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Effect of constant light and circadian entrainment of per^S flies: evidence for light-mediated delay of the negative feedback loop in *Drosophila*

Scott B.Marrus, Hongkui Zeng and Michael Rosbash[1]

Howard Hughes Medical Institute, National Science Foundation Science and Technology Center for Biological Timing, Department of Biology, Brandeis University, Waltham, MA 02254, USA

[1]Corresponding author

Light is the dominant environmental cue that provides temporal information to circadian pacemakers. In *Drosophila melanogaster* some *period* gene mutants have altered free-running circadian periods but entrain to 24 h light–dark cycles. To address the mechanism of light entrainment in *Drosophila*, we examined the effects of constant light on the *period* gene (*per*) and *timeless* gene (*tim*) products in wild-type and per^S flies. The results indicate that light affects three features of the PER–TIM program: PER and TIM phosphorylation, PER and TIM accumulation, and *per* and *tim* RNA cycling. A post-transcriptional effect on the PER–TIM complex is the likely primary clock target, which then delays the subsequent decrease in *per* and *tim* RNA levels. This is consistent with a negative feedback loop, in which the PER–TIM complex contributes to the decrease in *per* and *tim* RNA levels, presumably at the transcriptional level. There are enhanced constant light effects on the per^S mutant, which further support negative feedback as well as support its importance to entrainment of these flies to a 24 h cycle, far from their intrinsic period of 19 h. The per^S mutant leads to a truncated protein accumulation phase and a subsequent premature per^S RNA increase. A standard 24 h light–dark cycle delays the negative feedback circuit and extends the RNA and protein profiles, compensating for the accelerated RNA increase and restoring the rhythms to wild-type-like periodicity.

Keywords: circadian/clock/*Drosophila*/entrainment/light

Introduction

Circadian rhythms are present in most eukaryotic organisms and have been identified and characterized in some prokaryotes. They are endogenous in that they persist in the absence of temporal information from the environment. Endogenous periods are usually close to but not exactly 24 h. Light is the dominant time cue and normally resets the clock every day so that its period matches the precise 24 h environmental cycle (Edmunds,Jr, 1988).

The effect of light has been studied extensively in many circadian systems, for example by determining the pacemaker response to light pulses delivered at different times in the circadian cycle (the phase response curve; Johnson, 1990). It has also been studied by determining the circadian response to different entrainment regimes, for example the length of the light and dark phases of daily light–dark (LD) cycles (Pittendrigh, 1981; Saunders, 1982). Despite these many formal studies, there is little information about how light affects molecular aspects of pacemaker function. This is due to the fact that only in a few systems is there any indication of the existence and function of circadian clock components (e.g. Crosthwaite *et al.*, 1995).

In *Drosophila melanogaster*, the levels of *per* RNA and protein (PER) undergo circadian oscillations (Hardin *et al.*, 1990; Zerr *et al.*, 1990; Edery *et al.*, 1994b; Zeng *et al.*, 1994), and PER is a clock component (Edery *et al.*, 1994a). A similar if not identical situation obtains for *tim* RNA and protein (TIM), which is PER's heterodimeric partner *in vivo* (Gekakis *et al.*, 1995; Myers *et al.*, 1995; Zeng *et al.*, 1996). The *per* RNA oscillations are due at least in part to circadian changes in transcription; we assume the same is true for *tim* transcription, and there are indications that PER and TIM's biochemical functions include the regulation of transcription (Hardin *et al.*, 1990, 1992; Huang *et al.*, 1993). As PER and TIM influence the cycling of their mRNAs, an autoregulatory transcriptional feedback loop probably contributes to the molecular cycle that underlies behavioral circadian rhythms (Hardin *et al.*, 1990; Zeng *et al.*, 1994). Yet transcriptional regulation is insufficient to account for all of the features of PER and TIM cycling; they are also influenced by post-transcriptional mechanisms. PER and TIM undergo phosphorylation changes that are under temporal regulation (Edery *et al.*, 1994b; Zeng *et al.*, 1994, 1996), and it is likely that proteolysis is also under temporal control (Myers *et al.*, 1996; Zeng *et al.*, 1996; Dembinska *et al.*, 1997). This indicates that kinases, phosphatases and proteases may be among other as yet unidentified clock components.

Behavioral assays in constant darkness (DD) indicate that recurrent dawn and dusk transitions are not essential for rhythmicity, so one or both of them presumably function to entrain the rhythms to the environmental light cycle. An understanding of the relationship between light and the PER–TIM molecular rhythms may therefore clarify how light entrains the endogenous periods to the precise 24 h period of the light–dark cycle. It may also help explain how *per* mutant strains entrain to 24 h LD cycles, which are significantly different from their intrinsic periods (Hamblen-Coyle *et al.*, 1992).

There is emerging information on how light might interact with the *Drosophila* pacemaker and its components. Under conditions of constant intense light, *Drosophila* and many other organisms are arrhythmic (Winfree, 1974; Pittendrigh, 1981; Saunders, 1982; Sweeny and Hastings, 1987; Petersen *et al.*, 1988; Konopka *et al.*, 1989; Hamblen-Coyle *et al.*, 1992; Power *et al.*, 1995).

Constant intense light also inhibits PER cycling (Zerr *et al.*, 1990; Price *et al.*, 1995) and the absence of the molecular cycle is a likely cause of the behavioral arrhythmicity. *per* RNA, as well as *tim* RNA and protein, had not been examined under these conditions, so it was uncertain whether the low and constant protein levels are due to a more direct inhibitory effect of constant light (persistent, intense light) on transcription (cf. Crosthwaite *et al.*, 1995).

More importantly, the effect of constant light on *per* RNA has only recently been examined directly after lights would normally go off at ZT12 (ZT = zeitgeber time = time during a normal 24 h LD12:12 cycle; Qiu and Hardin, 1996). Therefore the extent to which all of the molecular events that normally occur in the first hours of the night are dependent on the light to dark (LD) transition at ZT12 is not known. The importance of LD is indicated, however, by the fact that behavioral rhythms are initiated by a single LD transition that defines the phase of locomotor activity during free-running conditions (i.e. no environmental cues = continuous darkness = DD; Pittendrigh, 1981; Saunders, 1982; Saunders *et al.*, 1994). Also there are rapid and potent molecular effects of light on the phosphorylation status of TIM and PER (Lee *et al.*, 1996; Zeng *et al.*, 1996), suggesting that lights off at ZT12 (or prolonged light exposure after ZT12) might also have rapid effects.

Based on these considerations, we examined the failure to experience lights off (constant light = LL) on the molecular rhythms of wild-type and per^S flies. The results indicate that constant light affects TIM and PER phosphorylation as well as TIM and PER half-lives. We speculate that darkness allows the proper PER and TIM post-translational program to take place, which is required for the proper timing of the decrease in *per* and *tim* RNA levels during the early night. The sequence of events as well as the enhanced effects observed in a per^S background suggest that PER or the PER–TIM complex participates in negative feedback regulation of transcription. The enhanced effects in per^S flies are consistent with the previously documented stronger light response of this genotype. The results indicate that light inhibits the level and phosphorylation status of PER and TIM, which then delays the negative feedback circuit and extends the RNA profiles. This light-mediated delay compensates for the accelerated RNA increase of the mutant strain and restores the rhythms to wild-type-like periodicities.

Results

The wild-type PER–TIM cycle in LD

PER oscillations during a 24 h LD cycle have been described (Figure 1; Zeng *et al.*, 1994). Protein levels increase modestly during the latter half of the day (ZT8–12) and then rise dramatically during the first half of the night. There is also an increase in PER's phosphorylation status preceding the decline in PER levels late at night, which then continues into the next day (Figure 2B; Edery *et al.*, 1994b). In the middle of the day (ZT8) there is almost no detectable protein, which is then followed by a new round of synthesis. The protein fluctuations follow comparable fluctuations in *per* RNA (Zeng *et al.*, 1994). TIM oscillations are very similar to those of PER (Zeng *et al.*, 1996). Both proteins also experience apparent progressive phosphorylation at approximately the same times of the cycle (Figures 2B and 3B; Zeng *et al.*, 1996). The only notable difference is that TIM accumulation is somewhat phase advanced (ca 2 hr) relative to PER (Figure 1). This earlier accumulation of TIM is also supported by a measurement of the TIM:PER ratio, which is higher at earlier times of the cycle (Zeng *et al.*, 1996).

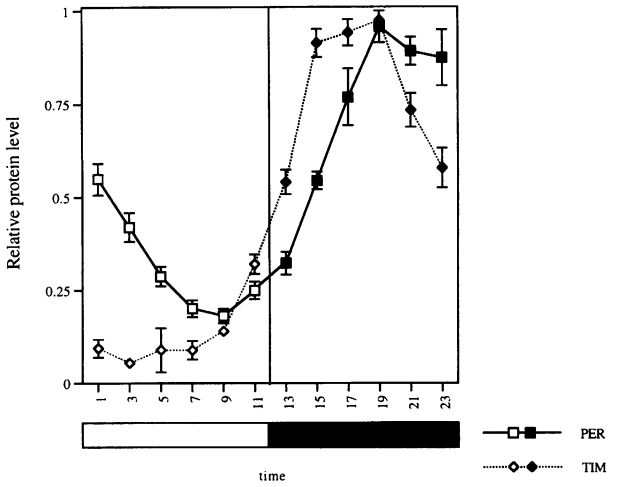

Fig. 1. *per* and *tim* protein cycling. Wild-type flies (CS) were entrained for 3 days in LD 12:12 and collected every 2 h. Western blot and quantification are described in Materials and methods. Four independent experiments were performed and the values were averaged; the standard error is indicated for each time point. The maximum value for each protein was normalized to one. Throughout the figures, open symbols correspond to samples collected in the light; closed symbols to samples collected in the dark.

Effect of constant light on the wild-type cycle

To examine the importance of the lights off transition to these oscillations, we initially asked what happens to PER in constant light (LL), i.e. when the lights fail to turn off at ZT12 (Figure 2). Surprisingly, the persistence of the light cycle (intense, saturating light ≥1000 lux) did not freeze the PER molecular cycle at the ZT12 value, the result expected from the steady-state consequences of constant light (e.g. Price *et al.*, 1995). Instead, the cycle continued for at least two days and was only modestly different from the well-documented control LD PER cycle (Figure 2A). The most obvious difference was a delay in the decline of PER levels in LL as compared to LD, which was also apparent by the phase delay of the next day's cycle (Figure 2A). Protein amplitude in the first day of LL was not dramatically affected [Figures 2A and B; an average of four experiments indicated that maximum PER levels in LL were 90% of those in LD (data not shown)], but a stronger and reproducible effect was apparent on the second day when the peak height was reduced compared to the first day (Figure 2A). The Western blots reveal another effect of LL on PER: the prominent phosphorylation which is normally most obvious after ZT18–20 (Edery *et al.*, 1994b) appears attenuated in LL (Figure 2B). This observation fits well with the reported effects of a light pulse on PER phosphorylation in the early night (Lee *et al.*, 1996).

We then asked, what happens to TIM when the lights fail to turn off at ZT12? The effect of this constant light

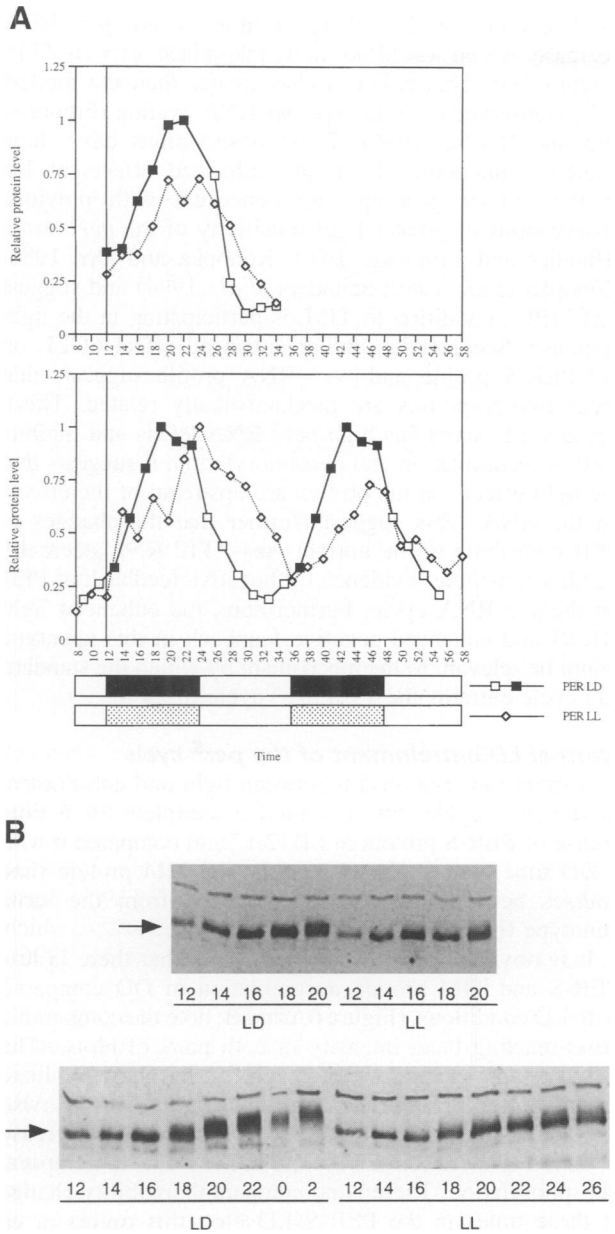

Fig. 2. PER protein in constant light. After standard entrainment, flies were transferred to constant light and collected at the indicated times. Control flies were maintained in LD. (**A**) Quantification of PER levels in flies collected in LD versus LL. Peak protein level is normalized to one. Top graph, generated from the bottom panel of Figure 2B. Bottom graph, from an independent LL experiment. The LD curve in the bottom graph comes from Figure 1 and is replotted for the second day. The stippled rectangles represent subjective night during constant light. (**B**) Western blot of PER in flies collected in LD versus LL showing the impact of LL on protein levels and phosphorylation state. Time of collection is indicated for each lane. Two independent experiments are shown.

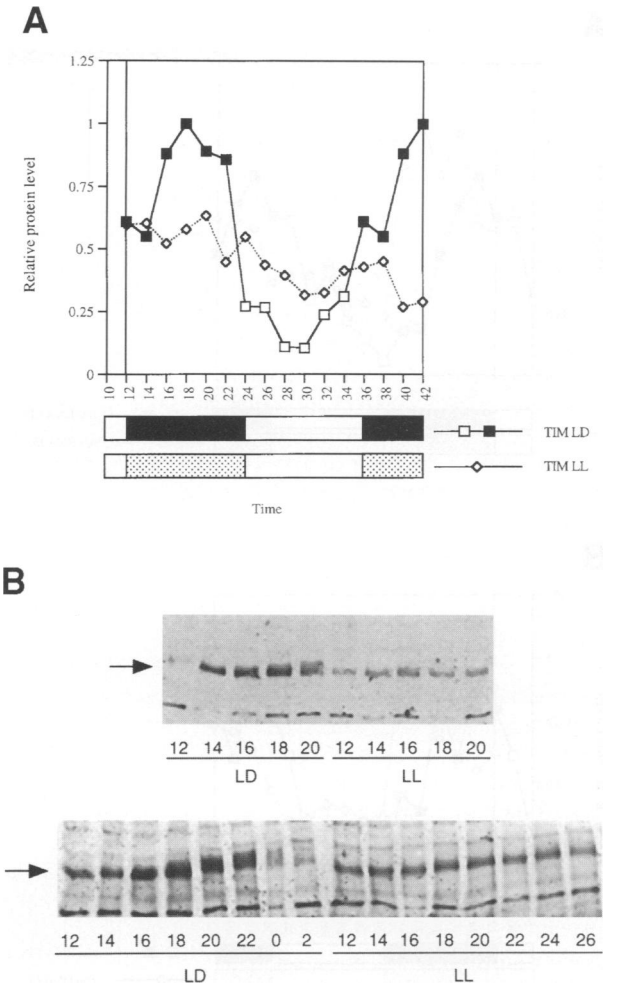

Fig. 3. TIM protein in constant light. Conditions are the same as for Figure 2, except that TIM levels were assayed. (**A**) Quantification of TIM in wild-type flies in LD versus LL. The gel that was quantitated is the bottom panel of Figure 3B. (**B**) Western blot of TIM in wild-type flies in LD versus LL. Time of each collection is indicated. Two independent experiments are shown.

regime is much more striking on TIM than on PER. TIM levels remain constant or nearly so during the first few hours in LL, instead of the substantial accumulation that would normally take place in the dark (Figure 3). The effect on TIM's temporal phosphorylation is also dramatic, as it is essentially undetectable in LL (Figure 3B). The normal night time accumulation and phosphorylation of TIM are therefore light-inhibited.

Next we examined the short-term effects of LL on *per* and *tim* RNA oscillations. As expected from the persistent PER fluctuations, RNA cycling continues for at least two days after the transfer to LL (Figure 4 and data not shown); the timing of the oscillations is delayed compared with LD control conditions. Like the LL-induced delays of the *per* protein cycle, the RNA cycle delays appear due to a delay in the decrease that takes place during the subjective night (what would be the night in LD; Figure 4). The amplitude of the second LL peak is also attenuated (Figure 4). These LL effects on the RNA profiles are identical to those recently published for *per* RNA by Qiu and Hardin (1996). Taken together, the effects of LL indicate that the molecular cycle is not frozen at ZT12 in the absence of lights-off, i.e. during the first hours of constant light. As substantial cycling continues under these conditions, the arrhythmicity characteristic of constant light takes place gradually rather than immediately, as also concluded by Qiu and Hardin (1996). Importantly, the modest effects of LL on *per* and *tim* RNA cycling parallel the modest effect on PER cycling. TIM cycling is the outlier and is undetectable in LL.

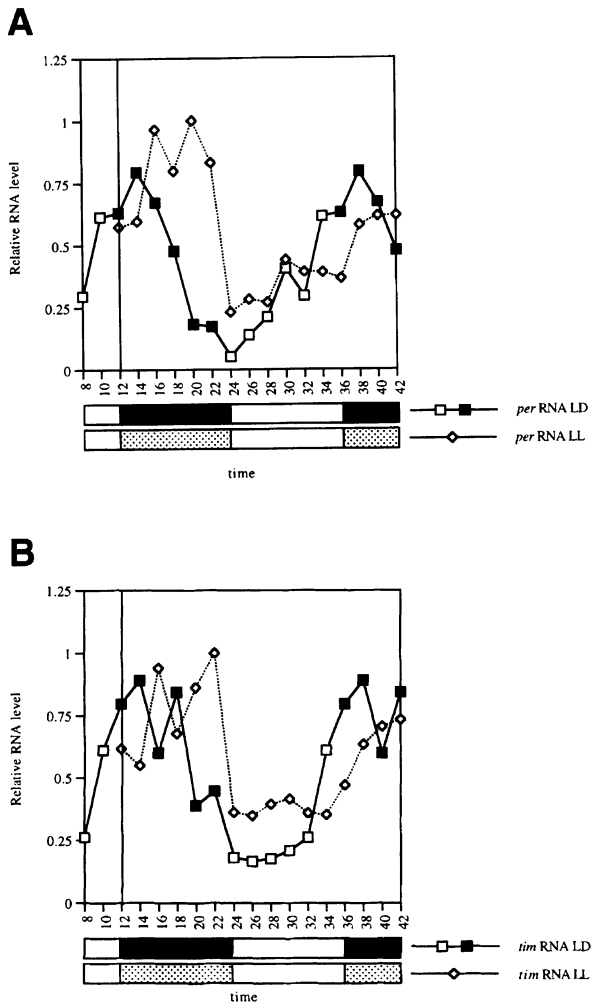

Fig. 4. *per* and *tim* RNA in constant light. Quantifications of RNase protection assays of *per* and *tim* RNA in wild-type flies. Typical results of multiple experiments are shown. (**A**) *per* RNA in LD versus LL. Peak RNA level is normalized to one. (**B**) Same as (A), for *tim* RNA.

Effect of constant light on the per^S cycle

To confirm and extend these correlations, we examined the effects of constant light on RNA and protein cycling in the *per^S* mutant genotype. These flies have an endogenous period of only 19 h instead of the 24 h characteristic of wild-type flies. Yet the mutants entrain well to 24 h LD cycles (Hamblin-Coyle *et al.*, 1992). The rhythmicity of *per^S* flies is also reported to be more light-sensitive than that of wild-type flies (Handler and Konopka, 1979; Konopka and Orr, 1980; Konopka *et al.*, 1989; Saunders *et al.*, 1994). Taken together, these two effects of light suggest that constant light treatment might be revealing about the relationship of the two protein cycles to the RNA cycles and about the mechanism of light entrainment.

Under LL conditions, there is little or no TIM cycling and little or no detectable TIM phosphorylation in *per^S* flies (Figure 5A). The effect is indistinguishable from what is observed in wild-type flies (Figure 3). LL also inhibits PER-S cycling and PER-S phosphorylation (Figure 5B and C). The effect, at least on PER-S accumulation, is considerably greater than that observed for wild-type PER (Figure 2). There is also a strong inhibition of *per^S* RNA cycling by LL; there is little or no *per^S* RNA decrease, which would normally take place after ca ZT16 (Figure 5D). This effect is also greater than the modest delays observed on wild-type *per* RNA cycling (Figure 4; Qiu and Hardin, 1996). These observations have three related implications. First, the enhanced effects of LL in the mutant genotype are consistent with previous observations of greater light sensitivity of the *per^S* strain (Handler and Konopka, 1979; Konopka and Orr, 1980; Konopka *et al.*, 1989; Saunders *et al.*, 1994) and suggest that PER, in addition to TIM, is participating in the light response. Second, the similar enhanced effect of LL on the PER-S profile and *per^S* RNA profile suggests that these two responses are mechanistically related. Third, because LL maintains high *per^S* RNA levels and inhibits PER-S accumulation and phosphorylation, it suggests that the light effects on the protein are upstream of the effects on the RNA. This suggests further that the changes in PER contribute to the normal post-ZT12 RNA decrease, i.e. this constitutes evidence for negative feedback of PER on the *per* RNA cycle. Furthermore, the enhanced light effects and enhanced negative feedback in this genotype might be relevant to the mechanism by which the standard LD cycle entrains the 19 h *per^S* cycle to 24 h.

Normal LD entrainment of the per^S cycle

To extend the relationship between light and entrainment of the *per^S* cycle, we measured a complete 24 h time course of PER-S protein in LD12:12 and compared it with a DD time course. We also compared TIM protein time courses between these two conditions from the same genotype (Figure 6).

It is obvious from the Western blots that there is less PER-S and TIM protein accumulation in DD compared with LD conditions. (Figure 6A and B; note the comparable cross-reacting band intensity in both pairs of blots.) The initial protein profiles are quite similar, but they terminate much earlier in DD than in LD (Figure 6A–C). Also visible on the DD blot of PER-S is a prominent decrease in mobility between CT11 and CT15, due to temporal phosphorylation. There is no comparable mobility change at these times in the PER-S LD blot; this occurs at ca ZT19 (Figure 6A). These differences are even more apparent in a side by side comparison from an independent experiment (Figure 6D). (i) protein levels until ZT15 or CT15 (LD or DD) are comparable, but thereafter LD levels are much higher than DD levels; (ii) the proteins begin to disappear earlier in DD than in LD; and (iii) for TIM as well as for PER-S, the phosphorylation profile is markedly delayed or inhibited in LD compared with DD conditions. None of these differences are comparable in the wild-type LD versus DD comparisons, for PER or for TIM (Zeng *et al.*, 1996; data not shown). As the mobility differences are already apparent at ZT11 versus CT11 when there is little difference in protein levels (Figure 6), it suggests that suppression of PER and TIM phosphorylation is an early light-mediated event (Figures 2, 3 and 5; Lee *et al.*, 1996; Zeng *et al.*, 1996).

The differences in the PER-S curves caused by the LD cycle are accompanied by equally striking differences in the *per^S* RNA curves (Figure 7). The LD curve is much broader than the DD curve, and this is due to an asymmetric effect of light on the profile: light has little or no effect

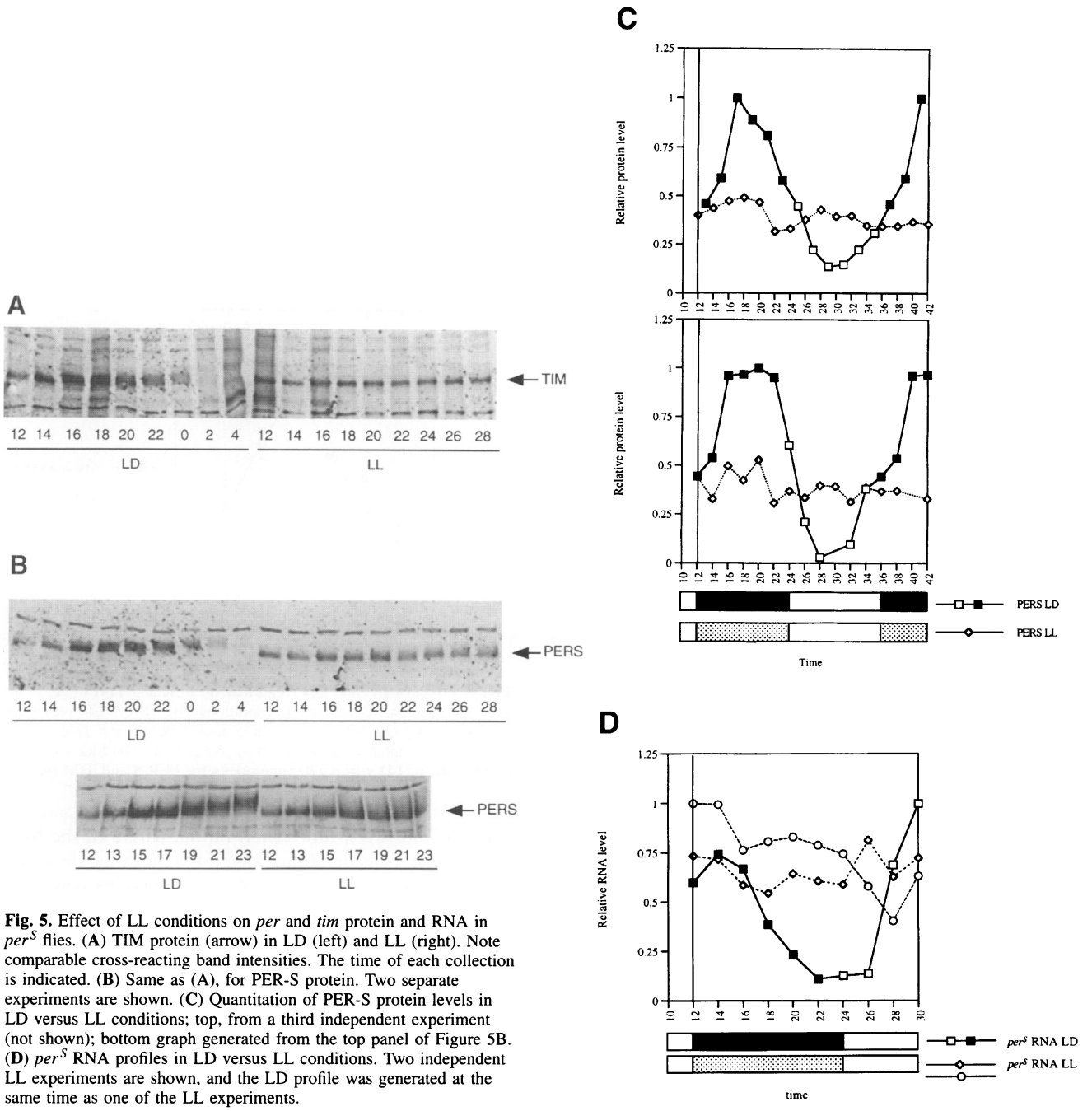

Fig. 5. Effect of LL conditions on *per* and *tim* protein and RNA in per^S flies. (**A**) TIM protein (arrow) in LD (left) and LL (right). Note comparable cross-reacting band intensities. The time of each collection is indicated. (**B**) Same as (A), for PER-S protein. Two separate experiments are shown. (**C**) Quantitation of PER-S protein levels in LD versus LL conditions; top, from a third independent experiment (not shown); bottom graph generated from the top panel of Figure 5B. (**D**) per^S RNA profiles in LD versus LL conditions. Two independent LL experiments are shown, and the LD profile was generated at the same time as one of the LL experiments.

on the rising phase of the RNA curve in the early morning, but the declining phase of the LD curve is delayed by several hours compared with the DD curve. As expected, the *tim* RNA profiles from this genotype are affected identically (data not shown). Given the effect of an extended light protocol (constant light) on the per^S RNA profile (Figure 5D), it is likely that the presence of light until ZT12 in LD conditions inhibits the decrease that the per^S RNA would otherwise begin to undergo between ZT10 and ZT12. The early decrease of the DD RNA curve is also likely to be relevant to the failure to continue to accumulate protein during the later parts of the night in DD (CT15–20) as compared with the same times (ZT15–20) in LD (Figure 6), i.e. there is much less mRNA template for synthesis at these times (Figure 7). In other words, light delays the per^S RNA decrease, which extends and raises protein levels.

If light delays the declining phase of the per^S RNA curve by ~4–6 h in LD conditions, it should be similar to the declining phase of the wild-type RNA curve under the same conditions and would fit with the daily adjustment in period difference between the two genotypes. Indeed, the two curves decrease at approximately the same time in the evening. This is consistent with the fact that the nuclear entry time of PER-S is indistinguishable from that of wild-type PER in LD conditions (Curtin *et al.*, 1995).

The earlier increase of per^S RNA compared with a wild-type RNA profile (Figure 7) indicates that much of the 4–5 h period difference between the two genotypes is already manifest in the first few hours of DD, after release

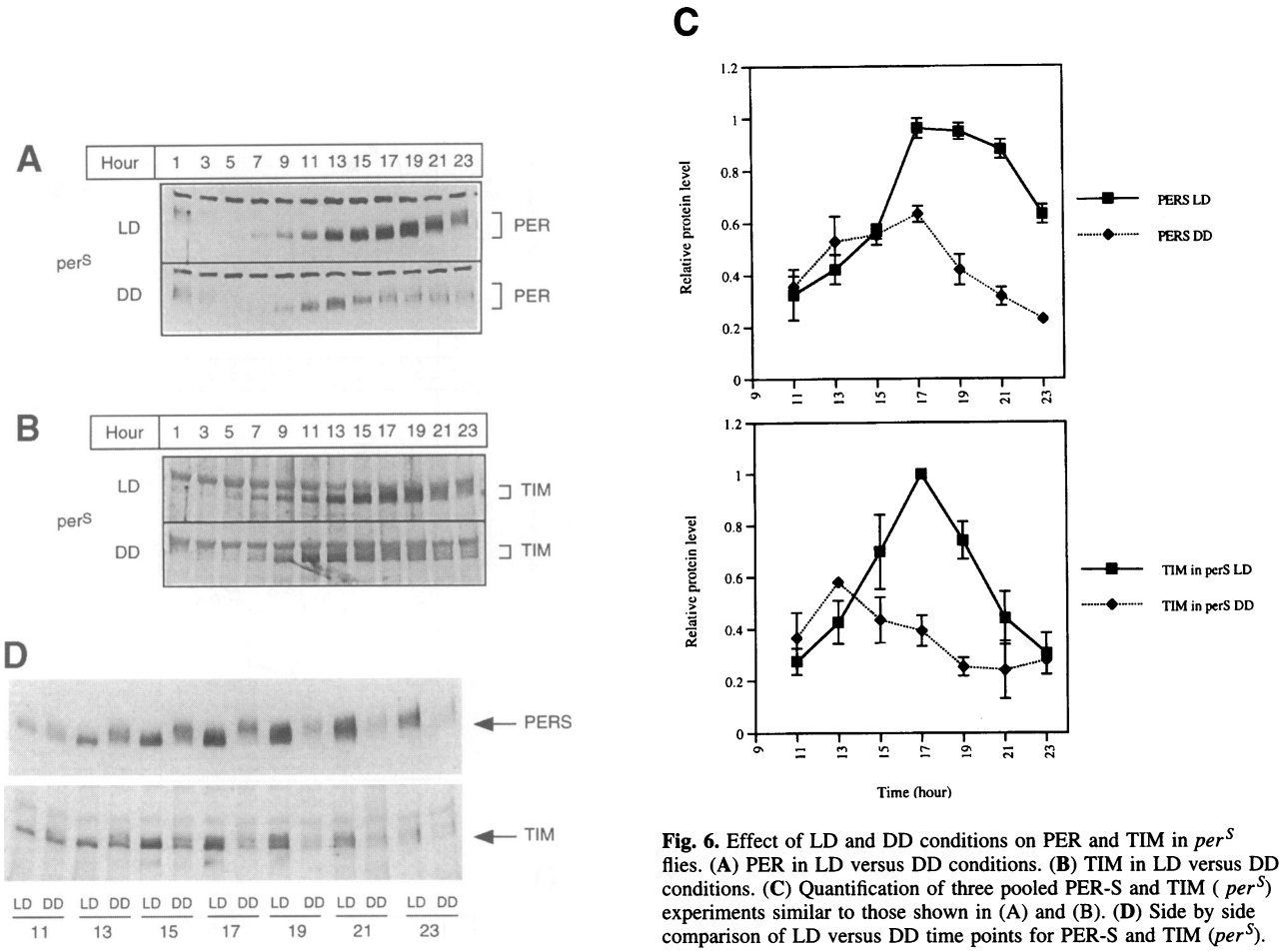

Fig. 6. Effect of LD and DD conditions on PER and TIM in per^S flies. (**A**) PER in LD versus DD conditions. (**B**) TIM in LD versus DD conditions. (**C**) Quantification of three pooled PER-S and TIM (per^S) experiments similar to those shown in (A) and (B). (**D**) Side by side comparison of LD versus DD time points for PER-S and TIM (per^S).

from the LD entrainment regime (also see Hardin *et al.*, 1990; Seghal *et al.*, 1995). As a comparable phase advance in per^S RNA accumulation takes place in LD conditions (Figure 7), the rise is largely unrelated to light entrainment and must be timed by some event in the previous cycle. A likely suspect is the disappearance of PER-S and TIM, which begins in the late night of the previous per^S cycle (Figure 8). Both proteins decrease more rapidly than wild-type PER and TIM, anticipating the premature rise in per^S RNA the next morning (Figure 7). PER-S reaches a minimum at ca ZT4 (Figure 8B) and (like the RNA rise) the timing is similar in LD and DD conditions (Figure 6A); wild-type PER reaches a minimum at ca ZT8 (Figure 8B; Zeng *et al.*, 1996). These considerations are consistent with a negative feedback view, that PER and/or TIM inhibit their own transcription (Hardin *et al.*, 1990, 1992; Zeng *et al.*, 1994; Lee *et al.*, 1996) and that the disappearance of the proteins relieves the inhibition.

Discussion

Previous work has shown that PER participates in a feedback loop which affects its own transcription (Hardin *et al.*, 1990, 1992; Zeng *et al.*, 1994). More recent studies support an expanded view, namely, that *tim* as well as *per* transcription is affected by one or more of their protein products: TIM, PER or the PER–TIM heterodimeric complex (Sehgal *et al.*, 1994, 1995; Gekakis *et al.*, 1995; Myers *et al.*, 1995; Lee *et al.*, 1996; Zeng *et al.*, 1996). There is, however, little detail on the nature of the loop. Theoretical considerations suggest a negative feedback loop, in which one or more of these proteins contribute to the downturn in *per* and *tim* transcription at ca ZT17. There is also one relevant experiment consistent with this view: overexpression of PER in the adult eye from a rhodopsin promoter causes non-cycling and low endogenous *per* RNA levels in this tissue (Zeng *et al.*, 1994). But this could be an indirect consequence of the stopping of the clock by high level PER expression rather than reflecting a dynamic, causal relationship between PER levels and *per* RNA cycling. Rather than contributing directly to negative feedback, the proteins might contribute solely to timing the next day's upswing in transcription, e.g. a positive or feedforward version of temporal autoregulation. The experiments presented here on the per^S genotype and the effects of constant light indicate that negative feedback regulation is indeed part of the PER–TIM autoregulatory circuit and contributes to entrainment.

A rapid effect of extended illumination (LL), manifest shortly after ZT12, is an inhibition of wild-type PER and TIM phosphorylation which would normally take place in the first few hours of the night. Even stronger effects take place in the mutant per^S strain. In both genotypes, TIM accumulation is strongly inhibited in LL, as TIM levels remain almost constant. As the *tim* RNA changes are modest, the LL effect on TIM levels as well as on phosphorylation must be post-transcriptional. Similar short-term effects were observed with light pulses in the

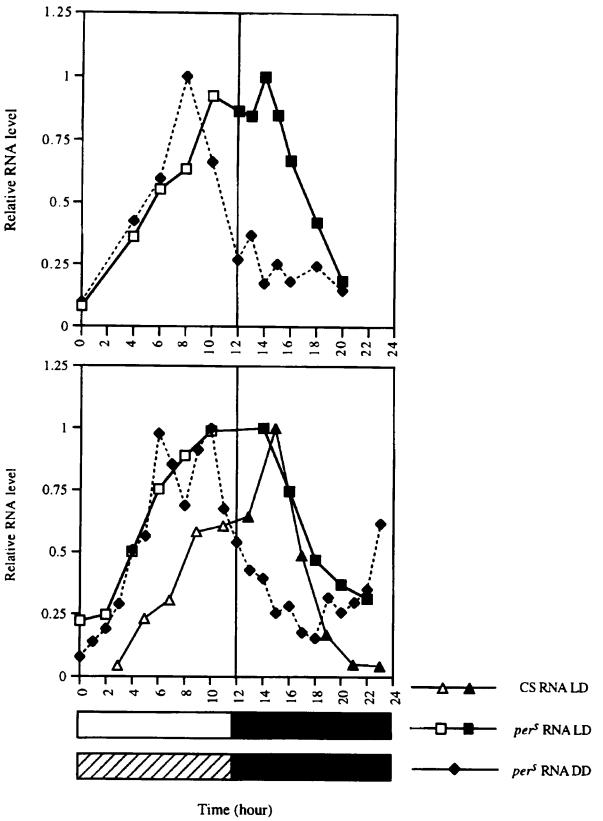

Fig. 7. *per*^{S} RNA in LD versus DD conditions. Two independent experiments are shown. The wild-type (CS) curve is from Zeng *et al.* (1994).

early night, where a preferential disappearance of the more highly phosphorylated forms of TIM and an inhibition of PER phosphorylation were observed (Lee *et al.*, 1996; Zeng *et al.*, 1996). This suggests that an early effect of LL is on the TIM and PER phosphorylation status, perhaps a light-mediated inhibition of the sequential phosphorylation program that normally takes place in the dark after ZT12 (Edery *et al.*, 1994b; Lee *et al.*, 1996; Zeng *et al.*, 1996).

We preferred the interpretation that a more primary effect of constant light is rapid proteolysis of the more highly phosphorylated forms of TIM (Zeng *et al.*, 1996). We assumed that the phosphorylation inhibition is a secondary effect, because the light effect on TIM levels is even stronger in the late night when PER and TIM have already undergone their early night phosphorylation programs (Zeng *et al.*, 1996) and because a qualitatively similar light effect on TIM levels is manifest in the absence of PER (Myers *et al.*, 1996; Zeng *et al.*, 1996). However, the enhanced light effects on PER-S levels and phosphorylation suggest that PER is not merely a passive partner but participates actively in the light response. All of the data point to the PER–TIM heterodimer as the light-sensitive target of the circadian clock. The circadian photoreceptor and the relevant signal transduction pathway to the PER–TIM complex remain unknown.

These post-translational effects on PER and TIM are accompanied and followed by extended RNA profiles and a prolonged presence of the proteins. The persistence of the template is presumably responsible for the persistence

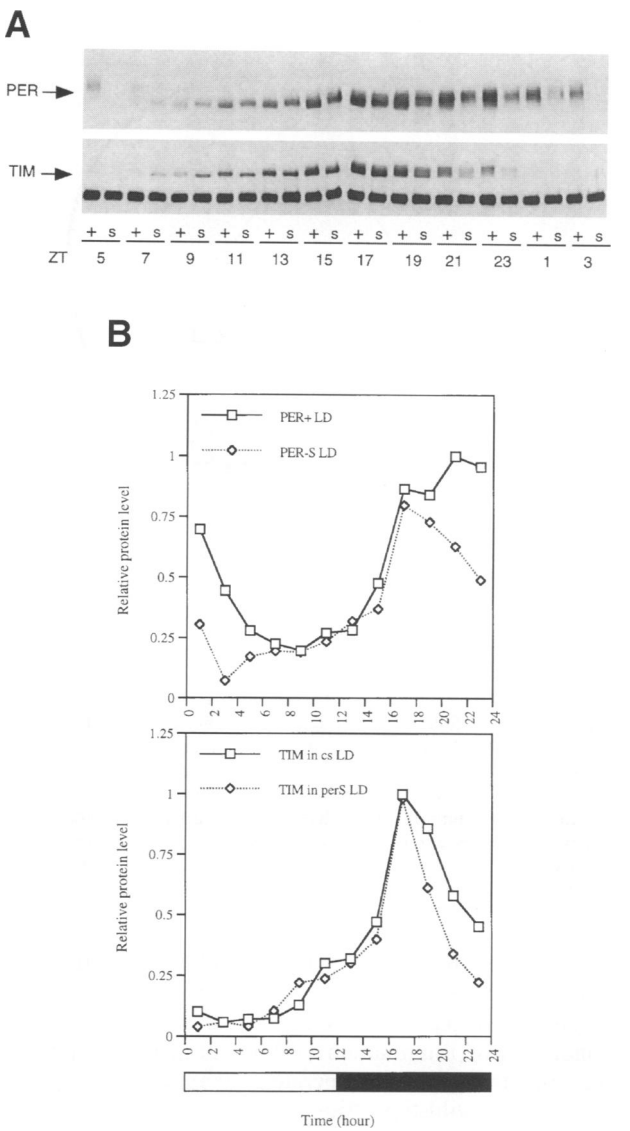

Fig. 8. Comparisons of PER and TIM between *per*^{S} and wild-type flies in LD conditions. (**A**) Side by side Western blot of PER and TIM from LD conditions. (**B**) Quantification of PER (top) and TIM (bottom) levels from (A).

of the proteins. This LL-mediated delayed downturn in *per* and *tim* RNA levels is identical to observations recently made by Qiu and Hardin (1996). Although the light effect on RNA might be direct, it is more likely in our view that it depends on a more primary post-translational effect on the PER–TIM system, revealing a negative feedback feature of the circuit (Figure 9). (i) In LD conditions *per* and *tim* RNA fail to cycle in *per*^{0} and *tim*^{0} flies (Hardin *et al.*, 1990; Sehgal *et al.*, 1994, 1995; Hunter-Ensor *et al.*, 1996); (ii) there are also no short-term light effects on *per* transcription in a *per*^{01} null background (data not shown; Hardin *et al.*, 1990); and (iii) the enhanced LL effect on *per*^{S} RNA levels (Figure 5D) provides strong support for an involvement of the PER–TIM system in negative feedback. Results from the *Neurospora* circadian clock, the only other molecularly well-characterized system, provide a different picture, namely, that light has potent effects on transcription of the *frequency* gene even in the absence of FRQ gene product (Crosthwaite *et al.*, 1995).

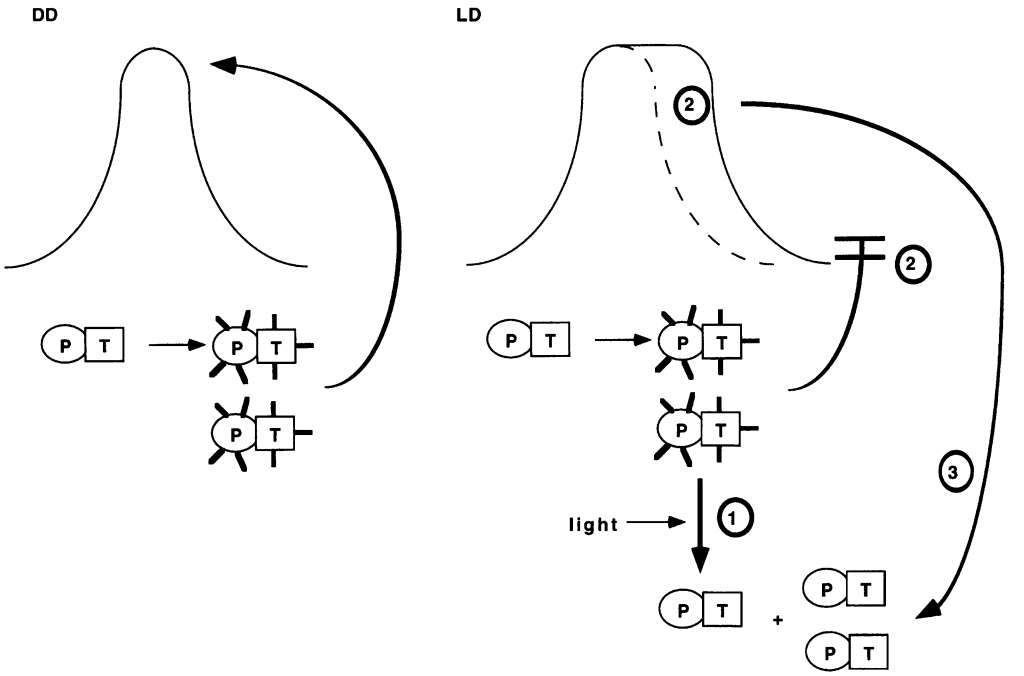

Fig. 9. Model of light-mediated delay of feedback regulation. On the left is the normal DD feedback regulation that governs per^S RNA cycling. The curve represents RNA cycling, and the more numerous and more phosphorylated PER–TIM dimers (PER, oval; TIM, square; phosphates, sticks) engage a negative feedback circuit that contributes to the decrease in per^S RNA levels (large curved arrow). The small straight arrow represents the accumulation of PER–TIM dimers and their phosphorylation as the cycle progresses. On the right is the delayed negative feedback regulation under entraining LD conditions. Light has a primary effect on the post-translational status of the PER-S–TIM dimers; light lowers protein levels and inhibits phosphorylation (1). This inhibits and delays the normal negative feedback regulation (2), which extends the cycle length. This prolongs high RNA levels (2), which leads to increased levels of underphosphorylated PER-S–TIM dimers (3). After the lights turn off at ZT12, these are eventually phosphorylated which engages the negative feedback circuit (not shown).

The early night (ZT12–17 or CT12–17) is the delay region of the phase response curve (PRC), i.e. the region where a light pulse causes a phase delay. Under identical conditions of illumination (≥1000 lux), a light pulse of only one min at ZT15 generates a maximum phase delay of ~3 h in wild-type flies; longer light pulses have no enhanced effect (data not shown). This is very similar to the LL-mediated delays observed in this study (cf. Qiu and Hardin, 1996), suggesting that they are of similar origin to light-pulse-generated delays. Importantly, light pulses in the delay zone not only delay PER/TIM accumulation and phosphorylation but also delay the decline in RNA levels (Lee *et al.*, 1996), consistent with the suggestion that the former causes the latter.

Although it is unknown which protein species participates more directly in the negative feedback (PER, TIM or the PER–TIM dimer), the night-time phosphorylation program suggests that the more highly phosphorylated species may be more potent transcriptional repressors or may lead to higher repressor concentrations. The modest inhibitory effect of LL on PER phosphorylation and on the RNA program is consistent with this view. More important are the enhanced LL effects on aspects of the per^S cycle: PER-S accumulation as well as phosphorylation is more strongly inhibited by LL than the wild-type PER program (Figure 5 versus Figure 2). The low level of relatively underphosphorylated PER-S is accompanied by a maintenance of high per^S RNA levels (Figure 5). In contrast, the LL effect on TIM in per^S is indistinguishable from the effect in a wild-type background (Figure 5 versus Figure 2). These observations not only support negative feedback but point to the accumulation and phosphorylation of PER as contributing to the inhibition of *per* and *tim* transcription that takes place after ZT15 (Figure 9).

The active species could be the PER–TIM heterodimer, or it could be the PER monomer. Biochemical analysis indicates that most PER is in the form of a PER–TIM heterodimer, even at late times in the cycle (Zeng *et al.*, 1996); this favors the former possibility. But the assay cannot exclude a small fraction of monomers; the *in vitro* nature of the assay also makes it impossible to assess the *in vivo* fraction of dimers and monomers. The LL accumulation of PER without a comparable change in TIM levels (Figures 2 and 3) suggests that PER monomers might be accumulating and be the active species that leads to the downturn in *per* and *tim* RNA levels. But it is still possible that the excess of TIM at ZT12 is sufficient to accommodate a full complement of PER in PER–TIM heterodimer form (Zeng *et al.*, 1996). If the PER monomer is the active component, it provides an attractive rationale for the phase advance of TIM accumulation compared with PER (Figure 1). Early in the cycle, newly synthesized PER is sequestered in inactive heterodimer form by the relatively high concentration of previously accumulated TIM. This can provide the delay required to sustain high amplitude oscillations of *per* and *tim* transcription (L.F.Abbott, H.Zeng and M.Rosbash, manuscript in preparation).

The per^S genotype has an endogenous circadian period of ca 19 h. Yet these flies manifest 24 h periods under standard 24 h LD conditions, albeit with an advanced evening activity phase (Hamblen-Coyle *et al.*, 1992). Why

is the endogenous period short, and how does light entrain the cycle to 24 h periodicity?

The per^S RNA profile rises in the early morning, several hours before the wild-type profile; the increase occurs indistinguishably in LD or DD conditions, i.e. light in the early morning plays no detectable role in timing this rise (Figure 7). The premature rise of per^S RNA is anticipated by the premature disappearance of PER and TIM several hours earlier (Figure 8). As the LD per^S cycle looks very similar to the wild-type cycle between ZT10–15 (Figure 8), a subsequent event is likely accelerated or bypassed so that the next ca 12 h take only 7–8 h. Although the primary effect of the per^S missense mutation is not known, it may accelerate progressive phosphorylation of the PER-S–TIM complex. Alternatively, the mutation may accelerate turnover of the mutant complex relative to the wild-type complex. This would also account for the residual phase advance despite 24 h periodicity in standard LD conditions (Hamblen-Coyle *et al.*, 1992). The effects of the per^S mutation on the molecular cycle therefore resemble those of premature light or a light pulse in the late night: premature light causes an early disappearance of TIM, an early liberation of PER monomers, a premature decline in PER levels, and a subsequent advance in the timing of the next round of *per* and *tim* transcription (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996; data not shown). Both premature light and the per^S mutation therefore manifest a feed-forward effect on the timing of the next cycle, which might simply be due to an early reversal of negative feedback.

The per^S RNA profile is broad in LD, due to a delayed decline compared with DD conditions (Figure 7). This suggests that the light-mediated delay is relevant to entrainment. It also suggests a mechanistic relationship to the delayed decline of the wild-type profile in constant light. The LD entrainment protocol exposes per^S flies to light during ZT0–12, a time interval that corresponds approximately to the beginning of the constant light protocol for wild-type flies during ZT4–16. This suggests that a primary effect of exposure of per^S flies to light between ZT8 and ZT12 is an inhibition of phosphorylation. Indeed, an inhibition of PER-S and TIM phosphorylation is already apparent at ZT11 (cf. Figure 6D), which presumably leads to a delay in the subseqent RNA decline (Figure 9).

Alternatively or in addition, hypersensitivity of per^S flies to light may contribute to the enhanced LD-delay of the per^S feedback circuit. In addition to a 4–5 h shorter period, per^S flies are arrhythmic at lower light intensities than wild-type flies (e.g. Konopka *et al.*, 1989). In the delay zone, the maximal response to a light pulse is approximately twice that of wild-type flies (Saunders *et al.*, 1994). This is also true when a saturating light pulse is delivered at ZT15 (data not shown). Although the primary cause of the enhanced light sensitivity of per^S is not known, it is probably related to the primary cause of the shorter cycle time. It is also probably relevant not only to the enhanced delays of the per^S protein and RNA profiles in LL but also to entrainment in standard LD conditions.

Entrainment of per^S is therefore explained by the mutant's advance and light's counteracting delay. The mutant leads to a truncated protein accumulation phase, which advances the subsequent per^S RNA rise. Light affects PER-S and TIM, which delay the mutant feedback cycle and extend the RNA and protein profiles (Figure 9), probably through an enhanced version of the mechanism that operates on wild-type flies in constant light. The delay restores the phase to a correct (wild-type-like) time of day and leads to 24 h periods.

Materials and methods

Preparation of fly heads

Flies were entrained for a minimum of 2 LD12:12 cycles and then transferred to other light conditions as indicated in the legends and as described previously (Zeng *et al.*, 1994). DD conditions were always the first day of DD. Flies were then collected on dry ice at the appropriate times. Heads were prepared and a maximum volume of 50 µl of heads was placed in cold (–20°C) acetone and left at –70° for at least 16 h. When needed, they were air-dried for 30 min at room temperature and used for RNA or protein extraction. The acetone treatment was omitted for samples assayed for TIM protein.

RNase protection assay

Total RNA was extracted from 30 heads for each time point, as described (Zeng *et al.*, 1994). The RNase protection assay was carried out with the RPA II RNase Protection Assay kit (Ambion) with one modification: the RNase was RNase One (Promega). The probes (*per*2/3 and RP49) were described previously (Zeng *et al.*, 1994). The *tim* probe was identical to that described in Sehgal *et al.*, 1995. Quantification was carried out with a PhosphorImager and the Phosphor Analyst software (Bio-Rad). Quantification involved subtraction of background values and normalization to the internal-control RP49 protected fragment.

Western blotting

Protein extractions and Western blotting were carried out as described (Zeng *et al.*, 1994) with the following modifications: for samples analyzed for TIM, after homogenizing 20 heads in 30 µl of extraction buffer, the extract was cleared twice by centrifugation, 10 µl 4× SDS buffer was added and the sample boiled. Samples were then chilled on ice and centrifuged briefly to remove particulate matter, cell debris and excess SDS. For samples analyzed for PER, 4× SDS buffer was added directly to the extract (the extract was not cleared). Equal numbers of heads (10–20) were loaded in each lane of a 5.7% polyacrylamide gel. Quantification of PER blots was carried out as described above. Quantification of TIM blots was carried out with a scanner (Arcus), and Adobe Photoshop and Molecular Analyst (Bio-Rad) software. Quantification included normalization to a cross-reacting band.

Light treatment

Light treatment was ⩾1000 lux of white light, a saturating intensity for phase shifting as well as for inducing arrhythmia (cf. Konopka *et al.*, 1989).

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