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Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*

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The *timeless* mutation (*tim*) leads to loss of circadian behavioral rhythms in *Drosophila melanogaster*. The effects of *tim* on rhythmicity involve interactions with *period* (*per*), a second essential clock gene, as the *tim* mutation suppresses circadian oscillations of *per* transcription and blocks nuclear localization of a PER reporter protein. In the present study it was found that the *tim* mutant constitutively produces a low level of PER protein that is comparable with that produced late in the day by wild-type flies. In addition, it was shown that *tim* suppresses circadian cycling of PER protein abundance and circadian regulation of PER phosphorylation. Transfer of wild-type flies to constant light also suppressed cycling of PER abundance and phosphorylation and produced constitutively low levels of PER. In the *tim* mutant there was no additional effect of constant light on PER. These results suggest that constant light and the *tim* mutation produce related changes in the underlying biological clock. We further suggest that the multiple effects of *tim* are due to a primary effect on *per* expression at the post-transcriptional level. The effects of *tim* on behavioral rhythms and *per* RNA cycling are therefore likely to involve effects on PER protein through previously proposed feedback mechanisms.

Keywords: biological clocks/molecular rhythms/phosphorylation/post-transcriptional regulation

Introduction

Circadian rhythms are found in organisms ranging in complexity from single cells to man (Pittendrigh, 1974). These rhythms are responsive to light and temperature cues, but they are also produced under conditions of constant temperature and darkness and are therefore considered to be endogenously generated. Genetic analyses in several organisms have sought to elucidate the underlying cellular and molecular mechanisms for these behaviors (Konopka and Benzer, 1971; Bruce, 1972; Feldman and Hoyle, 1973; Ralph and Menaker, 1988; Rosbash and Hall, 1989; Hall, 1990; Baylies *et al.*, 1993; Dunlap *et al.*, 1993; Jackson, 1993; McClung, 1993; Aronson *et al.*, 1994; Kondo *et al.*, 1994; Sehgal *et al.*, 1994; Vitaterna *et al.*, 1994; Vosshall *et al.*, 1994; Millar *et al.*, 1995).

Genetic screens have revealed two loci in *Drosophila* that are required for production of circadian rhythms: *period* (*per*) and *timeless* (*tim*). Mutations at either locus can eliminate behavioral rhythmicity or alter the period length of the rhythms (Konopka and Benzer, 1971; Sehgal *et al.*, 1994; A.Rothenfluh and M.Young, unpublished results). Circadian rhythms of *per* transcription have also been observed (Hardin *et al.*, 1990, 1992a,b; Sehgal *et al.*, 1994) and earlier work has shown that production of these molecular rhythms requires the functions of both *per* (Hardin *et al.*, 1990) and *tim* (Sehgal *et al.*, 1994).

per mutations that eliminate period protein (PER) block cycling of *per* RNA (Hardin *et al.*, 1990) and overproduction of PER also suppresses transcription from the *per* locus (Zeng *et al.*, 1994). These results indicate that PER may function in an autoregulatory loop in wild-type flies. Because PER is detected immunocytochemically in nuclei late at night, but not late in the day (Siwicki *et al.*, 1988; Zerr *et al.*, 1990; Vosshall *et al.*, 1994), it may be important that the *tim* mutation blocks nuclear localization of PER–β-galactosidase (β-gal) reporter proteins in addition to circadian cycling of *per* transcription (Sehgal *et al.*, 1994; Vosshall *et al.*, 1994). From these observations it has been suggested that in wild-type flies *tim* may regulate access of PER to the nucleus, permitting PER nuclear localization and function only at certain times of day (Vosshall *et al.*, 1994). Immunocytochemical experiments in wild-type flies have recently provided evidence for such temporal control (Curtin *et al.*, 1995). The PER protein also shows circadian cycling of its abundance and phosphorylation (Edery *et al.*, 1994).

In the work presented here we demonstrate that the *tim* mutation blocks the circadian oscillations of both PER abundance and phosphorylation. It is also shown that the *tim* mutation profoundly depresses PER levels. Constant light produces behavioral arrhythmicity in wild-type flies and elicits effects on PER protein that resemble those associated with the *tim* mutation. Constant light produces no obvious additional effect on PER in a *tim* mutant background. The results suggest that the effects of *tim* on *per* are mediated at the post-transcriptional level and that constant light and the *tim* mutation produce related changes in the circadian pacemaker.

Results

tim suppresses circadian cycling of PER protein abundance in light/dark cycles

To determine if *tim* affects the wild-type circadian oscillation in PER protein abundance (Edery *et al.*, 1994), extracts from the heads of wild-type and *tim* flies were resolved on SDS–polyacrylamide gels and PER was

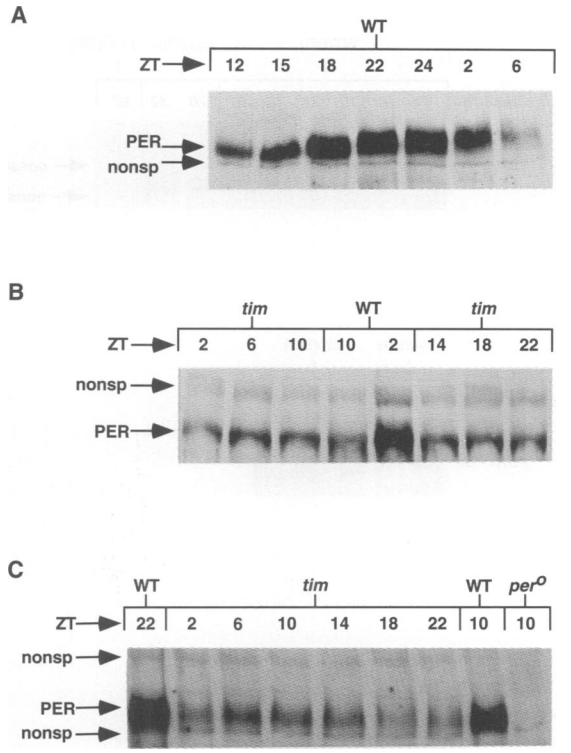

Fig. 1. Circadian oscillations in PER protein abundance and electrophoretic mobility are suppressed in *tim* mutant flies collected during light/dark (LD) cycles. Extracts were prepared from the heads of wild-type (WT), *tim* or per^0 flies frozen at the indicated time (ZT, where ZT 0–12 is the light period) in an LD cycle and PER was detected with anti-PER antibodies by Western blot analysis. Non-specific bands (nonsp), which are not derived from PER because they are present in per^0 flies (C, lane 9), are noted to draw attention to electrophoretic mobility shifts. The mobility of PER from wild-type flies shows cyclical changes according to time of day relative to these non-specific bands, while the relative mobility of PER in *tim* flies does not change. (**A**) PER in wild-type flies (the Canton S line; see Materials and methods for a complete description of strains) at 2–4 h intervals. (**B**) PER in *tim* flies at 4 h intervals (*tim*; ry^{506}). (**C**) PER in another *tim* strain [*tim* P(ry^+); ry^{506}]. Wild-type lanes are included in (B) and (C) to allow direct comparison of PER levels between wild-type and *tim* flies, since the exposure conditions used to detect PER in (A) were significantly less sensitive than those in (B) and (C).

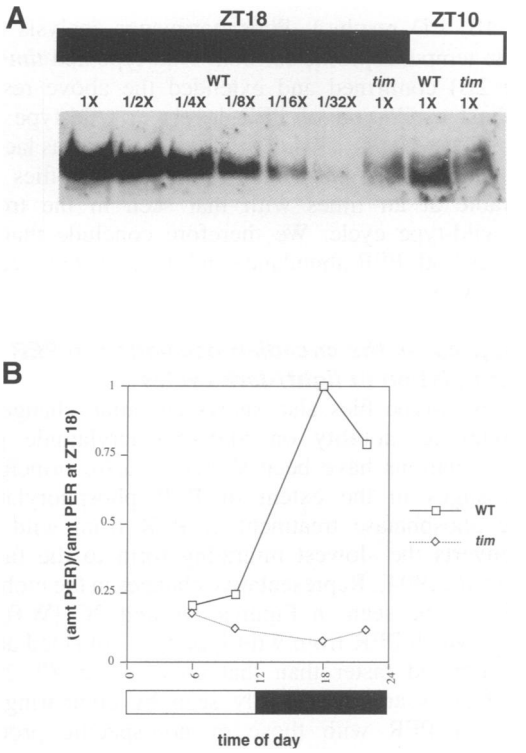

Fig. 2. Quantitation of relative PER levels in *tim* and wild-type flies collected during an LD cycle. Extracts were prepared from the heads of wild-type flies (WT) or *tim* flies and PER was detected with an anti-PER antibody by Western blot analysis (Materials and methods). (**A**) Extracts prepared from flies frozen in the dark at ZT 18 (filled bar over lanes) or in the light at ZT 10 (open bar over lanes). 1X represents the most concentrated, undiluted sample and contained 200 μg total protein (Materials and methods). The extract from wild-type flies was serially diluted as indicated and loaded into the left-most lanes. 1X amounts (200 μg total protein/sample) of ZT 10 and 18 extracts from the indicated genotypes were loaded in the right three lanes. (**B**) 1X amounts of the above samples were electrophoresed with 1X samples from other time points and the PER signals from these samples and those in (A) were quantitated with a phosphorimager. For this plot the PER signal in each sample was normalized to that obtained with the 1X amount of the wild-type ZT 18 sample. Quantitation of the serial dilution in (A) established the linearity of the signal (not shown).

detected by Western blotting. As previously described (Edery *et al.*, 1994) in wild-type flies, PER was expressed at the highest levels at night [Zeitgeber time (ZT) 12–24, with ZT 24 or 0 defined as the time at which the dark period ends and ZT 12 as the time at which the light period ends] and at the lowest levels during the day (ZT 0–12) in a 12 h light/12 h dark (LD) cycle (WT, Figures 1A and 2). The PER levels detected in *tim* flies showed no evidence of the robust circadian oscillation found in wild-type flies and were low at all times (Figures 1B and C and 2). To allow a comparison between PER levels in *tim* and wild-type flies, Figure 1B and C include wild-type extracts with representative lower levels (ZT 10) and higher levels (ZT 2 or 22) of PER. These wild-type controls demonstrate that the level of PER protein produced in *tim* flies is comparable with the low point of the wild-type oscillation, which normally occurs late in the day (ZT 6–10; cf Figure 1A). Because this low level of antigen was not detected in the heads of per^0 flies (Figure 1C, per^0; Edery *et al.*, 1994), it was PER rather than a cross-reacting antigen.

We performed seven experiments, all of which demonstrated these strong effects of *tim* on PER levels during LD cycles. In three of these experiments we quantitated the levels of PER in *tim* flies by serially diluting extracts from wild-type flies expressing peak levels of protein (ZT 18; see Figures 1A and 2 and Zeng *et al.*, 1994, for times of peak and trough levels) and comparing the levels with those in undiluted *tim* extracts and in extracts from wild-type flies expressing low levels of protein (ZT 10). The analysis in Figure 2A indicates that the peak level of PER in wild-type flies (ZT 18) is 8–16 times higher than the level of constitutively produced PER in the *tim* mutant. As in Figure 1, Figure 2A also shows that the amount of PER produced in the *tim* mutant is comparable with the low level of expression observed in wild-type flies at ZT 10. In all three experiments in which levels of PER in *tim* flies were quantitated by dilution there was always a <3-fold difference in PER levels between any two time points in *tim* flies (data not shown). Moreover, PER was always detected in *tim* flies at levels <25% of the ZT 18 level in wild-type flies (or ~33% of wild-type ZT 20 levels; see

Figure 4B, LD results.). Phosphorimager analysis of a complete temporal profile for both wild-type and *tim* flies (Figure 2B) confirmed and extended the above results. The robust oscillation of PER levels in wild-type flies (see also Edery *et al.*, 1994; Zeng *et al.*, 1994) was lacking in *tim* flies and the amount of PER in *tim* flies was comparable at all times with that seen in the trough of the wild-type cycle. We therefore conclude that *tim* suppresses both PER abundance and the circadian cycling of PER levels.

tim suppresses the circadian oscillation in PER phosphorylation in light/dark cycles

PER in wild-type flies also shows circadian changes in electrophoretic mobility on SDS–polyacrylamide gels. These oscillations have been shown to arise principally from changes in the extent of PER phosphorylation, because phosphatase treatment of PER from wild-type flies converts the slowest migrating form to the fastest (Edery *et al.*, 1994). Representative changes in the mobility of PER can be seen in Figures 1A and 3C [WT(LD) lanes], in which PER from wild-type flies collected at ZT 12–18 migrated faster than that collected at ZT 22–6. These changes are most easily seen by comparing the mobility of PER with those of non-specific proteins (nonsp), which did not show changes in mobility and which were present in extracts from per^0 flies.

In contrast to wild-type flies, there were no obvious changes in the mobility of PER in *tim* flies during the light/dark cycle (Figure 1B and C). Since all phosphorylation events do not lead to noticeable changes in protein mobility (Grasser and Konig, 1992), we cannot assert that all rhythmic phosphorylation has been eliminated, but some of the phosphorylation changes that give rise to the mobility shift in wild-type flies (Edery *et al.*, 1994) are evidently absent in *tim* flies.

The molecular oscillations of PER are also suppressed in tim flies in constant darkness

Wild-type and *tim* flies were entrained in LD. After the final lights off the flies were transferred to constant darkness (DD) for 62–82 h. Wild-type flies continue to express oscillations in PER abundance and electrophoretic mobility in DD, although these oscillations are less robust than those observed in LD (Figure 3A; Edery *et al.*, 1994). In *tim* flies there was no indication of a circadian oscillation in PER abundance in DD (Figure 3A–C). Furthermore, the amount of PER in *tim* flies at every time point was substantially less than that detected at peak times of the wild-type protein cycle [e.g. 70–74 h after transfer to constant darkness; Figure 3A, WT(DD)]. For all of the timed DD collections the amount of PER detected in *tim* flies was comparable with the amount of PER detected in *tim* flies in LD (Figure 3B). In contrast, the imposition of an LD cycle amplified the apparent oscillation of PER in wild-type flies, leading to higher amounts of PER at peaks of the LD cycle than in DD (Edery *et al.*, 1994).

Like *tim* flies collected in LD (Figure 1B and C), there was no apparent oscillation of PER electrophoretic mobility in *tim* flies in DD (Figure 3A and B). Figure 3B also indicates that the electrophoretic mobility of PER is the same in *tim* flies raised in LD and DD. Figure 3C compares the electrophoretic mobility of PER from *tim*

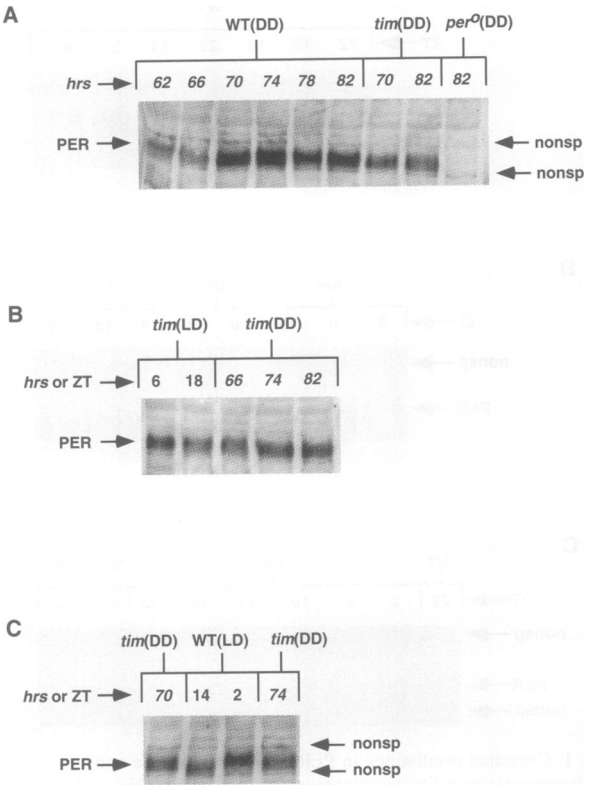

Fig. 3. Constant darkness (DD) affects neither the abundance nor the mobility of PER in *tim* flies. Extracts were prepared from the heads of wild-type (WT), *tim* or per^0 flies frozen at various ZT in LD or after a regime of DD. The flies collected in constant darkness had first been entrained to an LD cycle and were left in DD for the indicated number of hours (*hrs*) after the lights were turned off at the end of the final light period. PER was detected with anti-PER antibodies by Western blot analysis. Non-specific bands (nonsp), which were detected in flies lacking PER protein (per^0 in A), are also labeled in (C) to demonstrate the lack of mobility change in PER from *tim* flies as compared with representative fast migrating and slow migrating PER from WT flies (ZT 14 and ZT 2 in panel C). (**A**) Comparison of PER levels in wild-type flies collected in DD with representative PER levels of *tim* flies collected in DD. (**B**) Comparison of PER in *tim* flies collected in LD or DD. (**C**) Comparison of electrophoretic mobilities of PER from wild-type and *tim* flies.

flies with the mobility of PER from wild-type flies collected at opposite phases of an LD cycle. PER from *tim* flies migrates faster than PER from ZT 2 wild-type flies, but it migrates more slowly than PER from ZT 14 wild-type flies. Similarly, Figure 2A shows that PER from the *tim* mutant migrates more slowly than PER from wild-type flies collected at ZT 10.

Constant light suppresses PER abundance and cycling in wild-type flies, but has no effect on PER in tim flies

Earlier histochemical studies have indicated that constant light may depress the abundance of PER in wild-type flies (Zerr *et al.*, 1990). To directly assess effects of light on PER, wild-type and *tim* flies were entrained to an LD cycle and then transferred to constant light (LL) for 62–82 h. As shown in Figure 4A, neither the amount nor the electrophoretic mobility of PER in wild-type flies oscillated in LL. The level of PER expression was also quite

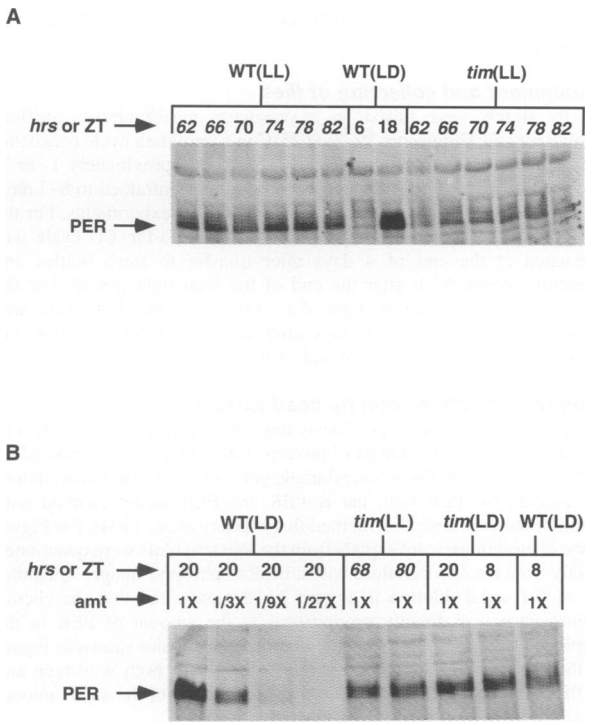

Fig. 4. Constant light (LL) suppresses the level and oscillation of PER in wild-type flies but has no apparent effect on PER in *tim* flies. Extracts were prepared from the heads of wild-type (WT) or *tim* flies frozen at various times (ZT) in an LD cycle or after exposure to LL. The flies collected in LL had first been entrained to LD cycles and were left in LL for the indicated number of hours (*hrs*) following the end of the final dark period. PER was detected with anti-PER antibodies by Western blot analysis. (**A**) Comparison of PER levels in wild-type flies exposed to LL with PER levels in *tim* flies in LL and wild-type flies collected at two time points in an LD cycle. (**B**) Comparison of PER levels in *tim* flies exposed to LL or LD cycles with those obtained in wild-type (WT) flies at times of high expression (ZT 20) and low expression (ZT 8) in LD. The relative amount (amt) of total extract loaded in each lane is indicated above the lane. The extract from wild-type flies collected at ZT 20 was diluted serially by the indicated amounts in lanes 2–4.

low [compare with PER from wild-type flies raised in LD and collected at ZT 18, WT(LD) 18 in Figure 4A].

In contrast to these effects on PER in wild-type flies, constant light produced no change in PER isolated from the *tim* mutant. The amounts and electrophoretic mobilities of PER in *tim* flies were indistinguishable in LL and LD (Figure 4B) and no oscillation in the amount or electrophoretic mobility of PER was detected in *tim* flies maintained in LL (Figure 4A).

Inspection of Figure 4A also shows that the electrophoretic mobility of PER derived from wild-type flies in LL [WT(LL)] resembles that observed for *tim* flies. Thus comparable shifts in the amount and electrophoretic mobility of PER are effected by mutation of *tim* and by exposure of wild-type flies to constant light. Because constant light fails to change the level or mobility of the PER protein in the *tim* mutant, but substantially alters the protein in wild-type flies in a manner similar to the effects of the *tim* mutation, the results suggest that constant light may produce changes in PER that are related to those generated by the *tim* mutation.

Discussion

The work presented in this study shows that the *tim* mutation suppresses the previously reported circadian oscillations in abundance and electrophoretic mobility of PER (Edery *et al.*, 1994; Zeng *et al.*, 1994). An earlier study demonstrated that *tim* also abolishes the nuclear localization of a reporter protein in which the N-terminal half of PER is fused to bacterial β-galactosidase (PER-SG; Vosshall *et al.*, 1994). The combined results of these studies argue that the *tim* mutation leads to: (i) cytoplasmic localization of PER proteins (Vosshall *et al.*, 1994); (ii) a level of PER comparable with the low points of the wild-type oscillation; (iii) loss or suppression of the circadian oscillation in PER abundance and (iv) an altered circadian regulation of PER phosphorylation.

Although earlier work has shown that *tim* also eliminates circadian cycling of *per* transcription (Sehgal *et al.*, 1994), the multiple effects of *tim* on PER suggest that effects on transcription are indirect. Since average *per* RNA levels are similar in *tim* and wild-type flies (albeit without evidence for oscillation in *tim* flies; Sehgal *et al.*, 1994), only the suppressed oscillation of PER protein levels would be expected to arise directly from a deregulation of *per* transcription. Thus the low abundance of PER protein, the cytoplasmic localization of PER-SG reporter proteins and the altered phosphorylation of PER in *tim* flies result, at least in part, from changes in one or more programs of post-transcriptional control. It is not possible to say with certainty which of the effects characterized here arises more directly from the *tim* mutation. The phosphorylation state of several proteins appears to regulate their nuclear localization (Moll *et al.*, 1991; Shuai *et al.*, 1994; Traenckner *et al.*, 1994) or proteolysis (McKinney *et al.*, 1993; Sherr, 1994; King *et al.*, 1994; Traenckner *et al.*, 1994). Accordingly, altered regulation of PER phosphorylation in *tim* flies might confer cytoplasmic localization or enhanced degradation of the protein or both, perhaps by constitutively exposing PER to cytoplasmic proteases involved in its post-transcriptional regulation. Alternatively, *tim* might influence PER dimerization (Huang *et al.*, 1993) or association of PER and a factor required for its stability, especially since PER contains a protein interaction domain, PAS (Crews *et al.*, 1988; Hoffman *et al.*, 1991; Burbach *et al.*, 1992; Reyes *et al.*, 1992; Huang *et al.*, 1993; Matsushita *et al.*, 1993), which has been implicated in the control by *tim* of PER-SG nuclear localization (Vosshall *et al.*, 1994). A key observation is that *tim* causes cytoplasmic localization of PER-SG in wild-type as well as per^0 flies (Vosshall *et al.*, 1994), which have no *per* activity. This suggests a rather direct effect of *tim* on PER protein.

Levels of the PER-SG reporter protein, which is expressed from the *per* promoter, are similar, if not identical, in wild-type and *tim* flies (Vosshall *et al.*, 1994; J.Price, M.Dembinska, M.Young and M.Rosbash, unpublished data), in contrast to the dramatic effect of *tim* on PER. The different effects of *tim* on PER and PER-SG are consistent with previous immunocytochemical observations of these proteins in wild-type and *tim* flies (Vosshall *et al.*, 1994). In wild-type flies both PER and PER-SG were detected in the nuclei of cells composing the eyes, optic lobes and central brain (Siwicki *et al.*,

1988; Liu *et al.*, 1988, 1992; Saez and Young, 1988; Zerr *et al.*, 1990; Vosshall *et al.*, 1994). In *tim* flies high levels of PER-SG were instead observed in the cytoplasm of *tim* flies in these locations and PER was not detectable in cytoplasm or in nuclei (Vosshall *et al.*, 1994). The biochemical data presented here show that the previous failure to detect PER by histochemical methods in *tim* flies is due, at least in part, to the very low protein levels. Since PER-SG is expressed at equal levels in *tim* and wild-type flies, replacement of the C-terminal half of PER with bacterial β-gal evidently abolishes this *tim*-mediated reduction in PER protein levels, for example by blocking proteolysis of PER or by removing sequences in the 3′-end of the *per* RNA that repress translation in the absence of wild-type *tim* gene product.

Constant light suppresses the oscillation and abundance of PER levels in wild-type flies, as does the *tim* mutation. Circadian cycling of phosphorylation is also suppressed by exposure to constant light and the electrophoretic mobility of PER produced in LL resembles that of PER recovered from *tim* flies. While the light regime (LD, DD or LL) affects expression of PER in wild-type flies, the expression of PER in the *tim* mutant is the same under all lighting conditions. Also like the *tim* mutation, constant light produces behavioral arrhythmicity (Pittendrigh, 1966; Konopka *et al.*, 1989). All of these observations suggest that constant light and the *tim* mutation may mediate similar molecular changes in the underlying circadian pacemaker.

When wild-type flies are transferred from LL to DD, circadian rhythmicity resumes with a phase that indicates a prior blockcade late in the day or early evening (Pittendrigh, 1966; Petersen *et al.*, 1988). At these times of day in an LD cycle low levels of PER are found and PER is predominantly in the cytoplasm rather than in nuclei of putative pacemaker cells in the central brain (Curtin *et al.*, 1995). Since the *tim* mutation blocks nuclear localization of PER–β-gal reporter proteins and similarly depresses the level of PER protein, possibly a *tim*-related function is suppressed by light or the circadian clock at this time of day in wild-type flies. Transfer of wild-type flies to constant light would then be expected to phenocopy changes in PER expression observed in a *tim* mutant background, while the *tim* mutant would not be expected to respond to differences in the environmental light regime. A less speculative connection between *tim* and constant light will require some molecular understanding of the relationship between *tim* and *per*, as well as some insight into the relationship between light and PER metabolism.

Materials and methods

Drosophila strains

For Figures 1A and C the strain of wild-type flies employed was Canton S, while the *tim* strain in Figure 1C was that isolated in the original eclosion screen of Sehgal *et al.* (1994). That strain contains a P-element insertion, carrying a wild-type *ry* gene, on the right arm of the second chromosome, which also contains the *tim* mutation. The third chromosome in this stock carries the ry^{506} mutation, so the genotype is *tim* P(ry^{+}); ry^{506}. The *tim* and wild-type strains used for all other figures were derived from this original *tim* strain by crossing it to a tim^{+}; ry^{506} strain in order to recover a recombinant *tim* chromosome which was therefore still arrhythmic but had lost the P-element (monitored by loss of the wild-type eye color). The wild-type strain was derived from this cross as a rhythmic strain which did not contain the P-element (*ry* mutant eye color).

Entrainment and collection of flies

The fly strains were raised on a yeast/corn meal/molasses medium (Ashburner and Thompson, 1978) at 25°C in a light/dark cycle consisting of 12 h light followed by 12 h darkness (LD). Approximately 1- to 7-day old flies were transferred to fresh pint bottles, entrained to 6–7 days of LD and frozen at the indicated times for the LD experiments. For the flies collected in constant darkness (DD; Figure 3) the LD cycle was terminated at the end of 4 days after transfer to fresh bottles and collections began 62 h after the end of the final light period. For the flies collected in constant light (LL; Figure 4) the LD cycle was terminated at the end of 4 days after transfer to fresh bottles and collections began 62 h after the end of the final dark period.

Detection of PER in total fly head extract

Extracts were prepared as previously described (Edery *et al.*, 1994). For each time point equal amounts of protein (100–200 μg) were resolved by electrophoresis on SDS–polyacrylamide gels, transferred to nitrocellulose and assayed for PER with the B6PER anti-PER serum coupled with chemiluminescent detection methodology (Edery *et al.*, 1994). For Figure 2B the chemiluminescent signals from the Western blots were quantitated directly from the nitrocellulose with a BioRad phosphorimager. Quantitation of the serial dilution in Figure 2A demonstrated that the chemiluminescence was directly proportional to the amount of PER in the sample, thereby validating the quantitation. For the plot shown in Figure 2B the quantitations of complete time courses for both wild-type and *tim* flies were normalized to the signal obtained from the same amount of extract from wild-type flies collected at ZT 18.

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