Neuron, Vol. 26, 505–514, May, 2000, Copyright ©2000 by Cell Press

A TIMELESS-Independent Function for PERIOD Proteins in the *Drosophila* Clock

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Summary

The mutation *timeless*^{UL} generates 33 hr rhythms, prolonged nuclear localization of PERIOD/TIMELESS^{UL} protein complexes, and protracted derepression of *period* (*per*) and *timeless* (*tim*) transcription. Light-induced elimination of TIM^{UL} from nuclear PER/TIM^{UL} complexes gives strong downregulation of *per* and *tim* expression. Thus, in the absence of TIM, nuclear PER can function as a potent negative transcriptional regulator. Two additional studies support this role for PER: (1) *Drosophila* expressing PER that constitutively localizes to nuclei produce dominant behavioral arrhythmicity, and (2) constitutively nuclear PER represses dCLOCK/CYCLE-mediated transcription of *per* in cultured cells without TIM. Conversion of PER/TIM heterodimers to nuclear PER proteins appears to be required to complete transcriptional repression and terminate each circadian molecular cycle.

Introduction

Circadian rhythms are generated by transcriptional autoregulatory loops in bacteria, fungi, plants, and animals (reviewed by Dunlap, 1999). In *Drosophila*, essential components of a molecular clock have been recognized in forward genetic screens. Two genes, *period* (*per*) and *timeless* (*tim*), are negatively regulated by their gene products, the PER and TIM proteins (reviewed by Hardin, 1998; Reppert, 1998; Young, 1998). PER and TIM interfere with the function of two activators of transcription, dCLOCK (dCLK) and CYCLE (CYC) (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). RNA and protein levels from the *per* and *tim* genes oscillate (Hardin et al., 1990; Sehgal et al., 1995), and this cycling is regulated by periodic formation of a PER/TIM complex (Gekakis et al., 1995; Sehgal et al., 1995). Heterodimerization of these proteins is required for their translocation to the nucleus, as certain PER and TIM sequences, cytoplasmic localization domains (CLDs), restrict monomers to the cytoplasm (Saez and Young, 1996). Heterodimerization also protects PER from the activity of a kinase encoded by *double-time* (*dbt*). DBT promotes phosphorylation and turnover of monomeric PER proteins, which delays cytoplasmic accumulation of PER/TIM complexes and fosters cycling nuclear function (Kloss et al., 1998; Price et al., 1998). DBT also regulates phosphorylation and turnover of nuclear PER proteins and therefore influences the duration of part of the molecular cycle (Price et al., 1998). PER, TIM, dCLK, and CYC are additionally involved in a second autoregulatory loop, whereby they directly mediate cycling expression of the transcription factor VRILLE (VRI). VRI oscillations are required for *per* and *tim* expression, for behavioral rhythmicity, and for accumulation of PDF (pigment-dispersing factor), a neuropeptide believed to regulate behavior (Blau and Young, 1999; Renn et al., 1999).

dClk and *cyc* encode bHLH-PAS-containing transcription factors, and antibodies to dCLK can coimmunoprecipitate PER and TIM in vivo (Lee et al., 1998). In vitro dCLK/CYC complexes directly bind to DNA sequences composing the *per* and *tim* promoters. This binding is inhibited by PER/TIM complexes (Lee et al., 1999). Coexpression of dCLK with PER and TIM in cultured *Drosophila* cells reduces dCLK-mediated transcription of *per* reporters (Darlington et al., 1998). Together, these results indicate that PER/TIM complexes act as negative autoregulatory components of the clock by directly associating with dCLK/CYC. However, PER and TIM proteins alone are able to interfere with dCLK/CYC DNA binding in vitro (Lee et al., 1999), raising the possibility that these monomers might also be involved in negative autoregulation in vivo.

Although circadian rhythms are self-sustaining in constant darkness (DD), light/dark (LD) cycles can affect the phase and period of the rhythm. Light induces rapid degradation of the TIM protein, which locks the phase and period of circadian molecular oscillation to the environmental cycle (reviewed by Young, 1998). CRYPTOCHROME (CRY), a novel blue light photoreceptor that can physically associate with TIM (Ceriani et al., 1999), appears to be an important regulator of light-dependent TIM degradation in some cell types (Emery et al., 1998; Stanewsky et al., 1998). TIM degradation is thought to involve phosphorylation, ubiquitination, and proteasome activity (Naidoo et al., 1999).

In the presence of the LD cycles of a solar day, dawn converts PER/TIM heterodimers to nuclear PER that persists for 4–6 hr. However, the role of these PER proteins has not been investigated. Since light-induced degradation of TIM is not associated with a rapid increase in *per* and *tim* RNA levels (Lee et al., 1996; Young et al., 1996), it is possible that nuclear PER proteins perform a distinct regulatory function each morning, at the end of the molecular cycle.

In this report, we characterize a new allele of *timeless* resulting in ultralong, 33 hr rhythms. The molecular phenotype of *tim*^{UL} is prolonged nuclear localization of the PER/TIM^{UL} complex, yet contrary to expectation, *per* and *tim* RNA levels remain moderately high for an extended time. Generation of nuclear PER by light-induced degradation of TIM^{UL} results in a decrease in *per* and *tim* RNA levels, suggesting that the PER/TIM heterodimer is defective in *tim*^{UL} flies and that the released PER proteins are sufficient for transcriptional repression of the *per* and *tim* genes. TIM-independent regulatory activity

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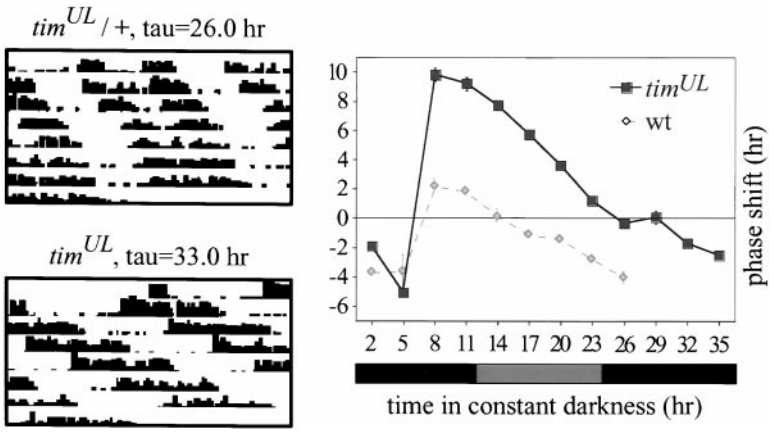

Figure 1. Behavioral Phenotypes of *tim*^{UL} Flies

(Left) *tim*^{UL} alters locomotor activity rhythms. Representative locomotor activity records of single flies in DD are shown, with the genotype and individual period length (tau) indicated above each plot. Previously entrained flies were assayed in DD and monitored for 8 days. Activity is double plotted for visual continuity, such that each horizontal line of the record shows a 48 hr interval that overlaps by 24 hr with the preceding line of the record. Increased activity is indicated by increased height of the vertical closed bars. Periods obtained were *tim*^{UL}: 32.7 ± 0.1 hr, n = 21; *tim*^{UL}/+: 26.1 ± 0.1 hr, n = 24; and +/+ (data not shown): 23.6 ± 0.1, n = 23.

(Right) Altered PRC of *tim*^{UL} flies. Flies were entrained to LD cycles for at least 3 days and then released into DD. Every 3 hr (starting 2 hr after the last lights off), a 10 min light pulse was administered to a different group of flies. The resulting phase of the locomotor rhythm was then compared with that of an unpulsed group, and a phase shift (y axis) was calculated as a function of the time when the pulse was given (x axis). Each time point was assayed two to five times, and error bars are shown (where bigger than the plot symbol). Note that for *tim*^{UL}, the time domain of phase-advancing shifts is greatly expanded (~18 hr for *tim*^{UL} as compared with ~8 hr for wild type), while the transition point (from phase delay to advance) is very similar to that of wild type, suggesting a specific late night defect in *tim*^{UL}. The gray bar indicates the previous LD entrainment regimen.

of PER was also suggested by studies in S2 cells; in the absence of TIM, expression of PER lacking its CLD repressed dCLK-mediated activation of the *per* promoter. This form of PER was also able to dominantly interfere with clock function in transgenic flies. The results indicate a TIM-independent role for nuclear PER in the termination of each molecular cycle.

Results

Isolation and Genetics of *tim*^{UL}

In our screen for dominant mutations affecting circadian locomotion rhythms (Price et al., 1998), we isolated a mutation that resulted in 26 hr periods in heterozygous flies and 33 hr rhythms in homozygotes (Figure 1, left). Both the original *tim*^{01} mutation and a deficiency (missing the *tim* locus), *Df(2L)tim*^{02} (Myers et al., 1995), failed to complement the ultralong 33 hr rhythm (data not shown), suggesting that we had isolated a novel allele of *tim*. This hypothesis was confirmed by two additional findings: (1) no recombination was observed between *tim*^{01} and the new mutation in 120 recombinant chromosomes (data not shown), and (2) determination of the DNA sequence of the *tim* gene in the mutant line revealed a missense mutation that distinguished it from the parental *Drosophila* stock. The mutation produced a change at amino acid 260 from Glu to Lys (numbering according to Myers et al., 1997; data not shown). Therefore, we termed this new allele *tim*^{UL}, for ultralong, since its period is considerably longer than that of any previously isolated mutant of a *Drosophila* clock gene (reviewed by Hall, 1998).

We then tested the genetic interaction of *tim*^{UL} with previously isolated *per* alleles. Double mutant flies with a short-period *per* allele, *per*^{S}*;tim*^{UL}, show close to wild-type period lengths of 24.5 ± 0.1 hr, n = 12, revealing an additive genetic interaction between these two alleles. An additive genetic interaction is also found with a long-period *per* allele: *per*^{L}*;tim*^{UL} flies have period lengths of 41.3 ± 0.3 hr, n = 22. This phenotype is very stable and fully penetrant without incidence of arrhythmicity, showing that the *Drosophila* clock can sustain rhythms that are almost 2 days long.

Late Night Defect in *tim*^{UL} Flies

To determine which part of the daily cycle is affected in *tim*^{UL} flies, we established a phase–response curve (PRC) for light. This PRC measures the light-induced phase change of the behavioral rhythm as a function of the time at which the pulse is administered in the cycle. Flies were entrained to LD cycles and then released into DD. Every 3 hr (starting 2 hr after the last lights off), a 10 min light pulse was administered to a different group of flies. The resulting phase of the locomotor rhythm was then compared with an unpulsed group.

Three different time domains are found in a wild-type PRC (reviewed by Young, 1998; Figure 1, right): at the beginning of the night, when PER and TIM protein accumulate in the cytoplasm, light pulses result in ~4 hr phase delays. Light pulses cause TIM degradation, thus PER/TIM complexes must reaccumulate after the light pulse, which results in a molecular and behavioral phase delay (Myers et al., 1996). Later at night, PER and TIM are nuclear. A light pulse accelerates the natural turnover of TIM and results in 2–3 hr phase advances. In wild-type flies, light pulses show little effect on the phase of locomotor activity rhythm if administered in the third time domain, which corresponds to subjective day. The latter result is expected because little TIM protein accumulates in this interval, even in DD (Young, 1998).

Comparison of the *tim*^{UL} and wild-type PRCs revealed substantial differences (Figure 1, right). During the advance domain, the amplitude of phase shifts and the effective duration of the domain were both increased. Significantly, the advance domain was extended by 8–10 hr, matching the increased period length (9 hr) of the behavioral rhythm. This result suggests that the *tim*^{UL} mutation specifically affects the late night part of the circadian cycle, when PER and TIM are nuclear.

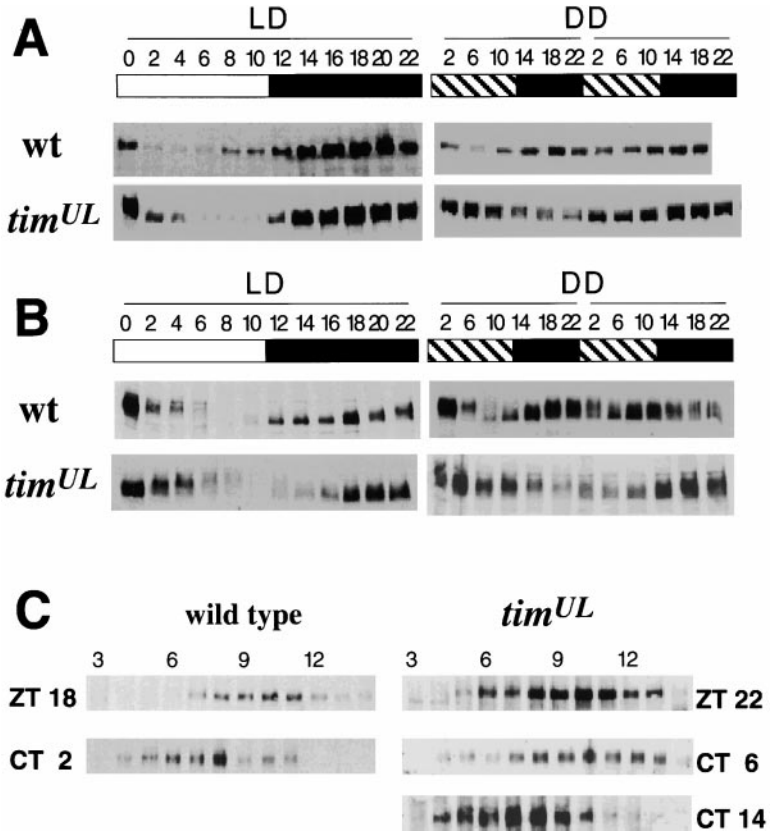

Figure 2. Lengthened TIM and PER Protein Oscillations in *tim*^{UL} Head Extracts

Western blot analysis of TIM (A) and PER (B) proteins from wild-type (wt) and *tim*^{UL} head extracts. Equal amounts of protein were loaded for each gel (controlled with a nonspecific cross-reacting band with anti-PER; data not shown). Differences in PER mobility have previously been shown to be due to protein phosphorylation (Edery et al., 1994); 1 day of LD (indicated by altering open and closed horizontal bars) and 2 days of DD (subjective day indicated by hatched bars) were assayed. Numbers indicate hours in a 24 hr cycle of LD or DD. For *tim*^{UL}, both TIM and PER protein show delayed accumulation in an LD cycle, and in DD, both proteins are detected for an extended time (see LD-to-DD transition). For *tim*^{UL}, a trough in protein levels occurs around CT 22 of the first day in DD, while wild type shows two troughs, both around CT 6. Thus, the periods of the protein oscillations in *tim*^{UL} are lengthened, reflecting the long-period behavioral rhythm, and the lengthened oscillations are associated with prolonged intervals of PER and TIM accumulation. Note that TIM^{UL} can be seen for a longer time at the beginning of the light phase than can wild-type TIM, probably reflecting increased TIM^{UL} protein and/or RNA levels (see also Figure 4). (C) shows sucrose gradient analysis of PER protein from wild-type and *tim*^{UL} head extracts; 5%–25% sucrose gradients were run with freshly isolated head extracts. Fractions were then collected from the top and run on SDS–PAGE. The times of extract collection are indicated to the left (wild type) or right (*tim*^{UL}) of each blot and indicate the hour of collection in a 24 hr LD cycle (ZT times) or the hour of collection during the first day in DD (CT times). Fraction numbers are indicated at the top of each blot. In wild-type flies, PER runs in a high molecular weight fraction at ZT 18 (median fraction = 8.7 ± 0.4, n = 5) and shifts to a lower fraction by CT 2 (median fraction = 7.4 ± 0.3, n = 5). In *tim*^{UL}, PER only shifts to a lower fraction well into the first day in DD (see CT 14), while it still runs in a high molecular weight fraction at CT 6. Two independent experiments gave similar results for *tim*^{UL} (with median fractions of 9 and 9.5 at ZT 22, 8.5 and 10 at CT 6, and 7 and 7.5 at CT 14). Note that weak PER staining in the *tim*^{UL} CT 6 blot is due to a short Western blot exposure and not to lower amounts of PER protein. Fraction numbers showing median staining for controls are (with molecular weight and sedimentation constant) BSA (67 kDa, 4.3 s), fraction = 3.3; aldolase (158 kDa, 7.8 s), fraction = 5.3; and catalase (232 kDa, 11.3 s), fraction = 8.8.

Prolonged Nuclear Localization of PER/TIM^{UL}

Light pulses late at night result in TIM degradation and earlier PER phosphorylation and turnover, advancing the molecular and behavioral cycles (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). In wild-type flies entrained to an LD cycle and then transferred to DD, light pulses no longer phase advance the cycle after 14 hr in DD (Figure 1, right). In contrast, *tim*^{UL} flies are still phase advanced by a light pulse 20 hr into DD (Figure 1, right), suggesting that TIM^{UL} protein levels are high for a prolonged time. To test this hypothesis, time courses of accumulation of TIM and PER proteins were analyzed on Western blots from *tim*^{UL} head extracts. As predicted from the PRC, TIM^{UL} was detected well into the first subjective day of DD (Figure 2A). In wild-type extracts, TIM levels were lowest at CT 6 of subjective day 1, whereas TIM^{UL} showed its first trough in DD at CT 18 (circadian time [CT] reflects the prior LD entrainment regimen of Zeitgeber time [ZT]: ZT 0 = lights on, and ZT 12 = lights off). Figure 2A also shows that TIM^{UL} protein levels were high for an additional 8–12 hr. The same result was found for PER protein in the mutant (Figure 2B), indicating that the *tim*^{UL} mutation affects both TIM and PER protein oscillations.

To find out whether PER and TIM^{UL} proteins remain associated during their prolonged accumulation, sucrose gradients were run with timed extracts, and Western blots were analyzed with anti-PER antibody. Using extracts from wild-type flies, PER was found in high molecular weight fractions at ZT 18 (in LD; Figure 2C). PER shifted toward low molecular weight fractions when TIM was removed by light (Zeng et al., 1996; data not shown). If wild-type flies were maintained in DD, PER shifted to low molecular weight fractions at CT 2 (first day in DD; Figure 2C). In contrast, PER from *tim*^{UL} extracts still migrated in high molecular weight fractions at CT 6 (first day in DD), and a shift toward lower molecular weight was not observed until CT 14 (Figure 2C). We therefore conclude that PER and TIM are physically associated for an extended time in *tim*^{UL}.

To test whether this prolonged association of PER and TIM occurs in the nucleus, wild-type and *tim*^{UL} head sections were stained with anti-TIM antibody during the first subjective morning. Figures 3A and 3B show that in wild-type sections, TIM disappears from the nuclei of

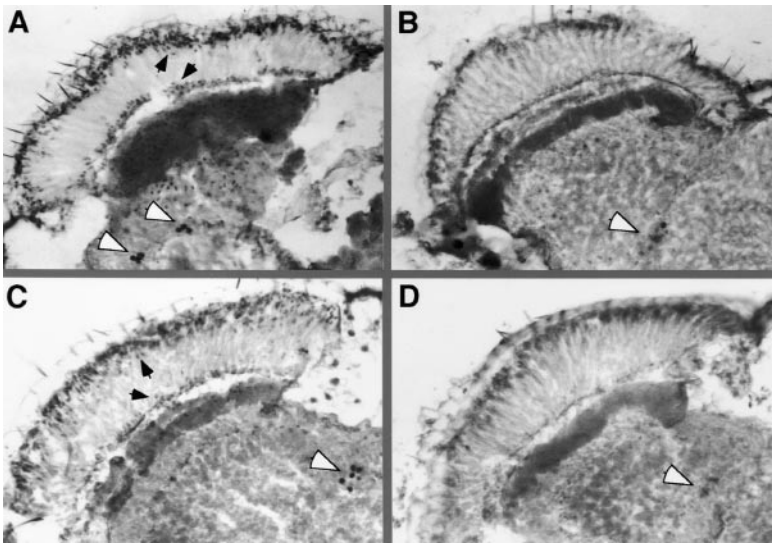

Figure 3. TIM Protein Localizations during the First Subjective Morning in tim^{UL} Head Sections

Antibody to TIM stained frontal head sections of wild-type (A and B) and tim^{UL} (C and D) flies in the first day of DD. Wild-type time points are CT 2 (A) and CT 8 (B). In wild-type heads, strong nuclear staining is visible in photoreceptors at CT 2 (closed arrowheads) but disappears by CT 8. The tim^{UL} time points are CT 10 (C) and CT 18 (D). Nuclear TIM^{UL} staining of photoreceptors is strong at CT 10 (comparable to wild type at CT 2; closed arrowheads) and largely disappears by CT 18. There is also some diffuse, cytoplasmic staining in tim^{UL} at most time points. Focused nuclear staining of the lateral neurons is seen in wild type at CT 2 (A), but less so at CT 8 (open arrows indicate positions of lateral neurons, [B]). In contrast, TIM^{UL} nuclear staining is still strong in lateral neurons at CT 10 (C).

photoreceptor cells by CT 8. However, strong nuclear staining of TIM is observed in tim^{UL} at CT 10 and does not disappear from photoreceptor nuclei until ~CT 18 (Figure 3D). These experiments indicate that tim^{UL} flies generate very long-lasting, nuclear PER/TIM^{UL} complexes.

Delayed Repression of *per* and *tim* Transcription in tim^{UL} Flies

Since nuclear PER/TIM complexes have been correlated with *per* and *tim* repression (Young, 1998; Dunlap, 1999), and the PER/TIM^{UL} heterodimer shows prolonged nuclear localization, we might expect prolonged repression of *per* and *tim* in tim^{UL} flies. However, the opposite was observed; peaks of *per* and *tim* RNA levels are extended in tim^{UL} (Figure 4). The first peak of *per* and *tim* RNA accumulation in LD is very broad and lasts for about 20 hr, compared with 6–8 hr in wild type. For wild-type flies in DD, troughs in the profile of *per* and *tim* RNA accumulation follow times of peak accumulation by 8–12 hr. RNA initially accumulates in tim^{UL} with normal kinetics in DD, but peak accumulation is again prolonged. In tim^{UL}, trough levels of *per* and *tim* RNA are reached after ~20 hr (see second day in DD, Figure 4). Thus, prolonged PER/TIM^{UL} complex in the nucleus is correlated with delayed repression of *per* and *tim*.

In Vivo Evidence for Nuclear PER Activity

In tim^{UL} flies shifted from LD to DD, *per* and *tim* RNA levels are fairly constant from ZT 20 to CT 6 of the first DD cycle (see Figure 4), and PER/TIM^{UL} complexes are nuclear during that time (data not shown). This pattern, however, does not apply to tim^{UL} flies maintained in LD; a decline of *per* and *tim* RNA was observed after lights on (compare the levels at ZT 2 and ZT 6 with those at CT 2 and CT 6; Figure 5A, replotted from Figure 4). After 6 hr in light, the *per* RNA level had fallen to ~50% of that found in flies transferred to DD, while *tim* RNA dropped to ~36% of the DD level. Since light results in TIM^{UL} degradation (Figure 2A) and thus release of PER, the drop in RNA levels suggests that the resulting nuclear PER is sufficient for transcriptional repression of *per* and *tim*. The effects of nuclear PER seen in tim^{UL} flies are within the range of repression observed in wild-type flies, in which transcription and RNA levels of *per* and *tim* fall to 30%–50% of peak levels in the 6 hr following the peak (So and Rosbash, 1997).

To confirm the correlation between PER's release from the PER/TIM complex and the decline in *per* and

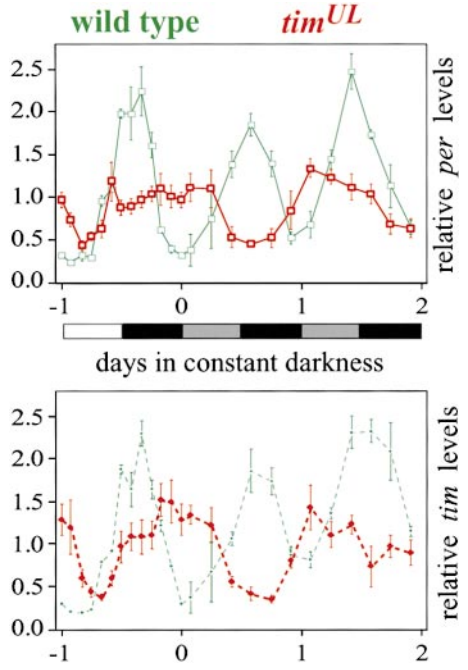

Figure 4. Prolonged Accumulation of *per* and *tim* RNA in tim^{UL}

per (top) and *tim* (bottom) RNA levels, isolated from tim^{UL} and wild-type heads, were measured over 3 days. Flies were entrained in LD for at least 4 days, with the first day of collection performed in LD. Subsequent collections were in DD (hatched bars indicate subjective day). RNase protections were performed and quantitated as described (Sehgal et al., 1994, 1995), and averages of three to five experiments per time point are shown with error bars. The first two peaks and troughs in tim^{UL} are separated by ~30–34 hr, corresponding to the long-period behavioral rhythm.

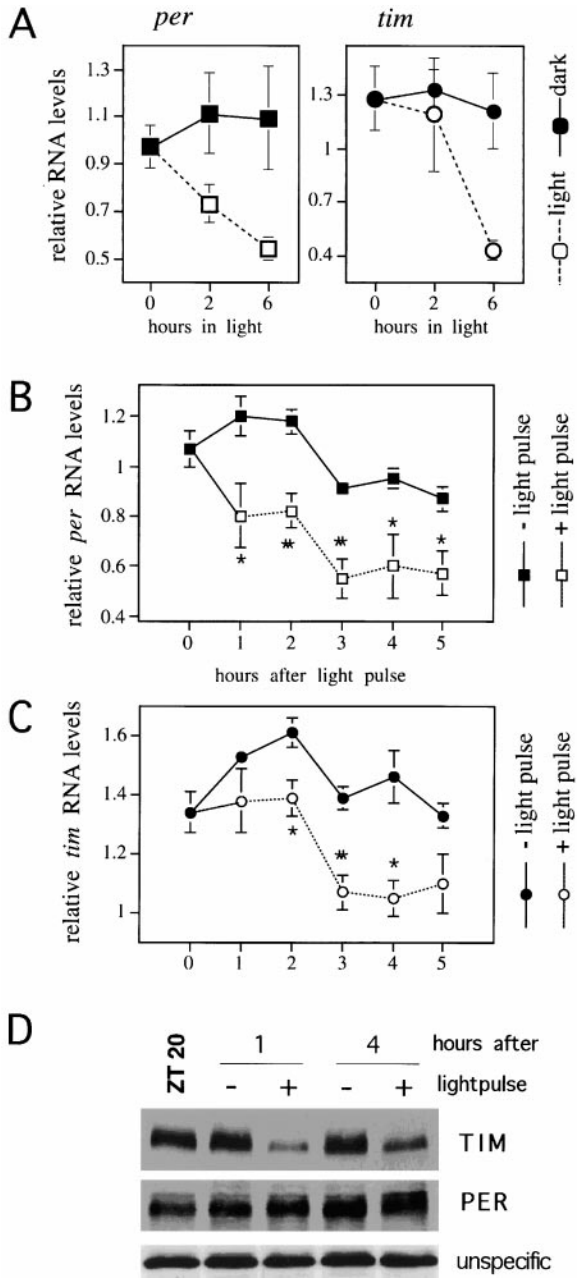

Figure 5. Effects of Light on *per* and *tim* RNA Levels in tim^{UL} Flies

(A) *per* and *tim* RNA levels decline when tim^{UL} flies are exposed to light. tim^{UL} flies were entrained to LD. At ZT 0 on the final day of entrainment, flies were either transferred to DD without any exposure to light (closed symbols) or kept in LD (open symbols), and *per* and *tim* RNA levels were measured relative to tubulin as described (Sehgal et al., 1994, 1995). Time points were taken simultaneously for LD and DD experiments, with the first time point collected immediately before lights on for flies maintained in LD. Both *per* and *tim* show a decrease after 6 hr in light, to 50% ± 15% of DD levels for *per* ($p < 0.07$, Student's t test) and to 36% ± 11% for *tim* ($p < 0.02$), respectively. Data are replotted from Figure 4.

(B and C) Effects of a 30 min light pulse on *per* and *tim* RNA levels. tim^{UL} flies were entrained to LD cycles and, at ZT 20, subjected to a 30 min light pulse. The pulsed flies (+) and an unpulsed control group (−) were then transferred to DD, and *per* and *tim* RNA levels were assayed. Quantitation of four to five experiments is shown in (B) for *per* and (C) for *tim* RNA (with error bars, where bigger than plot symbol). Significant decreases (**$p < 0.01$, *$p < 0.05$) were observed for both *per* and *tim*, respectively, after the light pulse. Maximum RNA reduction was seen after 3 hr for *per* (to 60% ± 10% of the unpulsed levels) and after 4 hr for *tim* (to 72% ± 9% of unpulsed levels). Note that the y axes from (A) through (C) and from Figure 4 are the same, to allow direct comparison between experiments.

(D) Western blot showing that loss of TIM^{UL} protein, but not PER protein, is induced by the light pulse. A nonspecific cross-reacting protein from the anti-PER blot is shown as loading control.

tim RNA levels in tim^{UL}, we administered a 30 min light pulse to tim^{UL} flies at ZT 20 and followed *per* and *tim* RNA levels over the next 5 hr (Figures 5B and 5C). This light pulse resulted in TIM^{UL} degradation (Figure 5D) and shifted PER toward low molecular fractions on a sucrose gradient (data not shown), indicating dissociation of PER from PER/TIM complexes. Within 1–2 hr, a significant decrease in RNA levels was observed for both *per* and *tim*. After 3–4 hr, *per* RNA titer was ~60%, and *tim* RNA ~72%, of the unpulsed level. Since flies maintained in light for 6 hr cannot reaccumulate TIM^{UL} (Figure 2A), whereas flies pulsed with light for 30 min can (Figure 5D), the results further indicate that the strength of repression is correlated with the diminution of TIM^{UL} levels. This supports the hypothesis that nuclear PER can negatively regulate *per* and *tim* RNA levels. In light of our studies of tim^{UL}, we have also noticed that the rise of *per* and *tim* RNA levels in wild-type flies is well correlated with the turnover of nuclear PER (compare Figures 2B and 4).

We note that Figures 5A–5C may also indicate a possible difference in the kinetics of *per* and *tim* repression in response to light. *per* RNA levels seem to be repressed more quickly than *tim* RNA levels by TIM-independent PER. Although our data on this point are not statistically significant, others have reported qualitative differences in regulation of *per* and *tim* RNA oscillations: *per* RNA levels are affected more strongly in tim^{rit} than are *tim* RNA levels (Matsumoto et al., 1999), and Majercak et al. (1999) have shown a strong effect of low temperatures on *tim*, but not *per*, RNA oscillations. These findings raise the possibility that unidentified factors contribute to differences in the transcriptional regulation of *per* and *tim*.

PER Represses Transcription in S2 Cells in the Absence of TIM

We have previously demonstrated that coexpression of PER and TIM in *Drosophila* S2 cells results in nuclear localization of both proteins (Saez and Young, 1996). In that study, we also identified a PER CLD. When this CLD is deleted, PER enters the nucleus in the absence of TIM. We used S2 cells expressing CLD-deficient PER (PERΔCLD) to determine whether PER alone is sufficient for repression of transcription. We adapted the cell culture assay developed by Darlington et al. (1998), whereby transfection of dCLK activates the *per* promoter (CYC is already expressed by S2 cells), while cotransfection with PER and TIM represses dCLK-mediated activation.

The activation of the *per* promoter by dCLK alone was compared with activation by dCLK when cotransfected

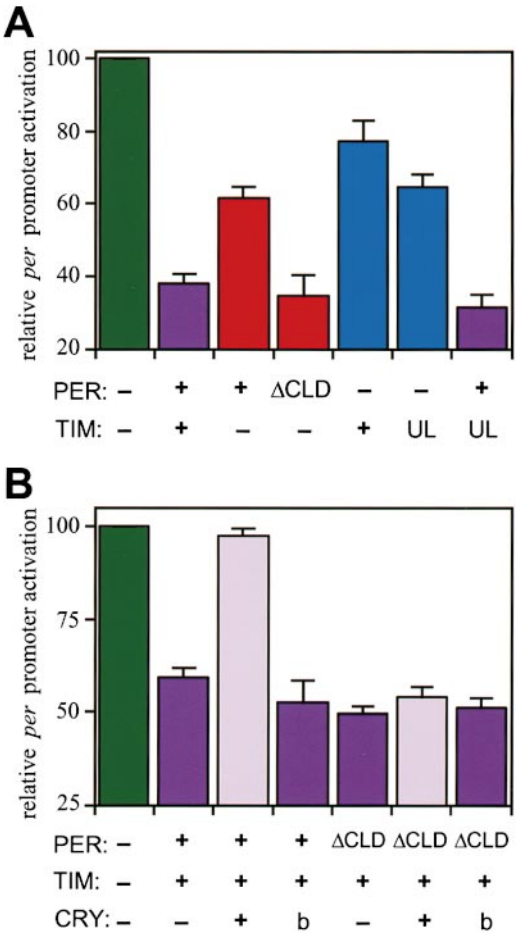

Figure 6. Nuclear PER Inhibits CLK-Mediated Transactivation in *Drosophila* Cells

Drosophila S2 cells were transfected with a reporter construct carrying the *luciferase* gene under control of the *per* promoter and/or a *per* gene lacking the CLD, *dClk*, *per*, and *tim*, all under control of pAct. For each transfection, the reporter activity is presented relative to the activity observed when S2 cells were cotransfected with pAct-*dClk* alone. The data were normalized to a cotransfected pAct-*lacZ* as described in Darlington et al. (1998), and displayed values are the mean with standard errors of four independent experiments. PER and PERΔCLD proteins were expressed to similar levels in the transfected S2 cells (Western blots and immunostaining not shown).

(A) A significant difference in the repression ($p < 0.01$, t test) was observed between PER and PERΔCLD, PER + TIM, or PER + TIM^{UL}. No significant difference was seen between PER + TIM and PER + TIM^{UL} repressor activity. TIM^{UL} represses slightly more strongly than TIM but not as strongly as either PERΔCLD, PER + TIM, or PER + TIM^{UL}. Note that TIM^{UL} cannot enter the nucleus without PER (data not shown).

(B) CRY and light abrogate the PER + TIM–mediated repression as previously described (Ceriani et al., 1999). This effect was seen neither with mutant CRY^{b} protein nor with PERΔCLD substituting for PER. The average of five experiments with standard errors is shown. The results for PER + TIM + CRY are significantly different ($p < 0.01$) from all other values (except dCLK induction alone).

with PERΔCLD, or with PER and/or TIM (Figure 6A). Coexpression of PERΔCLD and dCLK repressed transcriptional activation to ~35% of the value observed in the presence of dCLK alone. This level of repression was similar to that found for coexpression of PER + TIM and dCLK in this study (~38%, Figure 6A) and in the prior studies of Darlington et al. (1998) and Ceriani et al. (1999). Coexpression of wild-type PER and dCLK, or TIM and dCLK, affected transcription less strongly (activation was ~62% and ~77% of the control values, respectively), and both responses differ significantly from those obtained with PERΔCLD or PER + TIM ($p < 0.01$, Student's t test). Since dCLK-induced transcription is repressed equally well by PERΔCLD and by PER + TIM, these results supported our in vivo findings that PER can produce transcriptional repression in the absence of TIM protein.

Prior work has established that PERΔCLD translocates into S2 cell nuclei in the absence of coexpressed TIM (Saez and Young, 1996). To eliminate the possibility that in our experiment, trace amounts of TIM contribute to repression through formation of rare PERΔCLD/TIM complexes, we performed another S2 cell experiment. It has been established by others that, in this cell culture system, coexpression of CRY leads to physical association of the CRY protein with TIM (Ceriani et al., 1999). CRY expression also blocks the repressive activity of the PER/TIM complex in a light-dependent fashion, and this abrogation of PER/TIM repressor function is not seen with the CRY^{b} mutant protein (Ceriani et al., 1999; see also Figure 6B). As shown in Figure 6B, coexpressing CRY or CRY^{b} had no effect on the amount of repression seen with PERΔCLD, even in the presence of coexpressed TIM. Thus, CRY can abrogate repression of PER + TIM, but not of PERΔCLD + TIM, further indicating a TIM-independent activity of PERΔCLD in this established cell culture system.

We also tested the ability of TIM^{UL} protein to repress dCLK-induced activation. Figure 6A shows that there is no difference between the repressor activity of PER + TIM and PER + TIM^{UL}. Thus, the primary defect of TIM^{UL} protein does not seem to lie in reduced transcriptional repression activity.

Expression of PERΔCLD in Flies Interferes with the Clock

We reasoned that if PERΔCLD repressed transcription from the *per* promoter in the absence of TIM in S2 cells, in vivo expression of PERΔCLD might dominantly affect the circadian behavior of adult flies. To test this hypothesis, we generated transgenic flies expressing the *perΔcld* gene under control of the *per* promoter. Locomotor activities of four independent lines, each carrying two copies of the *perΔcld* transgene in a per^{+} background, were examined in DD after entrainment to LD cycles (Table 1). All four lines showed an unusually high number of arrhythmic flies, ranging from ~30% to 50% of all flies tested. This result contrasts with the low frequency of arrhythmic flies found in transgenic lines expressing wild-type *per* in a per^{+} background (8% for *y w;{per⁺}*; Table 1; cf. 7% in Petersen et al., 1988). A transgenic line containing four copies of *perΔcld* showed 65% arrhythmicity (*y w;{perΔcld}6;{perΔcld}8*), so there appears to be a correlation between *perΔcld* gene dosage and the frequency of arrhythmia. Even for flies that continue to show rhythmicity in the presence of *perΔcld*, period length is more variable than in wild-type controls (data not shown). Thus, unregulated nuclear entry of PER (i.e., TIM-independent nuclear entry

Table 1. Locomotor Activity Rhythms of Transgenic Flies Expressing PERΔCLD

Genotype	Tau	±SEM	R	AR	%AR
yw	23.5	±0.1	10	0	0
yw; {per⁺}	23.7	±0.1	11	1	8
yw;{perΔcld}1	23.9	±0.4	9	7	44
yw; {perΔcld}3	23.1	±0.2	9	8	47
yw; {perΔcld}6	23.1	±0.2	14	6	30
yw; {perΔcld}8	23.6	±0.2	11	5	31
yw; {perΔcld}6; {perΔcld}8	24.5	±0.4	8	15	65

Homozygous flies carrying two or four copies (*y w;{perΔcld}6; {perΔcld}8*) of transgenes encoding PERΔCLD were entrained to LD cycles for 3 days, and their locomotor activity was monitored in DD for 7 days. The period length (Tau) is indicated with the number of rhythmic (R) and arrhythmic (AR) individuals. The percentage of arrhythmic flies is also given (%AR). *y w* represents wild type with no transgene.

by PERΔCLD) appears to interfere with the function of the clock, even in a background of wild-type PER and TIM.

Discussion

Long-Period Rhythms from a *timeless* Allele

Four alleles of *tim* have now been reported: tim^{01}, which produces arrhythmia (Sehgal et al., 1994); tim^{SL}, which was found as a suppressor of per^{L} (Rutila et al., 1996); tim^{rit}, a temperature sensitive, long-period (26–30 hr) allele isolated from a natural population (Matsumoto et al., 1999); and tim^{UL}, which is shown in this study to generate rhythms with period lengths of ~33 hr. Thus, like certain mutations of *per* and *dbt* (Konopka and Benzer, 1971; Price et al., 1998), mutation of *tim* can substantially alter the period of the fly clock. Further characterization of *tim* mutations showed that the *Drosophila* pacemaker can support very long-period rhythms that are remarkably stable, as indicated by the 41 hr phenotype of per^{L};tim^{UL} double mutants and the ~45 hr period length of per^{L};tim^{rit}, obtained at high temperatures (Matsumoto et al., 1999).

The Molecular Defect in tim^{UL}

To learn which part of the molecular cycle is affected in tim^{UL}, a light-dependent PRC was generated. The tim^{UL} PRC indicates that the circadian oscillator has been altered with respect to a function(s) occurring late at night. The time domain associated with light-induced phase advances is extended by several hours in tim^{UL}. Analysis of PER and TIM protein levels in tim^{UL} confirmed such a change at the molecular level; both proteins can be detected for an extended period in DD. Sucrose gradient analyses showed that PER and TIM^{UL} are physically associated during the interval of prolonged protein accumulation, and immunocytochemical analysis showed that TIM^{UL} persists in photoreceptor cell nuclei for an extended time. Thus, the tim^{UL} mutation prolongs the duration of nuclear localization of the PER/TIM^{UL} complex.

Although we have not determined the mechanism responsible for prolonged association of PER and TIM^{UL}, it is possible that the tim^{UL} mutation specifically affects light-independent degradation of TIM^{UL}. In wild-type flies maintained in DD, most TIM is destroyed near subjective dawn, resulting in the accumulation of nuclear PER. PER is then phosphorylated and degraded over a period of 4–6 hr in response to the activity of a kinase, DBT (Kloss et al., 1998; Price et al., 1998; B. Kloss et al., unpublished data). As PER proteins are protected from degradation by association with TIM in wild-type flies (Vosshall et al., 1994; Price et al., 1995, 1998), increasing the half-life of TIM should prolong accumulation of PER. This was observed in tim^{UL} flies (Figure 2). An effect on TIM^{UL}, and PER/TIM^{UL} complex stability, might also explain our finding that reduction of *per* and *tim* expression to their lowest levels is severely delayed in tim^{UL} mutants. As discussed below, the latter mutant phenotype is likely to result from a failure to convert PER/TIM^{UL} complexes to nuclear PER.

The TIM^{UL} mutation involves a single amino acid substitution at position 260. Although this region of the TIM protein has not been characterized functionally, the mutation does not affect any of the protein fragments that were found in prior studies to independently bind to PER (Saez and Young, 1996; Sangoram et al., 1998). The TIM^{UL} region might contribute to the association of full-length PER and TIM proteins or allow association of an unidentified factor(s) influencing PER/TIM heterodimerization, dissociation, or TIM degradation in vivo.

Another attribute of the tim^{UL} phenotype is that cycling of *per* and *tim* RNA is less robust, and peak RNA levels are lower than in wild type. Since *per* and *tim* RNA levels drop to similar levels in tim^{UL} and wild-type flies, it appears that derepression of *per* and *tim* RNA in tim^{UL} is incomplete. We do not know the basis of this low-amplitude cycling in tim^{UL}. However, Bae et al. (1998) and Glossop et al. (1999) have demonstrated a role for the PER/TIM complex in the regulation of *dClk*, which itself is needed for activation of *per* and *tim* expression. Possibly, the tim^{UL} mutation will be found to specifically alter expression of *dClk*.

Transcriptional Repression by PER in the Absence of TIM

The PER/TIM complex is thought to repress transcription of its own genes by virtue of the following experiments: (1) overexpression of PER protein abolishes *per* RNA cycling and causes low levels of *per* RNA accumulation in transgenic flies (Zeng et al., 1994), (2) *per* and *tim* RNA levels begin to fall as the PER/TIM complex becomes nuclear (e.g., Qiu and Hardin, 1996), (3) coexpression of PER and TIM in cultured cells results in repression of dCLK/CYC-mediated transcription (Darlington et al., 1998; Figure 6), (4) the PER/TIM complex associates with dCLK/CYC and can prevent DNA binding of dCLK/CYC in vitro (Lee et al., 1999), (5) an antibody against dCLK can coimmunoprecipitate PER and TIM proteins from head extracts (Lee et al., 1998), and (6) light-dependent interaction of CRY and TIM blocks negative regulation of dCLK/CYC by PER/TIM complexes in cultured *Drosophila* cells (Ceriani et al., 1999).

Even though the PER/TIM^{UL} complex stays in the nucleus much longer than the wild-type complex does, we observed prolonged derepression of *per* and *tim* RNA. There are two possible explanations for this: (1) the PER/TIM^{UL} complex may not repress as efficiently as wild-type PER/TIM, or (2) the PER/TIM complex may not

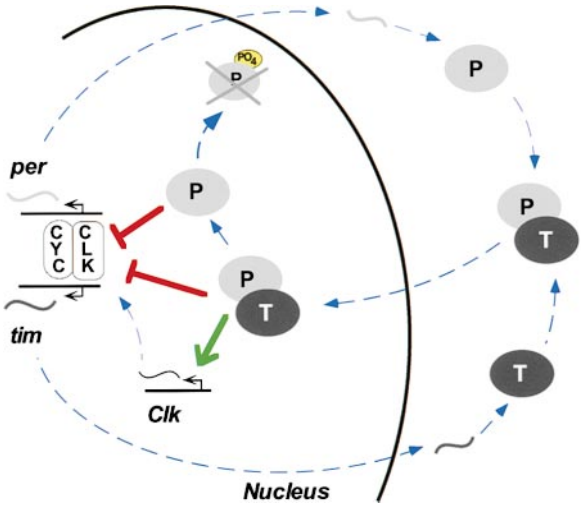

Figure 7. Roles of PER and the PER/TIM Complex in Transcriptional Regulation

In the model depicted, *per* and *tim* transcription promotes accumulation, with a delay, of heterodimeric complexes of PER and TIM proteins. The PER/TIM complex then translocates to the nucleus, initiates repression of *per* and *tim* transcription, and derepresses *dClk*. PER/TIM complexes are stable; however, specific degradation of TIM releases nuclear PER. In the absence of TIM, nuclear PER shows further repression of *per* and *tim* transcription, bringing *per* and *tim* RNA pools to their lowest levels. Phosphorylation of nuclear PER, regulated by DBT, leads to PER degradation, and the cycle starts anew (see text). Phosphorylation of nuclear PER may also promote its repressor function in the absence of TIM (Price et al., 1998). In this model, no role for TIM without PER is proposed because PER-independent TIM proteins have not been observed in wild-type nuclei.

be the only repressor. Since PER/TIM^{UL} complexes and PER/TIM complexes repressed dCLK-mediated *per* promoter activation in S2 cells equally well, we consider the second of the two explanations more likely. Light-induced formation of nuclear PER in *tim*^{UL} was associated with a strong reduction in *per* and *tim* RNA levels. Thus, our analysis of *tim*^{UL} indicated that nuclear PER is capable of transcriptional repression without TIM.

We extended our in vivo observations of a TIM-independent function of nuclear PER by examining PER function with and without TIM in cultured cells. We found that a CLD-deficient form of PER that can be transported to nuclei in the absence of TIM repressed dCLK/CYC-mediated transcription as effectively as the PER/TIM heterodimer. This form of PER also dominantly interfered with clock function when introduced into wild-type flies. Although the latter experiment does not resolve the mechanism of this interference, the results are consistent with a TIM-independent function for PERΔCLD in vivo that might correspond to this protein's function in S2 cells.

In wild-type flies, nuclear PER persists for several hours after TIM is degraded (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996; Price et al., 1998). In light of our results, we suggest that, in vivo, PER is active without TIM in wild-type flies at the end of each circadian molecular cycle and that nuclear PER brings transcriptional repression down to its trough levels. There are additional data supporting this hypothesis of a TIM-independent function for PER at the end of the night. First, transcription of *per* and *tim* does not start to increase until ZT 5 (So and Rosbash, 1997). This correlates better with the profile of PER protein, which is detected until ZT 5–7, than with those of PER/TIM complexes, which are dissociated at dawn (Marrus et al., 1996). Second, in *per*^{S} flies, PER^{S} protein levels decline about 4 hr prematurely, while TIM protein levels are affected less dramatically. This early disappearance of PER^{S} protein is correlated with an early rise in *per* and *tim* RNA levels (Marrus et al., 1996). Third, the opposite is observed in *dbt*^{L} flies, which show altered PER stability; PER proteins are detected for 2–4 hr longer in the morning than in wild-type flies, and this is correlated with a 2 hr delay in the rise of *per* and *tim* RNA levels (Price et al., 1998). Fourth, Lee et al. (1996) and Young et al. (1996) have previously reported a surprising small and transient decline in *per* and *tim* RNA levels immediately following light pulses. The effect of light on these RNA levels was difficult to further investigate in wild-type flies, because *per* and *tim* RNA levels were already in the declining phase. Such responses would now be explained if nuclear PER were sufficient for repression. We do not know the relative contributions of the PER/TIM complex and nuclear PER to repression in wild-type flies, but it is likely that the sequential appearance of PER/TIM complex followed by PER is functionally relevant. Our findings also suggest that prolonged derepression of *per* and *tim* in *tim*^{UL} mutants may be due to inefficient conversion of PER/TIM^{UL} complexes to nuclear PER.

A New Step in the Molecular Cycle Controlling *Drosophila* Circadian Rhythms

The clock mutants of *Drosophila* have provided insights into the sequential steps of a 24 hr molecular cycle. Cytoplasmic accumulation of PER and TIM, PER/TIM complex formation, and nuclear translocation are controlled steps in the cycle (reviewed by Young, 1998; Dunlap, 1999). The stability of PER in the nucleus is also a control point that influences period length (Zerr et al., 1990; Price et al., 1998). The present study suggests that dissociation of the PER/TIM complex to form nuclear PER is a further regulated element of the cycle and that in the case of the *tim*^{UL} mutant, interference with this step produces severely altered circadian rhythms.

What is the relevance of controlling the step from PER/TIM complex to PER? Toward the end of each molecular cycle, nuclear PER that is released from PER/TIM complexes becomes increasingly phosphorylated in a fashion dependent on the kinase DBT (Kloss et al., 1998; Price et al., 1998; B. Kloss and M. W. Y., unpublished data). When this phosphorylation is suppressed by *dbt* mutants, nuclear PER shows greatly increased stability (Price et al., 1998). Thus, phosphorylation should regulate the duration of repression by nuclear PER (see Figure 7, model). As both PER/TIM complexes and nuclear PER can repress *per* and *tim* transcription, but only phosphorylated PER proteins are significantly degraded, termination of each molecular cycle should be triggered by the conversion of PER/TIM complexes

to PER. While periodic degradation of TIM will be precisely set by an LD cycle, sustained molecular oscillations and behavioral rhythmicity close to 24 hr must be set in DD by light-independent turnover of TIM. This specific downregulation of TIM can be seen to occur in DD several hours before a corresponding diminution in the level of PER (Figures 2A and 2B, compare wild-type patterns of PER and TIM in day 1 of DD). Thus, a light-independent mechanism effecting nuclear TIM degradation should be a key determinant of period length. As indicated above, tim^{UL} may affect this mechanism.

Our studies raise the possibility that nuclear PER and the PER/TIM complex can perform distinct functions. For example, the observation that *per* and *tim* expression is decreased by removing TIM from PER/TIM^{UL} complexes indicates that quantitative or qualitative differences distinguish the activities of TIM-independent and TIM-associated forms of PER in vivo. This also suggests that, in vivo, full repression of *per* and *tim* expression requires the activity of nuclear PER at the end of each molecular cycle. There may also be different contributions to the regulation of *dClk* expression. dCLK protein negatively regulates *dClk* RNA accumulation (Glossop et al., 1999), which cycles with a phase distinct from that of *per* and *tim* (Bae et al., 1998; Darlington et al., 1998). PER and TIM block this dCLK activity (Glossop et al., 1999), such that PER/TIM nuclear translocation is associated with increased *dClk* RNA synthesis. However, *dClk* RNA levels fall at dawn, suggesting that the conversion of PER/TIM dimers to nuclear PER restores the autoregulatory activity of dCLK. Accordingly, PER may regulate *per* and *tim* expression, while PER/TIM complexes control transcription of *per*, *tim*, and *dClk* (see Figure 7). Such a mechanism could provide a general basis for establishing molecular oscillations with a variety of phases from a single clock.

Experimental Procedures

Genetics and Locomotion Analysis

Fly husbandry was performed according to standard procedures. For the mutagenesis screen, males were mutagenized with ethyl methanesulfonate (denoted by asterisk) as described (Lewis and Bacher, 1968) and mated to virgin females. Mating schemes were as follows: (1) *cn bw*/cn bw** males × *Sb/TM3,Ser* virgins; (2) assay locomotor activity of male progeny, and if aberrant phenotype is displayed, cross *cn bw*/+;*/Sb* single F_1 male × *Sb/TM3,Ser* and *Sp/SM5* virgins; and (3) rescreen locomotor activity of male progeny, and if aberrant phenotype is reproduced, establish lines with isogenous second or third chromosomes, (*cn bw** or +)/+;**/TM3,Ser* F_2 males × *Sb/TM3,Ser* virgins or (b) *cn bw*/SM5;+/Sb* males × *Sp/SM5* virgins.

For locomotion analysis, flies were generally entrained to LD cycles and then monitored in DD at 25°C for 6–9 days. Analysis was performed as described in Price et al. (1998). The PRCs were obtained with 10 min light pulses, as described in Myers et al. (1996).

The *per*Δ*cld* transforming plasmid was constructed by inserting the 4.8 kb genomic DNA containing the CLD deletion (Saez and Young, 1996) into the *per* 12.3 kb SphI-Xho construct (Baylies et al., 1992). The reconstructed *per*Δ*cld* gene was cloned into pCasper4 vector for P element–mediated germline transformation of *y w* flies, which was carried out as described by Rubin and Spradling (1982), and multiple independent transformed lines were generated.

Biochemical Methods

Western blot analysis was performed on adult head extracts as described (Price et al., 1998), and 30–50 μg of total protein was loaded onto 5.6% minigels. Sucrose (11 ml, 5%–25% gradients) was poured with a gradient maker from BioRad (Hercules, CA) and loaded with 50 μl of fresh head extract. They were spun overnight at 36 krpm, and 350 μl fractions were collected from the top, TCA precipitated, resuspended in 20–40 μl SDS gel loading buffer, and run on minigels as described above (see also Zeng et al., 1996). Anti-TIM immunocytochemistry on head cryosections was performed as described in Myers et al. (1996).

Molecular Biology and Cell Culture Experiments

RNase protection assays on total adult head RNA was performed as previously reported on 12–20 μg of total RNA (Sehgal et al., 1995). All RNase protections included wild-type LD extracts, and quantitations were normalized so that a value of 1 corresponded to the mean of a wild-type LD oscillation. For the light pulse experiment, flies were entrained in LD cycles for at least 4 days and then transferred to DD with or without a 30 min light pulse (of ~3000 lux) at ZT 20. The S2 cell transfection and analysis were carried out as described by Darlington et al. (1998) and Ceriani et al. (1999), except that the cells were plated in a 24-well plate and transfected using calcium phosphate. Cells were kept in constant light; they were harvested 48 hr after transfection, and enzyme activities were measured, using Promega Luciferase Assay System and standard liquid β-galactosidase assays.

Acknowledgments

We are very grateful to Marla Abodeely for her excellent technical assistance at many stages of the project. We thank Jeff Price and Evelyn Icasiano for their help during the genetic screen. We thank Ralf Stanewsky and Jeff Hall for anti-PER antibody, and Tom Darlington and Steve Kay for sharing materials and data prior to publication. We also thank Jeff Hall and Brian Kloss for valuable suggestions, and, especially, Justin Blau and Cedric Wesley for many helpful discussions, critical reading of the manuscript, and help in generating the P element lines. A. R. was supported by a Beckman fellowship. This work was supported by National Institutes of Health grant GM-54339 and the National Science Foundation Science and Technology Center for Biological Timing (M. W. Y.).

Received January 28, 2000; revised March 17, 2000.

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