Period protein from the giant silkmoth Antheraea pernyi function as a circadian clock element in Drosophila melanogaster

ARTICLE *in* NEURON · AUGUST 1995

Impact Factor: 15.05 · DOI: 10.1016/0896-6273(95)90072-1 · Source: PubMed

5 AUTHORS, INCLUDING:

Ivo Sauman
Academy of Sciences of the Czech Republic
40 PUBLICATIONS **1,329** CITATIONS
SEE PROFILE

F. R. Jackson
76 PUBLICATIONS **3,680** CITATIONS
SEE PROFILE

Neuron, Vol. 15, 147–157, July, 1995, Copyright © 1995 by Cell Press

Period Protein from the Giant Silkmoth Antheraea pernyi Functions as a Circadian Clock Element in Drosophila melanogaster

Joel D. Levine,* Ivo Sauman,†‡ Michelle Imbalzano,* Steven M. Reppert,† and F. Rob Jackson*
*Neurobiology Group
Worcester Foundation for Experimental Biology
Shrewsbury, Massachusetts 01545
†Laboratory of Development Chronobiology
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts 02114

Summary

Homologs of the Drosophila clock gene *per* have recently been cloned in Lepidopteran and Blattarian insect species. To assess the extent to which clock mechanisms are conserved among phylogenetically distant species, we determined whether PER protein from the silkmoth Antheraea pernyi can function in the Drosophila circadian timing system. When expressed in transgenic Drosophila, the silkmoth PER protein is detected in the expected neural cell types, with diurnal changes in abundance that are similar to those observed in wild-type fruitflies. Behavioral analysis demonstrates that the silkmoth protein can serve as a molecular element of the Drosophila clock system; expression of the protein shortens circadian period in a dose-dependent manner and restores pacemaker functions to arrhythmic *per*^{0} mutants. This comparative study also suggests that the involvement of PER in different aspects of circadian timing, such as period determination, strength of rhythmicity, and clock output, requires distinct molecular interactions.

Introduction

Circadian clocks, which synchronize physiological, behavioral, and molecular processes with environmental cycles, display similar functional properties in unicellular organisms, plants, and animals (Edmunds, 1988). All circadian pacemakers, for example, have an endogenous period close to 24 hr, which is stable over a physiological range of temperatures (i.e., temperature compensated; Pittendrigh, 1974) and can be reset by light and other environmental stimuli. There are also demonstrable similarities among pacemakers at the level of cellular mechanism. For instance, circadian pacemaker neurons or neural ensembles in the molluscan eye and the rodent suprachiasmatic nucleus have similar electrophysiological properties (Rosenwasser, 1988). In both types of pacemaker tissues, there are circadian rhythms in electrical activity with daily peaks during the subjective day, and this patterning of electrical activity occurs in nocturnal and diurnal species (Rosenwasser, 1988). In addition, in a variety of animal species, the same intracellular signaling pathways may mediate the resetting response of neural pacemakers (Eskin et al., 1982; Prosser and Gillette, 1989; Prosser et al., 1989, 1994; Ralph et al., 1992; Takahashi et al., 1993; Levine et al., 1994; Colwell et al., 1994). These similarities in integrative function and physiological mechanism in vertebrates and invertebrates suggest that the molecular mechanisms underlying pacemaker function might be conserved among different species. Hence, studies in different model systems, with distinct experimental advantages, might provide a more general understanding of circadian pacemaker mechanisms.

The fruitfly Drosophila melanogaster has been employed extensively as a model genetic system for the analysis of circadian rhythms (reviewed in Jackson, 1993). In particular, the Drosophila *period* (*per*) gene has been studied in great detail and shown to encode a molecular element of the circadian pacemaker (see Hall and Rosbash, 1993). Mutations of the *per* gene can abolish behavioral rhythmicity (*per*^{0}), lead to abnormally long (*per*^{L}) or short (*per*^{S}) circadian period, perturb the clock resetting response, and disrupt temperature compensation (Konopka et al., 1989). Changes in *per* gene dosage also have predictable effects on the circadian clock (Smith and Konopka, 1982).

Both *per* mRNA and PER protein exhibit regular changes in abundance during the circadian cycle (Siwicki et al., 1988; Hardin et al., 1990), and it has been postulated that *per* gene products constitute a negative feedback transcriptional loop that is part of the circadian clock (Hardin et al., 1992; Zeng et al., 1994). Consistent with a role in transcriptional regulation, the PER protein is known to have a nuclear localization within neurons of the adult brain (Liu et al., 1992), including a group of neurons in lateral regions of the protocerebrum that are thought to be cellular elements of the circadian clock (Ewer et al., 1992; Frisch et al., 1994).

Homologs of the *per* gene have been cloned in various Dipteran species (Colot et al., 1988; Nielsen et al., 1994), and functional studies using D. pseudoobscura (Petersen et al., 1988) or D. simulans (Wheeler et al., 1991) sequences have demonstrated interspecies rescue of rhythms in D. melanogaster *per* mutants. Interspecific comparisons of Drosophila PER proteins have identified conserved domains, and one of these conserved regions (c-2; Colot et al., 1988) includes the locations of the *per*^{L} and *per*^{S} mutations, as well as the PAS domain (Huang et al., 1993), which may mediate protein associations. Both the amino-terminal (c-1) and carboxy-terminal (c-6) coding regions of *per* are also highly conserved. Though these comparative studies show that PER function is conserved in Drosophila species, they do not indicate whether such clock mechanisms are employed in more distant species.

Recently, two of us (S. M. R. and I. S.) cloned *per* gene

‡Permanent address: Institute of Entomology, Czech Academy of Sciences, Czech Republic.

relatives in a number of phylogenetically distant insects, including the giant silkmoth Antheraea pernyi (Reppert et al., 1994), which diverged from Dipteran species 240 million years ago. Given the previous studies of the silkmoth circadian clock (Truman, 1971, 1972, 1974) and the presence of large, identifiable neurons in the brain, this species might have tremendous advantages for the cellular analysis of circadian pacemaker mechanisms. The neural expression pattern of *per* product has already been documented in the silkmoth, and it has been shown that both the mRNA and protein exhibit circadian fluctuations in abundance (Reppert et al., 1994) that are similar to those observed in Drosophila (Siwicki et al., 1988; Hardin et al., 1990). However, for the further pursuit of molecular investigations in this species, it would be valuable to examine the silkmoth protein functionally to demonstrate that it is a molecular element of the pacemaker, which regulates circadian rhythms. Here, we ask whether the A. pernyi PER protein can function as an element of the Drosophila circadian pacemaker.

Results

Generation of Transgenic Drosophila Carrying a Fly-Moth Chimeric Gene

A chimeric gene construct was prepared by placing the coding region for the silkmoth *per* gene homolog under the control of Drosophila *per* regulatory sequences. This construct, called M4 (Figure 1A), was introduced into the Drosophila genome by P element–mediated DNA transformation (Spradling and Rubin, 1982; see Experimental Procedures). Strains were established from each of three putative transformants, and it was apparent in the next generation that all three P element inserts mapped to the autosomal genome. Whole-genome Southern analysis with DNAs from transgene-bearing flies and control siblings demonstrated that silkmoth coding sequences were present and verified that the three lines represented independent P element insertion events (Figure 1B). One of these lines, M4-2, was used to generate additional independent genomic insertions of the M4 transgene by the method of "transposase hopping" (see Experimental Procedures). Two independent transgene-bearing lines, M4-2 and M4-15, were selected for detailed immunohistochemical and behavioral analysis. In all of our experiments, M4 transgene–bearing flies were compared with siblings of similar genetic background that lacked the transgene.

The Silkmoth PER-Related Protein Cycles in Abundance in Drosophila

In D. melanogaster and A. pernyi, PER protein immunoreactivity exhibits circadian changes within photoreceptor nuclei of the visual system (Siwicki et al., 1988; Reppert et al., 1994). In addition, PER protein cycles are in abundance in two clusters of neurons (dorsal [LNd] and ventral [LNv]) within the lateral portion of the Drosophila protocerebrum, and these lateral neurons are thought to be essential for normal circadian pacemaker function (Ewer et al., 1992; Frisch et al., 1994). In contrast, PER protein cannot

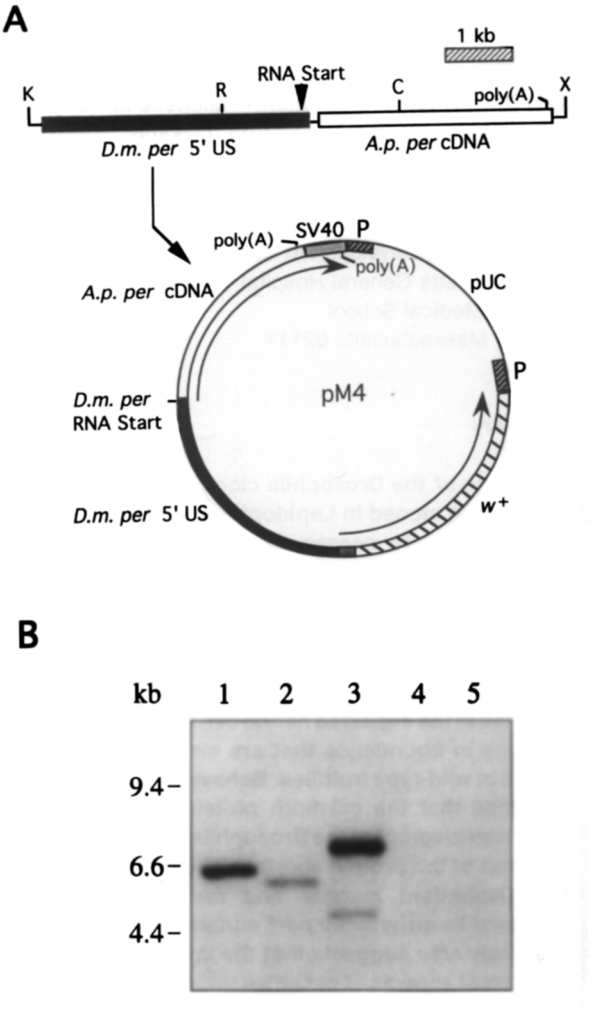


Figure 1. Diagram of the pM4 P Element Plasmid and Southern Blot Analysis of Drosophila Transgenic Strains

(A) Construction of the fly–moth *per* gene (top) and the pM4 plasmid (bottom). Closed and open rectangles indicate, respectively, Drosophila *per* upstream regulatory sequence (including the fly RNA initiation site) and Antheraea *per* coding sequence and 3′ untranslated region with a polyadenylation signal. The miniwhite gene used to select transformants is depicted by the hatched rectangle, and P element sequences are shown by shaded diagonal stripes (see Experimental Procedures).

(B) Southern blot analysis of EcoRI-digested genomic DNA from transgenic strains M4-1, M4-2, and M4-3. Lanes 1–3, genomic DNA from flies carrying transgenes M4–1, M4–2, and M4–3, respectively; lanes 4–5, DNA from siblings of lines M4-1 and M4-2, respectively, lacking the transgene. The blot was hybridized with labeled silkmoth *per* cDNA sequences. Different size classes of hybridizing bands are detected in each strain, indicating that each P element insertion is flanked by unique sequences and hence is an independent event. On genetic criteria, strain M4-3 apparently carries two inserts of the transgene (data not shown), and this is consistent with the presence of multiple hybridizing DNA fragments. As expected, DNA from siblings without the transgene does not hybridize to this probe (lanes 4–5), but did hybridize to a fly *per* probe (data not shown). Digestion with XhoI and BglII provided comparable evidence for independent insertion events. PCR analysis with genomic DNAs using primers flanking the fly–moth ligation junction also indicated that these transgenic lines carried silkmoth coding sequences.

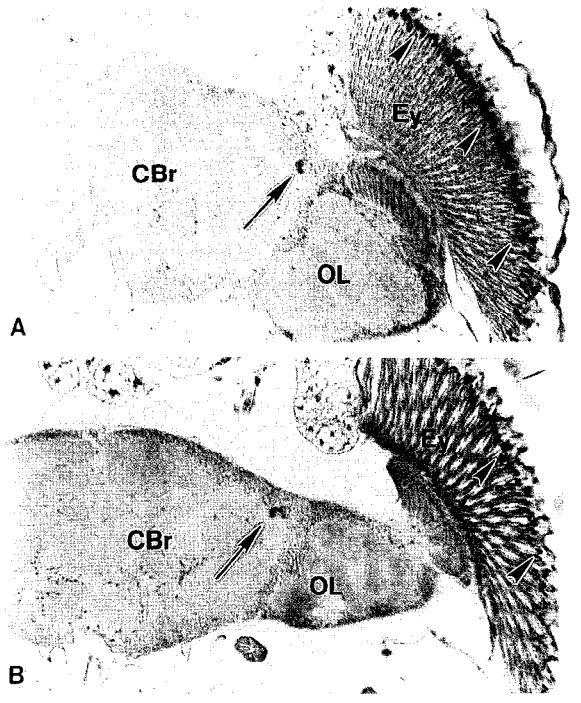
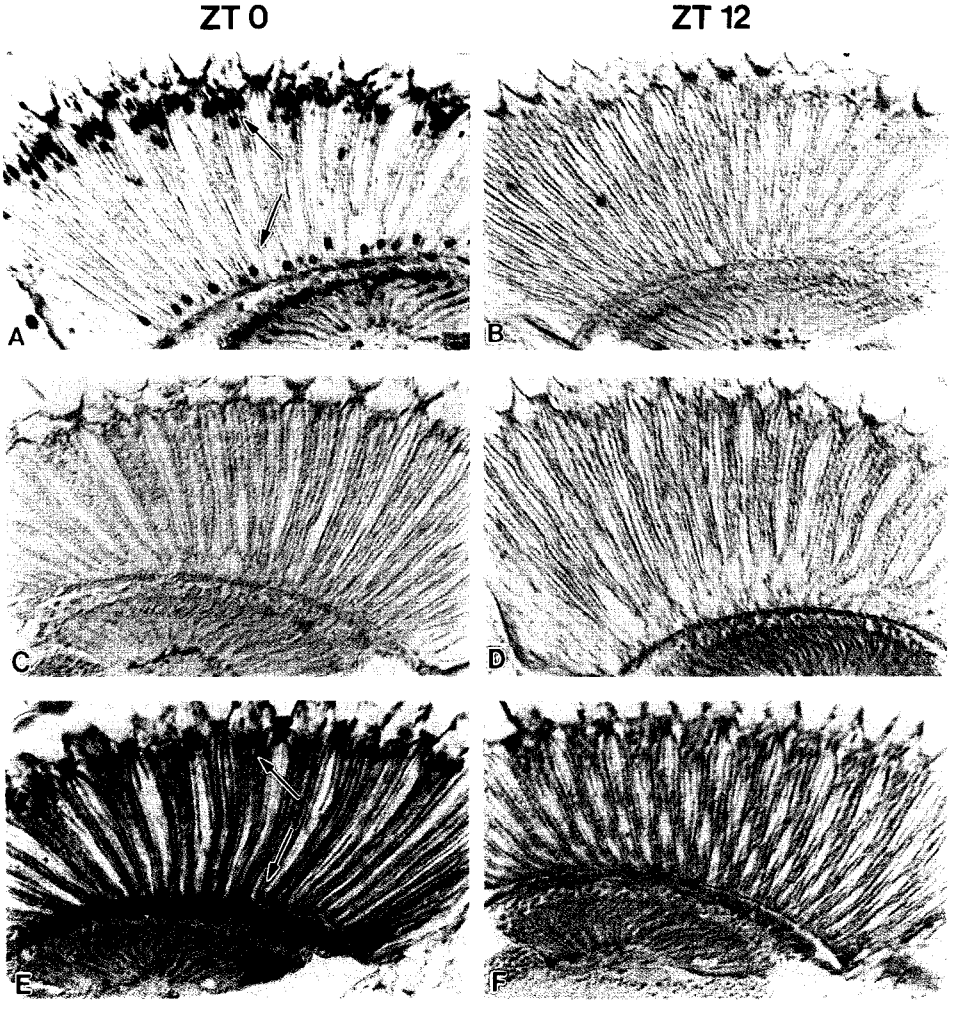


Figure 2. Spatial Distribution of PER-Immunoreactive Product in the Nervous Systems of Wild-Type and Transgenic Flies at ZT 0

(A) Wild type; (B) *per*^{0}; M4-2.
Arrowheads indicate the nuclei of photoreceptors 1–7. Arrows show straining in the dorsal group of lateral neurons. CBr, central brain; Ey, eye; OL, optic lobe. Magnification, 200 x .

Figure 3. PER Immunohistochemical Staining in the Compound Eyes of Wild-Type, *per*^{0}, and *per*^{0}; M4 Transgenic Individuals at Two Different Times of Day

(A and B) *per*$^{+}$; (C and D) *per*^{0}; (E and F) *per*^{0}; M4-2.
(A), (C), and (E) show tissue sections from flies dissected at ZT 0 (lights on), whereas (B), (D), and (F) are from flies dissected at ZT 12 (lights off). Per staining in photoreceptor nuclei is shown by the arrows. Magnification, 560 x .



be detected in the visual system or CNS of *per*^{0} null mutants that are arrhythmic (Siwicki et al., 1988).

Immunohistochemical staining demonstrated the presence of silkmoth PER protein in *per*^{0} flies carrying the M4 transgene, with a neuroanatomic distribution similar to the wild type. For example, in two different strains carrying independent insertions of the M4 transgene (M4-2 and M4-15), expression of the moth PER protein was detected in nuclei of the photoreceptors and lateral neurons. Figure 2 documents the spatial expression pattern in wild-type and *per*^{0}; M4-2 flies, and a similar pattern of protein expression was observed in *per*^{0} mutants carrying the M4-15 insertion (data not shown). Figure 3 and Figure 4 show high magnification views of the photoreceptors and lateral neurons, respectively. In general, the number of PER-immunoreactive lateral neurons was similar, although not identical, in wild-type and *per*^{0}; M4-2 transgenic flies. In the wild type, there were 5–7 cells in the LNd group and 4–7 in the LNv group (see Ewer et al., 1992 for comparison), whereas transgenic flies had 7–10 cells in LNd and 5–9 in LNv. We cannot assess the significance of this small difference in cell number between wild-type and transgene-bearing *per*^{0} mutants. Transgenic flies also expressed moth PER protein in glia of the CNS and optic lobes, similar to the wild type (data not shown). Although the moth protein might be expressed in nonneural tissues, as is fly PER, expression outside of the nervous system was not examined in the present study.

We also looked for evidence that the silkmoth protein was rhythmically expressed in transgenic individuals. Both in *per*^{0}; M4-2 and *per*^{0}; M4-15 flies, clear immunostaining was observed in the nuclei of photoreceptors and lateral neurons at the time of lights on (ZT 0) in the light–dark (LD) 12:12 cycle in which animals were maintained. There was a low level of expression at ZT 12 (Figure 3 and Figure 4; data not shown). Mutants carrying the M4-2 transgene insertion were sectioned for immunostaining at 2 hr intervals throughout the cycle, starting at ZT 0 (see Experimental Procedures). Those studies demonstrated a marked diurnal variation in PER staining in the nuclei of both the photoreceptors and lateral neurons of *per*^{0}; M4-2 flies; representative photomicrographs from ZT 0 and ZT 12 are shown in Figure 3 and Figure 4. Staining was most intense within the photoreceptor nuclei of *per*^{0}; M4–2 individuals between ZT 20 and ZT 2 and least intense between ZT 6 and ZT 16 (Figure 3). The same temporal pattern of PER staining was observed in the the nuclei of the lateral neurons of *per*^{0}; M4-2 flies (Figure 4).

The data shown in Figures 3E and 3F and Figures 4E and 4F represent *per*^{0}; M4-2 flies carrying two copies of the transgene. Similar results were observed for *per*^{0}; M4-2 flies carrying only a single copy, but the immunostaining was less intense than that seen in the two-dose siblings, suggesting a dose-dependent expression of the silkmoth protein (data not shown). As expected, PER immunoreactive product was not detected at any time of day within photoreceptor nuclei (Figures 3C and 3D) or lateral neurons (Figures 4C and 4D) of *per*^{0} sibling flies that lacked the M4-2 transgene.

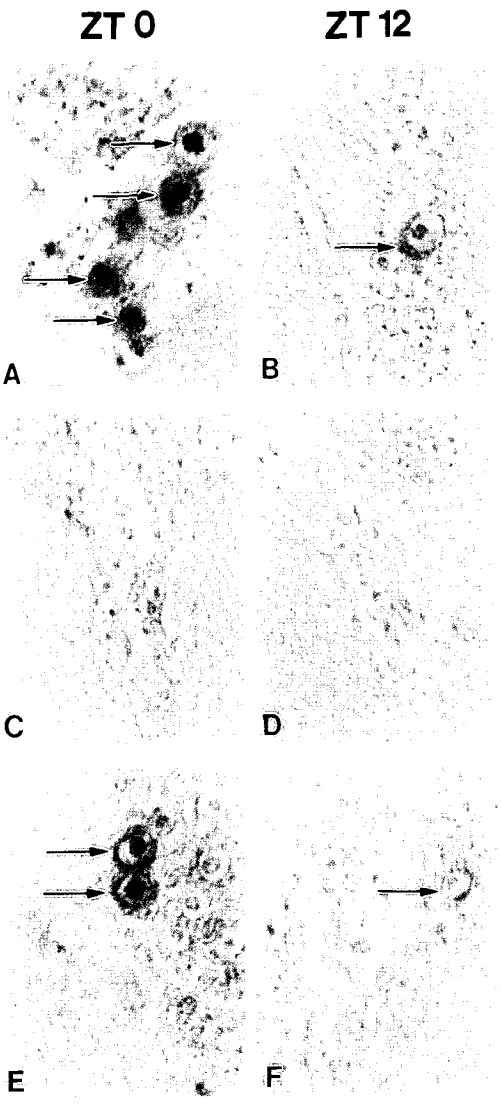


Figure 4. Per Immunohistochemical Staining in the Lateral Neurons of Wild-Type, *per*^{0}, and *per*^{0}; M4 Flies

(A–F) are as described in the legend to Figure 3. PER staining in lateral neurons is indicated by the arrows. Magnification, 970×.

Behavioral Studies of M4 Transgenic Flies

We used three behavioral criteria to determine whether the silkmoth protein functioned as an element of the Drosophila circadian clock. One criterion was whether the M4 transgene shortened circadian period, since earlier studies had shown that circadian period is shortened in a dose-dependent manner by additional copies of Drosophila *per*$^+$ or *per*^{S} (Smith and Konopka, 1982). A second criterion is transgenic rescue of behavioral rhythmicity in *per*^{0} mutants, which has previously been documented for Drosophila *per* gene sequences (Bargiello et al., 1984; Zehring et al., 1984; Petersen et al., 1988). For the first two types of studies, we evaluated rhythms in constant darkness to assess whether the silkmoth protein participated

Table 1. Dose-Dependent Shortening of τ by the M4 Transgene in *per*$^+$ and *per* Mutant Genetic Backgrounds

Genotype	M4 Dosage	n	τ (hr)[a]	% Arrhythmic
per$^+$; M4-2	0	15	23.3 ± 0.1	0
	1	22	23.0 ± 0.1[b]	0
	2	14	22.0 ± 0.1	0
per$^+$; M4-15	0	8	23.7 ± 0.1	50
	1	13	22.7 ± 0.3[c]	53.8
	2	19	21.8 ± 0.2	36.8
per^L; M4-2	0	23	27.8 ± 0.1	0
	1	23	27.4 ± 0.1	0
	2	18	26.6 ± 0.1	16.6
per^L; M4-15	0	26	28.5 ± 0.1	3.8
	1	29	28.1 ± 0.1	3.4
per^L; M4-17	0	23	27.5 ± 0.1	8.7
	1	22	24.4 ± 0.5	54.5
	2	25	24.6 ± 1.0	92.0
per^L; M4-20B	0	25	27.6 ± 0.1	12
	1	24	26.9 ± 0.2	4.1
	2	21	25.1 ± 0.4	19.0
per^S; M4-2	0	10	19.0 ± 0.1	0
	1	11	18.8 ± 0.1[d]	0
	2	12	18.1 ± 0.5	75
per^S; M4-15	0	24	19.0 ± 0.1	8.3
	1	12	18.3 ± 0.1	25

[a] Period values for flies carrying 1 or 2 copies of the M4 transgene were compared with 0 copy siblings using two-tailed t test. All differences were significant at $p \leq .01$ unless otherwise noted.
[b] $p < .05$
[c] $p = .02$
[d] Not significant at $p = .07$.

in endogenous pacemaker functions (Figure 3 and Figure 4; Table 1). Finally, we analyzed the entrainment of M4 individuals in a LD 12:12 cycle as an indication of light-induced resetting of the circadian pacemaker.

M4 Shortens Circadian Period in a *per*$^+$ Background

Initially, *per*$^+$; M4-2 flies were examined to determine whether the presence of the transgene affected circadian period. The period of the adult locomotor activity rhythm was clearly shortened by the M4-2 insert in a dose-dependent manner (Figure 5; Table 1). In *per*$^+$ flies carrying one copy of M4, on average, a slight shortening of period was observed relative to siblings (0 dose flies) lacking the transgene (Table 1). In flies carrying two copies of M4-2, however, period was shortened by 1.3 hr (Table 1). Moreover, one remarkable *per*$^+$ individual bearing a single copy of M4-2 displayed robust rhythmicity with a period of only 13.5 hr (data not shown). A comparable dose-dependent shortening of ciradian period was also observed in *per*$^+$ flies carrying the M4-15 insert (Table 1), which is located in a different chromosomal position (see Experimental Procedures). Therefore, the observed effects on circadian period are not dependent on the genomic location of the M4 transgene. One difference between these two lines was the high frequency of arrhythmicity observed in *per*$^+$; M4-15 flies, but this phenotype was not correlated with the presence of the M4-15 insert (see Discussion).

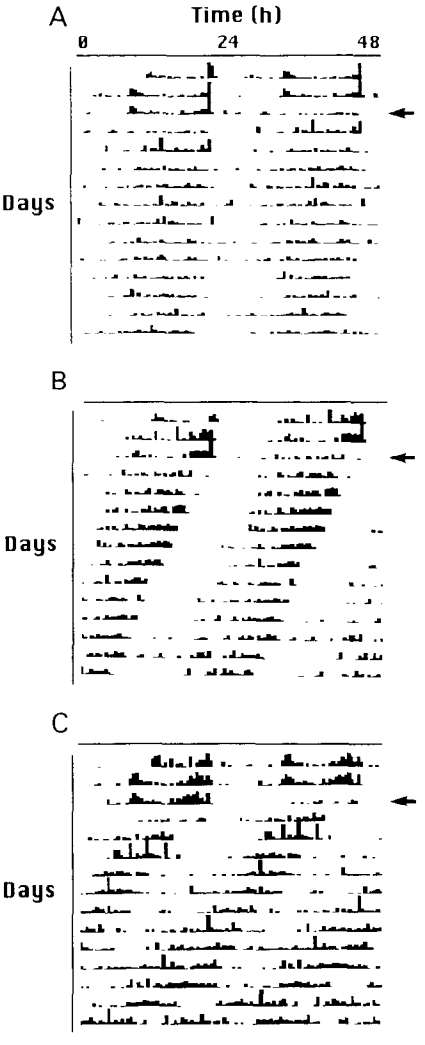


Figure 5. Activity Plots Showing a Dose-Dependent Shortening of Circadian Period in *per*$^+$; M4 Flies

In this figure and Figure 6, locomotor activity is double-plotted modulo 24 hr, with successive days depicted one beneath the other. Histograms represent activity events during each 30 min period of time. Arrows indicate the transition from LD 12:12 to constant darkness.
(A) *per*$^+$ fly without the transgene; τ = 23.6 hr.
(B) *per*$^+$; M4-2 fly carrying one copy of the transgene; τ = 22.8 hr.
(C) *per*$^+$; M4-2 fly with two copies of the transgene; τ = 21.3 hr.

M4 Shortens Circadian Period in *per*^{L} and *per*^{S} Backgrounds

Similar results were obtained with *per*^{L} flies carrying one or two copies of M4-2; that is, there was a slight shortening of period in *per*^{L} flies with one copy of M4-2 but a 1.2 hr decrease in period in flies with two copies of M4-2 relative to control siblings (Table 1). The M4-15 insertion also shortened circadian period in a *per*^{L} background (Table 1), although we did not examine mutants carrying two cop-

ies of this transgene. However, at least two other independent genomic insertions of the M4 transgene (M4-17 and M4-20B) did shorten the circadian period of per^L flies, and one of these had a dose-dependent effect on period (Table 1).

In per^S flies carrying either the M4–2 or M4–15 insertions, a shortening of circadian period was also observed (Table 1). For M4-2, a single copy of the transgene shortened period by only 0.2 hr (in this one case, not a statistically significant effect), whereas two copies decreased period by 0.9 hr (Table 1). Similar to the results with per^L, there was an increase in the percentage of arrhythmic flies with increased M4-2 copy number.

The M4 Transgene Can Restore Behavioral Rhythms to per^0 Mutants

To determine whether the M4 transgene could rescue an arrhythmic phenotype, per^0 mutants carrying the transgene were examined in constant darkness. In those experiments, we screened flies carrying the M4 transgene in 19 independent genomic locations, including the M4-2 and M4-15 inserts, since a complete rescue of the null phenotype might require high levels of *per* expression, and the expression level of the silkmoth transgene could be subject to position effects (data not shown; see Experimental Procedures). Although rhythmic individuals were observed in several of these lines, including M4-2, rescue was most consistently seen in the M4-15 line. In this particular line, 22.9% (11 of 48) of per^0 individuals carrying one copy of the transgene displayed rhythmicity in the circadian range (Figure 6A), with an average period of 20.9 ± 0.5 hr. For these rhythmic individuals, the average peak amplitude from periodogram analysis was 67.9 ± 7.5 (arbitrary units). All per^0 siblings from this line were arrhythmic (Figure 6B) with the exception of a single individual (1 of 27) with significant periodicity in the circadian range. Thus, at least in certain genomic locations, the M4 transgene can rescue the per^0 phenotype.

per^0; M4 Flies Entrain to LD 12:12

When the wild-type circadian pacemaker is entrained to a LD 12:12 cycle, bimodal dawn and dusk bouts of activity are observed (Figure 7A). According to oscillator theory, short-period and long-period mutants should entrain to a 24 hr cycle such that activity bouts are abnormally early or late, respectively (Pittendrigh, 1981), and this has been demonstrated for per^S and per^L mutants (Hamblen-Coyle et al., 1992). In contrast, per^0 flies do not entrain to LD cycles; they exhibit bursts of activity associated with the lights-on and lights-off signals (a so-called masking effect; Wheeler et al., 1993) but no anticipation of the dawn and dusk transitions (Figure 7C).

To assess the effects of the M4-2 transgene on LD entrainment (i.e., clock resetting), behavioral rhythms were examined for 7 days in LD 12:12 in several types of flies: per^+ males from the parental stock used for P element transformation, per^0; M4 males carrying one or two copies of the transgene, and per^0 sibling males. The summary profiles of activity for populations of per^+ and per^0 flies (Figures 7A and 7C) entirely replicated previous reports (Hamblen-Coyle et al., 1992). Similar to per^+ flies and unlike per^0 siblings, per^0; M4 flies entrained to LD 12:12, exhibiting anticipatory activity prior to subjective dawn and dusk (Figure 7). However, both the dawn and dusk bouts of activity in the transgene-bearing flies were extremely early compared with per^+ control flies, occurring in the middle of the night or day, respectively (compare Figure 7B with 7A). In separate experiments, we examined per^0; M4-15 flies, and these individuals also entrained to LD 12:12 with a similar early phase phenotype (data not shown).

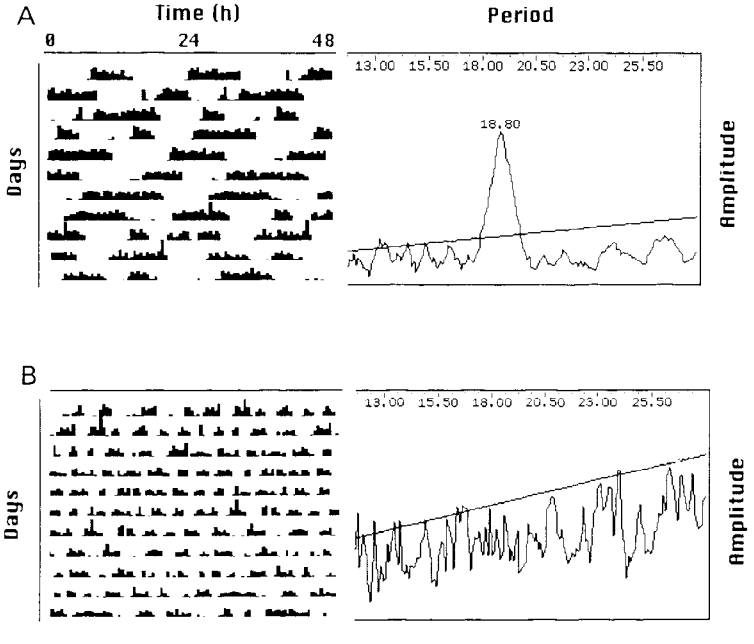


Figure 6. Rhythmic Activity in a per^0 Fly Carrying the M4 Transgene

(A) per^0; M4–15 fly with one copy of the transgene; τ = 18.8 hr.

(B) Arrhythmic per^0 sibling.

The periodograms next to the two activity plots indicate significant rhythmicity for the transgene-bearing fly (A) and arrhythmicity for the per^0 sibling (B).

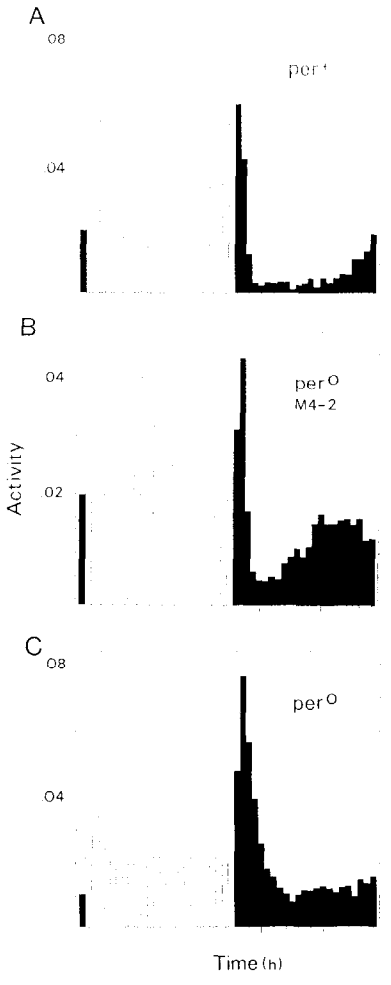


Figure 7. Population Summary Plots of Locomotor Activity for *per*^{0}; M4 and Control Flies in LD 12:12

(A) *per*$^+$ males without the transgene; n = 16.
(B) *per*^{0}; M4–2 males carrying two copies of the transgene; n = 26.
(C) *per*^{0} siblings without the transgene; n = 16.
In these plots, histograms represent the average activity in 30 min bins for the population expressed as a percentage of total daily activity. The open and closed bins indicate activity during the day and night, respectively. In (A), bouts of activity immediately anticipate lights on and lights off, whereas in (B), they are significantly early. The peaks of activity evident for *per*^{0} flies (C) are simply due to a masking effect (see text). *per*$^+$ females and *per*^{0}; M4 flies carrying one copy of the transgene were also characterized; their behaviors were similar to those shown in (A) and (B), respectively. Although we show population averages for *per*$^+$ and *per*^{0}; M4–2 flies, the behavior of individuals was the same; i.e., for both genotypes, 100% of the flies entrained to the LD cycle.

Discussion

Silkmoth PER Is Expressed in Appropriate Spatial and Temporal Patterns in Transgenic Flies

We have shown that PER protein from the silkmoth A. pernyi can mediate clock functions in the fruitfly D. melanogaster. These functions are presumably dependent on proper neuroanatomic and temporal patterns of PER protein expression, which we have demonstrated for flies carrying the M4 transgene. Because the silkmoth protein is expressed appropriately in the fly, it is possible to analyze its function in the proper cellular and biochemical contexts.

The Silkmoth Protein is a Short-Period Form of PER

In addition to mediating rescue of *per*^{0} mutants and entrainment to a LD cycle, a transgene expressing silkmoth protein shortens circadian period in a dose-dependent manner. Smith and Konopka (1982) previously demonstrated similar but not identical dosage effects using the wild-type Drosophila *per* gene. Their results indicated that extra copies of *per*$^+$ shortened period in *per*$^+$ and *per*^{L} backgrounds, but lengthened period in *per*^{S} mutants. They also showed that extra copies of Drosophila *per*^{S} shortened period when carried in a *per*^{S} mutant. As this last result is similar to the effect of the M4 transgene, we conclude that the moth protein functions in Drosophila as a short-period form of PER. This interpretation is strengthened by the observation of short-period behavior in rhythmic *per*^{0}; M4 flies.

Three different short-period *per* alleles have been identified in Drosophila: *per*^{T}, *per*^{S}, and *per*^{Clk}, with average circadian periods of about 16, 19, and 22.5 hr, respectively (see Konopka et al., 1995). Only the first two of these mutations have been molecularly characterized. The *per*^{S} allele is a missense mutation that results in the substitution of asparagine for serine at position 589 (Baylies et al., 1987; Yu et al., 1987). This mutation identified a short-period mutable domain from amino acids 585–601, in which single substitutions lead to a short-period phenotype (Baylies et al., 1992; Rutila et al., 1992). Although the exact lesion has not yet been defined, the *per*^{T} mutation is thought to result from deletion of 3′ gene sequences (J. Hall, personal communication), suggesting that the carboxy terminus of the protein might be affected. In this regard, it is known that the silkmoth protein is truncated at its carboxy terminus relative to fly PER protein and lacks conserved regions c-4 to c-6 (Reppert et al., 1994). Thus, it is possible that both per^T and the silkmoth proteins lack carboxy terminal amino acid sequence that is important for the determination of normal circadian period in Drosophila. An alteration of this sequence might contribute to the short-period phenotypes observed in *per*^{T} and M4 transgenic flies, although the D. melanogaster and silkmoth per proteins also differ in other regions, including the short-period mutable domain. Interestingly, the short-period mutable domain of D. virilis (but not D. melanogaster) is quite similar to that of A. pernyi (Reppert et al., 1994), and D. virilis adults also exhibit circadian periods shorter than those of D. melanogaster (M. Hamblen-Coyle and J. Hall, personal communication).

Effects on Period and Strength of Rhythmicity Are Separable in M4 Transgenic Flies

In several different lines carrying independent insertions of the M4 transgene, we observed a dose-dependent increase in arrhythmicity in *per*^{L} or *per*^{S} backgrounds. Such an effect was not observed in a *per*$^+$ background, although

relative to M4-2, flies from the *per*$^+$; M4–15 line were more frequently arrhythmic, irrespective of whether they carried the transgene. Since the M4 transgene shortens period in a *per*$^+$ background without a correlated increase in arrhythmicity, the observed effects on period and strength of rhythmicity are formally separable. Such separable effects on period and rhythmicity were also reported by Liu et al. (1991) in their studies of Drosophila transgenic strains. Though the effects on either parameter are not understood in molecular terms, it seems probable that both involve changes in the normal spectrum of PER protein interactions. It is known, for example, that PER protein can form homotypic and heterotypic associations that are mediated by a conserved region known as the PAS domain (Huang et al., 1993), although the putative in vivo partners of per have not been identified.

The PAS domain is highly conserved among different species, and thus it is likely that silkmoth PER protein can directly interact with Drosophila PER in addition to potential downstream targets such as transcription factors. It is known that *per*^{L} mutations result in amino acid substitutions within this domain that affect intramolecular and intermolecular PER protein interactions (Huang et al., 1993, 1995). Although *per*^{S} mutations do not affect the formation of PER homodimers (Huang et al., 1995), it is possible that they alter heterotypic interactions. As indicated previously, silkmoth PER protein has dose-dependent effects on circadian period, yet causes significant arrhythmicity only in mutant backgrounds. Therefore, it is likely that the protein interactions underlying period determination are distinct from those responsible for strength of rhythmicity. Perhaps the silkmoth protein interferes to a greater extent with the protein interactions required for normal rhythmicity because of an altered affinity of mutant protein for downstream targets.

Silkmoth PER Protein Mediates Free-Running Rhythmicity and Entrainment in Drosophila

Approximately 23% of *per*^{0}; M4-15 transgenic flies expressed coherent activity rhythms in constant darkness, indicating that silkmoth PER protein, by itself, can mediate clock function. The remainder of these flies, however, were arrhythmic, suggesting that interactions of the silkmoth protein with other components of the circadian system are compromised in some way. In theory, such aberrant protein interactions might disrupt the actual generation of circadian period or, alternatively, affect clock output (i.e, coupling of the clock to the activity rhythm). It is clear that the clock itself is not completely normal in the rescued *per*^{0}; M4-15 flies, since they exhibit average circadian periods of 20.9 hr.

The entrainment phenotype of *per*^{0}; M4 individuals in LD 12:12 is more likely to be a consequence of altered clock output, rather than an effect on the pacemaking mechanism. We have shown that silkmoth PER protein cycles in the lateral neurons and photoreceptors of these transgenic flies in a manner that is similar to that observed for the endogenous fly protein (Zerr et al., 1990). It is of interest that Zerr et al. were able to observe a phase difference between wild-type and *per*^{S} flies with regard to PER protein staining intensity. Given the number of transgenic flies examined immunohistochemically in the present study, it was not possible to determine whether moth PER cycling was slightly early relative to the wild type. However, protein cycling was not phase advanced in transgenic flies to the same extent as behavior, which was approximately 7 hr early compared with control *per*$^+$ individuals. This indicates that PER protein cycling and its regulation of behavior are separable molecular functions. It is possible that this alteration of the steady-state phase of activity reflects an abnormal interaction of silkmoth PER protein with downstream effector molecules of the clock output pathway.

In this context, it is intriguing to note that entrainment profiles in LD 12:12 are nearly identical for *per*^{0}; M4 transgenic flies and Drosophila *per*^{T} mutants (Konopka et al., 1995), both of which have short circadian periods. As mentioned earlier, it is known that the silkmoth PER protein is truncated relative to the wild-type fly protein, and the per^T protein might also be lacking carboxy terminal sequence. In the aggregate, these data suggest that the early-phase phenotype observed in both the transgenic flies and *per*^{T} might result from an alteration of carboxy terminal sequence.

Comparison of Fly and Silkmoth Clock Systems

The overt expression of circadian rhythms differs between the fruitfly D. melanogaster and the silkmoth A. pernyi, which are diurnal and nocturnal, respectively (Saunders, 1982). Whereas the fruitfly activity rhythm is bimodal (see Figure 7A), in the silkmoth, there is a single bout of activity during each cycle that occurs near the middle of the night when animals are entrained to LD 12:12 (Truman, 1974). In addition to exibiting short circadian periods, *per*^{0}; M4 flies express abnormally early bouts of activity, one of which is near the middle of the subjective night. It is tempting to speculate that these effects on period and phase might, in part, reflect an interspecific transfer of clock properties.

Experimental Procedures

Culture and Maintenance of Drosophila

For all studies, Drosophila strains were reared in a LD 12:12 cycle at 24°C and ~60%–70% relative humidity. Adults were then maintained in LD 12:12 for a minimum of 5–7 days prior to immunohistochemical or behavioral analysis. Two different Drosophila *per*^{0} strains, *y w per*^{01} and *w per*^{01} *sn*^{3} were used in these studies. Both the *per*^{L} and *per*^{S} mutations were carried on *w sn*^{3} chromosomes.

Construction of Plasmids

Plasmids for P element–mediated DNA transformation were generated using D. melanogaster *per* regulatory sequences and the coding region of the A. pernyi *per* gene. A 4 kb BamHI–SalI DNA fragment including the Drosophila sequences was obtained from pEMBL18.1, which was kindly provided by P. Hardin. It contains the Drosophila *per* transcription initiation site and extends from approximately −4000 to +25 relative to this site (Hardin et al., 1992). To generate our construct, the clone was digested with SalI, and then this site was filled in using Klenow. Following BamHI digestion of the clone and gel purification of the BamHI–blunt fragment, it was ligated into the pBluescript vector adjacent to a 3.4 kb blunt–PstI silkmoth cDNA fragment that contains

167 bp of 5′ untranslated sequence and the entire silkmoth coding domain (Reppert et al., 1994). Flanking KpnI and XbaI sites in pBluescript were employed to excise the fly–moth *per* cassette and directionally ligate it into a modified pCaSpeR–AUG–β-galactosidase P element vector (Thummel et al., 1988) from which the β-galactosidase coding region had been removed by KpnI/XbaI digestion. The resulting P element construct is known as M4 (see Figure 1); it contains Drosophila *per* gene flanking sequences adjacent to the silkmoth cDNA with about 20 bp of intervening plasmid polylinker sequence. This chimeric gene was ligated into the P element vector such that its 3′ end is next to SV40 sequences containing a small t intron and a polyadenylation signal (Thummel et al., 1988). The construction of Bluescript and P element plasmid vectors was verified by sequencing all ligation junctions.

Production of Transgenic Strains

Embryo injections were performed using the w^{1118} strain as host and the Δ2–3 P element helper plasmid, which produces transposase but cannot insert in the genome. Both plasmids were purified by CsCl density centrifugation and then dialyzed extensively in 10 mM Tris, 1 mM EDTA prior to injections. They were injected into preblastoderm embryos (Spradling and Rubin, 1982) at concentrations of 0.7 mg/ml and 0.15 mg/ml for the M4 and helper plasmids, respectively, using a Picospritzer II pressure injection system (General Valve Corp) and a Leitz micromanipulator. Three independent transgene-bearing strains were derived from the progeny of 99 surviving G_0 adults. One of these three strains, M4-2, was characterized in most detail; it carries a single P element insertion in region 57EF of chromosome 2R.

Transposase hopping was employed to generate additional M4-bearing strains with P element inserts in other genomic positions (Baylies et al., 1987; Robertson et al., 1988). To establish new lines, we took advantage of the ability to distinguish insert-bearing heterozygotes from homozygotes on the basis of eye color. This is possible because flies carrying two copies of the miniwhite gene in pCaSpeR and other P element vectors have significantly darker eye color than their siblings with one copy of the P element insert (Klemenz et al., 1987).

To mobilize the M4-2 insert, transgene-bearing males were crossed to *y w/y w*; *Ki Δ2–3/Ki Δ2–3* females carrying a genomically stable source of P transposase (Robertson et al., 1988). F1 males of the genotype *y w/Y*; $w^+/+$; *Ki Δ2–3/+* were then crossed to attached-X, *y w* females in vials. F2 males or females with significantly darker eye color than that observed in M4-2 flies were collected as progeny that potentially carried new inserts. Only a single w^+ fly was collected per vial to ensure that each established strain carried an independent genomic insert. Approximately 50% of the transposase hopping crosses yielded at least one progeny fly with darker eye color, indicating that the transgene had moved to a new genomic location. To establish genetically balanced strains, the F2 w^+ males were individually crossed to *y w* per^{01}/*y w* per^{01} females and then F3 *y w* per^{01}/*Y*; w^+ males were mated to attached-X, *y w* females. This was possible for almost all new insertion events, because the majority of them were autosomal. F2 females carrying new inserts (which also had attached-X chromosomes) were crossed to *y w* per^{01} males to generate balanced strains. In all, 29 new transgene-bearing lines were established. Homozygotes from three of these new lines, M4–15, M4–17 and M4–20B, have almost wild-type red eye color, in contrast to flies from the parental M4-2 line, which have orange eye color. The M4-15 insert, which partially rescues the per^{01} mutation, is located in region 50 of chromosome 2R.

Molecular Analysis of Transgenic Strains

Isolation of genomic DNAs, Southern blot analysis, and DNA hybridizations were conducted as previously described (Newby and Jackson, 1993). In situ hybridizations to larval polytene chromosomes were performed according to the method of Engels et al. (1986). Standard methods were used to carry out PCR analysis of genomic DNAs. For those studies, a pair of primers was designed to amplify an approximately 300 bp segment of the chimeric transgene that spans the junction between the Drosophila and silkmoth sequences.

Genetic Crosses and Behavioral Assays

Flies carrying a *per* mutation (per^{01}, per^S, or per^L) in combination with an autosomally linked M4 transgene were generated by crossing *per*/*per* mutant females to transgene-bearing males. The behavior of *per*; M4 male progeny was compared with that of *per* male siblings lacking the transgene. Flies were handled and locomotor activity rhythms were monitored exactly as described in previous studies (Newby and Jackson, 1993; Levine et al., 1994).

Immunohistochemistry

Two independently generated polyclonal antisera against Drosophila per protein were used: anti-S directed against a PER peptide near the site of the per^S mutation (Siwicki et al., 1988) and a second PER antiserum prepared against a baculovirus-expressed recombinant PER protein (provided by Lino Saez, Rockefeller University). The anti-S antiserum has been used previously to detect the endogenous silkmoth per protein (Reppert et al., 1994). These antisera were used at dilutions of 1:400 (anti-S) and 1:500. In control experiments, heat-inactivated normal serum was substituted for the primary antibodies, or the primary anti-S antibody was preincubated with its original peptide antigen. The temporal and spatial pattern of PER immunoreactivity was virtually identical with both antisera. For transgene-bearing flies and siblings, 3–4 individuals were stained with antibody and examined at each of 12 different timepoints (ZT 0 to ZT 22 at 2 hr intervals). For the lateral neuron cell counts reported in the results, 15 wild-type and 4 per^0; M4 individuals were examined.

In preparation for immunostaining, flies were anesthetized with triethylamine at desired timepoints. The head with attached thoracic segments was surgically removed and fixed overnight at 4°C in modified Bouin-Hollande solution without acetic acid but supplemented with 0.7% mercuric chloride. Standard techniques were employed for sample dehydration, embedding in paraplast, sectioning at 5–7 μm, and deparaffinization. For transgene-bearing flies and siblings, at least 3–4 individuals were sectioned at each of 12 different timepoints. After rehydration, the slides were treated with Lugol's iodine followed by 5% sodium thiosulfate to remove residual heavy metal ions from the fixed tissues. Sections were washed in distilled water and phosphate-buffered saline supplemented with 0.2% Tween 20 and 0.1% bovine serum albumin (PBS-TB), blocked with 10% heat-inactivated goat serum in PBS-TB, and then incubated overnight at 4°C with primary antiserum in PBS-TB. After rinsing with PBS-TB (3 × 10 min), they were incubated with goat anti-rabbit-XX-biotin conjugated secondary antibody (Molecular Probes; 1:800 dilution in PBS-TB, 1 hr at room temperature), rinsed with PBS-TB (3 × 10 min), and finally incubated with NeutraLite avidin-horseradish peroxidase conjugate (Molecular Probes; 1:200 in PBS-TB, 1 hr at room temperature) before DAB (0.25 mM) staining. Tissue sections were dehydrated and mounted in Accu-Mount-60 medium.

Acknowledgments

We thank Ginka Genova for help in the initial stages of this work, Joel Richter for the use of a pipet puller, Bob O'Connell for the loan of a Leitz micromanipulator, Linda Hall for Δ2–3 P element transposase plasmid DNA, Jeff Hall and Mike Young for *per* mutants, Al Handler for advice about P element–mediated transformation, Carl Thummel for P element plasmids, Paul Hardin for Drosophila *per* gene regulatory sequences, Kathy Siwicki, Lino Saez, and Mike Young for per antisera, Jeff Hall, Melanie Hamblen-Coyle, and Joan Rutila for rhythm-analysis programs and advice, and Kathy Matthews (Indiana University Drosophila Stock Center) for fly strains. This research was supported by National Institutes of Health grants NS30386 (F. R. J.), RR09767 (F. R. J.), DK42125 (S. M. R.), and National Research Service Award F32 GM15907 (J. D. L.).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisment*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received February 3, 1995; revised April 25, 1995.

References

Bargiello, T. A., Jackson, F. R., and Young, M. W. (1984). Restoration of circadian behavioral rhythms by gene transfer in Drosophila. Nature *312*, 752–754.

Baylies, M. K., Bargiello, T. A., Jackson, F. R., and Young, M. W. (1987). Changes in abundance or structure of the *per* gene product can alter periodicity of the Drosophila clock. Nature *326*, 390–392.

Baylies, M. K., Vosshall, L. B., Sehgal, A., and Young, M. W. (1992). New short period mutations of the Drosophila clock gene *per*. Neuron *9*, 575–581.

Colot, H. V., Hall, J. C., and Rosbash, M. (1988). Interspecific comparison of the *period* gene of Drosophila reveals large blocks of non-conserved coding DNA. EMBO J. *7*, 3929–3937.

Colwell, C. S., Whitmore, D., Michel, S., and Block, G. D. (1994). Calcium plays a central role in phase shifting the ocular circadian pacemaker of *Aplysia*. J. Comp. Physiol. *175*, 415–423.

Edmunds, L. N., Jr. (1988). Cellular and Molecular Bases of Biological Clocks. (New York: Springer-Verlag).

Engels, W. R., Preston, C. R., Thompson, P., and Eggleston, W. B. (1986). In situ hybridization to Drosophila salivary chromosomes with biotinylated DNA probes and alkaline phosphatase. Focus *8*, 6–8.

Eskin, A., Corrent, G., Lin, C.-Y., and McAdoo, D. J. (1982). Mechanisms for shifting the phase of a circadian rhythm by serotonin: involvement of cAMP. Proc. Natl. Acad. Sci. USA *79*, 660–664.

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the *period* clock gene within different cell types in the brain of Drosophila adults and mosaic analysis of these cells influence on circadian behavioral rhythms. J. Neurosci. *12*, 3321–3349.

Frisch, B., Hardin, P. E., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1994). A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the Drosophila nervous system. Neuron *12*, 555–570.

Hall, J. C., and Rosbash, M. (1993). Oscillating molecules and how they move circadian clocks across evolutionary boundaries. Proc. Natl. Acad. Sci. USA *90*, 5382–5383.

Hamblen-Coyle, M. J., Wheeler, D. A., Rutila, J. E., Rosbash, M., and Hall, J. C. (1992). Behavior of period-altered circadian rhythm mutants of *Drosophila* in light:dark cycles (Diptera: Drosophilidae). J. Insect Behav. *5*, 417–446.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1990). Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature *343*, 536–540.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1992). Circadian oscillations in period gene mRNA levels are transcriptionally regulated. Proc. Natl. Acad. Sci. USA *89*, 11711–11715.

Huang, Z. J., Edery, I., and Rosbash, M. (1993). PAS is a dimerization domain common to Drosophila period and several transcription factors. Nature *364*, 259–262.

Huang, Z. J., Curtin, K. D., and Rosbash, M. (1995). PER protein interactions and temperature compensation of a circadian clock in Drosophila. Science *267*, 1169–1172.

Jackson, F. R. (1993). Circadian rhythm mutants of Drosophila. In Molecular Genetics of Biological Rhythms, Cellular Clocks Series, M. W. Young, ed. (New York: Marcel Dekker), pp. 91–121.

Klemenz, R., Weber, U., and Gehring, W. J. (1987). The white gene as a marker in a new P-element vector for gene transfer in Drosophila. Nucleic Acids Res. *15*, 3947–3959.

Konopka, R. J., Pittendrigh, C. S., and Orr, D. (1989). Reciprocal behaviour associated with altered homeostasis and photosensitivity of *Drosophila* clock mutants. J. Neurogenet. *6*, 1–10.

Konopka, R. J., Hamblen-Coyle, M. J., Jamison, C., and Hall, J. C. (1995). An ultrashort clock mutation at the *period* locus of *Drosophila melanogaster* that reveals some new features of the fly's circadian system. J. Biol. Rhythms, in press.

Levine, J. D., Casey, C. I., Kalderon, D. D., and Jackson, F. R. (1994). Altered circadian pacemaker functions and cyclic AMP rhythms in the Drosophila learning mutant *dunce*. Neuron *13*, 967–974.

Liu, X., Zwiebel, L. J., Hinton, D., Benzer, S., Hall, J. C., and Rosbash, M. (1992). The *period* gene encodes a predominantly nuclear protein in adult Drosophila. J. Neurosci. *12*, 2735–2744.

Newby, L. M., and Jackson, F. R. (1993). A new biological rhythm mutant of *Drosophila melanogaster* that identifies a gene with an essential embryonic function. Genetics *135*, 1077–1090.

Nielsen, J., Peixoto, A. A., Piccin, A., Costa, R., Kyriacou, C. P., and Chalmers, D. (1994). Big flies, small repeats: the "Thr-Gly" region of the *period* gene in *Diptera*. Mol. Biol. Evol. *11*, 839–853.

Petersen, G., Hall, J. C., and Rosbash, M. (1988). The *period* gene of Drosophila carries species-specific behavioral instructions. EMBO J. *7*, 3939–3947.

Pittendrigh, C. S. (1974). Circadian oscillations in cells and the circadian organization of multicellular systems. In The Neurosciences, Third Study Program, F. O. Schmitt and F. G. Worden, eds. (Cambridge: MIT Press), pp. 437–458.

Pittendrigh, C. S. (1981). Circadian organization and the photoperiodic phenomena. In Biological Clocks in Seasonal Reproductive Cycles, B. K. Follett and D. E. Follett, eds. (New York: Halsted Press), pp. 1–35.

Prosser, R. A., and Gillette, M. U. (1989). The mammalian circadian clock in the suprachiasmatic nuclei is reset in vitro by cAMP. J. Neurosci. *9*, 1073–1081.

Prosser, R. A., McArthur, A. J., and Gillette, M. U. (1989). cGMP induces phase shifts of a mammalian circadian pacemaker at night, in antiphase to cAMP effects. Proc. Natl. Acad. Sci. USA *86*, 6812–6815.

Prosser, R. A., Heller, H. C., and Miller, J. D. (1994). Serotonergic phase advances of the mammalian circadian clock involve protein kinase A and K^+ channel opening. Brain Res. *644*, 67–73.

Ralph, M. R., Khalsa, S. B. S., and Block, G. D. (1992). Cyclic nucleotides and circadian rhythm generation in *Bulla gouldiana*. Comp. Biochem. Physiol. *101*, 813–817.

Reppert, S. M., Tsai, T., Roca, A. L., and Sauman, I. (1994). Cloning of a structural and functional homolog of the circadian clock gene period from the giant silkmoth *Antheraea pernyi*. Neuron *13*, 1167–1176.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D., Benz, W. K., and Engels, W. R. (1988). A stable genomic source of *P* element transposase in *Drosophila melanogaster*. Genetics *118*, 461–470.

Rosenwasser, A. M. (1988). Behavioral neurobiology of circadian pacemakers: a comparative perspective. Prog. Psychobiol. Physiol. Psychol. *13*, 155–226.

Rutila, J. E., Edery, I., Hall, J. C., and Rosbash, M. (1992). The analysis of new short-period circadian rhythm mutants suggests features of *D. melanogaster period* gene function. J. Neurogenet. *8*, 101–113.

Saunders, D. S. (1982). Insect Clocks. (Oxford: Pergamon Press).

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., and Hall, J. C. (1988). Antibodies to the *period* gene product of *Drosophila* reveal diverse tissue distribution and rhythmic changes in the visual system. Neuron *1*, 141–150.

Smith, R. F., and Konopka, R. J. (1982). Effects of dosage alterations at the *per* locus on the *period* of the circadian clock of Drosophila. Mol. Gen. Genet. *185*, 30–36.

Spradling, A. C., and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. Science *218*, 341–347.

Takahashi, J. S., Kornhauser, J. M., Koumenis, C., and Eskin, A. (1993). Molecular approaches to understanding circadian oscillations. Annu. Rev. Physiol. *55*, 729–753.

Thummel, C. S., Boulet, A. M., and Lipshitz, H. D. (1988). Vectors for *Drosophila P* element-mediated transformation and tissue culture transfection. Gene *74*, 445–456.

Truman, J. W. (1971). The role of the brain in the ecdysis rhythm of silkmoths: comparison with the photoperiodic termination of diapause.

In Biochronometry, M. Menaker, ed. (Washington D. C.: National Academy of Sciences), pp. 483–504.

Truman, J. W. (1972). Physiology of insect rhythms. II. The silkmoth brain as the location of the biological clock controlling eclosion. J. Comp. Physiol. *81*, 99–114.

Truman, J. W. (1974). Physiology of insect rhythms. IV. Role of the brain in the regulation of the flight rhythm of the giant silkmoths. J. Comp. Physiol. *95*, 281–296.

Wheeler, D. A., Kyriacou, C. P., Greenacre, M. L., Yu, Q., Rutila, J. E., Rosbash, M., and Hall, J. C. (1991). Molecular transfer of a species-specific behavior from *Drosophila simulans* to *Drosophila melanogaster*. Science *251*, 1082–1085.

Wheeler, D. A., Hamblen-Coyle, M. J., Dushay, M. S., and Hall, J. C. (1993). Behavior in light-dark cycles of Drosophila mutants that are blind, arrhythmic, or both. J. Biol. Rhythms *8*, 67–94.

Yu, Q., Jacquier, A. C., Citri, Y., Hamblen, M., Hall, J. C., and Rosbash, M. (1987). Molecular mapping of point mutations in the *period* gene that stop or speed up biological clocks in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *84*, 784–788.

Zehring, W. A., Wheeler, D. A., Reddy, P., Konopka, R. J., Kyriacou, C. P., Rosbash, M., and Hall, J. C. (1984). P-element transformation with *period* locus DNA restores rhythmicity to mutant, arrhythmic Drosophila melanogaster. Cell *39*, 369–376.

Zeng, H., Hardin, P. E., and Rosbash, M. (1994). Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. EMBO J. *13*, 3590–3598.

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of *period* protein immunoreactivity in the CNS and the visual system of Drosophila. J. Neurosci. *10*, 2749–2762.