REFERENCES AND NOTES

1. S. Gottlieb, A. Ernst, L. Litt, K. Q. Schwarz, R. S. Meltzer, *J. Am. Soc. Echocardiography* **3**, 238 (1990); J. R. Saphiro, S. A. Reisner, G. S. Lichtenberg, R. S. Meltzer, *J. Am. Coll. Cardiol.* **16**, 1603 (1990); K. Sugibayashi, Y. Morimoto, T. Nadai, Y. Kato, *Chem. Pharmacol. Bull.* **25**, 3433 (1977).
2. V. P. Torchilin *et al.*, *Fed. Am. Soc. Exp. Biol. J.* **6**, 2716 (1992); L. Illum, L. O. Jacobsen, R. H. Müller, R. Mak, S. S. Davis, *Biomaterials* **8**, 113 (1987); J. Lee, P. A. Martic, J. S. Tan, *J. Colloid Interface Sci.* **131**, 252 (1989); D. D. Lasic, F. J. Martin, A. Gabizon, S. K. Huang, D. Papahadjopoulos, *Biochim. Biophys. Acta* **1070**, 187 (1991); M. Donbrow, Ed., *Microcapsules and Nanoparticles in Medicine and Pharmacy* (CRC Press, Boca Raton, FL, 1992); T. M. Allen and C. Hansen, *Biochim. Biophys. Acta* **1068**, 133 (1991); R. H. Müller, K. H. Wallis, S. D. Tröster, J. Kreuter, *J. Controlled Release* **20**, 237 (1992); T. Verrecchia *et al.*, paper presented at VIII Journees Scientifiques du Groupe Thematique de Recherche sur les Vecteurs, Nancy, France, 6 to 10 December 1993; B. G. Müller and T. Kissel, *Pharm. Pharmacol. Lett.* **3**, 67 (1993); D. Bazile *et al.*, *Yanuzaigaku* **53**, 10 (1993).
3. A. Schindler *et al.*, *Contemp. Top. Polym. Sci.* **2**, 251 (1977); R. J. Kelly, *Rev. Surg.* **2**, 142 (1970).
4. S. W. Kim, R. V. Petersen, J. Feijen, in *Polymeric Drug Delivery Systems*, A. J. Ariens, Ed. (Academic Press, New York, 1992), p. 193.
5. R. Gref, Y. Minamitake, M. T. Peracchia, V. Torchilin, R. Langer, unpublished data.
6. S. J. Holland, B. J. Tighe, P. L. Gould, *J. Controlled Release* **4**, 155 (1986).
7. E. Wagner, M. Zenke, M. Cotten, H. Beug, M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3410 (1990).
8. G. Blume *et al.*, *Biochem. Biophys. Acta* **1149**, 180 (1993).
9. L. R. Beck, V. Z. Pope, D. R. Cowsar, D. H. Lewis, T. R. Tice, *Adv. Contracep. Delivery Syst.* 1, 79 (1986).

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Loss of Circadian Behavioral Rhythms and *per* RNA Oscillations in the *Drosophila* Mutant *timeless*

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Eclosion, or emergence of adult flies from the pupa, and locomotor activity of adults occur rhythmically in *Drosophila melanogaster*, with a circadian period of about 24 hours. Here, a clock mutation, *timeless* (*tim*), is described that produces arrhythmia for both behaviors. The effects of *tim* on behavioral rhythms are likely to involve products of the X chromosome–linked clock gene *period* (*per*), because *tim* alters circadian oscillations of *per* RNA. Genetic mapping places *tim* on the left arm of the second chromosome between *dumpy* (*dp*) and *decapentaplegic* (*dpp*).

Fruit flies show circadian regulation of several behaviors (*1*, *2*). When populations of *Drosophila* are entrained to 12 hours of light followed by 12 hours of darkness (LD 12:12), adults emerge from pupae (eclose) rhythmically, with peak eclosion recurring every morning. The eclosion rhythm persists when the entraining cues are removed and behavior is monitored in constant darkness, thus indicating the existence of an endogenous clock. Adult locomotor activity is also controlled by an endogenous clock and recurs rhythmically with a 24-hour period.

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Several mutations that affect eclosion and locomotor activity have been isolated in behavioral screens (*2*–*4*). The best characterized, and those with the strongest phenotypes, are mutations at the X chromosome–linked *period* (*per*) locus (*3*). Missense mutations at *per* can lengthen or shorten the period of circadian rhythms, whereas null mutations abolish circadian rhythms altogether. The *per* gene is expressed in many cell types at various stages of development. In most cell types, the *period* protein (PER) is found in nuclei (*5*, *6*). A domain within PER is also found in the *Drosophila single-minded* protein (SIM) and in subunits of the mammalian aryl hydrocarbon receptor (*7*), and this domain (PAS, for PER, ARNT, and SIM) mediates dimerization of PER (*8*). The amounts of both PER protein and RNA oscillate with a circadian period, which is affected by the *per* mutations in the same manner as behavioral rhythms are affected (*6*, *9*). Given the homologies to *sim* and the aryl hydrocarbon receptor (which are thought to regulate transcription), the effects of *per* on behavioral rhythms have been postulated to depend on circadian regulation of gene expression, including that of *per* itself (*9*). However, neither direct proof of this postulate nor elucidation of *per*'s actual biochemical function has been forthcoming.

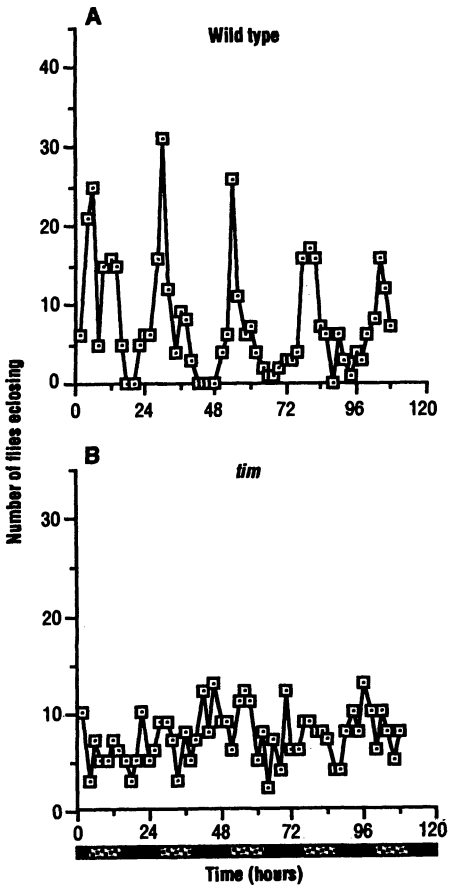


Fig. 1. Assessment of eclosion in *tim* flies. (**A**) Eclosion of wild-type flies in constant darkness (DD). (**B**) Eclosion of *tim* flies in DD. At the bottom of (B) are phases of LD cycles during entrainment (hatched boxes represent subjective day during collection). We entrained flies by maintaining them in LD 12:12 at 25°C for 4 days. Twenty hours before the first collection, lights were turned off. Newly emerged adults were collected and counted every 2 hours. A safelight that blocks wavelengths less than 600 nm (15-W bulb with a Kodak GBX-2 filter) was used to collect the eclosing adults.

Because an analysis of the molecular mechanisms that underlie circadian rhythms requires the identification of other components in the pathway, we conducted a genetic screen in order to isolate new mutations affecting biological rhythms in *Drosophila*. The mutagenesis was based on the mobilization of single P transposable elements, as described (*10*). The transposase-encoding, Δ2-3 P element from the Engels 2 strain (*11*) was used to mobilize a

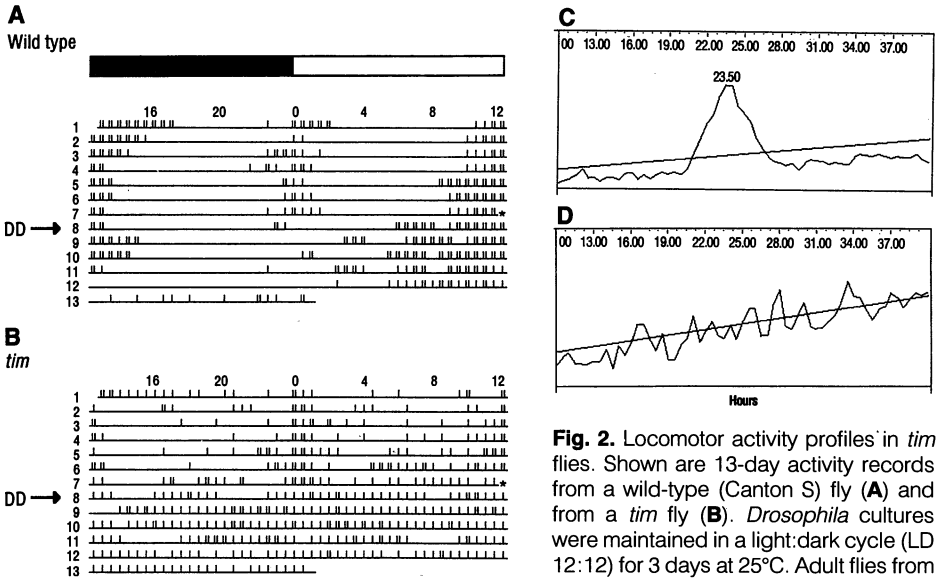


Fig. 2. Locomotor activity profiles in *tim* flies. Shown are 13-day activity records from a wild-type (Canton S) fly (**A**) and from a *tim* fly (**B**). *Drosophila* cultures were maintained in a light:dark cycle (LD 12:12) for 3 days at 25°C. Adult flies from these cultures were etherized and placed individually in cylindrical glass tubes. Activity was then monitored at 25°C (*15*) in the same LD 12:12 cycle for 7 days, and subsequently for 6 days in constant darkness (DD). Horizontal lines represent successive 24-hour intervals (top to bottom for each record). Activity corresponds to vertical deflections from the time lines. Time of day is indicated at the top of the records in Zeitgeber or circadian time (ZT0 = lights on, ZT12 = lights off during entrainment). For both records, the time of transition to DD is indicated by an asterisk. The first 24-hour interval in constant darkness is also indicated for each record with an arrow (DD). Portions of the records shown in (A) and (B) corresponding to constant darkness were subjected to χ square periodogram analysis (**C** and **D**, respectively) (*15*) (software obtained from Mini Mitter). Records were analyzed for evidence of periodicity in the 10- to 40-hour range. For each periodogram, the lower limit of statistical significance ($P < 0.05$) is indicated by a sloping line. Prior analysis of several hundred wild-type flies by these methods indicated that ~90% produce robust, single peaks of activity with periodicities of 23 to 25 hours (*21*) like that shown in (C). In contrast, periodogram analysis of 407 of 436 *tim* flies (93%) showed no evidence of periodicity or showed weak multiple periodicities throughout the circadian and noncircadian range [the latter behavior is illustrated in (D)]. Twenty-nine of the 436 *tim* flies (7%) produced weak single periodicities that were seldom in the circadian range. In no case was circadian rhythmicity evident from visual inspection of the 436 records. Results similar to those for *tim* flies have been observed for *per*^{o} flies (*13, 15*).

Table 1. Recombination mapping of *tim* relative to markers on the left arm of chromosome 2. The genotypes of chromosomes that had recombined between *tim*, *Sp*, and *J* (cross 1), between *tim* and *dp* (cross 2), or between *tim* and *dpp* (cross 3) are shown (recombinant chromosomes). Also indicated are the frequencies of such events (number of lines with the indicated recombinant genotype versus the total number of lines tested). The data indicate a location for *tim* that is approximately 17 map units distal to *Sp*, 5 map units distal to *dp*, and 3 map units proximal to *dpp*. Comparable tests with other markers on chromosome 2 (including *fy, nub, b, cn, L, Pin,* and *or*) were consistent with this placement of *tim* (*13*).

Parental chromosomes	Recombinant chromosomes	Frequency
	Cross 1	
+ *Sp* *J*	+ + +	15/126
tim + +	+ + *J*	1/126
	tim Sp J	6/126
	tim Sp +	0/126
	+ *Sp* +	11/126
	tim + *J*	2/126
	Cross 2	
al + *dp b*	*al* + + +	0/36
+ *tim* + +	+ *tim dp b*	2/36
	Cross 3	
ast dpp + *cl*	*ast dpp tim* +	0/94
+ + *tim* +	+ + + *cl*	3/94

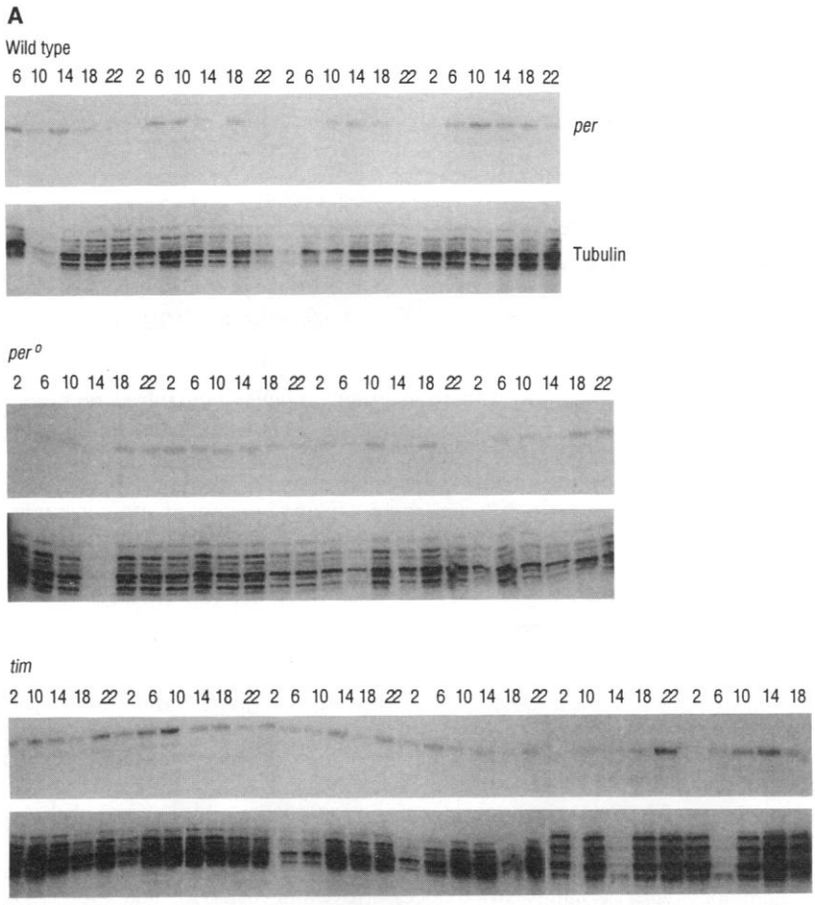
P(*ry*$^+$) mutator element derived from strain R702.1 (*12*). Strains containing single new insertions of P(*ry*$^+$) were made homozygous for the insertion.

Approximately 7000 homozygous second or third chromosome lines were screened for altered eclosion rhythms as described (*3*). Because wild-type flies emerge near dawn, cultures with large numbers of night-emerging adults were expanded for further analysis. Flies from one second chromosome line did not exhibit any preference for night or day emergence (*13*). We confirmed the arrhythmic eclosion pattern of the new mutant line by monitoring eclosion at 2-hour intervals for 5 days in constant darkness (DD) after exposure to three cycles of LD 12:12 (Fig. 1). Wild-type flies under these conditions showed peaks of emergence at subjective dawn (Fig. 1A). No such discernible rhythm was found in the mutant flies in constant darkness (Fig. 1B).

Some *Drosophila* clock mutants have effects on both eclosion and locomotor activity rhythms (*2–4*). To determine whether the new mutation also affects locomotor activity, we monitored mutant flies for 7 days in LD 12:12 and subsequently for 6 days in DD. Wild-type flies showed rhythmic behavior in LD 12:12, with peaks of activity each morning and evening (Fig. 2A). These rhythms persisted in wild-type flies in DD, albeit with a less obvious bimodality (Fig. 2A). In contrast, although they were responsive to LD cycles, flies from the mutant line became arrhythmic when the LD cycle was removed (Fig. 2B). In this regard, the behavior of the new mutant resembles that produced by *per*^{o} (*3, 13, 14*). The rhythmic activity displayed by flies of both genotypes in the presence of light:dark cycles may be a forced response to LD as described (*14*) and not indicative of an underlying circadian clock (*14*). Thus far, over 400 flies of the mutant line have been analyzed for evidence of locomotor activity rhythms. Periodogram analysis (*15*) and visual inspection of locomotor activity records failed to detect clear circadian rhythmicity in DD in any of the *tim* flies (Fig. 2).

The new clock mutation was localized on chromosome 2 by genetic recombination (Table 1) and is recessive. Although a site of P(*ry*$^+$) hybridization mapped to the right arm of the affected chromosome (53A-C) and genetic recombination placed the *ry*$^+$ marker at the same position, the site of insertion was not associated with the clock mutation (*16*). Rather, mapping studies with various dominant and recessive markers showed that the behavioral mutation is located on the left arm of chromosome 2 distal to *Sternopleural* (*Sp*), which is located at 2-22 (Table 1). Further recombination mapping was carried out with markers in the region extending from 2-0 to 2-16. The mutation, which we refer to as *timeless* (*tim*), maps between *decapentaplegic* (*dpp*) and *dumpy* (*dp*) (Table 1). None of the previously isolated clock mutations of *Drosophila* maps to this chromosomal interval (*2–4*). Furthermore, the *tim* mutation has not been associated with any obvious morphological or developmental change. Preliminary inspection of the anatomy of the nervous system in embryos, and of the brain and visual system in adults, also failed to distinguish *tim* mutants from wild-type *Drosophila* (*13*).

Both *per* mRNA and protein are expressed with a circadian rhythm in adults (*6, 9*). These oscillations are detectable in total RNA extracted from adult heads and have an altered phase in *per*^{s} and *per*^{L} flies. The oscillations are abolished in *per*^{o} flies. The cycling RNA and protein, therefore,



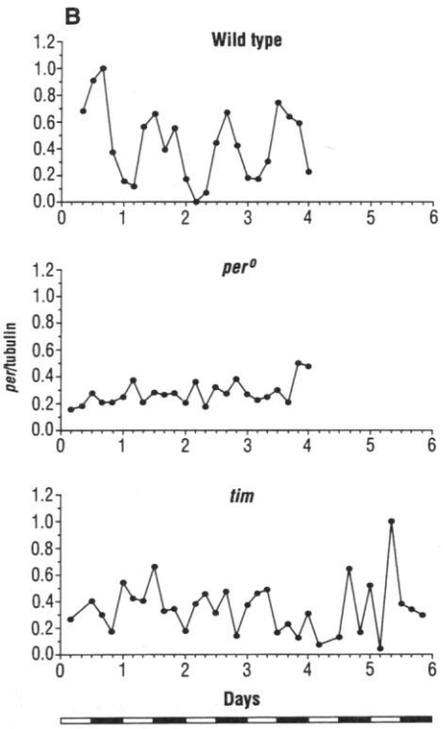


Fig. 3. Amounts of *per* RNA accumulation in the heads of wild-type, *per°*, and *tim* flies in LD cycles. The *per* RNA amounts were assayed in RNase protection assays (*17*, *20*) (**A**) RNase protection experiments showing amounts of *per* and tubulin RNA at different Zeitgeber times in wild-type (Canton S), *per°*, and *tim* flies. The repeating series of numbers marking the lanes at the top of each autoradiograph indicates Zeitgeber time over four successive LD 12:12 cycles for wild-type and *per°* flies and six cycles for *tim* flies. (**B**) Quantitative analysis of *per* RNA amounts in Canton S, *per°*, and *tim* flies. For the plots presented in (B), the autoradiographs shown in (A) were analyzed with a phosphorimager, and *per* and tubulin bands were quantitated. The plot shows *per*/tubulin ratios over a 4-day interval for wild-type and *per°* flies, and over a 6-day interval for *tim*. For the ordinates, 1.0 represents the highest *per*/tubulin ratio measured in wild-type flies. All other *per*/tubulin ratios were normalized to *per*/tubulin amounts in the wild-type maximum. Data points on days 5 and 6 of the *tim* record were normalized relative to the highest *per*/tubulin ratio on those days (*17*). The phase of the LD 12:12 cycle is presented at the bottom of (B). Open boxes indicate lights on; closed boxes indicate lights off.

are useful molecular markers for circadian rhythmicity and may be molecular components of a central circadian pacemaker that drives downstream behavioral rhythms. Because *tim* mutants are arrhythmic for both eclosion and locomotor activity, *per* mRNA cycling was assessed in *tim* flies (*17*). In agreement with Hardin *et al.* (9), wild-type flies showed peaks of *per* RNA accumulation each evening, at about Zeitgeber time 14 (ZT14; ZT0 = lights on, ZT12 = lights off). The smallest amount of accumulation occurred around ZT2. This cycling can be seen in the autoradiographs of protected *per* mRNA (Fig. 3A) and in the graph of *per* mRNA abundance (Fig. 3B), in which data from Fig. 3A were quantitated with a phosphorimager and graphically plotted. As previously shown (9), Fig. 3 also indicates that *per°* flies, when monitored over a 4-day interval in LD 12:12, did not exhibit circadian oscillations of *per* RNA.

The pattern of *per* mRNA accumulation in *tim* flies also differed from the wild-type pattern. Although fluctuations of *per* RNA were observed in the *tim* mutant over six LD 12:12 cycles (Fig. 3, A and B), these fluctuations were usually of much lower magnitude than in the wild type and showed no evidence of circadian or noncircadian rhythmicity. Similar results were obtained in each of four independent experiments (including that depicted in Fig. 3) with RNA collections representing 16 LD 12:12 cycles. In no case did *per* mRNA levels appear to be rhythmic in a *tim* background.

The effect of *tim* on *per* RNA oscillation suggests an interaction between these two loci. More evidence for this interaction has been obtained by studying the expression of PER protein in *tim* flies (*18*), where the *tim* mutation was found to block nuclear localization of the PER protein. Because PER protein has been proposed to transcriptionally regulate expression of its own RNA (9), perhaps its exclusion from the nucleus in *tim* flies accounts for the lack of *per* RNA oscillation. These data establish a functional interaction between *tim* and *per* that appears to be central to the control of circadian rhythms in *Drosophila*.

REFERENCES AND NOTES

1. C. S. Pittendrigh, in *The Neurosciences Third Study Program*, F. O. Schmitt and F. G. Worden, Eds. (MIT Press, Cambridge, MA, 1974), chap. 38.
2. F. R. Jackson, in *Molecular Genetics of Biological Rhythms*, M. W. Young, Ed. (Dekker, New York, 1993), pp. 91–121.
3. R. J. Konopka and S. Benzer, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2112 (1971); M. Rosbash and J. C. Hall, *Neuron* **3**, 387 (1989); M. K. Baylies, L. Weiner, L. B. Vosshall, L. Saez, M. W. Young, in *Molecular Genetics of Biological Rhythms*, M. W. Young, Ed. (Dekker, New York, 1993), pp. 123–153.
4. F. R. Jackson, *J. Neurogenet.* **1**, 3 (1983); M. S. Dushay, M. Rosbash, J. C. Hall, *J. Biol. Rhythms* **4**, 1 (1989); M. S. Dushay *et al.*, *Genetics* **125**, 557 (1990); R. J. Konopka, R. F. Smith, D. Orr, *J. Neurogenet.* **7**, 103 (1991).
5. A. A. James, J. Ewer, P. Reddy, J. C. Hall, M. Rosbash, *EMBO J.* **5**, 2313 (1986); X. Liu, L. Lorenz, Q. Yu, J. C. Hall, M. Rosbash, *Genes Dev.* **2**, 228 (1988); L. Saez and M. W. Young, *Mol. Cell. Biol.* **8**, 5378 (1988); X. Liu *et al.*, *J. Neurosci.* **12**, 2735 (1992).
6. K. K. Siwicki, C. Eastman, G. Petersen, M. Ros-

bash, J. C. Hall, *Neuron* **1**, 141 (1988); D. M. Zerr, J. C. Hall, M. Rosbash, K. K. Siwicki, *J. Neurosci.* **10**, 2749 (1990); I. Edery, L. J. Zwiebel, M. E. Dembinska, M. Rosbash, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2260 (1994).
7. S. T. Crews, J. B. Thomas, C. S. Goodman, *Cell* **52**, 143 (1988); E. C. Hoffman *et al.*, *Science* **252**, 954 (1991); K. M. Burbach, A. Poland, C. A. Bradfield, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8185 (1992); H. Reyes, S. Reisz-Porszasz, O. Hankinson, *Science* **256**, 1193 (1992).
8. Z. J. Huang *et al.*, *Nature* **364**, 259 (1993).
9. P. E. Hardin, J. C. Hall, M. Rosbash, *ibid.* **343**, 536 (1990); *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11711 (1992).
10. L. Cooley *et al.*, *Science* **239**, 1121 (1988).
11. H. M. Robertson *et al.*, *Genetics* **118**, 461 (1988).
12. A. C. Spradling and G. M. Rubin, *Cell* **34**, 47 (1983).
13. A. Sehgal, J. L. Price, B. Man, M. W. Young, unpublished results.
14. C. Helfrich and W. Engelmann, *Z. Naturforsch.* **42C**, 1335 (1987); M. J. Hamblen-Coyle *et al.*, *J. Insect. Behav.* **5**, 417 (1992); D. A. Wheeler, M. J. Hamblen-Coyle, M. S. Dushay, J. C. Hall, *J. Biol. Rhythms* **8**, 67 (1993).
15. M. Hamblen *et al.*, *J. Neurogenet.* **3**, 249 (1986); H. B. Dowse, J. C. Hall, J. M. Ringo, *Behav. Genet.* **17**, 19 (1987).
16. Hybrid dysgenesis can induce P element excision mutations that ultimately have few or no P element sequences. See M. G. Kidwell, in Drosophila: *A Practical Approach*, D. B. Roberts, Ed. (IRL Press, Oxford, 1986), chap. 3; S. B. Daniels and A. Chovnick, *Genetics* **133**, 623 (1993); and W. R. Engels, D. M. Johnson-Schlitz, W. B. Eggleston, J. Sved, *Cell* **62**, 515 (1990).
17. Wild-type (Canton S), per^o, and *tim* flies were contemporaneously entrained as follows: 0- to 5-day-old adult flies were transferred to fresh bottles and maintained in LD 12:12 at 25°C for 3 days. Beginning on the fourth day, collections were made at ZT2, 6, 10, 14, 18, and 22 (ZT0 = lights on). Each collection consisted of shaking approximately 1500 flies into a 15-ml conical polypropylene tube kept in dry ice. Total RNA was extracted from the heads, which we separated from the bodies by shaking frozen flies in sieves at temperatures below −20°C. A ^{32}P-labeled riboprobe that protects 316 nucleotides (nt) of *per* mRNA (nt 123 to 438 of the *per* mRNA coding sequence) (*3*) was transcribed from cloned *per* DNA. A tubulin riboprobe also was produced that protects 86 nt (nt 57 to 142 from the start of transcription) of α1 tubulin mRNA (*19*). Ribonuclease (RNase) protection experiments were carried out as described (*20*) with minor variations. Ten micrograms of head RNA was used for each time point, and digestions with RNase were for 30 min at 25°C. Hybridizations and RNase digestions were performed contemporaneously for wild-type, per^o, and *tim* RNA samples representing the first 4 days. For days 5 and 6 of the *tim* record, RNA hybridizations and digestions involved newly labeled riboprobes, so RNA from day 4 was again processed to allow direct comparison of *per* RNA amounts measured in the two sets of experiments. Digested samples were separated by electrophoresis on 5% polyacrylamide–8 M urea gels. Dried gels were exposed to x-ray film and also analyzed with a phosphorimager (Molecular Dynamics) for quantitation.
18. L. B. Vosshall, J. L. Price, A. Sehgal, L. Saez, M. W. Young, *Science* **263**, 1606 (1994).
19. W. E. Theurkauf, H. Baum, J. Bo, P. C. Wensink, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8477 (1986).
20. K. Zinn, D. DiMaio, T. Maniatis, *Cell* **34**, 865 (1983).
21. A. Sehgal, J. Price, M. W. Young, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1423 (1992).
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Block in Nuclear Localization of *period* Protein by a Second Clock Mutation, *timeless*

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In wild-type *Drosophila*, the *period* protein (PER) is found in nuclei of the eyes and brain, and PER immunoreactivity oscillates with a circadian rhythm. The studies described here indicate that the nuclear localization of PER is blocked by *timeless* (*tim*), a second chromosome mutation that, like *per* null mutations, abolishes circadian rhythms. PER fusion proteins without a conserved domain (PAS) and some flanking sequences are nuclear in *tim* mutants. This suggests that a segment of PER inhibits nuclear localization in *tim* mutants. The *tim* gene may have a role in establishing rhythms of PER abundance and nuclear localization in wild-type flies.

Mutations in the *Drosophila period* (*per*) gene disrupt circadian rhythms of pupal eclosion and adult locomotor behavior (*1*). Although *per* has been cloned and sequenced and its pattern of expression has been analyzed (*2*, *3*), the biochemical function of the PER protein is unknown. PER shares some homology with a family of transcription factors (*4*–*6*) that possess a common sequence motif called the PAS domain. The PAS domain consists of two repeats of approximately 50 amino acids within a homology region of 258 to 308 amino acids (*7*).

Immunocytochemical experiments demonstrated that PER is a nuclear protein in a variety of *Drosophila* tissues (*8*, *9*). In cells of the adult fly visual and nervous systems, the amount of PER protein fluctuates with a circadian rhythm (*10*), the protein is phosphorylated with a circadian rhythm (*10*), and PER is observed in nuclei at night but not late in the day (*8*). The expression of *per* RNA is also cyclic. However, peak mRNA amounts are present late in the day, and the smallest amounts are present late at night (*11*, *12*). Three mutant alleles—per^o, per^s, and per^L—cause arrhythmic behavior or shorten or lengthen periods, respectively (*1*). These mutations also produce corresponding changes in the rhythms of *per* RNA and protein amounts (*10*–*12*) and PER immunoreactivity in nuclei (*8*). This suggests a possible role for molecular oscillations of *per* in the establishment of behavioral rhythms (*11*). Recently, a new mutation, *timeless* (*tim*), was isolated that produces arrhythmic behavior and suppresses the circadian oscillation of *per* RNA (*12*). Here, we examine the effect of *tim* on the expression and localization of PER protein.

We compared PER protein expression in wild-type, per^o mutant, and *tim* mutant flies by staining head sections with PER antibody. Because the amounts of PER protein staining in eye and brain nuclei fluctuate daily (*8*), sections were prepared at four time points. Nuclear staining in wild-type photoreceptor cells was most prominent at Zeitgeber times 2 and 20 (ZT2 and 20) (*13*), intermediate at ZT7, and absent at ZT13 (Fig. 1A). Because the per^o mutation introduces a stop codon in the PER reading frame (*2*, *3*), PER antibody specificity was demonstrated by the absence of staining in sections from this null mutant at each time point (Fig. 1C). In *tim* mutants, nuclear staining was not seen at any time point (Fig. 1B).

PER–β-galactosidase (PER–β-gal) fusion proteins have been used extensively to study patterns of *per* locus expression (*9*, *14*, *15*). Such fusion proteins have also allowed the functional dissection of the gene and the encoded protein (*9*, *14*, *15*). To explore the effect of *tim* on PER protein expression and to map elements of the *per* locus responding to *tim*, we compared patterns of expression of a PER–β-gal fusion protein (PER-SG) (*9*) in transgenic flies with a wild-type or *tim* mutant genetic background. PER-SG contains

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