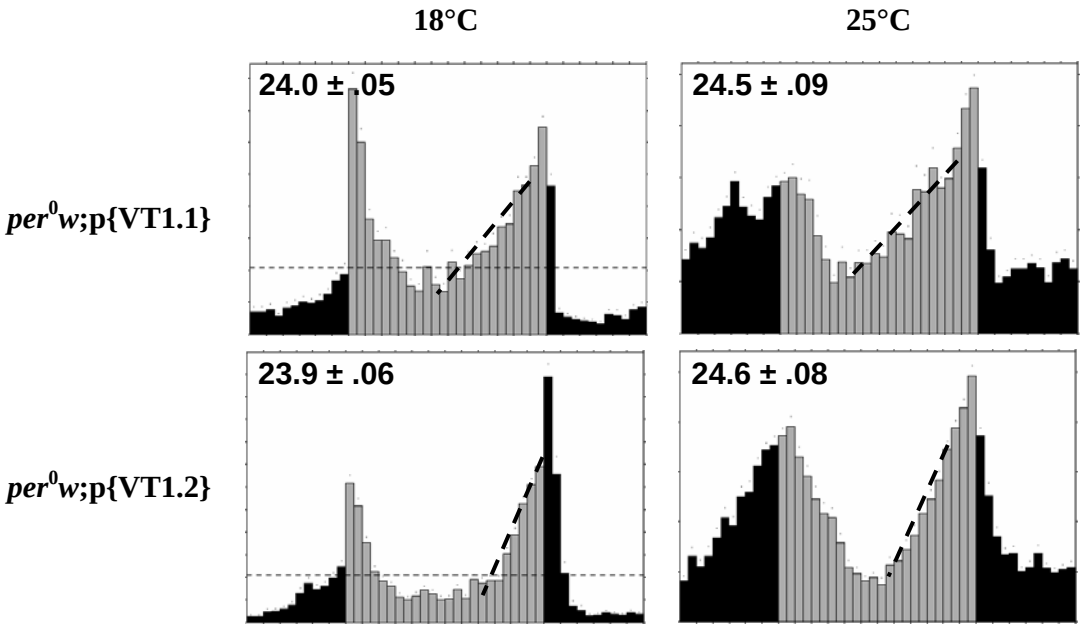

Figure 3.6. Daily Locomotor activity rhythms of *per*^{0}*w*;p{VT1.1} and *per*^{0}*w*;p{VT1.2} transgenic flies at different temperatures. Shown are the daily distribution of locomotor activity averaged over the third and fourth days of LD for male flies. The flies were entrained to 12:12LD at either 18°C (left column) or 29°C (right column). Shown at the upper left corner are the periods (± SEM) for the behavior of the same groups of flies kept in complete darkness after the LD cycles. No significant difference between the free-running periods was found for the transgenic flies at each temperature. The dashed lines indicate the approximate rising phase of evening activity of the flies. The phases of both evening peak and 50% evening behavior onset for these flies are summarized in Table 3.2.

Table 3.2. *per*^{0}*w*;p{VT1.1} transgenic flies exhibit earlier evening activity onsets and peaks compared to *per*^{0}*w*;p{VT1.2} flies. [a]

		per^{0}w;p{VT1.1}	per^{0}w;p{VT1.2}	
18°C	Peak ± sem (N)	10.4 ± 0.13 (61)	11.3 ± 0.09 (64)	**[b]
	50% onset ± sem (N)	7.6 ± 0.27 (61)	9.1 ± 0.15 (64)	**
25°C	Peak ± sem (N)	10.4 ± 0.17 (40)	11.0 ± 0.10 (40)	**
	50% onset ± sem (N)	7.6 ± 0.21 (39)	8.3 ± 0.14 (39)	**

[a] Shown in the table are phase data extracted from the same experiment as in Figure 3.6.

[b] Double stars (**) indicate that the P-values are <0.01 for student's *t*-test in the above four tests between the *per*^{0}*w*;p{VT1.1} and *per*^{0}*w*;p{VT1.2} flies.

i.e., increased splicing from a VT1.2 background or decreased splicing from a VT1.1 background (Figure 3.7). As positive controls, we showed that dmpi8 splicing of pAct-Luc-VT1.1 and pAct-Luc-VT1.2 constructs transfected into S2 cells at 22°C recapitulates the splicing results from the inbred lines and the transgenic flies, i.e., dmpi8 in the VT1.1 background splices more efficiently (compare Figure 3.7B left two bars with Figure 3.2A and Figure 3.5).

Consistent with previous results from transgenic flies, single SNP mutations in the VT1.2 genetic background give significantly different splicing efficiencies from the pAct-Luc-VT1.1 but not the pAct-luc-VT1.2 construct (Figure 3.7B, compare left 2 bars with the right four bars). While none of the tested single mutations in the VT1.1 background produces different dmpi8 splicing efficiency, several double mutations in the VT1.1 background exhibit reduced dmpi8 splicing. Notably, the pAct-Luc-VT1.1S34 construct demonstrates similar levels in splicing efficiency to that of the VT1.2 haplotype. These data further confirmed the observation with transgenic flies that switching at least two polymorphisms between VT1.1 and VT1.2 haplotypes is required to render a change in dmp8 splicing efficiency. Because SNP3 and SNP4 are at least 74 nt downstream from the intron, it's unlikely that these two SNPs directly affect the strength of any splice site for dmpi8 according to *Drosophila* splice site consensus (99).

Furthermore, dmpi8 splicing efficiency is significantly enhanced for both pAct-Luc-VT1.1 and the pAct-luc-VT1.2 constructs when temperatures are lowered (Figure 3.7C), indicating that SNP3 and SNP4 regulates dmpi8 splicing efficiency without affecting the thermosensitivity of the dmpi8 intron. This is in contrast to other unpublished data from our lab showing that strengthening the 5' and 3' splice sites of dmpi8 intron

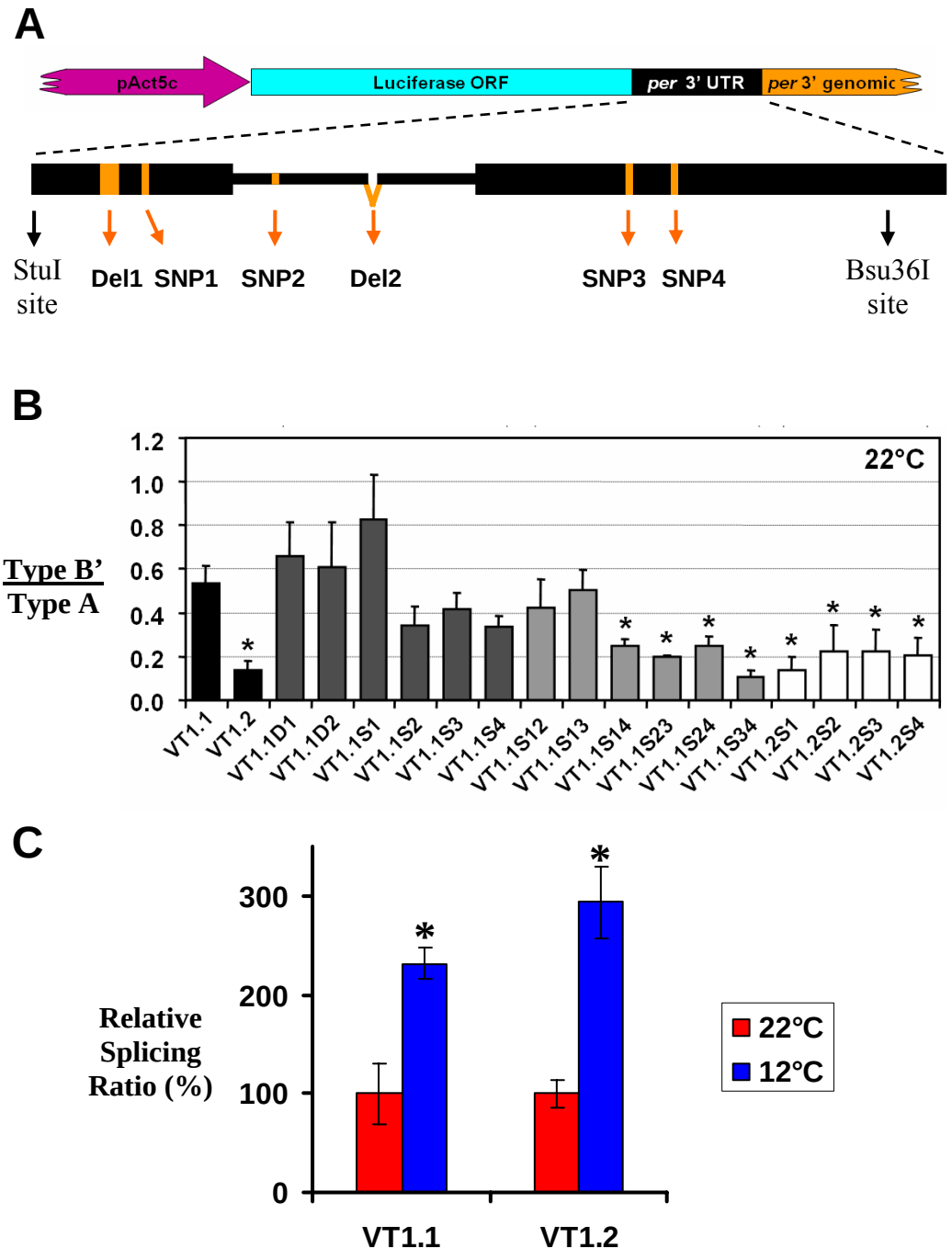

Figure 3.7. Multiple SNPs contribute to the different dmpi8 splicing efficiency between the VT1.1 and VT1.2 *per* 3' UTRs in S2 cells. (A). Schematic view of the pAct-Luc-UTR constructs for transient dmpi8 splicing assay in S2 cells. An actin promoter drives the transcription of a luciferase open reading frame, which is followed by

Figure Legend 3.7 Continued

the entire *per* 3' UTR and approximately 100 bp of 3' *per* genomic DNA. The constructs were generated by swapping the mutated VT1.1 or VT1.2 3' UTRs into the pAct-Luc-UTR backbone taking advantage of the unique StuI and Bsu36I sites. (B). Constructs were transiently transfected for two days before the S2 cells at 22°C were collected for dmpi8 splicing assay. Shown are the averages of five replicates (± sem). The brief construct names shown start with the genetic background of a haplotype followed by the SNP introduced, e.g., VT1.1S34 is a construct in the VT1.1 background with SNP3 and SNP4 from VT1.2. The stars on top of the bars indicate significant different in splicing efficiency against the VT1.1 construct ($P<0.05$). Fillings of bars: black, the VT1.1 and VT1.2 haplotypes as positive controls; dark gray, single polymorphism mutation in VT1.1 background; light gray, double mutations in VT1.1 background; white, single SNP mutations in VT1.2 background. (C). About one day after transient transfection, groups of cells were placed at 12° or 22°C for at least 24 hours and subsequently collected. Relative dmpi8 splicing ratios were calculated with the value at 22°C set to 100 and the values at 12°C normalized. Shown are the averages of two independent experiments. The stars on top of the bars indicate significant difference in splicing efficiencies at 12°C compared to 22°C ($P<0.05$).

enhances dmpi8 splicing but reduces or abolishes its thermosensitivity (K.H. Low, unpublished results).

Together, my work identified natural polymorphisms in the per 3' UTR that affect splicing efficiency and the timing of evening activity onset but without affecting the strengths of the 5' and 3' splice sites or the thermosensitivity of dmpi8 splicing.

Splicing of dmpi8 appears to enhance *per* mRNA nuclear export

Exon junction complex (EJC) is a protein complex that deposits 20-24 nucleotides upstream of mRNA exon-exon junctions as a result of splicing (75). Direct interaction between REF, a component of EJC, and the Nuclear Export Factor 1 (NXF1) has been described in both human and yeast systems (116, 136). These and other pieces of evidence from the *S. cerevisiae* and *X. laevis* models demonstrate that mRNA export is coupled to splicing and splicing is sufficient but not necessary to promote mRNA export (31).

To investigate whether enhanced dmpi8 splicing promotes *per* mRNA nuclear export, cytoplasmic and nuclear RNAs were fractionated from the S2 cells that had been transfected 2 days before with pAct-Luc-VT1.1 or pAct-Luc-VT1.2 constructs the same way as for dmpi8 splicing assay in S2 cells. Relative splicing ratios were then measured for the cytoplasmic and nuclear RNAs with the total RNAs from the same experiments as controls. Although splicing occurs in the nucleus, the VT1.1 haplotypes improves the apparent dmpi8 splicing ratio in the total RNA and cytoplasmic RNA but not the nuclear RNA, suggesting that the enhanced splicing in the VT1.1 haplotype facilitated nuclear spliced *per* mRNAs export to the cytoplasm (Figure 3.8 and Table 3.3). When comparing the dmpi8 splicing ratio among different fractions, cytoplasmic RNA fractions contain more spliced *per* than the nuclear fraction (Figure 3.8 and Table 3.3), indicating that

enhanced *per* mRNA export happens in both pAct-Luc-VT1.1 and pAct-Luc-VT1.2 constructs. Although no RNA marker (e.g., U6 snRNA as nuclear RNA marker) was included in this experiment to control for possible contamination of fractions, if there was leakage of nuclear material into the cytoplasmic fraction, the actual difference between the dmpi8 splicing efficiency in nuclear and cytoplasmic fractions should be larger than currently estimated. Due to the fractionation of the cytoplasmic and nuclear RNAs, no RNA can adequately serve as an internal RNA level control such as *cbp20* previously used in the fly head dmpi8 splicing assay (84). We calculated the relative *per* mRNA levels in the cytoplasm and nucleus from the splicing ratio data (Table 3.3, note b for formula). About 59% of total *per* mRNA in the pAct-Luc- VT1.1 transfected S2 cells exists in the cytoplasm, compared with significantly lower 38% cytoplasmic *per* mRNA for the pAct-Luc-VT1.2 transfected cells (Table 3.3). Together, these results suggest that the splicing of dmpi8 enhances *per* mRNA export, a coupled event that is important in accelerating the accumulation of cytoplasmic *per* transcripts that are ready for PER protein translation by two fold effects: 1) increasing the total *per* mRNA levels, and 2) exporting a higher proportion of *per* mRNA to the cytoplasm.

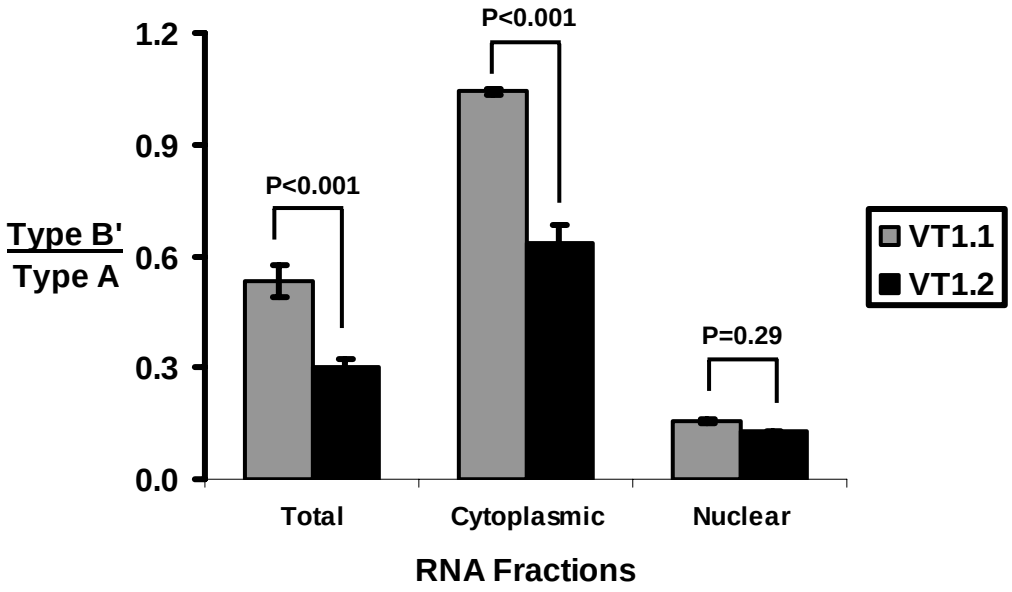

Figure 3.8. Splicing of dmpi8 appears to affect nuclear *per* mRNA export in S2 cells. The chart is plotted with the data shown in Table 3.3. Bars in gray, RNA fractions from S2 cells transfected with the pAct-luc-VT1.1 construct; Bars in black, RNA fractions from the S2 cells transfected with the pAct-luc-VT1.2 construct. Both total RNA and cytoplasmic RNA transcribed from the pAct-luc-VT1.1 construct showed significantly better splicing efficiency in the dmpi8 intron than those transcribed from the pAct-luc-VT1.2 construct ($P<0.001$), whereas no significant difference in the splicing efficiency of dmpi8 intron is found between the nuclear RNAs transcribed from the above two constructs ($P=0.29$).

Table 3.3. Splicing of dmpi8 apparently affects nuclear *per* mRNA export in S2 cells.[a]

RNA Fractions	VT1.1			VT1.2		
	ratio	SEM	calculated RNA levels (%) [b]	ratio	SEM	calculated RNA levels (%) [b]
Total	0.55	0.024	100	0.29	0.018	100
Cytoplasmic	1.03 [c]	0.009	59 [d]	0.63 [c]	0.027	38 [d]
Nuclear	0.16	0.005	41	0.14	0.011	62

[a] S2 cells were transiently transfected with pAct-luc-VT1.1 or pAct-luc-VT1.2 constructs and allowed to recover for 2 days at 22°C before harvesting. Data shown here are the averages from duplicates of two independent dmpi8 splicing assay experiments (N = 4).

[b] Calculated percentage of cytoplasmic *per* transcript = $[(1 + Rc) \times (Rt - Rn)] / [(1 + Rt) \times (Rc - Rn)] \times 100$, where Rt: total splicing ratio; Rc: cytoplasmic splicing ratio; Rn: nuclear splicing ratio.

[c] Splicing efficiency for cytoplasmic *per* RNAs is significantly higher than that of nuclear ones for both genotypes (VT1.1: $P<0.0001$, VT1.2: $P<0.01$).

[d] Calculated percent of *per* RNA found in cytoplasm is significantly higher in VT1.1 genotype than in VT1.2 ($P<0.05$).

Discussion

Prior work from our lab showed that temperature, clock and photoperiod influence the splicing efficiency of dmpi8, contributing to the evening activity onset in *Drosophila melanogaster* (84, 85). Findings described in this chapter indicate that natural polymorphisms outside of the dmpi8 region influence the splicing efficiency of the intron and consequently the evening behavior onset of the flies. Furthermore, the VT1.2 flies exhibit different photic sensitivity to late night light pulses than the VT1.1 flies. The results suggest that clock amplitude is modulated by the *per* mRNA levels which is enhanced by dmpi8 intron removal and supports the model that clocks with lower amplitudes are more sensitive to environmental stimuli. Lastly, the steady state dmpi8 splicing in S2 cells suggests that higher dmpi8 splicing efficiency leads to a higher proportion of total *per* mRNA being exported to the cytoplasm. Together with previous results that stronger splicing leads to higher *per* mRNA accumulation in fly heads, much higher *per* mRNA levels are available in the cytoplasm for PER protein translation during the late day/early night in flies.

Regulation of dmpi8 splicing by natural polymorphisms

Different lines of evidence, including splicing assays with RNAs from inbred flies, transgenic flies, and S2 tissue culture cells, confirmed that the natural polymorphism haplotypes VT1.1 and VT1.2 result in different splicing efficiencies for the dmpi8 intron (Figure 3.2, Figure 3.5, and Figure 3.7). Consistent with our previous model, enhanced splicing of dmpi8 advances evening activity onset in both VT1.1 inbred flies and $per^{0}w$;p{VT1.1} transgenic flies compared with the counterpart with VT1.2 3' UTRs

(Figure 3.3 and Figure 3.6). Interestingly, the VT1.2 flies, which express lower level of *per* mRNAs due to weaker dmpi8 splicing activity, demonstrate 4 hrs greater phase advance following a light pulse at ZT20 compared with the VT1.1 flies (Figure 3.4). A possible molecular explanation could be that the enhanced *per* mRNA levels in the VT1.1 flies give rise to faster PER and TIM protein accumulation and earlier progression of PER and TIM phosphorylation, as observed by Majercak et al (85). A light pulse during the late night time would reset the clock to a molecular state (i.e., degradation of PER and TIM) that will otherwise take less time to reach for those flies with more progressed phosphorylation of PER and TIM, and thus a shorter phase advance. But how the difference in *per* mRNA levels only generate difference with light pulse at ZT20 but not ZT18 is not clear.

Comparison of dmpi8 splicing efficiency with constructs containing various mutated polymorphisms in S2 cell system indicates that swapping of more than two SNPs between the VT1.1 and VT1.2 haplotypes are required to observe a significant change in splicing efficiency (Figure 3.7). Notably, swapping SNP3 and SNP4 of VT1.2 into the VT1.1 genetic background completely switched the *per* transcripts from the pAct-Luc-VT1.1S34 construct to transcripts with dmpi8 splicing efficiency resembling that of pAct-Luc-VT1.2. Owing to the fact that SNP3 and SNP4 are only 11 bp away from each other, it's rather possible that a stretch of RNA including the SNP3 and SNP4 loci is a binding site for unknown *trans*-acting protein factors, which indirectly regulate the dmpi8 splicing efficiency. All the SNPs discussed in this study are popular polymorphisms in flies from America, Africa and Europe (unpublished data from Cecilia Lim, personal communication). It would be very interesting to look for possible clines by which the flies

adapted to their environments.

Enhanced splicing of the dmpi8 intron apparently promotes *per* mRNA export

It is now widely accepted that mRNA export is coupled to splicing through the exon junction complex (EJC) and splicing is sufficient but not necessary to promote mRNA export (31, 116, 136). In the S2 cell splicing assay, VT1.1 3' UTR promotes 20% more *per* mRNA presence in the cytoplasm than VT1.2 3' UTR does (Figure 3.8 and Table 3.3). As the method only measures the steady state levels of *per*, the apparent promotion of cytoplasmic proportion by dmpi8 splicing could be due more efficient *per* mRNA export the stabilization of *per* mRNA in the cytoplasm. We favor the model that the *per* mRNA nucleocytoplasmic transport coupled to dmpi8 splicing plays a significant role in this observation. Together, improved splicing of the dmpi8 intron promotes clock phase advances through both increasing total *per* mRNA level and stimulating *per* mRNA export, thereby accelerating the accumulation of *per* mRNA levels in the cytoplasm and the appearance of PER protein (85).

Chapter 4. Clock-gated photic stimulation of *timeless* expression at cold temperatures and seasonal adaptation in *Drosophila*

Introduction

Circadian (≅24 hr) pacemakers or clocks play a major role in regulating the daily timing of activity in animals (54). The natural light-dark cycle is likely the predominant entraining agent (zeitgeber) in nature, synchronizing circadian clocks to local time and evoking daily adjustments in the approximately 24-hr endogenous periods of these oscillators such that they precisely match the 24-hr solar day. Although circadian clocks are synchronized to local time, changes in photoperiod (day length) and ambient temperature can modulate the daily patterns of activity in animals. While some of these environmental influences are 'direct' (termed, masking effects; (100), photoperiod and temperature also adjust the dynamics (phase and amplitude) of the clock, leading to changes in the daily distributions of clock-controlled rhythms. This feature of clocks not only enables them to play a role in tracking the passage of daily time but also in seasonal adaptation.

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 (85). Under standard daily cycles of 12 hr light followed by 12 hr dark (12:12LD) at 25°C, *D. melanogaster* exhibit a bimodal activity pattern with peaks centered around the lights-on ('morning' peak) and lights-off ('evening' peak) transitions (e.g., (46). A large portion of the morning peak appears to be due to a visually based masking effect of light that directly stimulates activity ('startle' response) (115, 154). The

duration of the photoperiod and ambient temperature have strong effects on the timing of evening activity. Over a wide range of photoperiods, the evening peak occurs around the light-to-dark transition (85, 113, 115, 124). As a result, lengthening the photoperiod delays the evening activity relative to the prior sunrise. Although the evening activity is centered around the light-to-dark transition, ambient temperature adds another layer of regulation by modulating the proportion of evening activity that occurs during either daytime or nighttime hours. As temperature increases the evening activity becomes progressively more nocturnal (85), an adaptive response that for small insects likely minimizes the risks of desiccation during the hot midday. 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 (139).

We showed that splicing of the terminal intron in the RNA product from the key clock gene termed *period (per)* is an important aspect of how the *Drosophila* circadian clock adapts to seasonal changes in temperature (85). At low temperatures a greater proportion of the intron (termed dmpi8) is spliced. The enhanced removal of dmpi8 at low temperatures leads to an advance in the timing of the *per* mRNA and protein accumulation phases, contributing to the preferential daytime evening activity of flies during cold days. More recent findings show that short photoperiods enhance the splicing efficiency of dmpi8 (23, 84). Thus, both low temperatures and short days increase the proportion of the spliced *per* RNA variant, acting in concert to advance the accumulation phase of PER and hence the timing of evening activity. This environmental synergism makes biological 'sense' because at temperate latitudes short days are normally associated with cooler

temperatures. We view the cold stimulated advance in the *per* RNA rhythm as an adaptive response to endow flies with the ability to exhibit daytime activity despite the early onset of dusk. Indeed, others and we proposed that the dual photoperiodic and temperature regulated splicing of dmpi8 acts as a seasonal sensor that modulates the timing of activity (23, 84, 85).

In addition to the temporal regulation of *per* mRNA levels, the daily accumulation of PER protein is strongly influenced by posttranslational regulation (reviewed in, (53, 85). A central feature of this regulation involves a second clock gene termed *timeless* (*tim*) (123). Both *per* and *tim* mRNA levels undergo daily cycles that manifest similar phases and are co-regulated by the same mechanism (reviewed in, (51). CLOCK (CLK) and CYCLE (CYC) are members of the basic helix-loop-helix (bHLH)/PAS (PER-ARNT-SIM) superfamily of transcription factors that heterodimerize to activate *per* and *tim* expression by binding to E-box elements in the promoter regions of these genes. Transcription of *per* and *tim* begins in the early-to-mid day resulting in peak mRNA levels being reached in the early night. During the late day/early night as the levels of PER and TIM increase they interact, an event that stabilizes PER and somehow promotes the nuclear accumulation of both proteins. Once in the nucleus, PER (and less likely TIM) interacts with the CLK-CYC transcription factor blocking its activity. In the absence of *de novo* synthesis, nuclear localized PER and TIM undergo progressive phosphorylation events leading to the rapid degradation of hyperphosphorylated isoforms. Reductions in the nuclear levels of PER relieves autoinhibition of CLK-CYC activity, initiating another round of *per*/*tim* expression. A second loop that regulates the cycling of *Clk* expression presumably adds robustness to the oscillatory mechanism.

Numerous lines of evidence indicate that the light-induced degradation of TIM is the primary clock-specific photoresponse in the entrainment of *Drosophila* clocks to daily light-dark cycles (63, 78, 101, 138, 158, 163). The putative blue-light photoreceptor CRYPTOCHROME (CRY) is required for the light-induced degradation of TIM in most, if not all, cells (37, 44, 61, 67, 131).

In this report we describe novel effects of light on *tim* expression that further demonstrate a strong interaction between photic and temperature signals by circadian pacemakers. In a mechanism that appears closely integrated with the dual photic and temperature regulation of dmpi8 splicing efficiency, we show that at cold but not warm temperatures light acutely stimulates expression of *tim* in a temporally gated manner. As a result, on cold short days the accumulation phases of *tim* and *per* transcripts are accelerated, working in concert to advance the phase of the clock. Notwithstanding the similar co-regulation of *per* and *tim* transcription, light does not acutely modulate the levels of *per* mRNA even though the photostimulation of *tim* expression at cold temperatures occurs at the transcriptional level. These results reveal seasonal adaptation in the molecular circuitries regulating clock gene expression.

Materials and Methods

Fly strains and collections

The wildtype (CS), *per*^{01}, *tim*^{0}, *cyc*^{0}, *Clk*^{Jrk}, *cry*^{b} and *norpA* flies used in this study were previously described (3, 72, 119, 131). The *tim-luciferase* transgenic lines used in this study were previously described (91). All flies were reared at room temperature (22-25°C) and maintained in vials containing standard agar-cornmeal-sugar-yeast-tegosept media. Vials containing ~100 young (2-6 day old) adult flies were placed in controlled environmental chambers (Percival) at the indicated temperature and exposed to at least three 24 hr photoperiods of alternating light:dark cycles [LD; where zeitgeber time 0 (ZT0) is defined as lights-on], and in some cases subsequently maintained in the constant darkness (DD). Cool white fluorescent light (~2000 lux) was used during LD and the temperature did not vary by more than 0.5°C between the light and dark periods. At selected times during LD and DD flies were collected by freezing.

RNase protection assay

For each time point, total RNA was extracted from ~10 μl of fly heads using TriReagent (Sigma) and following manufacturer's recommended procedure. The levels of *per*, *tim* and *luciferase* (*luc*) transcripts were determined by RNase protection assays (52). *per* and *tim* RNA levels were determined using the *per* 2/3 probe (52) and a *tim* probe (127), respectively. To measure *luc* RNA levels in *tim-luc* transgenic flies (91) we used PCR to generate an antisense probe. PCR was performed with genomic DNA isolated from the heads of timp^{FL-LUC}/TER1 flies (91) as template in the presence of the following

primers: (i) 5'-AAT**AAGCTT**GATTACCAGGGATT TCAGTCG-3' (termed luc-p1; an engineered HindIII site is shown in bold and luciferase sequences are underlined) and (ii) 5'-TCCGAGTGTAGTAAACATTCC-3' (termed luc-p2). The 5' primer (luc-p1) contains *luciferase* sequences between 772 and 792 nt, whereas the 3' primer (luc-p2) contained *luc* sequences between 1044 and 1064 nt (numbering according to GeneBank acc. no. M15077). The PCR product was inserted into the pGEM-T Easy vector (Promega). The resulting plasmid (pGEM/luc) was linearized with HindIII and radiolabeled antisense RNA produced *in vitro* using T7 RNA polymerase and [^{32}P]-UTP. Following RNase protection assay, this probe protects a 293 nt fragment of *luc* mRNA. As a control for RNA loading in each lane, a ribosomal protein probe (RP49) was included in each protection assay (52). Protected bands were quantified using a Phosphorimager from Molecular Dynamics.

Results

Light evokes rapid increases in the levels of *tim* mRNA at cold temperatures

We previously showed that although robust rhythms in the abundance of *per* mRNA are observed at all temperatures during either light:dark cycles (LD) or constant dark conditions (DD), the *tim* mRNA rhythm quickly dampens to near trough amounts during DD at cold (i.e., 18°C) but not warmer (i.e., 25° or 29°C) temperatures (85) (Figure 4.3A). This raised the possibility that during cold days light stimulates *tim* expression.

To determine whether light has acute effects on *tim* expression, wildtype Canton-S (CS) flies were kept at 18°C and exposed to several days of 12:12LD followed by two days in complete darkness. Subsequently, flies were treated with light-pulses at different times during the second day of DD when *tim* expression is constitutively low (85) (Figure 4.1). Light-pulses evoked rapid increases in the abundance of *tim* mRNA (Figure 4.1A and B), whereas *per* transcript levels exhibited little or no changes (Figure 4.1C). After ~4-6 hr of continuous light exposure *tim* RNA reached maximal levels of about 2-4 fold greater than control non-treated samples, followed by a gradual return to baseline values after termination of the light treatment (Figure 4.1 and data not shown). As previously reported, light-pulses did not lead to acute changes in the transcript levels of *per* or *tim* at standard warmer temperatures (e.g., 25°C) (78, 122); and data not shown).

Differential effects of clock mutants on light-induced increases in the abundance of *tim* transcripts

We sought to determine whether the photoinducibility of *tim* expression at cold temperatures is dependent on the clock proteins CLK, CYC, PER and TIM previously shown to participate in interconnected transcriptional feedback loops required for daily

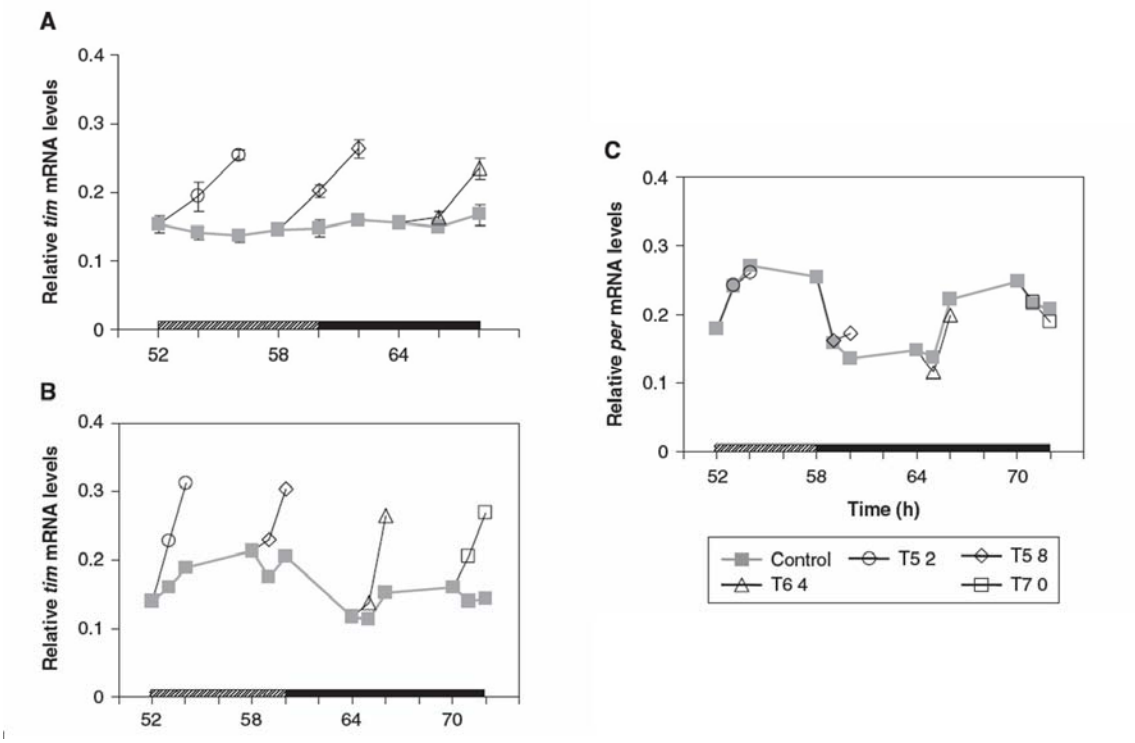

Figure 4.1. Light evokes rapid increases in *tim* but not *per* mRNA levels at 18°C. Wildtype flies were exposed to four cycles of 12:12LD at 18°C and on the second day of DD were treated with light-pulses; another group of flies served as non-treated controls. Light exposure was initiated at the indicated times (T; hr after the last lights-on transition) and flies were collected at 2 and 4 hr after the beginning of the light treatment (A), or 1 and 2 hr after the beginning of the light treatment (B and C). Shown are the relative levels of *tim* (A and B) or *per* (C) transcripts in head extracts measured by RNase protection assays. Grey symbols; non-treated controls; open symbols, light-treated flies. Hatched horizontal bar, subjective day; black horizontal bar, subjective night. (A) Data from three independent experiments were pooled and average values (±SEM) shown. (B and C) Results from a representative experiment are shown (n=3). *tim* and *per* RNA levels were measured from the same extracts.

cycles in *per* and *tim* mRNA levels (see Introduction). To this end we used arrhythmic mutant flies that either abolish (per^{01}, tim^{0} and cyc^{0}) (72, 119, 123) or at the very least severely impair (Clk^{Jrk}) (3) the known activities of these genes.

We observed differential effects on *tim* photoinduction in the different mutants tested at 18°C; (1) no or little increases in cyc^{0} and Clk^{Jrk} flies, (2) attenuated but significant increases in tim^{0} flies, and (3) robust responses in per^{0} flies (Figure 4.2, and data not shown). These results are similar to other light-sensitive rhythmically expressed core clock genes such as *frequency* (*frq*) in *Neurospora* (15, 16, 21, 24, 25, 79) and *mPer1/2* in mammals (106, 126, 147) whose acute photoresponses are preferentially dependent on elements from the 'positive' but not 'negative' limbs operating within their respective clockworks (see Discussion). Similar to the situation at standard warmer temperatures, the overall levels of *tim* RNA are lower in the Clk^{Jrk} and cyc^{0} mutants compared to per^{0} and tim^{0} flies (Figure 4.2B and data not shown) (3, 119), indicating a critical role for CLK-CYC-mediated transactivation of *tim* expression over a wide range of temperatures.

High-amplitude photic induction of *tim* RNA in cry^{b} and *norpA* mutants

To determine whether the putative blue-light photoreceptor CRY has a role in the photostimulation of *tim* mRNA levels on cold days we used cry^{b} flies which, at best, have low levels of CRY activity (37, 131). Light treatments at 18°C increased the levels of *tim* RNA in cry^{b} flies to greater amplitudes compared to wildtype controls (Figure 4.2A). Thus, although we cannot rule out the possibility of residual CRY activity in cry^{b} flies, it is likely that CRY is not a major player in the photoreception pathway leading to light-mediated increases in *tim* transcript levels at cold temperatures. This is in sharp contrast to a key role for CRY in the photosensitivity of TIM protein stability (131).

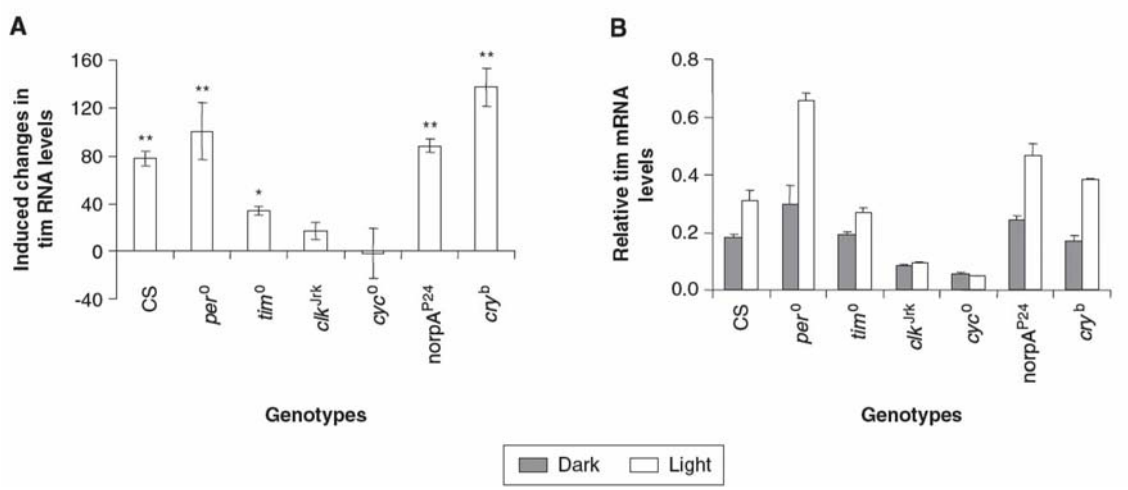

Figure 4.2. Differential effects of clock and phototransduction mutants on light-induced increases in *tim* mRNA levels. Wildtype (CS) and mutant (per^0, tim^0, Clk^{Jrk}, cyc^0, $norpA^{P24}$ and cry^b) flies were exposed to four days of 12:12LD at 18°C and on the second day of DD flies were exposed to light for 2 hr beginning at T52; another group of flies served as non-treated controls. Results from three independent experiments were pooled and are depicted in two different formats (A and B). (A) For each genotype the abundance of *tim* transcripts in non-treated controls was set to 0 and the light-treated values normalized to yield the percent induction by light. Significant difference between light-treated and control values using student's t-test; *, $P<0.05$; **, $P<0.01$. (B) Relative levels of *tim* in non-treated (grey bars) and light-treated (open bars) flies.

Recent findings indicate that in concert with PER, CRY acts as a transcriptional repressor of CLK-CYC-mediated expression in peripheral pacemakers (22). This could explain why we observe larger magnitude photoinduction of *tim* RNA levels in the cry^b mutant (Figure 4.2A; see Discussion).

To examine whether classic visual phototransduction pathways are involved in regulating the light-mediated stimulation of *tim* expression we used the well-characterized *no-receptor-potential-A* (*norpA*) mutant termed $norpA^{P24}$, which inactivates phosphoinositol phospholipase Cβ (PI-PLCβ) and renders the compound eyes and ocelli unable to transduce photic signals (92, 108); although see, (48). Although the opsin-based ocular phototransduction pathway is not necessary for circadian rhythmicity or its entrainment to daily light-dark cycles, it contributes to several different aspects of circadian responses and sensitivity to photic treatments (61, 67, 86, 93, 115, 131, 160). Light-induced increases in *tim* RNA levels were similar or greater in *norpA* mutants (Figure 4.2). This was also the case when using anatomically blind flies such as *eyes absent* (*eya*) (11, 149) (data not shown).

Day length regulates the timing of *tim* photosensitivity

To further examine the effects of the clock and light on *tim* expression at cold temperatures we entrained flies to either short (6:18LD) or long (12:12LD) photoperiods (Figure 4.3). As might be expected the overall rhythm in *tim* RNA abundance was advanced approximately 6 hr (relative to lights-on) under 6:18LD compared to 12:12LD (Figure 4.3A). Likewise, the timing in the daily accumulation of PER and TIM proteins are also advanced under the shorter photoperiod (85). However, we noticed that in flies maintained under 12:12LD conditions *tim* RNA levels are essentially flat for several hrs

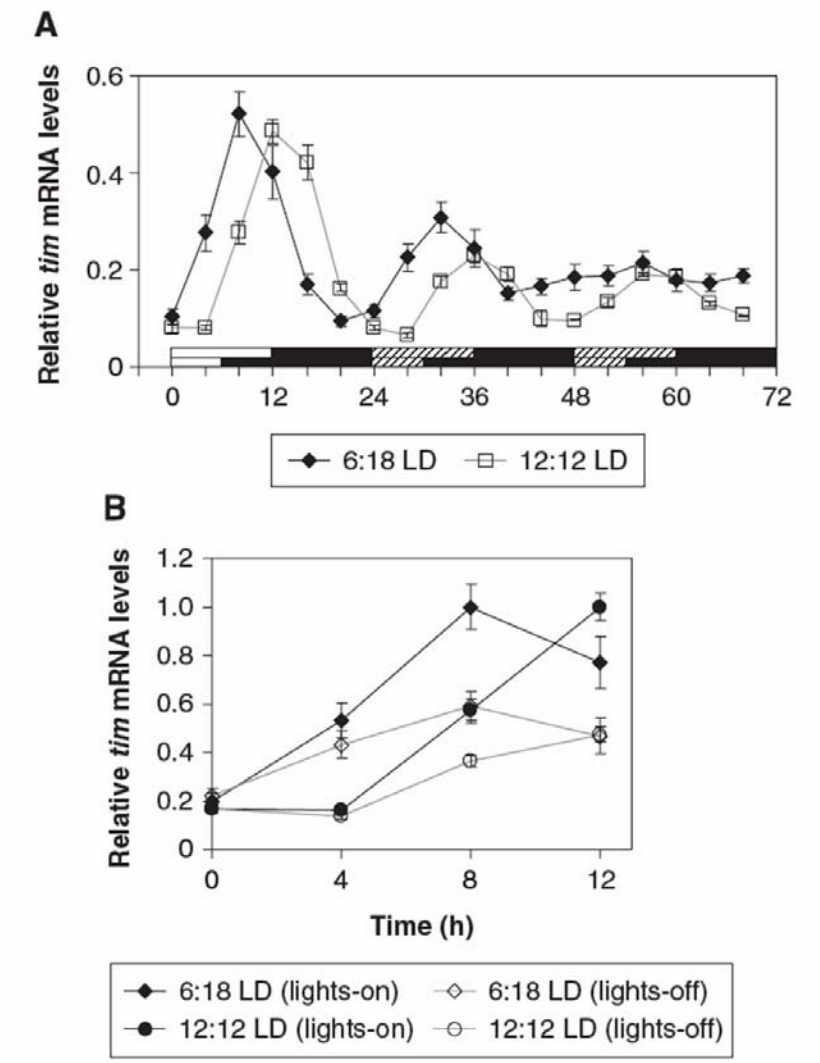

Figure 4.3. Photoperiod affects the waveforms of the *tim* mRNA abundance profiles. (A) Wildtype flies were kept at 18°C, exposed to four days of different photoperiods (6:18LD or 12:12LD as indicated, right of panels) and collected during the last day of LD and the next two days of DD. The relative levels of *tim* transcripts in head extracts were measured by RNase protection assays. White horizontal bar, lights on; black horizontal bar, lights off; hatched horizontal bar, subjective day. (B) Wildtype flies were kept at 18°C, exposed to three days of either 6:18LD or 12:12LD. Subsequently, one group of flies was collected during ZT0-12 on the fourth day of LD (lights-on), whereas another group did not experience lights-on during the last LD cycle but was maintained in the dark (lights-off) and collected during CT0-12. Shown is an overlay of *tim* mRNA levels for

Figure Legend 4.3 Continued

lights-on and lights-off treated flies. This analysis better illustrates the relatively constant levels of *tim* mRNA during the first 4 hr in 12:12LD entrained flies even in the presence of light. Data from four independent experiments were pooled and average values (±SEM) shown.

after the lights-on transition. In contrast, the levels of *tim* RNA are rising during the early daytime hours in the short photoperiod, suggesting that in daily LD cycles the duration of the photoperiod modulates the ability of light to acutely stimulate *tim* RNA levels following sunrise.

This is further illustrated by overlaying the *tim* RNA accumulation phases of short and long day entrained flies that were either collected during the last LD cycle (referred to as 'lights-on') or did not experience lights-on but rather remained in the dark during the last LD cycle (i.e., first day of DD; referred to as 'lights-off', Figure 4.3B). In 12:12LD entrained flies the levels of *tim* RNA remain relatively flat during the first 4 hr into the day/subjective day whether the flies are exposed to light or not (Figure 4.3B, filled and open circles). Under these entrainment conditions, light-exposed flies begin to exhibit higher levels of *tim* RNA after 4 hr following lights-on (ZT4), coinciding with the start of the clock-driven *tim* RNA accumulation phase—revealed by the temporal pattern in flies that did not experience lights-on during the last LD cycle (Figure 4.3B, compare filled and open circles). Otherwise stated, the acute photostimulation of *tim* expression appears restricted to times in the day when the endogenous clock drives the rising phase in *tim* RNA levels (Figure 4.3B). Thus, it appears that under long photoperiods *tim* expression is refractory to stimulation by light during the early morning hrs. In contrast, under the short photoperiod the start of the *tim* RNA accumulation phase occurs around lights-on (ZT0/CT0).

To better establish the possibility of temporal gating in the photosensitivity of *tim* expression, flies were entrained to either 6:18LD or 12:12LD and exposed to brief light-pulses at different times during the last dark phase of LD and the first subjective day

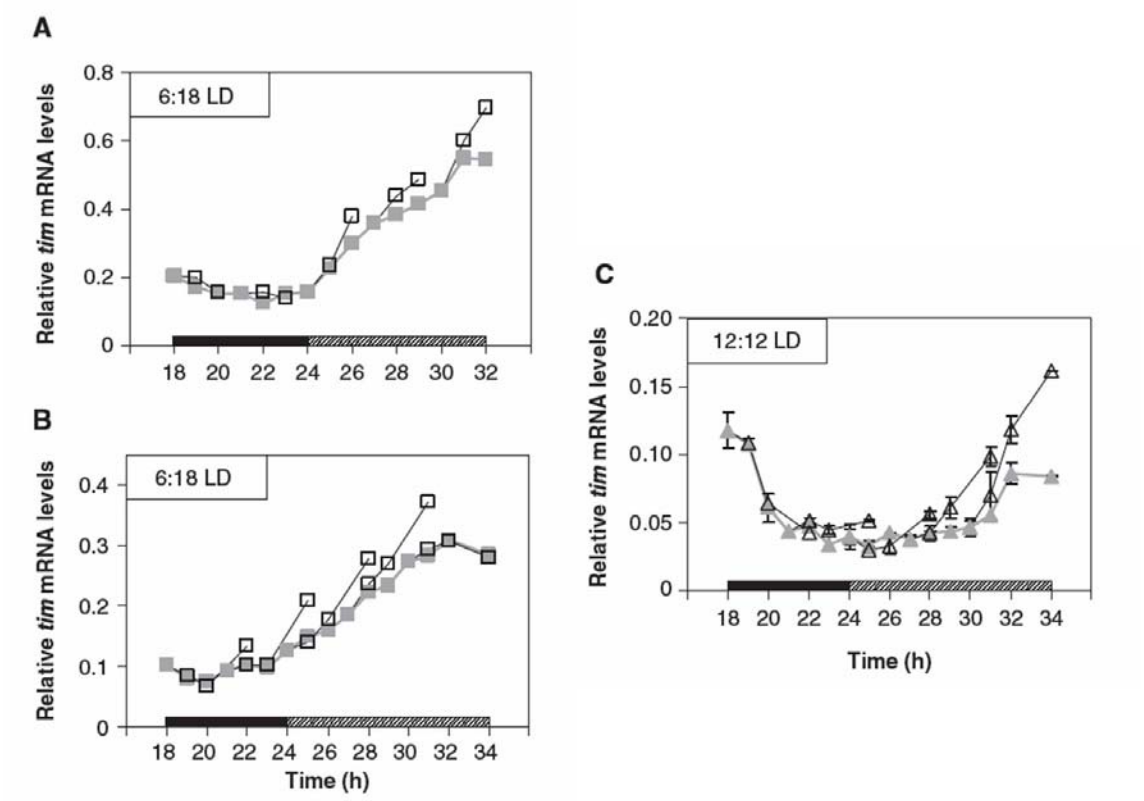

Figure 4.4. Light induction of *tim* transcript levels is temporally gated. Wildtype flies were exposed to four cycles of either 6:18LD (A and B) or 12:12LD (C) at 18°C and subsequently maintained in DD. During the last dark cycle of LD and the first subjective day of DD, a group of flies was exposed to light and collected 1, 2 or 4 hr after the beginning of the light treatment; another group served as non-treated controls. The relative levels of *tim* transcripts in head extracts were measured by RNase protection assays. Black horizontal bar, night; hatched horizontal bar, subjective day. For the 6:18LD conditions two independent experiments are shown (A and B), whereas for the 12:12LD data values from two independent experiments were pooled (in some cases pooling of data to show light-induced effects was difficult due to slight differences in the RNA profiles of non-treated control flies).

of DD (Figure 4.4). The results clearly indicate that in both photoperiods, acute increases in the abundance of *tim* transcripts by brief light stimulation are mainly or solely evoked during the accumulation phase of the *tim* RNA rhythm. For example, whereas light-pulses at time 24 lead to substantial increases in the levels of *tim* RNA in flies entrained to 6:18LD (Figure 4.4A and B), such was not the case under the longer photoperiod (Figure 4.4C). These findings are very consistent with the more low and flat levels of *tim* RNA during the first 4 hr after lights-on in 12:12LD compared to 6:18LD (Figure 4.4B). Thus, the photoperiod regulates the relative alignment between dawn and the start of the rising phase in *tim* expression, which in turn determines the timing of when light can begin to acutely stimulate *tim* mRNA levels during the day. We propose that on long days that are unseasonably cold this photoperiodic gating mechanism ensures that *tim* expression is not prematurely induced by sunrise. It is likely that circadian gating in the magnitude of the photic response in *tim* expression was more difficult to observe in the experimental paradigm where flies were treated after two days in DD (Figure 4.1) because under these conditions the *tim* RNA rhythm quickly dampens, which might increase its 'effective' photosensitivity (see Discussion).

Transcriptional regulation of *tim* photoresponses at cold temperatures

Under standard conditions of 25°C, daily oscillations in the abundance of *tim* mRNA are largely regulated at the transcriptional level (91, 105, 128, 132, 151). The 5' non-transcribed regions of *per* and *tim* each contain at least one *cis*-acting canonical E-box element (CACGTG) that is critical for high level expression and likely acts as a direct binding site for the CLK-CYC transcription factor (28, 47, 77, 83, 91, 151). To determine whether transcriptional regulation contributes to light-induced increases in the abundance

of *tim* mRNA at cold temperatures we used a set of previously generated transgenic flies bearing *tim* 5' regulatory sequences fused to a *luciferase* (*luc*) reporter gene (91). We first analyzed *luc* RNA levels in $timp^{FL\text{-}LUC}$ flies, which harbor a transgene containing a 756-bp *tim* promoter fragment that begins upstream of the transcription start site followed by the first untranslated exon, first intron, and noncoding parts of the second exon fused to the Luc open reading frame. Prior work showed that under standard conditions of 12:12LD at 25°C, Luc reporter activity exhibits high amplitude rhythms in $timp^{FL\text{-}LUC}$ flies (91).

At 18°C the levels of *luc* RNA during LD exhibited robust cycles that dampened somewhat during the first day of DD (Figure 4.5A and B). These results are similar to those observed for endogenous *tim* mRNA except that the amplitude of the *luc* RNA rhythm during LD is approximately 50% that of native *tim* mRNA and the relative decrease in amplitude from LD to DD is less for the *luc* RNA rhythm (Figure 4.5B).

To probe for acute photoresponses in the *tim-luc* transgene versions, we used the more sensitive experimental paradigm of exposing flies to light for several hr during the second day of DD (Figure 4.6A). Exposure to light evokes acute photostimulation of *luc* RNA levels (Figure 4.6, panels A and C). Although the behavior of *luc* and endogenous *tim* transcripts were similar following nocturnal light exposure, we observed differences in the magnitude and circadian gating of this response (Figure 4.6, compare panels B and C). It is likely that the more sustained cycling of *luc* RNA levels in DD compared to the relatively flat and low expression of endogenous *tim* is enhancing time-of-day differences in the amplitudes of the light-induced increases in the levels of *luc* compared to *tim* transcripts. Also, differences in the cycling patterns and inferred photosensitivities of *luc* compared to *tim* RNAs could result from variations in the stabilities of the different

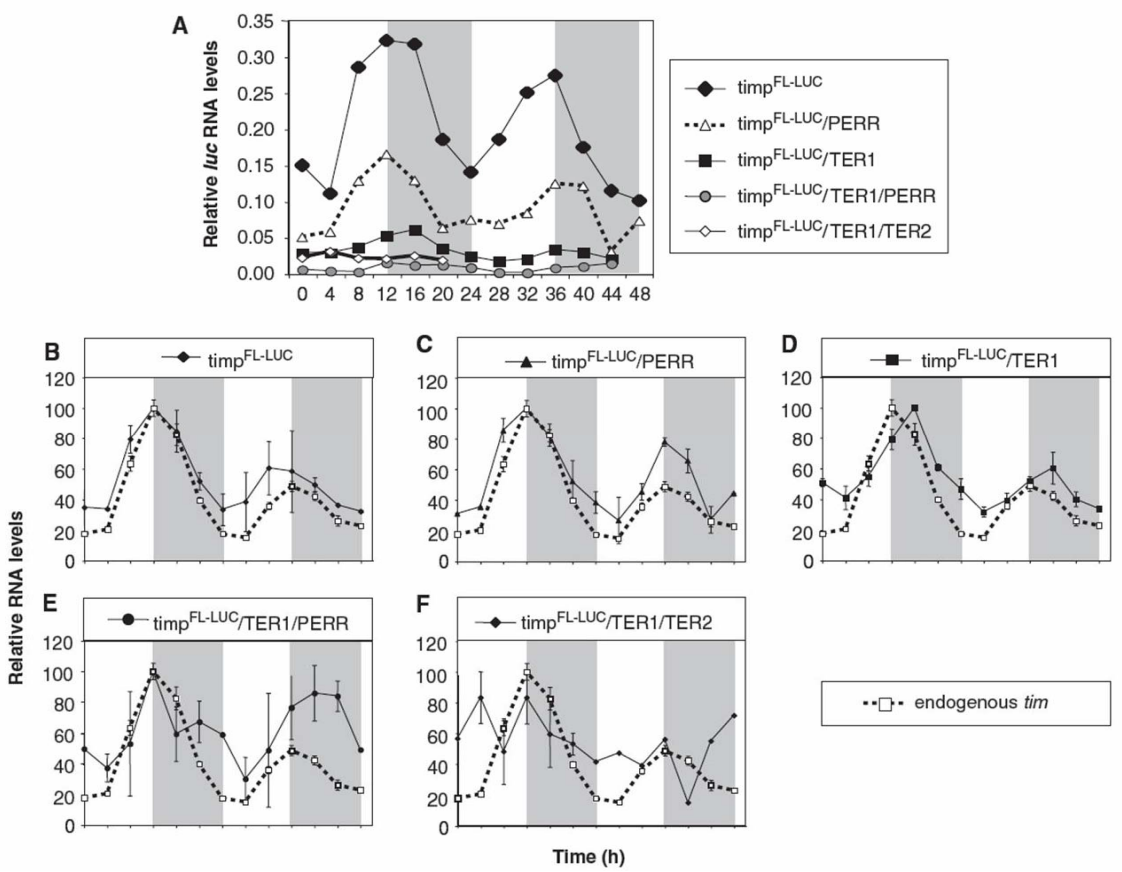

Figure 4.5. Transcriptional regulation contributes to the daily oscillations in the levels of *tim* mRNA observed during LD cycles at cold temperatures. Transgenic flies bearing different *tim-luc* transgenes (as indicated, right or top of panels) were kept at 18°C, exposed to four days of 12:12LD and collected during the last day of LD and the first day of DD. The relative levels of *luc* and *tim* transcripts in head extracts were measured by RNase protection assays. (A) Relative levels of *luc* RNA for each of the genotypes evaluated in this study. (B-F) For each genotype, peak values for either *luc* or *tim* RNA levels were set to 100 and all other values normalized. Shown are overlays of the profiles for *luc* (solid line) and *tim* (dashed line) transcript levels. All the different transgenic lines displayed highly similar profiles in endogenous *tim* RNA cycles. Values from at least two independent experiments were pooled.

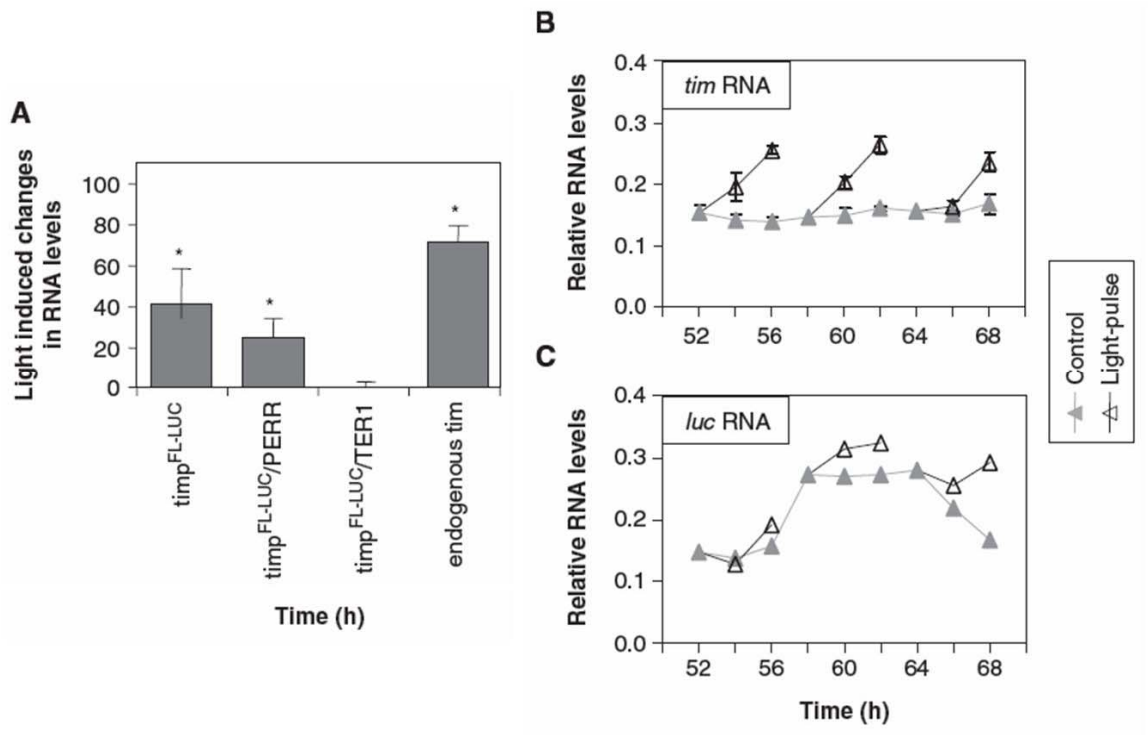

Figure 4.6. Acute light induction of reporter gene expression driven by *tim* 5' regulatory elements. Several different transgenic flies bearing different *tim-luc* transgenes were exposed to four cycles of 12:12LD at 18°C and on the second day of DD exposed to light; another group of flies served as non-treated controls. (A) Light exposure was initiated at T52 and flies collected 2 hr after. For each genotype (bottom) the abundance of *luc* (black bars) or endogenous *tim* (white bar) transcripts was set to 0 and the light-treated values normalized to yield the percent induction by light compared with non-treated controls. Values were pooled from at least two independent experiments. Significant difference between light-treated and control values using student's *t*-test; *, $P<0.01$. (B and C) $timp^{FL\text{-}LUC}$ flies were exposed to light at the indicated times. Shown are relative RNA levels of endogenous *tim* (B) and *luc* (C) in light-treated (open symbols) and non-treated controls (grey symbols).

transcripts. Likewise, differences were observed in the photic responses of endogenous mouse *Per1* transcripts and an *mPer1-luc* reporter that were suggested to be based on posttranscriptional events (156). Another possibility is that other regions of the *tim* promoter not present in the timp^{FL-LUC} transgene are required to mimic wildtype *tim* expression. Indeed, at 25°C whereas the phase in the bioluminescence rhythm from timp^{FL-LUC} flies is early, flies transformed with a *tim-luc* construct containing a larger fragment of the *tim* 5' non-transcribed region drives reporter gene expression that more faithfully reflects the temporal pattern of endogenous *tim* expression (132). Nonetheless, the results clearly show that at cold temperatures transcriptional regulation makes a strong contribution to the photic-induced increases in the levels of *tim* mRNA. This is consistent with the observation that in LD cycles *tim* mRNA levels are low and constant in the *Clk*^{Jrk} and *cyc*^{0} mutants (Figure 4.2 and data not shown).

Besides generating the timp^{FL-LUC} flies, McDonald et al., (2001) altered *tim* regulatory sequences and identified several closely spaced *cis*-acting DNA elements in addition to the canonical E-box that regulate the expression of *tim* in flies and/or *Drosophila* cultured cells. These include two 11-bp *tim* E-box-like repeats (TER1 and TER2) and a 10-bp sequence conserved in the *per* promoter (PERR). They showed that in flies maintained at 25°C, Luc activity is not affected by mutations in PERR. However, although cycles in Luc activity are observed in TER1 mutants, peak values are reduced to about 50%. In the TER1/TER2 double mutant luciferase activity is even lower, approximately 20 to 30% of the wildtype control (timp^{FL-LUC}), and cycling is nearly abolished. Finally, the TER1/PERR double mutant is only slightly different from the TER1 mutant, indicating that TER1 and TER2 play a major role in *tim* transcription.

We determined the behavior of *luc* RNA in LD cycles and in response to light-pulses in the different *tim-luc* transgenic flies (Figure 4.5 and Figure 4.6). We are wary of comparing our results with those previously reported at standard temperatures by McDonald et al., (2001) because we measured *luc* RNA levels in head extracts using RNase protection assays, whereas they measured Luc protein activity in whole flies. Nonetheless, our results are fairly consistent with the prior work because TER1 and TER2 but not PERR are important to manifest high-level expression of *tim* (Figure 4.5A). The main exception is that compared to the TER1 mutant, the TER1/PERR double mutant showed more severe effects on overall levels (Figure 4.5A) and cycling amplitudes (compare panels D and E).

Under a variety of conditions (e.g., altering duration of light-pulse and time in the day when the light exposure was initiated) we routinely observed light-induced increases in the levels of *luc* RNA in the PERR mutant but not in the TER1 mutant, either singly or in combination with another mutation (Figure 4.6A, and data not shown). Although the results suggest that TER1 and/or TER2 play key roles in the rapid light-mediated induction of *tim* expression at cold temperatures, it is possible that the attenuated photosensitivities are an indirect consequence of the overall inefficiency of these modified *tim* promoters (Figure 4.5A). Future work will be required to test for the possibility of one or more dedicated photoresponsive *cis*-acting elements in *tim* regulatory sequences. The fact that circadian cycling and light responses in *tim* expression are abolished in the Clk^{Jrk} and cyc^{0} mutants (Figure 4.2 and data not shown) indicates that both phenomena are dependent on CLK-CYC-induced transactivation. Likewise, the light and clock regulation of *frq* expression in *Neurospora* are both dependent on the White Collar-1 (WC-1)/White

Collar-2 (WC-2) complex (WCC) (15, 21, 24, 55, 79).

Discussion

In prior work we showed that the cold stimulated splicing of the *per* dmpi8 intron advances the accumulation phase in the levels of *per* mRNA (85). Findings in this study expand on this model by suggesting an integrated circuit whereby light stimulates the daytime rise in the abundance of *tim* transcripts at cold temperatures. Thus, cold short days accelerate the daytime accumulation rates of both *per* and *tim* mRNA and protein levels, events that contribute to the earlier onset of evening activity in flies as temperatures drop (85). Together, the results indicate complex integration of photic and temperature signals by the *Drosophila* clock. We propose that the dual thermal and photic regulation of dmpi8 splicing and *tim* expression act as seasonal sensors by fine-tuning the trajectories in the daytime rising phases of the *per*/*tim* RNA cycles, endowing flies with the ability to optimally align their physiological and behavioral rhythms to the prevailing environmental conditions.

Current model for how the *Drosophila* clock responds to seasonally cold days

The timing of the clock-controlled evening activity in *Drosophila* is regulated by the length of the photoperiod and temperature. Although decreasing the photoperiod tends to increase the proportion of evening activity occurring during the nighttime (85, 115, 124, 143), temperature appears to be the main environmental cue determining whether the evening activity will be mostly diurnal or nocturnal (85, 142, 161). At cold temperatures the enhanced splicing of the *per* dmpi8 intron and the photostimulation of *tim* expression adjust the daily upswing in the *per*/*tim* RNA and protein accumulation phases such that they rise faster. Earlier increases in PER/TIM protein levels likely underlie the preferential

manifestation of daytime evening activity in *Drosophila* during cold days (85). We view the rapid induction of *tim* expression at dawn and the enhanced splicing of dmpi8 as cold-specific responses that alter the dynamics of the clock in 'anticipation' of a short photoperiod, which is characteristic of spring/winter seasons in temperate regions. Although highly speculative, this scenario would imply that it is the *de novo* synthesized PER/TIM in the cytoplasm that is most intimately involved in regulating the timing of evening activity.

The photostimulation of *tim* expression on cold days is gated by the clock such that it is mainly restricted to the accumulation phase of the *tim* mRNA abundance rhythm (Figure 4.3B and Figure 4.4). Because photoperiod modulates the dynamics of the clock, changes in day length will affect the relative alignment between the start of the rise in *tim* mRNA levels and the timing of dawn. On long days, *tim* mRNA levels begin to increase several hours after sunrise (Figure 4.3). Hence, the circadian gating of *tim* photostimulation would ensure that on long days that are unseasonably cold the dawn associated sunlight does not prematurely advance the accumulation phase of *tim* RNA abundance. A similar integration of appropriate photic (short days) and temperature (cold) signals are also observed for maximal splicing efficiency of dmpi8 (23, 84, 85).

A critical role for the daily rhythms in *per* and *tim* mRNA levels has been questioned because functional clocks can be designed with constant expression of *per* and *tim* (e.g., (159). It was proposed that the strong posttranslational regulatory mechanisms that enable PER and TIM levels to cycle in the absence of oscillating *per* and *tim* RNA levels are sufficient to drive molecular and behavioral rhythms. While cyclical *per* and *tim* expression is not necessary for the manifestation of circadian rhythms, these clock are

defective with low amplitudes and long periods. In addition, it is now clear that rhythms in the RNA levels of key clock genes play an important role in generating optimal phase alignments in natural conditions. Indeed, even though light stimulates the rapid degradation of TIM and TIM regulates the stability of PER, alterations in the rising phases of the *per*/*tim* RNA cycles can alter the kinetics of the PER/TIM accumulation phases, key events in the progression of the clock (e.g., (85). As such the timing of PER and TIM activities are not solely dictated by a fixed amount of time following sunrise or sunset (e.g., 'hour-glass' timing mechanism) but are subject to seasonal adjustments.

Transcriptional regulation of *tim* mRNA levels by light

The abundance of *tim* mRNA levels at cold temperatures is strongly controlled by light with some contribution from circadian regulation since low amplitude cycling occurs for several days in constant dark conditions (Figure 4.3A) (85). Based on the analysis of a previously generated set of *tim-luc* transgenic flies (91), we conclude that transcriptional regulation likely makes significant contributions to high-amplitude cycling of *tim* mRNA levels during light-dark cycles and its rapid photoinduction at cold temperatures (Figure 4.5 and Figure 4.6; and data not shown).

How might light evoke rapid increases in *tim* mRNA levels? Prior work has shown that light can induce the rapid accumulation of *tim* and *per* transcripts in transgenic flies bearing a variant form of PER that appears to be defective in autoinhibition (122). It was suggested that in these mutant flies TIM can still enter the nucleus where it is sufficient to mediate downregulation of CLK-CYC-mediated transactivation. Under these conditions the rapid degradation of TIM by light would quickly relieve autoinhibition of *per*/*tim* expression. However, in the normal context although the light mediated degradation of

TIM enhances the instability of PER by promoting its hyperphosphorylation, the decline in the abundance of nuclear PER is a relatively slower process compared to that of TIM (71, 78, 117, 152, 163). As a result hyperphosphorylated PER, which is very effective at transcriptional repression (104), remains in the nucleus for several hours after TIM disappears presumably explaining why light does not evoke rapid changes in *per*/*tim* mRNA levels. It is possible that at cold temperatures light-mediated changes in TIM metabolism somehow evoke rapid reductions in the ability of PER to function as a transcriptional inhibitor. This could explain why photoinducibility of *tim* expression is attenuated in the tim^0 mutant; i.e., in the absence of TIM, PER can still engage in transcriptional repression but not in a photosensitive manner.

Relevant to this discussion are recent results indicating that in addition to its role in photic entrainment, CRY functions as a repressor of CLK-CYC-mediated transcription in peripheral clocks, similar to its role in mammalian circadian pacemakers (22). It was also suggested that whereas PER and CRY are both needed to effectively repress *per* expression, either PER or CRY can reduce *tim* expression, especially within the context of a weak promoter setting. Perhaps such is the scenario at cold temperatures where overall *tim* expression is reduced (i.e., possibly reflecting low transcriptional efficiency) (85). This might explain why *tim* photoinduction is relatively greater in per^0 and cry^b mutants compared to wildtype controls (Figure 4.2); i.e., by themselves PER or CRY have reduced inhibitory potential that can be further abrogated by photic signals. While in the case of per^0 flies the enhanced photoinduction of *tim* expression can be easily explained as a result of the light-induced degradation of CRY (80), how PER protein levels/activity might be modulated by light in the cry^b mutant is not clear. In any event, mechanisms based solely

on photic regulation of autoinhibitory components (e.g., PER, CRY) do not easily explain why *tim* and not *per* is rapidly induced by light (Figure 4.1A and B).

A possible explanation for the differential acute effects of light on *per* and *tim* expression is that there is a light-inducible element in the *tim* promoter that is functionally relevant at cold temperatures. There are several examples where gene expression is regulated by combinations of acute effects of light, duration of photoperiod and circadian pathways, and in some cases distinct *cis*-acting elements that mediate light and clock regulation have been identified (4, 9, 38, 94, 97, 98, 137). Our analysis of a series of *tim-luc* fusions were difficult to interpret because all the transgenes that showed attenuated photosensitivity also had markedly reduced expression levels (Figure 4.5 and Figure 4.6).

However, even if there is a cold-dependent light-inducible element(s) driving *tim* photoinduction, it is not clear why the *tim* RNA rhythm strongly dampens in constant dark conditions when robust cycling of *per* transcript abundance is maintained (Figure 4.1 and Figure 4.3) (85). This 'dark' phenotype is curious because similar to standard warmer conditions, at low temperatures daytime increases in the levels of *tim* and *per* transcripts are highly dependent on CLK/CYC (Figure 4.2). For unknown reasons at cold temperatures the *tim* RNA cycle is behaving like a rapidly dampening oscillator. This is in stark contrast to the behavior of *tim* expression at higher temperatures. As the temperature rises peak levels in *tim* mRNA increase and high amplitude rhythms that are sustained in constant dark conditions are observed (85). Indeed, the ability of cold temperatures to lower the amplitude of the clock-controlled cycle in *tim* expression might be part of the molecular logic enabling its acute photic induction. Mathematical modeling posits that low amplitude rhythms are more sensitive to perturbation by environmental cues (e.g.,

(110). Clearly, although *tim* expression on cold and warm days is clock regulated via a mechanism that involves CLK-CYC-mediated transactivation, there is a temperature dependent switch in the mechanism governing this molecular rhythm. Future work will be required to explain why at cold temperatures light has acute effects on *tim* but not *per* expression, and why in daily LD cycles the photostimulation of *tim* mRNA levels is mainly restricted to the rising phase of this molecular rhythm. Identification of a putative light-responsive element would also facilitate studies to understand the contributions of the acute photostimulation of *tim* expression on the seasonal adaptation of behavioral rhythms.

It is interesting to note that the waveform of *tim* mRNA levels on cold days as a function of photoperiod is somewhat reminiscent of that observed for the genes encoding chlorophyll *a*/*b*-binding proteins (*CAB* genes) (97). Light acutely induces *CAB* expression but the accumulation phase begins prior to dawn in short photoperiods and the apparent amplitude of the induction varies as a function of the duration of the daylength. As mentioned above, a simple framework that might explain the relationship between photoperiodic responses and acute effects of light on gene expression is that changes in the duration of the photoperiod differentially entrain the circadian clock, and the clock modulates acute responses to light (97). Circadian gating in the magnitude of light-induced increases in clock gene expression has also been shown for other clock genes such as *frq* in *Neurospora* (56). The fact that *Drosophila* also has a *bona fide* clock gene that is rapidly induced by light, indicates that similarities and differences between dipartite clock mechanisms (32) might be conditional. It also suggests that the core circuitry underlying circadian timekeeping devices can accommodate dramatic changes in design principles in response to changes in external conditions.

Although not the focus of this study, we show very robust photoinduction of *tim* expression in *norpA* and cry^b mutants (Figure 4.2). It is possible that NORPA and CRY have redundant functions within the context of *tim* photoinduction at cold temperatures and that a double mutant (*norpA*;cry^b) would not exhibit light-induced increases in the levels of *tim* RNA. Alternatively, the ability of light to evoke acute effects on the levels of *tim* RNA at low temperatures might involve a non-NORPA/non-CRY dependent pathway(s) previously suggested to play a role in circadian photoresponses (e.g., (61, 93, 115).

Differential effects of 'negative' and 'positive' factors on molecular and behavioral photic responses?

An intriguing aspect of our results is that they seem to follow a broader pattern of differential effects on photic responses by clock components that are mainly depicted as functioning in the negative versus positive limbs of the transcriptional-translational feedback loops that underlie many eukaryotic circadian clocks. This is clearly observed at the behavioral level in *Drosophila* whereby arrhythmic mutants can be placed into two groups; negative (i.e., *per* and *tim*) or positive (i.e., *Clk* and *cyc*) factors with regards to a variety of light-dependent responses, such as; 1) startle or masking responses (3, 119), 2) light-associated temperature preference (161) and 3) and larval photophobic behavior (89). Although the mechanism underlying the differential effects of CLK/CYC and PER/TIM on photic responses is not known, prior work has shown that gene expression of direct and indirect targets are differentially regulated by inactivating the negative and positive elements in *Drosophila* (e.g., (20, 41, 82). Mutations in *Clk*/*cyc* compared to *per*/*tim* clamp the clock at two different molecular states, most likely explaining why they have essentially opposite effects on the basal RNA levels of rhythmically expressed genes.

Such a difference in one or more key factors functioning in photic and/or temperature responses could underlie the differential light-mediated phenotypes observed in the two groups of mutants. Moreover, it is noteworthy that inactivating clock genes also leads to changes in the overall levels of many RNAs that are thought to be either constitutively expressed or light-regulated (20, 82, 90, 107, 144). Differential effects of *Clk*/*cyc* compared to *per*/*tim* mutants on basal and/or light-regulated gene expression could account for the differences in behavioral photic responses in *Drosophila*.

In this context it is noteworthy that light-mediated induction of *frq* in *Neursopora* and *mPer1,2* in mammals exhibit a similar dependency to photostimulation of *tim* expression at cold temperatures, requiring factors that function in the positive but not negative limbs of the relevant clockworks (15, 16, 21, 25, 79, 106, 126, 147). In *Neurospora* it is likely that all light-responses are dependent on the WC-1/WC-2 complex, which also plays a key role in the clockworks by driving cyclical expression of the negatively acting *frq* gene. Moreover, the ability of light to 'directly' inhibit or 'mask' the activity of mice appears to be highly compromised in mutants where the positive factors BMAL1/MOP3 (homolog of CYC in *Drosophila*) or CLOCK are impaired (12, 114). This might reflect more ancient roles for these positively acting transcription factors in mediating cellular photic responses (24). In any event, although recent work has questioned the need for any transcriptional regulation in the design of circadian clocks (103), it is likely that the ability of temperature and day length to modulate the cycling profiles of one or more key clock RNAs is a key feature underlying daily and seasonal adjustments in clock dynamics.

Chapter 5. Summary

The daily timing of circadian (congruent with 24-hrs) controlled activity in many organisms exhibits seasonal adjustments, responding to changes in photoperiod (day length) and temperature so that "early birds get the worm" and "flowers blossom in spring warmth"(a Chinese idiom).

In a previous study, Majercak *et al* (85) showed that the splicing of dmpi8 intron plays an important role in advancing the steady state of *per* mRNA and protein cycles at cold temperature. To investigate the role of dmpi8 splicing in regulating seasonal behavior of *Drosophila*, we carefully optimized an RT-PCR splicing assay employing one single PCR reaction to evaluate both relative abundance and the ratio of type B' and type A *per* mRNA, as validated by RNase protection assays (*Materials and Methods* in Chapter 2, Figure 2.1) (84). Using this new splicing assay, it was further demonstrated that dmpi8 splicing is also regulated by the circadian clock in a manner that depends on the photoperiod (day length) and temperature. Shortening the photoperiod enhances dmpi8 splicing and advances its cycle and higher *per* mRNA peak values are obtained (Figure 2.1 and Figure 2.2), responses that are likely mediated via the effects of day length on the phase and amplitude of clock. The amplitude of the clock-regulated daytime decline in dmpi8 splicing increases as temperatures rise (Figure 2.4). This suggests that at elevated temperatures the clock has a more pronounced role in maintaining low splicing during the day, a mechanism that likely minimizes the deleterious effects of daytime heat on the flies by favoring nocturnal activity during warm days. Light also has acute inhibitory effects, rapidly decreasing the proportion of dmpi8-spliced *per* transcript, a response that does not

require a functional clock (Figure 2.4A, B and Figure 2.5). While low dmpi8 splicing in *cry*^{b} mutants have excluded CRY as a possible photoreceptor in modulating light mediated inhibition of dmpi8 splicing, our results identify a novel non-photic role for NORPA, a phospholipase C, in the temperature regulation of dmpi8 splicing (Figure 2.6). Although light-pulses still evoke rapid inhibition of dmpi8 splicing in *norpA* mutant flies and a greater dmpi8 splicing is still observed at 18°C compared to 29°C, it appears that inactivation of PLC 'locks' the splicing behavior of dmpi8 into a pattern of high overall splicing with low amplitude rhythm, characteristic of that observed on cold days.

Given that the majority of *per* mRNA in the head extract is coming from fly eyes where the majority of NORPA expresses, we sought to determine whether the effects of NORPA in inhibiting dmpi8 splicing is through its role in *Drosophila* visual phototransduction pathway. Surprisingly, unlike the *norpA*^{P41} mutant, other mutants that also disrupt the phototransduction pathway do not affect dmpi8 splicing efficiency (Figure 5.1), suggesting that NORPA does not regulate dmpi8 splicing through light.

To further investigate the roles of NORPA in this regard, we generated the UAS-norpAi and UAS-FHT-norpA transgenic flies. By crossing them to flies that drive the expression of the GAL4 transcription factor in a tissue specific manner, NORPA levels will be either interfered by RNAi or enhanced by overexpression, thereby allowing us to pinpoint where in the head NORPA functions to modulate the dmpi8 splicing efficiency. Intriguingly, preliminary data showed that RNAi knockdown of NORPA expression in the key PDF expressing pacemaker neurons dramatically advances fly evening behavior onset at both 18°C and 29°C, compared to overexpression of NORPA in the same cells (Figure 5.2). Crossing w^{1118} flies, the wild type genetic background of the transgenic flies, to the

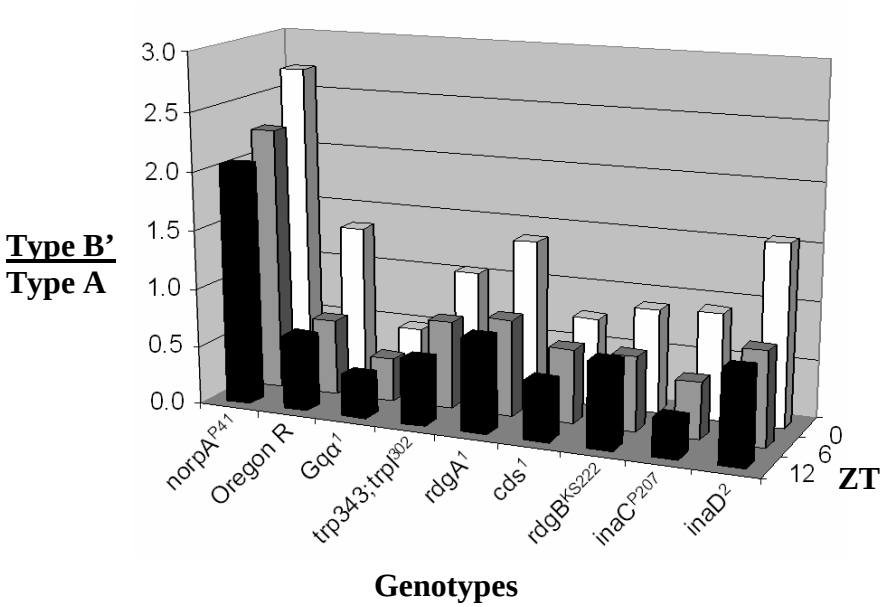

Figure 5.1. The effect of NORPA on dmpi8 splicing is not through the visual phototransduction pathway. Flies from the visual phototransduction pathway mutants (*norpA*^{P41}, *Gqα*^{1}, *trp*^{343};*trp*^{302}, *rdgA*^{1}, *cds*^{1}, *rdgB*^{ks222}, *inaC*^{P207}, and *inaD*^{2}) were entrained to 12:12LD, 29°C together with the Oregon R wild type flies and collected for dmpi8 splicing assay at ZT0, 6, and 12 on the 4th LD. Data from different ZT times are shown in bars of different colors: ZT0, white; ZT6, gray; ZT12, black.

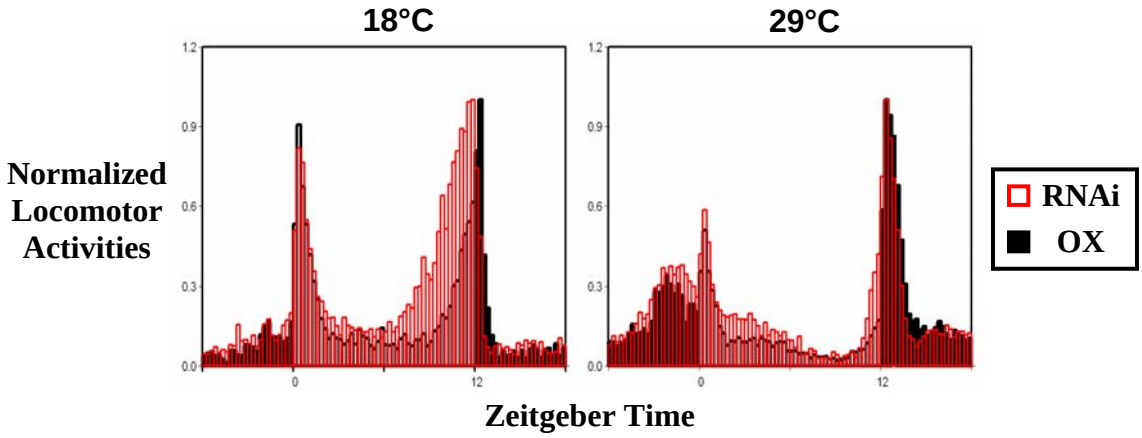

Figure 5.2. RNAi-mediated inhibition of *norpA* in the PDF-expressing cells advances *Drosophila* evening behavior onset. UAS-norpAi and UAS-FHT-norpA transgenic flies were generated for norpA RNAi knock down or over expression, respectively. Female UAS-norpAi (RNAi, red), UAS-FHT-norpA (OX, black) flies were crossed to male pdf-GAL4 flies to knockdown or overexpress NORPA in the PDF expressing neurons. Female transgenic host w^{1118} were similarly crossed to pdf-GAL4 as control (not shown) Male progenies were entrained to 12:12LD at 18°C or 29°C. Behavior data were averaged for each genotype and normalized against the evening activity peaks before the profiles were plotted using a 3D bar chart in Excel. The behavior profile of norpA RNAi flies is plotted in red open bars to facilitate comparison of the profiles.

pdf-GAL4 flies generate similar results with the overexpression transgene (data not shown). Interestingly, the PDF neuropeptide is only expressed in a small number of pacemaker cells in the large and small ventral lateral neurons (LNv) which have extensive arborization in the optic lobe and the brain (58, 60). These LNv pacemaker cells have recently been described as "morning" cells that control the phase of morning activity onset (43, 133-135). Intriguingly, at 29°C, flies with NOPRA knockdown in the presumed "morning" cells show an advanced evening peak despite showing a delayed morning peak (Figure 5.2, right panel). Recent work showed that speeding up the "morning" cells also led to a speeding up of the "evening" cells, suggesting that the pace of the morning clock regulates the pace of the evening clock in light-dark cycles (133). However, our data appears inconsistent with that model because whereas the morning peak is delayed the evening peak is advanced. Future work will be needed to better address this apparent inconsistency.

In the process of investigating whether there is a latitudinal cline in dmpi8 splicing, we isolated two haplotypes of *per* 3' UTRs with six polymorphisms from the VT97.1 isofemale line (Figure 3.1). To study this further, we generated inbred lines from the isofemale line. VT1.1 flies showed better dmpi8 splicing and consequently higher *per* mRNA peak levels than VT1.2 flies at various photoperiod and temperature conditions (Figure 3.2 and data not shown). Consequently, evening activity of the VT1.1 flies rise with an earlier timing compared to the VT1.2 flies. Interestingly, light pulses at ZT20 at 18°C generated more dramatic clock phase advance in the VT1.2 flies than the VT1.1 flies (Figure 3.4). To clarify that the observed differences are not due to the variation in genetic background inherited from the inbred crosses, transgenic flies were generated with *per*

transgenic constructs containing the VT1.1 and VT1.2 3' UTRs. More efficient splicing of dmpi8 and earlier behavior onset in the *per*^{0}*w*;p{VT1.1} transgenic flies were observed compared to the *per*^{0}*w*;p{VT1.2} flies, confirming that the genotypic differences in the *per* 3' UTRs are responsible for the observed differences between VT1.1 and VT1.2 flies (Figure 3.5 and Figure 3.6). Further investigation in the S2 cells with pAct-Luc-UTR reporter system have pinpointed the *cis*-elements to SNP3 and SNP4 as the key modulators of dmpi8 splicing between the VT1.1 and VT1.2 3' UTRs (Figure 3.7A and B). Prior work showed that the 5' and 3' splice sites of dmpi8 are weak (17, 23, 84, 85). In addition, unpublished work in our lab showed that increasing the splice site strength of the 5'ss or 3'ss enhances splicing efficiency, reduces splicing thermosensitivity and advances the timing of fly evening activity (K.H. Low, unpublished data). My work identified two natural polymorphisms in the *per* 3' UTR, SNP3 and SNP4, that also affect splicing efficiency and the timing of evening activity but without affecting the strengths of the 5' and 3' splice sites or the thermosensitivity of dmpi8 splicing (Figure 3.7B and C). We propose that the weak 5' and 3' splice sites are the main determinants regulating the thermosensitivity of dmpi8 splicing, whereby low temperature enhances the docking of the spliceosome to the pre-mRNA via increased snRNA/pre-mRNA interactions, whereas SNPs outside of these regions can regulate the overall splicing efficiency without affecting the temperature sensitivity of dmpi8 splicing.

Also, our data suggest that VT1.1 3' UTR promotes higher proportion of total *per* mRNA export than the VT1.2 (Figure 3.8), revealing another level of regulation by dmpi8 splicing to advance the phase of the clock.

Numerous lines of evidence indicate that the primary clock-specific photoresponse

daily light-dark cycles is the light-induced degradation of TIM (63, 78, 101, 138, 158, 163). Here I also describe a novel effect of light on *tim* expression, acutely stimulating the transcription of *tim* at cold but not warm temperatures (Figure 4.1). This cold-specific photoinduction of *tim* mRNA abundance is regulated at the transcriptional level, as revealed by the *tim* promoter driven induction of luciferase mRNA (Figure 4.5 and Figure 4.6). Although *per* and *tim* are thought to be activated with a similar mechanism by CLK-CYC heterodimer, no light-induced stimulation of *per* mRNA transcription is observed (Figure 4.1). This photoinduction occurs in flies defective for the classic visual phototransduction pathway or the circadian-relevant photoreceptor CRYPTOCHROME (CRY). Moreover, light-induced increases in the levels of *tim* RNA are abolished or greatly reduced in the absence of functional positive factors, CLOCK (CLK) or CYCLE (CYC), but not the negative factors, PER or TIM (Figure 4.2), likely owing to the fact that the positive and negative factors have essentially opposite effects on the basal RNA levels of rhythmically expressed genes. Importantly, this acute effect of light on *tim* expression is essentially restricted to the daily rising phase of *tim* mRNA levels (Figure 4.4). Because the start of daily upswing in *tim* expression begins a few hours after dawn in long photoperiods (day length) (Figure 4.3), this gating mechanism ensures that sunrise does not prematurely stimulate *tim* expression during unseasonably cold long days. This result suggests that the photic stimulation of *tim* transcription at low temperature is part of a seasonal adaptive response that helps advance the phase of the clock on cold days, enabling the flies to exhibit preferential daytime activity despite the earlier onset of dusk (Figure 5.3).

In conclusion, this dissertation shows that temperature and photoperiod integrate to

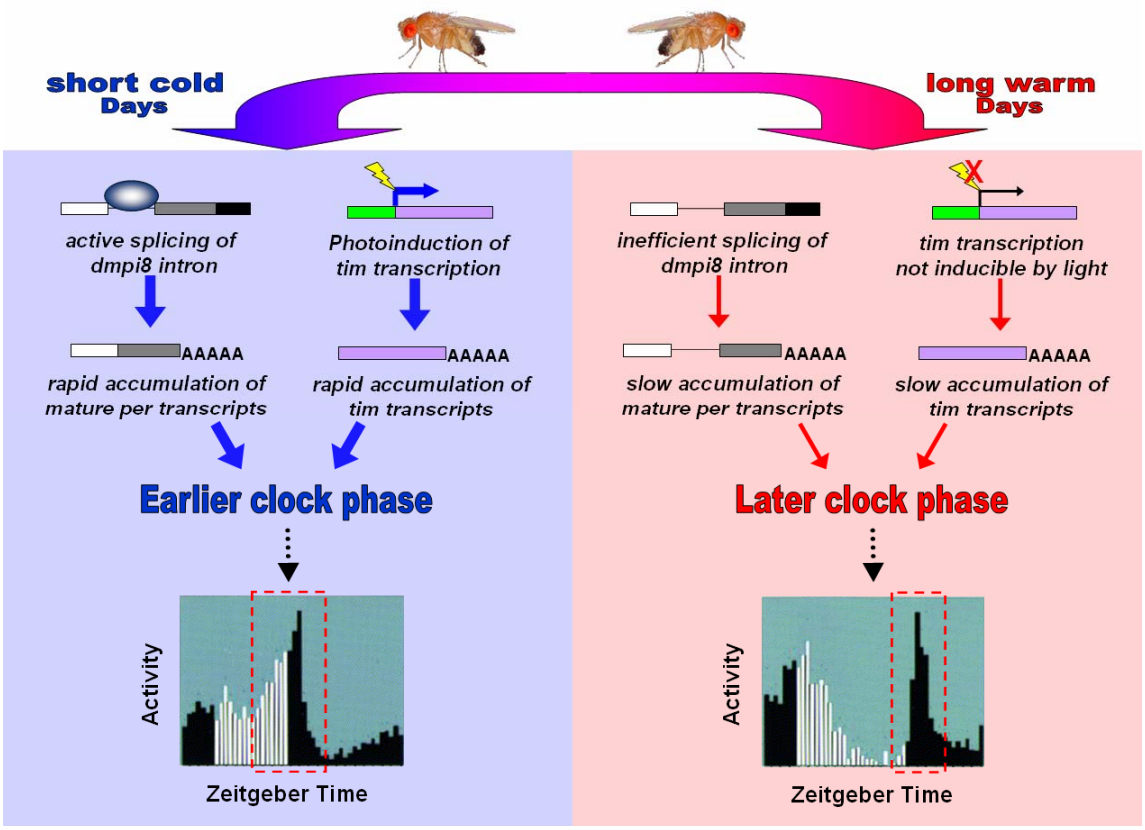

Figure 5.3. Current model for seasonal adaptation of daily activity patterns in *Drosophila melanogaster*. Daily accumulation of *per* and *tim* mRNA transcripts are modulated by seasonal environmental cues, namely, day length and temperature. (Left panel), In seasonably short cold days, short day length and cold temperature stimulate active splicing of dmpi8 intron and photoinduction of *tim* transcription, leading to rapid accumulation of mature *per* and *tim* transcription, thereby advancing the clock phase. Consequently, flies show early evening activity onset. (Right panel), On the other hand, long day length and warm temperature inhibit the splicing of dmpi8 and abolish the photoinducibility of *tim* mRNA, leading to slow accumulation of both *per* and *tim* mRNA, which lead to a delayed clock phase that push back the evening behavior onset.

regulate the daily upswing of *per* and *tim* mRNA by modulating the splicing efficiency of *per* 3' intron and cold-specific photoinduction of *tim* transcription, respectively, thereby facilitating the seasonal adaptation of *Drosophila melanogaster* daily activity (Figure 5.3). On "winter-like" short cold days, both short day length and cold temperature stimulate splicing of the 3' terminal intron in *per* 3' UTR, leading to rapid accumulation of mature *per* transcripts coupled with active mRNA export to the cytoplasm. Association of short day length with cold temperatures enables the stimulation of clock-gated photoinduction of *tim* mRNA transcription. Rapid increases of mature *per* and *tim* transcripts advances the clock phases by accelerating the appearance of PER and TIM proteins (85), endowing the flies to be active during the early evening time, when it is warmer. Conversely, on "summer-like" short warm days, long day length and warm temperature both inhibit dmpi8 splicing, leading to slow *per* transcript buildup. Also, *tim* mRNA transcription is not light-inducible at higher temperatures. Thus, both *per* and *tim* mRNA accumulations are delayed, leading to late/nocturnal evening activity and a longer mid-day siesta time, presumably to avoid the deleterious effects of the hot midday hours.

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