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double-time Is a Novel *Drosophila* Clock Gene that Regulates PERIOD Protein Accumulation

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Summary

We have isolated three alleles of a novel *Drosophila* clock gene, *double-time* (*dbt*). Short- (dbt^S) and long-period (dbt^L) mutants alter both behavioral rhythmicity and molecular oscillations from previously identified clock genes, *period* and *timeless*. A third allele, dbt^P, causes pupal lethality and eliminates circadian cycling of *per* and *tim* gene products in larvae. In dbt^P mutants, PER proteins constitutively accumulate, remain hypophosphorylated, and no longer depend on TIM proteins for their accumulation. We propose that the normal function of DOUBLETIME protein is to reduce the stability and thus the level of accumulation of monomeric PER proteins. This would promote a delay between *per*/*tim* transcription and PER/TIM complex function, which is essential for molecular rhythmicity.

Introduction

Patterns of activity, with periodicities of approximately 24 hr, are termed circadian rhythms and appear to be universal components of animal behavior (Pittendrigh, 1967, 1974). These behaviors can be entrained to a "zeitgeber" (most commonly light) but are sustained under conditions of constant darkness and temperature, revealing activity of an endogenous biological clock. Circadian physiological rhythms are not limited to the animal kingdom, and genetic screens have identified clock genes in *Drosophila melanogaster* (Konopka and Benzer, 1971; Sehgal et al., 1994; Allada et al., 1998; Rutila et al., 1998), *Chlamydomonas* (Bruce, 1972), *Neurospora crassa* (Feldman and Hoyle, 1973; Crosthwaite et al., 1997), cyanobacteria (Kondo et al., 1994), *Arabidopsis thaliana* (Millar et al., 1995), hamster (Ralph and Menaker, 1988), and mouse (Vitaterna et al., 1994).

Our current understanding of the molecular regulation of circadian rhythmicity in *Drosophila* comes from integrating genetics and molecular biology. Null mutations in either of two genes, *period* (*per*) and *timeless* (*tim*), abolish behavioral rhythmicity, while alleles encoding proteins with missense mutations have been recovered at both loci and show either short- or long-period behavioral rhythms (Konopka and Benzer, 1971; Sehgal et al., 1994; Rutila et al., 1996; A. R., M. A., J. L. P., and M. W. Y., unpublished data). The RNA and protein products of the genes oscillate with a circadian rhythm in wild-type flies. These molecular rhythms are abolished by null mutations of either gene, and the periods of all molecular rhythms are correspondingly altered in each long- and short-period mutant, indicating a regulatory interaction between these genes (Hardin et al., 1990; Edery et al., 1994; Sehgal et al., 1994, 1995; Vosshall et al., 1994; Price et al., 1995; Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996).

Production of these molecular cycles appears to depend on the rhythmic formation and nuclear localization of a complex containing the PER and TIM proteins (Gekakis et al., 1995; Sehgal et al., 1995; Lee et al., 1996; Saez and Young, 1996; Zeng et al., 1996). A physical interaction of PER and TIM is required for nuclear localization of either protein, and nuclear activity of these proteins coordinately regulates *per* and *tim* transcription through a negative feedback loop (Sehgal et al., 1994; Vosshall et al., 1994; Gekakis et al., 1995; Sehgal et al., 1995; Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Saez and Young, 1996; Zeng et al., 1996). A complex of two transcription factors, CLOCK and BMAL1, positively regulates both *per* and *tim* transcription (Allada et al., 1998; Darlington et al., 1998; Gekakis et al., 1998; Hogenesch et al., 1998; Rutila et al., 1998), and this positive regulation is suppressed by nuclear PER/TIM proteins (Darlington et al., 1998). Studies of per^L, a mutation that lengthens the period of behavioral rhythms (Konopka and Benzer, 1971) and delays nuclear localization of PER protein (Curtin et al., 1995), have shown that the PER^L protein has reduced affinity for TIM (Gekakis et al., 1995). This suggests that rates of PER/TIM association influence the period of the molecular cycle in mutant and wild-type flies.

Sehgal et al. (1995) proposed a model for the *Drosophila* clock in which delayed formation of PER/TIM complexes ensures separate phases of *per*/*tim* transcription and nuclear function of the encoded proteins. Recent mathematical treatments of the *Drosophila* data are consistent with this model (Leloup and Goldbeter, 1998). Entrainment of this oscillator is regulated through the TIM protein, which is rapidly eliminated from the nucleus and cytoplasm of pacemaker cells when *Drosophila* are exposed to daylight (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Studies of transgenic *Drosophila* have shown that adult behavioral rhythms can be linked to *per* and *tim* expression in a small group of central brain cells, the lateral neurons (LNs; Ewer et al., 1992; Frisch et al., 1994; Vosshall and Young, 1995). *per* and *tim* are also expressed in larval brain cells that are most likely the larval LNs (Kaneko et al., 1997), suggesting a basis for larval entrainment to light/dark cycles (Sehgal et al., 1992). Oscillations of *per* and *tim* RNA, and PER and TIM proteins have been found outside of the head in a variety of tissues (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997). Some of the latter oscillations were observed in vitro with isolated tissues, further indicating a cell

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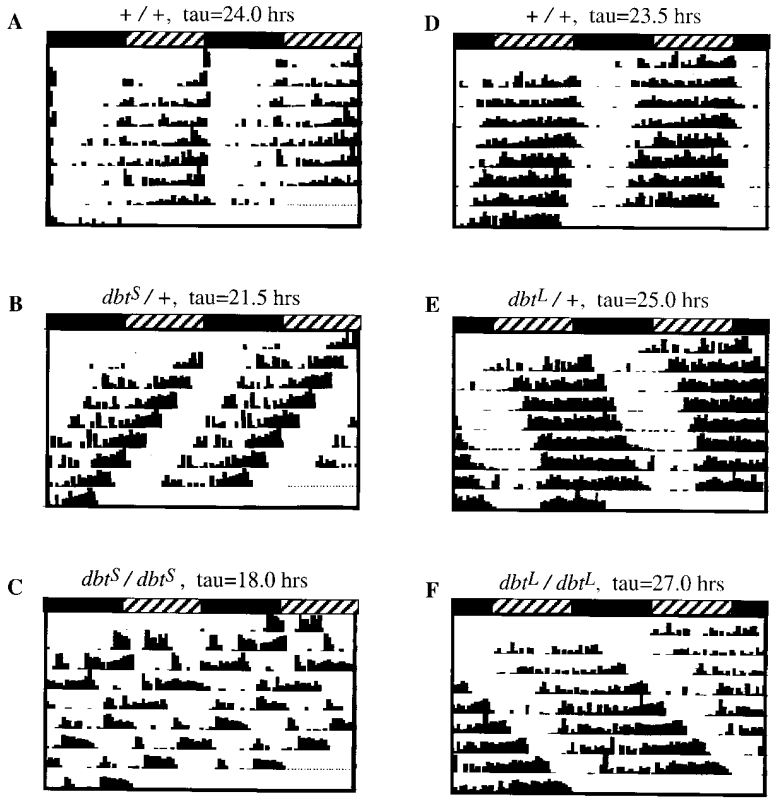


Figure 1. dbt^S Shortens and dbt^L Lengthens Periods of Locomotor Activity

Representative locomotor records of single flies of (A) wild type (+/+), (B) dbt^S/+, (C) dbt^S/dbt^S, (D) wild type, (E) dbt^L/+, and (F) dbt^L/dbt^L in DD. Adult flies were entrained in a 12 hr light:12 hr dark cycle for more than 3 days, and then locomotor activity was monitored in constant darkness. The phase of the previous light:dark regime is indicated at the top of each record with the hatched boxes indicating the time of the photophase. Horizontal lines are 48 hr intervals, and activity is denoted by closed bars, with the level of activity indicated by the height of each bar. The record for each 24 hr period composing the right half of each line is plotted again on the left half of the line underneath for visual continuity. The period of the rhythm, calculated by chi-square periodogram analysis, and the genotype of each fly are indicated on top of the records. Records in each column are from sibling flies. Activity events occur later on successive days in records with long periods, while they occur earlier on successive days in records with short periods. Note that both dbt^L and dbt^S are semidominant. Periods obtained were dbt^S, 18.0 ± 0.1 hr, n = 20; dbt^S/+, 21.8 ± 0.2 hr, n = 10; dbt^L, 26.8 ± 0.1 hr, n = 19; dbt^L/+, 24.9 ± 0.1 hr, n = 16; and wild type, 23.6 ± 0.1 hr, n = 15.

autonomous mechanism (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997). Mammalian homologs of *per* have recently been identified (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997), and CLOCK and BMAL1 were first described in mammals (King et al., 1997; Ikeda and Nomura, 1997), suggesting that the molecular basis of circadian rhythms may be conserved from flies to mammals. A related circadian oscillator has also been described at the molecular level in *Neurospora* through the detailed work of Dunlap and colleagues (reviewed by Dunlap, 1996).

Although some key features of the *Drosophila* clock have been identified, the involvement of additional, essential factors is suspected from prior work. PER fails to accumulate in the absence of TIM even in the presence of high *per* RNA levels (Vosshall et al., 1994; Price et al., 1995), pointing to the existence of an activity that destabilizes cytoplasmic PER monomers. Both PER and TIM are phosphorylated with a circadian rhythm (Edery et al., 1994; Zeng et al., 1996) indicating unidentified kinases. PER, in particular, becomes progressively phosphorylated over many hours, and the timing of its phosphorylation is changed in period-altering mutants (Edery et al., 1994), suggesting either circadian regulation of PER phosphorylation or a role in establishing rhythmicity.

In this paper, we report the identification and genetic characterization of the clock gene *double-time* (*dbt*). We have isolated alleles that either shorten or lengthen the periods of behavioral and molecular rhythms. We have also recovered a strongly hypomorphic *dbt* allele that is associated with pupal lethality and blocks circadian oscillations of *per* and *tim* gene products in larvae. *dbt* is therefore a central clock component alongside *per* and *tim*. *dbt* period-altering alleles alter the kinetics of PER phosphorylation and degradation. The hypomorphic allele constitutively produces unusually high levels of PER proteins that are hypophosphorylated. Thus, a normal function of *dbt* appears to be regulation of PER accumulation.

Results

double-time Is a New Gene that Sets the Period of Locomotor Activity Rhythms of *Drosophila*

Ethyl methane sulfonate (EMS) mutagenesis (Lewis and Bacher, 1968) was used to induce new clock mutations affecting the period length of locomotor activity rhythms in homozygous or heterozygous flies. Screening of heterozygous phenotypes was performed because all known clock mutations that affect period length in *Drosophila*, *Neurospora*, *Arabidopsis*, mice, and hamsters are semidominant (reviewed by Dunlap, 1996).

The locomotor activity of individual flies, each bearing heterozygous or homozygous mutagenized chromosomes, was monitored under constant darkness (DD) to reveal free-running period length (Experimental Procedures). From a screen of ~15,000 second and third chromosomes, three lines were recovered carrying long-period alleles of *timeless* (A. R., M. A., J. L. P., and M. W. Y.; unpublished data). Two additional lines were isolated that contained mutations in a novel clock gene, which we refer to as *double-time* (*dbt*), because the

first mutant allele that we isolated (dbt^S) dramatically shortens the behavioral period (described below).

Flies that are heterozygous for the dbt^S mutation ($dbt^S/+$) produce locomotor rhythms with an average period of 21.8 hr in DD, while homozygous flies (dbt^S/dbt^S) produce locomotor rhythms with an average period of 18.0 hr (Figure 1). Flies that are heterozygous for a second allele, dbt^L ($dbt^L/+$) produce locomotor rhythms with an average period of 24.9 hr, and homozygous flies (dbt^L/dbt^L) produce locomotor rhythms with 26.8 hr periods (Figure 1). Because $dbt^S/+$ and $dbt^L/+$ flies have shorter and longer periods, respectively, than wild-type controls, but not as short or long as homozygous mutant flies, dbt^S and dbt^L are semidominant. Homozygous dbt^S and dbt^L flies can be entrained by an imposed 12 hr light:12 hr dark cycle (LD 12:12) since they exhibit 24 hr periodicity under such conditions (data not shown). Analysis of several hundred locomotor activity records from homozygous dbt^S and dbt^L flies indicated complete penetrance of the mutant phenotypes.

dbt^S and dbt^L were also tested for aberrant circadian rhythms of eclosion (emergence of the adult fly from the pupal case) to determine whether *dbt* mutations affect this phenotype as previously observed for *per* and *tim* (Konopka and Benzer, 1971; Sehgal et al., 1994). Although eclosion occurs only once in the lifetime of an individual fly, it occurs repeatedly and rhythmically in a population of flies of diverse ages. In DD, the period of the dbt^S eclosion rhythm was shorter than the rhythm of the wild-type population. Peaks of eclosion occurred progressively earlier in dbt^S as compared to wild type over the 5-day interval tested (Figure 2A). For dbt^L, a longer period rhythm was obtained as compared to wild type (Figure 2B). The similar effects of the dbt^S and dbt^L mutations on two behavioral outputs of the *Drosophila* circadian clock are consistent with an effect on the central pacemaker mechanism rather than on a specific output pathway. In this regard, the effects of *dbt* mutations on rhythmicity are comparable to those of *period* and *timeless* mutations (see also below).

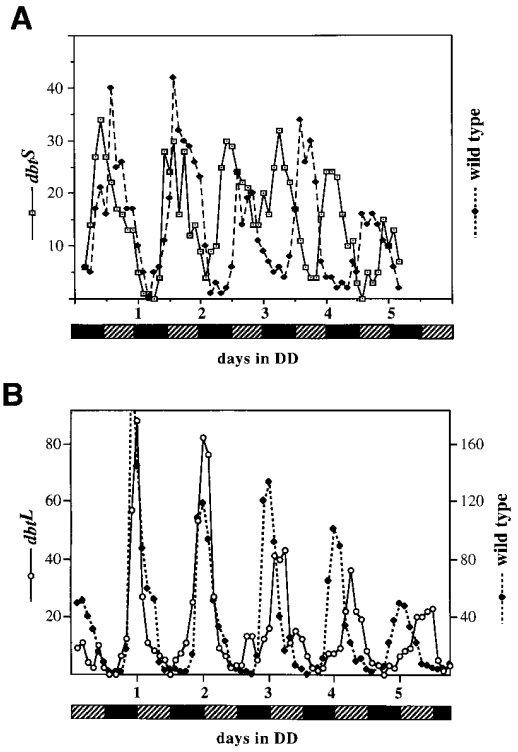


Figure 2. dbt^S Shortens and dbt^L Lengthens Periods of Eclosion Rhythms

Wild-type (wt), dbt^S (A), and dbt^L (B) flies were entrained in LD 12 hr:12 hr for at least 5 days and then transferred to constant darkness (DD). Newly emerged adults were cleared and counted every 2 hr for wild type and dbt^S. Eclosing dbt^L flies were counted every half hour and the numbers pooled for the 2 hr interval plot. The phase of the previous light:dark regime is indicated at the bottom, with the hatched boxes indicating the time of the photophase. Period length was calculated from all data collected after a first day of adaptation to DD. For dbt^S, the period was ~20 hr, and for dbt^L, ~27 hr. Wild-type controls each gave ~24 hr periods. We note that on shifting dbt^S and dbt^L flies from LD to DD, short- and long-period eclosion rhythms are evident only after 1–2 days of adaptation to DD. Eclosion patterns of per^S and tim^L mutants were also examined and showed a similar delay during LD-to-DD transitions (A. R., unpublished data). Such delays were not seen in measurements of the locomotor activity rhythms.

Genetic Tests Colocalize the dbt^S and dbt^L Mutations to the Right Arm of the Third Chromosome

Flies from the dbt^S stock were crossed to *Drosophila* stocks containing multiple third chromosome mutations and individual recombinant F_2 progeny used to establish lines. Locomotor activity rhythms were analyzed for several representatives from each recombinant line to verify the presence or absence of the dbt^S mutation. These recombination tests placed the dbt^S mutation near the tip of the right arm of chromosome 3 between *claret* (*ca*) and *brevis* (*bv*), and closely linked to *loboid* (*ld*) (data not shown).

dbt^S and dbt^L were also genetically localized by complementation analyses with a series of deficiencies affecting the right arm of chromosome 3. One deficiency, *Df(3R)tll-g*, produced flies with 19.1 hr locomotor rhythms in dbt^S/Df heterozygotes, and 27.2 hr rhythms in dbt^L/Df heterozygotes (Table 1). *Df(3R)tll-g*/+ heterozygotes produced locomotor rhythms with approximately wild-type periods (~24 hr). These results argue that this deficiency lacks the wild-type allele of *dbt*. The deficiency is missing polytene chromosomal region 99E-F to 100B, which is distal to *ca*. The results are consistent with the recombination analysis of dbt^S.

Duplication analysis was also performed. dbt^S/dbt^S flies carrying *Dp(3;1)124P*, which includes chromosomal region 99E-100F, produced locomotor activity rhythms with a period of 21.2 hr (Table 1). Hence, *dbt* is contained within the duplicated region. The duplication had no significant effect on the period of the rhythm of wild-type flies (data not shown), indicating that an essentially wild-type rhythm is obtained with 1, 2, or 3 doses of wild-type *dbt*.

dbt Mutations Alter the Periods of *per* and *tim* RNA and Protein Oscillations

To investigate whether *dbt* period-altering alleles change the molecular oscillation of known clock components, we first looked at PER and TIM protein time courses on

Table 1. Complementation Analysis of *dbt*

Genotype	tau ± SEM (#AR/n)
$dbt^S/Df(3R)tll\text{-}g$	19.1 ± 0.04 (1/25)
$dbt^S/TM6B$	21.7 ± 0.1 (1/11)
$Df(3R)tll\text{-}g/TM3$	24.8 ± 0.2 (3/5)
dbt^S/dbt^P	19.0 ± 0.1 (1/11)
$dbt^S/TM3$	21.8 ± 0.3 (1/4)
$dbt^P/TM3$	23.8 ± 0.1 (0/4)
$Dp(3;1)124P;\ dbt^S/dbt^S$	21.2 ± 0.1 (1/7)
dbt^S/dbt^S	18.4 ± 0.1 (3/10)
$Dp(3;1)124P;\ dbt^S/+$	22.5 ± 0.2 (0/5)
$dbt^S/+$	22.4 ± 0.1 (0/4)
$dbt^L/Df(3R)tll\text{-}g$	27.2 ± 0.1 (0/16)
$dbt^L/TM3$	25.1 ± 0.1 (1/14)
$Df(3R)tll\text{-}g/TM3$	24.7 ± 0.3 (1/6)
dbt^L/dbt^P	26.6 ± 0.1 (0/15)
$dbt^L/TM3$	25.0 ± 0.1 (0/14)
$dbt^P/TM3$	24.4 ± 0.3 (0/8)
$dbt^S/TM3$	21.5 ± 0.1 (0/19)
dbt^L/dbt^S	22.9 ± 0.1 (0/15)
$dbt^L/TM3$	24.9 ± 0.1 (0/13)
$Df(3R)tll\text{-}g/+$	23.9 ± 0.1 (1/7)
$dbt^P/Df(3R)tll\text{-}g$	†

Lines with the three alleles of *dbt* (dbt^S, dbt^L, and dbt^P) were crossed to each other and to lines containing *Df(3R)tll-g* or *Dp(3;1)124P*, and progeny with the indicated genotypes were tested in locomotor assays in DD. The average period (tau) of each genotype is indicated. Genotypes in each group came from the same cross. #AR/n, number of arrhythmic flies/total number of flies assayed. (†) indicates that no progeny survived to adulthood.

Western blots. We assayed 1 day of LD and 2 days of DD for wild-type, dbt^S, and dbt^L genotypes (Figure 3). Overall, the levels of expression of both PER and TIM are not grossly altered. However, in LD, both proteins oscillate with a slight phase advance in dbt^S, and phase delay in dbt^L. In DD, the proteins oscillate with a period length corresponding to the locomotor activity rhythms of the mutants. This is especially clear if cycling patterns of PER phosphorylation are examined, which are immune to differences in the loading of the protein samples (Edery et al., 1994). For dbt^S in DD, sharp transitions from hyperphosphorylated to hypophosphorylated PER occur between CT6 and CT10, next between CT22 and CT2, and on the last cycle between CT18 and CT22, giving an average periodicity of 18 hr (Figure 3B). Wild-type controls show hyper-to-hypophosphorylated PER transitions between CT6 and CT10 in both cycles, giving a 24 hr period (Figure 3B). Although shifts in mobility are less dramatic in dbt^L, transitions appear to occur between CT10 and CT14, and subsequently between CT14 and CT18, giving a 28 hr period (Figure 3B). For TIM, mobility differences for all genotypes are much smaller, but the strong phase differences among the genotypes (Figure 3A), and period differences in RNA expression patterns (below), indicate that period is likely altered as for PER.

A closer inspection of dbt^S reveals that both PER and TIM disappear prematurely in an LD cycle. Forms of PER with lowest electrophoretic mobility, which have been associated with highest levels of PER phosphorylation (Edery et al., 1994), appear earlier than in wild type (Figure 3C). In contrast, in dbt^L, both PER and TIM are detectable for an extended period of time in DD (see LD-to-DD transition, Figures 3A and 3B), and the appearance of low-mobility forms of PER is delayed (Figure 3C). In contrast to wild-type flies, we also detect persistence of PER in the absence of TIM after lights-on in an LD cycle in dbt^L (Figure 3B). This effect suggests unusual persistence of monomeric PER proteins after TIM is eliminated by light. The higher level of PER from ZT2–6 (ZT, zeitgeber time, indicates time in LD cycles) is not simply due to increased PER levels in dbt^L, since a side-by-side comparison of PER proteins in wild type and dbt^L at ZT0 shows roughly equal amounts of PER (Figure 3C). Thus, there seems to be an increase in PER stability in dbt^L. Conversely, dbt^S may cause premature degradation of both PER and TIM.

Figures 3D–3F show that *per* and *tim* RNA cycling can also be altered by mutation of *dbt*. Patterns of RNA cycling were followed in dbt^L mutants and in wild-type flies (only quantitation of wild-type data is shown). The first day of sampling (−1 to 0 in Figures 3E and 3F) occurred in LD, with subsequent days followed in DD. Although the initial LD cycles of *per* and *tim* RNA expression occurred with essentially the same phase in dbt^L and wild type, dbt^L gave three complete molecular cycles in ~3.5 days of DD, while wild-type flies produced three cycles in ~2.5–3 days of DD. Peaks of *per* and *tim* RNA accumulation occurred with an ~27 hr periodicity in dbt^L, and an ~23 hr periodicity in wild type (Figures 3E and 3F). Chi-square periodogram analysis of the data (TAU, Mini-Mitter, Sunriver, OR) gave estimates of ~27 and ~22 hr for dbt^L and wild type, respectively. The lower amplitude of the RNA rhythm in dbt^L as compared to wild type also resembles the molecular cycling observed in tim^L mutants (A. R., unpublished data). As peaks of RNA expression are not always coincident for *per* and *tim* in dbt^L, and the oscillations are of reduced amplitude, it is possible that the molecular rhythms are less stable in the mutant. However, the simplest interpretation of the data is that *per* and *tim* cycle together in the mutant with an ~27 hr period.

Isolation of a P-Element Insertion in the *dbt* Locus

Drosophila strains containing P-element insertions on the right arm of the third chromosome were screened for failure to complement the original dbt^S mutation. One strain, (derived from $P\{lacW\}l(3)j3B9^{j3B9}$, Berkeley Drosophila Genome Project), and referred to hereafter as dbt^P, behaved like the *Df(3R)tll-g* deficiency. dbt^S/dbt^P flies produced locomotor activity rhythms with a period of 19 hr, while $dbt^P/+$ flies had wild-type periods (23.8 hr; Table 1). Similarly, dbt^L/dbt^P flies had locomotor activity rhythms of 26.6 hr, similar to those obtained in $dbt^L/Df(3R)tll\text{-}g$ flies (Table 1). The P element is therefore likely to result in a large reduction, or even absence, of *dbt* gene products (demonstrated in the accompanying manuscript, Kloss et al., 1998, this issue of *Cell*). The finding that dbt^P fails to complement both dbt^S and dbt^L indicates that the latter mutations affect the same gene, a conclusion that has been confirmed by molecular studies of Kloss et al. (1998).

Recessive lethality is associated with the dbt^P strain,

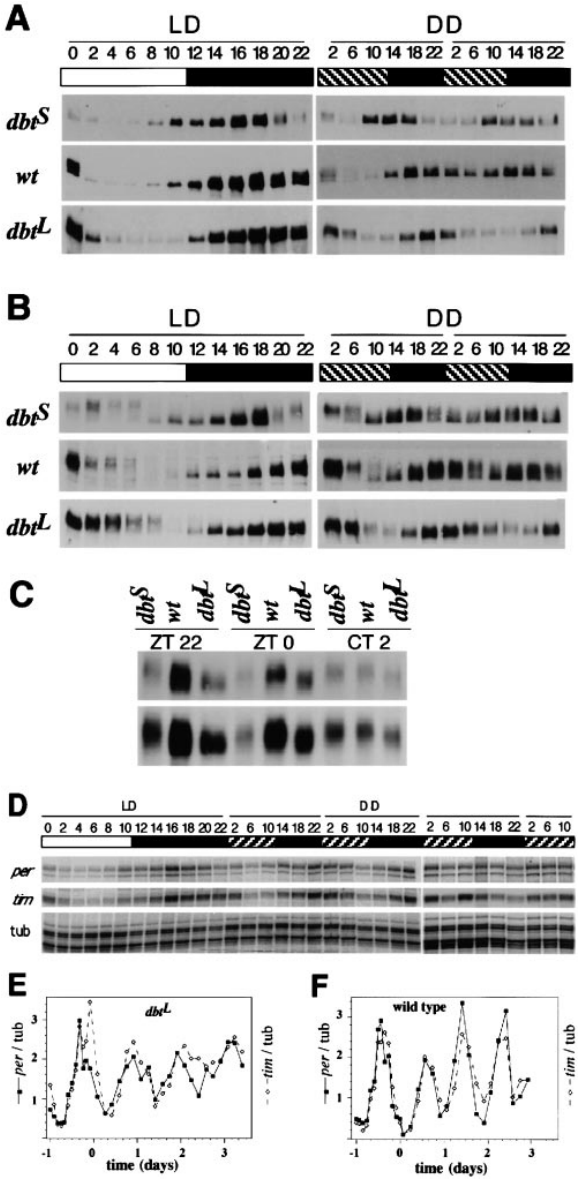


Figure 3. The Timing of *per* and *tim* RNA and Protein Oscillations Is Altered in dbt^S and dbt^L Adult Heads

Western blot analysis of (A) TIM and (B) PER proteins from dbt^S (top), wild type (wt, middle), and dbt^L (bottom) fly head extracts during 1 day of LD and 2 days of DD. The times of collection are indicated above the blots. Total protein levels in all lanes of a panel were judged to be similar by comparing a slower running cross-reacting band (data not shown). Note the successive phase changes in LD for dbt^S, wild type, and dbt^L, respectively. In additional, the PER phosphorylation rhythms differ in the three genotypes giving 18, 24, and 28 hr periods for dbt^S, wild type, and dbt^L, respectively (see text). Experiments for dbt^S and wild type were performed three times. dbt^L protein cycling was tested twice with comparable results. For dbt^S and wild-type images in (B), contrast was increased to clarify the signal for lanes CT6 and CT10 of day 1 in DD. (C) Side-by-side analysis of PER proteins at ZT22, ZT0, and CT2 from dbt^S, wild type, and dbt^L shows that, relative to wild type, PER phosphorylation is advanced in dbt^S and delayed in dbt^L. Note that three times as much total protein was used in the dbt^S lanes in this panel. Different exposures of the same gel are presented to clarify protein migration differences. The same result was obtained in two experiments. (D) Adult dbt^L flies were entrained to four LD cycles and then transferred to constant darkness. Collections were made at 2 hr intervals on the last day of LD, and at 4 hr intervals in subsequent DD. RNase protection assays were performed as described (Sehgal et al., 1995). Numbers above the lanes indicate zeitgeber time (ZT) in hours for LD and circadian time (CT) for DD. The samples were exposed and analyzed by a phosphorimager (Molecular Dynamics). (E–F) *per* and *tim* levels were assessed in reference to tubulin RNA, the arbitrary ordinate numbers representing proportional changes within the same genotype only. *per* and *tim* RNA peaks occur with ~27 hr periodicities for dbt^L, and ~23 hr periodicities for wild type (see text). The pattern of *per* and *tim* RNA cycling was determined in two experiments for dbt^L, and in five experiments for wild type with comparable results.

as no adults of the genotype dbt^P/dbt^P or *Df(3R)tll-g/* dbt^P have been recovered (Table 1). A strain, dbt^P/TM6, which produces homozygous larvae and pupae distinguishable from their heterozygous siblings by virtue of the dominant marker *Tubby* (on TM6), was constructed. Most third instar homozygous dbt^P larvae pupate, but they die later in pupal development. Proof that *dbt* function is required for both viability and circadian rhythmicity has come from reversion studies of dbt^P (Kloss et al., 1998).

Using Third Larval Instar Brain Clock Cells to Analyze dbt^P

We reasoned that the strongly hypomorphic allele dbt^P might show the most dramatic effects on clock gene cycling. Although dbt^P embryos take longer to develop into third instar larvae than do their heterozygous siblings, the foraging motility of these larvae and their touch sensitivity appear normal. In the analyses below, we only used clearly motile larvae.

Behavioral studies have demonstrated that a circadian clock is active in *Drosophila* larvae (Sehgal et al., 1992). It has recently been shown that a specific group of central brain cells is likely to compose the larval pacemaker (Kaneko et al., 1997). In each hemisphere of the third instar larval brain, four to five cells coexpress PER and TIM with circadian oscillations that are in phase with the oscillations of these proteins in adult pacemaker cells (Figures 4A and 5A; Kaneko et al., 1997). The only detectable staining in larval brain hemispheres for pigment-dispersing hormone (PDH), a marker for adult lateral neurons (Helfrich-Forster, 1995), is found in the cell bodies and axons of these PER-TIM expressing cells (Kaneko et al., 1997; Figure 5A; J. B., unpublished data). Therefore, the PER-TIM-PDH coexpressing larval brain cells can be considered larval lateral neurons (lvLNs). In per^o larvae, TIM is constitutively cytoplasmic in lvLNs (Figure 4C), and in tim^{01} larvae, PER is undetectable in these cells by immunocytochemistry (J. B., unpublished data). Both of these mutant phenotypes are characteristics of adult clock cells (Vosshall et al., 1994; Myers et al., 1996). PER and TIM oscillations can also be seen in two groups of cells at the anterior of the third instar larval brain, but in one of these groups, the oscillations are out of phase with the lvLNs and may be regulated by activity of the lvLNs (Kaneko et al., 1997). Since there are no good markers to distinguish clearly between these two anterior cell types, we have focused on the lvLNs in the analysis below.

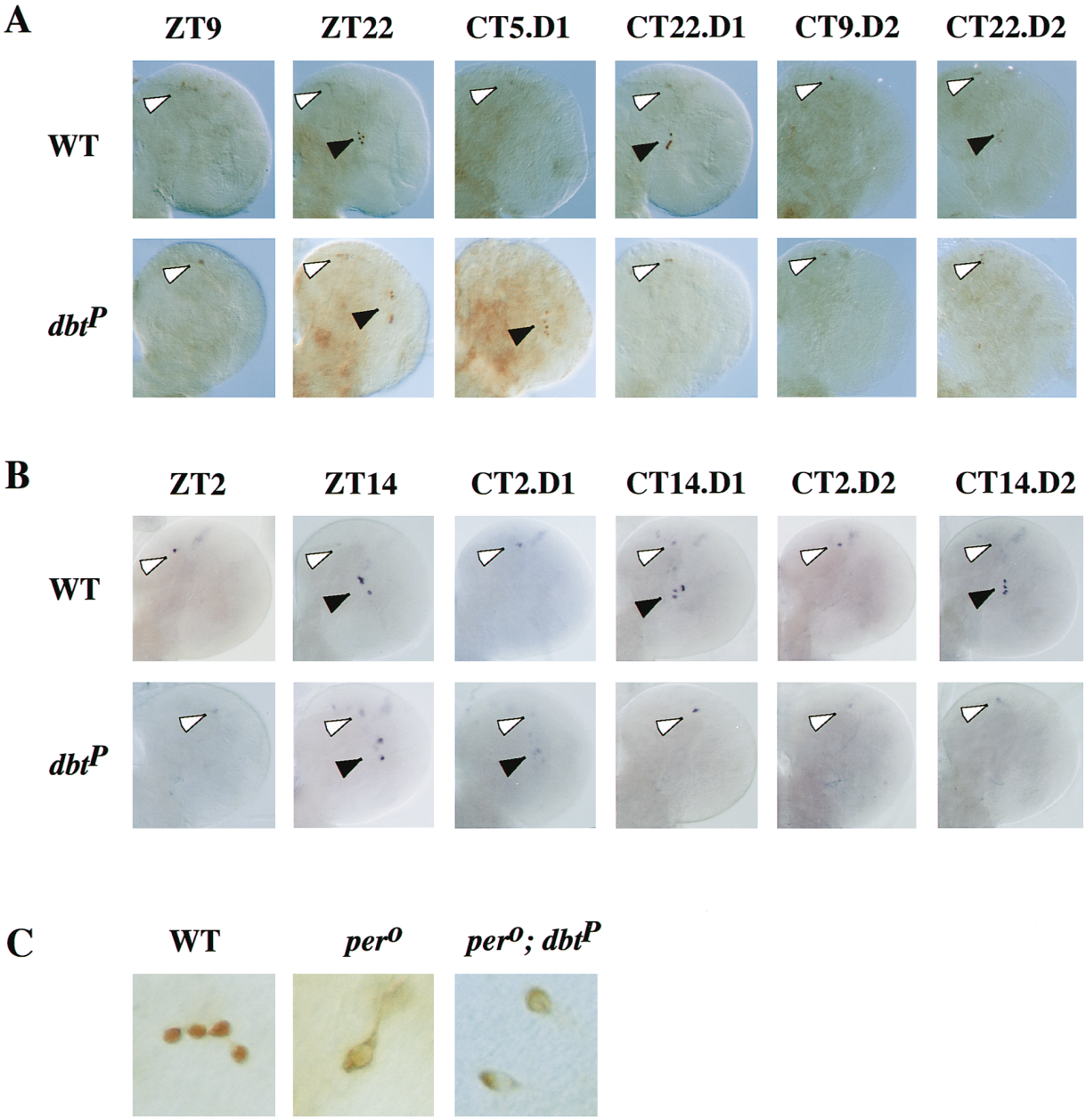


Figure 4. Circadian Oscillations of *tim* RNA and Protein Are Blocked in *dbt*^{P} Third Instar Larval Brains

Larvae were entrained for at least 3 days in standard LD cycles, with some transferred to DD for either 1 (D1) or 2 (D2) further days. Third instar larval brains were isolated at the times shown (ZT and CT indicate time in LD and DD cycles, respectively) and processed for either TIM protein (A and C) or *tim* RNA (B) as described in Experimental Procedures. Representative single hemispheres are shown in (A) and (B). Closed arrowheads indicate lvLNs, and open arrowheads indicate TIM-expressing anterior brain cells, which include cells that oscillate both in and out of phase with the lvLNs. Wild-type brains (WT, top panels in [A] and [B]) show robust oscillations of TIM protein and *tim* RNA in lvLNs in both LD and DD cycles. In contrast, lvLNs of *dbt*^{P} brains (bottom panels in [A] and [B]) show oscillations of TIM protein and *tim* RNA only in LD. On transfer to DD, TIM protein and *tim* RNA persist weakly in lvLNs at CT5 or CT2, respectively, and are subsequently not detected. Staining in the anterior brain cells serves as a control for the procedure. (C) High magnification of lvLNs showing TIM protein in wild type (left), *per*^{o} (center), and *per*^{o}; *dbt*^{P} (right) strains at ZT22. TIM is nuclear in wild type but forms cytoplasmic rings in *per*^{o} and *per*^{o}; *dbt*^{P}.

We first checked that lvLNs are present in *dbt*^{P} larvae. We detected PDH staining in the cytoplasm of four cells in each hemisphere (these are rarely detected in the same focal plane) in all *dbt*^{P} larval brains examined at different times in LD and DD cycles (Figure 5A and data not shown), as seen for wild-type larvae (Kaneko et al., 1997). The axons of these *dbt*^{P} lvLNs fasciculate and head to the anterior of the brain as in wild type. However, *dbt*^{P} lvLNs are found slightly more peripherally than in wild type, as seen in the staining patterns of PER and

TIM in Figures 4A, 4B, 5B, and 5F. This probably indicates a subtle developmental effect of *dbt* on the architecture of the brain, which might be expected given that the dbt^P mutation causes lethality before completion of pupal development.

Regulation of TIM's Light Sensitivity and Nuclear Localization Are Not Affected by dbt^P

In an LD cycle, TIM protein oscillates in the lvLNs in both wild-type and dbt^P larvae and is detected only during the dark phase of the cycle (Figure 4A, compare ZT9 and 22). Therefore, we conclude that the light sensitivity of TIM in lvLNs is not affected in a strongly hypomorphic *dbt* background. In a minority of dbt^P lvLNs counterstained with PDH, TIM is not detected at ZT22 (data not shown), suggesting that the TIM oscillation is not as robust in dbt^P as in wild type, and this is not surprising given the results found in DD below. We also examined TIM accumulation in per^o and per^o; dbt^P larvae. TIM accumulated at night in the cytoplasm of lvLNs in both strains in contrast to the predominantly nuclear localization observed in wild type (Figure 4C). A similar cytoplasmic accumulation of TIM is observed in the photoreceptors and LNs of adult per^o *Drosophila* (Myers et al., 1996; J. B., unpublished data). Therefore, *dbt* has no role in retaining TIM in the cytoplasm in the absence of PER (Myers et al., 1996; Saez and Young, 1996).

Oscillations of TIM Protein and *tim* RNA Cease in dbt^P Larval Brains in Constant Darkness

When wild-type larvae are transferred to constant darkness, TIM continues to oscillate robustly with only night time TIM accumulation in the lvLNs (Figure 4A; Kaneko et al., 1997). In contrast, in dbt^P larvae transferred to DD, TIM is weakly detected in lvLNs in the first subjective morning at CT5 (CT, circadian time, indicates time in DD), disappears by CT10 (data not shown), and is undetectable thereafter in the lvLNs (Figure 4A). TIM is always detected in dbt^P in the anterior larval brain cells, which serves as a positive control for the procedure. For the lvLNs, the differential effects of dbt^P on TIM in LD and DD shown in Figure 4A are not due to selecting larvae from slightly different developmental stages since identical results were derived from larvae that had been synchronized developmentally (data not shown).

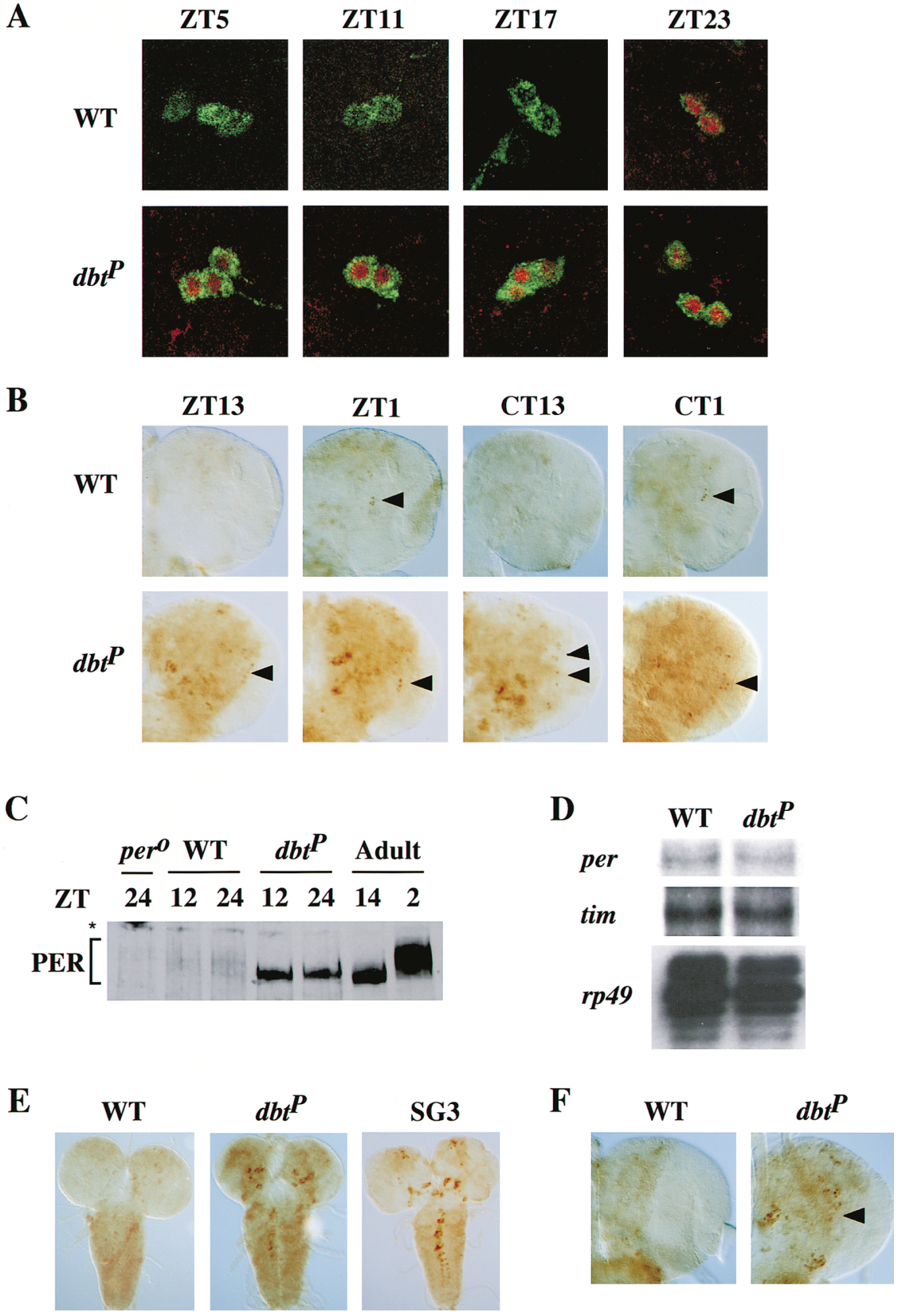
In wild-type larvae, *tim* RNA, as detected by in situ hybridization with an antisense *tim* probe, shows robust oscillations in the lvLNs in both LD and DD (Figure 4B). *tim* RNA levels oscillate in the lvLNs of dbt^P mutants in an LD cycle (Figure 4B), indicating that PER/TIM complexes can still negatively regulate *tim* gene expression in dbt^P larvae and that this regulation can be blocked by light-dependent degradation of TIM. When dbt^P larvae are transferred to DD, *tim* RNA is weakly detected in lvLNs on the first subjective morning (CT2) but is undetectable thereafter in these cells (Figure 4B). Thus, the effects of the dbt^P mutation on levels of TIM protein (Figure 4A) probably reflect more direct effects of dbt^P on *tim* RNA levels (Figure 4B).

PER Is Constitutively Expressed at High Levels in dbt^P Larval Brains

PER protein levels oscillate in wild-type lvLNs in an LD cycle, reaching peak levels at ZT23 of the four time points shown in Figure 5A, in agreement with Kaneko et al. (1997). In DD, PER continues to cycle in wild-type lvLNs and is detected at CT1 but not CT13 (Figure 5B; Kaneko et al., 1997). PER proteins produced by dbt^P larvae show three significant differences from wild type (Figures 5A and 5B). First, PER is constitutively expressed in lvLNs in LD and DD cycles. Second, the intensity of staining in the lvLNs is stronger in dbt^P than in wild-type larvae (samples shown were processed identically on the same day). Third, the pattern of expression in dbt^P is widened to include regions of the brain not significantly stained in a wild-type background (also seen clearly in Figure 5E). The elevated level of PER in dbt^P was confirmed by Western blotting using extracts from dissected larval brains collected in LD (Figure 5C). The latter results show that PER protein accumulation is dramatically increased by the dbt^P mutation (compare dbt^P to wild-type larvae, where PER is difficult to detect, Figure 5C), and the high levels of accumulated PER protein do not show significant differences between ZT12 and ZT24 (Figure 5C) in LD cycles in dbt^P. In addition, the electrophoretic mobility of PER proteins is relatively high and uniform in dbt^P larvae, in contrast to the broad spectrum of lower PER protein mobilities observed in wild-type larvae and adult heads (Figure 5C). As the spectrum of protein mobilities in wild-type *Drosophila* reflects PER protein phosphorylation (Edery et al., 1994), the results suggest that PER is hypophosphorylated in dbt^P mutants.

To determine whether the high levels of PER protein found by Western blotting of dissected dbt^P larval brains reflected altered *per* RNA levels, we used RNase protection to detect *per* RNA in these tissues at ZT14–16 (time of expected peak *per* RNA accumulation in wild-type *Drosophila*). The results in Figure 5D show that *per* RNA is expressed at similar levels in wild-type and dbt^P larval brains. Thus, the aberrant accumulation of PER proteins in dbt^P mutants does not reflect increased *per* transcription or *per* RNA stability but must be downstream of these events.

The pattern of PER expression in dbt^P is similar to a PER-β-galactosidase fusion protein, PER-SG, expressed from the *per* promoter (Figure 5E). In adults, *per*-SG RNA oscillates, but PER-SG protein does not. In fact, the PER-SG protein accumulates over progressive cycles, suggesting that it is a stable protein (Vosshall et al., 1994; Dembinska et al., 1997). PER-SG, detected with an antibody against β-galactosidase, is expressed in larvae in the lvLNs and other cell clusters in the brain hemispheres, as well as cells adjacent and close to the ventral ganglion midline (Figure 5E; Kaneko et al., 1997). The presence of the noncycling PER-SG fusion protein therefore marks cells in which the *per* promoter is active, or has been active, during development. Comparable patterns were seen with two independent SG lines (Kaneko et al., 1997). PER in wild-type larvae has also been detected at very low levels in these cells (Kaneko et al., 1997). Thus, the pattern of staining for PER we see in


A
ZT5
ZT11
ZT17
ZT23
WT
dbtP
B
ZT13
ZT1
CT13
CT1
WT
dbtP
C
perO
WT
dbtP
Adult
ZT
24
12
24
12
24
14
2
*
PER
D
WT
dbtP
per
tim
rp49
E
WT
dbtP
SG3
F
WT
dbtP

dbt^P reflects the normal spatial expression of *per*, but this pattern is only easily visible with a stable fusion protein or in a *dbt*^{P} background.

PER Is Stable in *dbt*^{P} Larvae in Constant Light

PER is detected at high levels in *dbt*^{P} larval brains in DD as in LD (Figure 5B). The persistence of PER proteins in lvLNs in DD is presumably occurring in the presence of very low levels of the TIM protein, which, as indicated above, fall below immunocytochemical detection in these cells in DD (Figure 4A). In *dbt*$^+$ larvae and adults, PER accumulation is suppressed in the absence of TIM (Vosshall et al., 1994; Price et al., 1995; J. B., unpublished data). It therefore seemed likely that PER in *dbt*^{P} had become less dependent on TIM for its accumulation than in wild type, especially since PER is detectable in brain cells where TIM is not detected in wild-type or *dbt*^{P} larvae (Figures 4A and 5E; J. B., unpublished data; Kaneko et al., 1997). Ideally we would have tested this possibility using *tim*^{01}; *dbt*^{P} larvae. However, it has not been possible to obtain third instar larvae from two different *tim*^{01}; *dbt*^{P}/TM6 lines. This may indicate a shift in the lethal phase of *dbt*^{P} by the *tim*^{01} mutation (J. B., unpublished data). An alternative to *tim*^{01} was possible as constant light (LL) in a wild-type background produces a *tim*^{01} phenocopy through light-dependent degradation of TIM (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Figure 5F shows that in wild-type larvae, PER accumulation is suppressed in response to LL as previously seen for adults. In contrast, in *dbt*^{P} larvae raised in LL, PER continues to be strongly detected in the lvLNs and the other PER-expressing cells (Figure 5F). The persistence of PER in DD and LL indicates that PER proteins can accumulate in *dbt*^{P} mutants even with very low levels of TIM.

Discussion

What is the Role of *dbt* in the Clock?

tim RNA and protein cycle in LD, and TIM remains cytoplasmic in *per*^{o}; *dbt*^{P}, indicating that at least some regulatory features of the *Drosophila* clock are unaffected in *dbt*^{P} mutants. In contrast, PER is constitutively expressed at very high levels in LD and DD cycles in *dbt*^{P} mutants, with increased levels also seen in new regions of the larval brain. These observations, and the finding that increased PER accumulation is not due to increased *per* RNA production, suggest that *dbt* affects circadian rhythmicity through PER protein. Altered patterns of *tim* RNA and TIM protein accumulation in *dbt*^{P} are presumably secondary effects derived from the substantially increased accumulation of PER.

dbt Appears to Affect Stability of the PER Protein

The high levels of PER observed in *dbt*^{P} are due either to increased *per* RNA translation or increased PER stability. Altered patterns of PER degradation in *dbt*^{S} and *dbt*^{L} mutants could also reflect changes in either translational control or stability: PER in *dbt*^{S} starts to accumulate 2 hr before wild type, but most PER disappears at least 6 hr before loss of the wild-type protein (Figure 3B). This is consistent with decreased stability of PER in *dbt*^{S} mutants, although we cannot rule out the possibility that in *dbt*^{S}, *per* RNA available at ZT20 is translated significantly less efficiently than at other times of day. In wild-type flies, light degrades TIM, which promotes elimination of PER. However, in *dbt*^{L} mutants, PER remains for about 4 hr longer than in wild type. Since these are times of day when little *per* RNA is present (Figure 3D), the results suggest that the *dbt*^{L} mutation reduces the rate of PER degradation. The low electrophoretic mobility observed for PER in *dbt*^{L} mutants collected from ZT2 to ZT6 indicates hyperphosphorylation of the persistent PER proteins, which also suggests that these proteins were translated many hours earlier (Edery et al., 1994).

The electrophoretic mobility of PER in *dbt*^{P} mutants differs significantly from wild type: its migration in relation to PER proteins formed in wild-type larval brains and in adult heads suggests that it is constitutively hypophosphorylated (Figure 5C and data not shown). Since a substantial portion of the PER accumulating in *dbt*^{P} larval brains is derived from nonclock cells, we cannot rule out the possibility that PER is more highly phosphorylated in lvLNs, but slower-migrating PER proteins have never been observed in *dbt*^{P} mutants despite high protein levels. We suggest that *dbt* mutants affect PER stability and circadian rhythmicity by altering PER phosphorylation. This is also consistent with the results of

Figure 5. PER Is Constitutively Expressed and Accumulates to Unusually High Levels in *dbt*^{P} Third Instar Larval Brains

(A) Brains were dissected at different times of one LD cycle from entrained third instar larvae, incubated with antibodies against PER (shown in red) and PDH (green), and visualized by confocal microscopy (see Experimental Procedures). PDH is a highly specific marker for the lvLNs. PER oscillates in wild-type (WT) lvLNs but is constitutively expressed in *dbt*^{P} mutants.

(B) Whole brain hemispheres are shown with PER protein detected as described in Experimental Procedures. Arrowheads indicate lvLNs. PER protein oscillates in wild-type (WT) lvLNs, with staining only at ZT1 and CT1. PER is detectable in *dbt*^{P} lvLNs at all four time points shown, as well as strongly in other cell clusters.

(C) Western blot of extracts from ten larval brains for *per*^{o}, wild-type, and *dbt*^{P} larvae at ZT12 and ZT24 (ZT24 coincides with peak PER accumulation in wild type) run alongside 2 μg protein from wild-type adult heads from either ZT14 or ZT2. The asterisk marks a cross-reacting band that serves as a loading control, confirming that PER accumulates to higher levels in *dbt*^{P} than in wild type. PER is difficult to detect in wild-type brain extracts, but a PER signal distinguishes wild type from the *per*^{o} control. (D) RNase protection of *per*, *tim*, and *rp49* RNA from 10 μg wild type (left) or *dbt*^{P} (right) larval brain RNA at ZT14–16 (predicted peak for *per* RNA expression in wild type). Relative to *rp49*, *per* and *tim* levels are similar in wild type and *dbt*^{P}.

(E) Whole brains of wild-type (left) and *dbt*^{P} larvae (center) at ZT1 stained with anti-PER, and SG3 (right) stained with anti-β-gal antibodies. The PER-SG3 pattern is very similar to PER in *dbt*^{P}, indicating that the pattern of PER protein seen in *dbt*^{P} larval brains reflects the normal activity of the *per* promoter. (F) Brain hemispheres from wild-type (left) or *dbt*^{P} larvae (right) raised in constant light from embryogenesis onward and stained for PER show that PER accumulates to high levels in lvLNs and many additional cells in *dbt*^{P} mutants but is not detected in wild type.

dbt sequence analysis, which has shown that the DBT protein is very closely related to human casein kinase Iε (Kloss et al., 1998). Therefore, the most likely role for DBT in the *Drosophila* clock is as a mediator of post-translational modification that determines PER stability. We cannot rule out that *per* translation is also altered in the *dbt* mutants, but we have no evidence for this.

PER in dbt^P behaves much like the PER-β-galactosidase fusion protein, PER-SG, in wild-type flies. PER-SG is expressed widely in the larval brain and is detectable at high levels in adults even in the absence of TIM (Vosshall et al., 1994) as well as in constant light in adults and larvae (J. L. P. and J. B., unpublished data). In adults, *per*-SG RNA cycles, but the protein does not, indicating that PER-SG is either more stable than PER or its translation is much more efficient. Adding an additional 231 amino acids of PER to PER-SG to make the fusion protein PER-BG results in cycling of this larger fusion protein (Dembinska et al., 1997). If the 231 amino acid difference between PER-SG and PER-BG indeed confers instability, one interpretation of our data is that DBT works through this region of PER. We are currently testing this hypothesis.

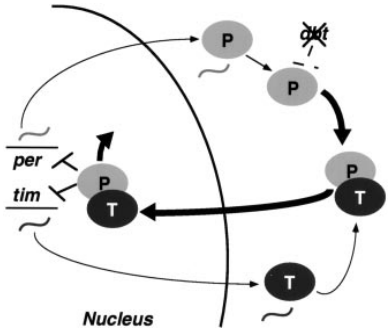


Figure 6. Model Depicting Influence of *dbt* on Circadian Rhythms through Altered Stability of PER

In dbt^P mutants, loss of DBT activity results in increased stability of monomeric PER proteins. In constant darkness, high titers of stable PER promote constitutive nuclear translocation of PER/TIM heterodimers. An equilibrium is established in which constitutive production of nuclear PER/TIM dimers is balanced with constitutively low levels of expression of *per* and *tim* RNA. High stability of PER in dbt^P leads to a pool of nuclear PER monomers as TIM turns over in nuclei.

Where Does DBT Act in the Cell?

In tim^{01} mutants, which block PER nuclear translocation, PER is unstable (Vosshall et al., 1994; Price et al., 1995), indicating the presence of a cytoplasmic activity that destabilizes PER monomers. In wild-type adults, constant light suppresses TIM, which subsequently results in very low levels of PER (Price et al., 1995), and we see the same result in wild-type third instar larvae raised in constant light (Figure 5F). However, in dbt^P mutants, similar high levels of PER accumulate in LD, DD, and LL. Since the dbt^P allele allows comparable PER accumulation with either high or low levels of TIM, we conclude that dbt^P allows TIM-independent PER accumulation and that DBT is a component of the cytoplasmic activity that destabilizes PER monomers in wild-type and tim^{01} flies. Consistent with this conclusion, we observe predominantly cytoplasmic accumulation for the expanded PER pattern in dbt^P larval brains (J. B., unpublished data). *tim* does not appear to be expressed in this expanded pattern in wild-type larvae (Kaneko et al., 1997), so expanded accumulation of PER monomers in dbt^P, but not wild-type, larval brains indicates novel cytoplasmic stability for PER in the mutant.

There is also evidence that DBT influences stability of nuclear PER proteins. PER is detected immunocytochemically in nuclei of dbt^S and dbt^L lvLNs prior to its disappearance (J. B., unpublished data), suggesting that the differing kinetics of PER degradation in wild type, dbt^S, and dbt^L reflect different rates of PER elimination from the nucleus. Increased nuclear stability of PER monomers is also apparent from our analyses of dbt^P lvLNs: PER is lost from wild type but persists in dbt^P nuclei after exposure to light has removed most TIM proteins (Figures 5A and 5F; J. B., unpublished data). Thus, *dbt* may affect the stability of both cytoplasmic and nuclear PER monomers. This does not mean that DBT must function in both subcellular compartments, since a posttranslational modification generated in the cytoplasm could have delayed effects in the nucleus. We are generating antibodies to determine the subcellular location of DBT.

A Basis for Light-Driven Molecular Cycles in dbt^P Mutants

If PER is constitutively expressed in the nuclei of lvLNs in dbt^P, why are *tim* RNA oscillations seen in LD cycles, but not in DD? Prior work has indicated that suppression of *per* and *tim* RNA levels requires activity of a PER/TIM complex (reviewed in Rosbash et al., 1996; Young et al., 1996). Because light eliminates TIM proteins, LD cycles will periodically eliminate PER/TIM complexes but generate a large pool of long-lived, monomeric PER proteins in dbt^P mutants. The latter proteins evidently have little or no influence on *per* and *tim* transcription. One interpretation of our results is that in DD, dbt^P mutants produce a low level of *tim* transcription that is maintained by constitutive formation, nuclear translocation, and nuclear activity of PER/TIM complexes. In wild-type *Drosophila*, PER's cytoplasmic instability allows high levels of *per* and *tim* RNA to accumulate prior to formation of PER/TIM complexes, promoting oscillations of RNA and protein accumulation. Stable accumulation of cytoplasmic PER monomers in dbt^P mutants should allow an equilibrium to be established in DD in which constitutive transfer of PER/TIM complexes to nuclei produces two effects: low levels of RNA expression and a sizeable pool of nuclear PER monomers that are evidently much more stable than TIM (Figures 4A, 5A, and 5B). Although we do not detect *tim* RNA or protein in dbt^P in DD immunocytochemically, this probably reflects how even a very low level of TIM (which is stable in the absence of light) is sufficient to maintain continuous nuclear entry of PER/TIM complexes when PER proteins are stabilized (further evidence is discussed below). We suggest that an important difference between wild type and dbt^P *Drosophila* is that most PER monomers are degraded with only a small fraction of the proteins escaping to form PER/TIM complexes in

wild-type flies, whereas stable PER proteins in dbt^P mutants now bind TIM without temporal constraints. This interpretation is summarized in Figure 6.

In constant light, we do see PER translocation to the nucleus in the dbt^P lvLNs, but this probably reflects that light is less effective at removing TIM than the tim^{01} mutation and that increased *tim* transcription during the period of lights-on can offset some light-induced TIM depletion (Figure 4B). The results also indicate that a very low level of residual TIM protein can translocate highly stabilized PER to the nucleus. A precedent for nuclear translocation of PER in constant light exists with the PER-β-gal fusion protein, PER-SG, which is stable cytoplasmically in tim^{01} but can be found in the nucleus in tim^+ LNs and photoreceptors in constant light (J. L. P., unpublished data).

In summary, we have characterized *double-time*, a novel gene required for circadian rhythms in *Drosophila*. Evidence has been presented that *dbt* contributes to circadian rhythmicity by determining the stability of PER. We have proposed that DBT activity in wild-type flies promotes molecular cycles of *per* and *tim* expression by ensuring the instability of monomeric PER proteins, thus allowing PER accumulation only in conjunction with high titers of TIM.

Experimental Procedures

Locomotor and Eclosion Assays

Monitoring and analysis of locomotor activity of individual flies, and chi-square periodogram analysis, were performed as described previously (Sehgal et al., 1992). For eclosion assays, populations of flies with the indicated genotypes were reared in pint-size plastic bottles and entrained in LD at 25°C. They were then transferred to DD, and emerging adults collected and counted every 2 hr. Bottles in DD were handled under a darkroom red light (15 W bulb, Kodak GBX-2 filter), the wavelength of which has no effect on *Drosophila* circadian rhythms. Alternatively, emerging flies were counted with a TriKinetics eclosion monitoring system (Konopka et al., 1994) in DD. Period estimates were obtained by chi-square periodogram analysis.

Larval Immunostaining and In Situ Hybridization

Larvae were entrained for at least 3 days before an experiment in a standard 12 hr:12 hr light:dark cycle at 25°C. Larval brains were processed for immunostaining using a modified protocol of Kunes et al. (1993). Brains were dissected from entrained wandering third instar larvae, treated with 1 mg/ml Sigma blend collagenase in PBS with 50 μM $CaCl_2$ for 15 min at room temperature, and then fixed for 30 min in 4% paraformaldehyde in PBS. Brains were permeabilized and rinsed in PBS, 1% Triton for 2 periods of 20 min and then blocked in PBS, 0.5% Triton (PBT) with 10% heat-inactivated goat serum for 1 hr or longer. Primary antibodies were incubated overnight at 4°C in blocking solution. For immunocytochemistry, anti-TIM 316, anti-PER (Stanewsky et al., 1997), and anti-lacZ (Promega) antibodies were used at dilutions of 1:1500, 1:16,000, and 1:1000, respectively. For immunofluorescence, rat anti-PER (made against PER amino acids 876–1224) and anti-PDH antibodies were used at dilutions of 1:200 and 1:50,000, respectively. PER and TIM antisera were preabsorbed against per^o or tim^{01} embryos, and neither antibody gave significant staining on the relevant control. The rest of the procedures were carried out at room temperature according to standard procedures and viewed either by light microscopy for immunocytochemistry or by confocal microscopy for immunofluorescence. The results shown are representatives of at least 15 larvae analyzed for each time point shown, from at least two independent experiments, which all gave consistent results.

In situ hybridizations were performed as described in Tautz and Pfeifle (1989), except that proteinase K was used at 5 μg/ml. The TIM probe was synthesized from pSK-TIM2 (Myers et al., 1997) using DIG RNA labeling mix (Boehringer Mannheim).

RNase Protection and Western Blotting

RNase protection assays were performed as previously described for adult head RNA samples (Sehgal et al., 1995). For larval brains, 10 μg RNA (isolated from approximately 50 dissected brains) were used for one lane of an experiment using the Ambion RPAII kit. The *per* riboprobe protects nucleotides 1426–1749 of the *per* cDNA; the *tim* probe was as in Myers et al. (1997); and the *rp49* probe protects nucleotides 323–381 of the *rp49* cDNA. *per* and *tim* probes were synthesized with only radioactive UTP, while *rp49* probe synthesis included 5 μM nonradioactive UTP.

For adult head Western blots, extracts were made as in Edery et al. (1994), and 100 μg protein was loaded per lane. For larval brain extracts, 10 larval brains were dissected in PBS, frozen, boiled in 30 μl 1.1× SDS loading buffer, and spun, and the supernatant was loaded. All samples were run on 5.7% (75:1) SDS-polyacylamide gels (Edery et al., 1994) and transferred with either a semidry or wet transfer apparatus (Bio-Rad). Blots were incubated overnight at 4°C with either anti-PER (Stanewsky et al., 1997) or anti-TIM (Myers et al., 1996) at dilutions of 1:10,000 or 1:2000, respectively. For washes, secondary antibody incubation, and ECL (Amersham), we followed standard procedures.

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References

Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. (1997). A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. Cell *91*, 1055–1064.

Allada, R., White, N.E., So, W.V., Hall, J.C., and Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian clock disrupts circadian rhythms and transcription of period and timeless. Cell *93*, 791–804.

Bruce, V.G. (1972). Mutants of the biological clock in *Chlamydomonas reinhardi*. Genetics *70*, 537–548.

Crosthwaite, S.K., Dunlap, J.C., and Loros, J.J. (1997). *Neurospora wc-1* and *wc-2*: transcription, photoresponses, and the origins of circadian rhythmicity. Science *276*, 763–769.

Curtin, K.D., Huang, Z.J., and Rosbash, M. (1995). Temporally regulated nuclear entry of the *Drosophila period* protein contributes to the circadian clock. Neuron *14*, 365–372.

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S., and Kay, S.A. (1998). Closing the circadian feedback loop: CLOCK induced transcription of its own inhibitors, *period* and *timeless*. Science *280*, 1599–1603.

Dembinska, M.E., Stanewsky, R., Hall, J.C., and Rosbash, M. (1997). Circadian cycling of a PERIOD-β-galactosidase fusion protein in *Drosophila*: evidence for cyclical degradation. J. Biol. Rhythms *12*, 157–172.

Dunlap, J.C. (1996). Genetic and molecular analysis of circadian rhythms. Annu. Rev. Genet. *30*, 579–601.

Edery, I., Zwiebel, L.J., Dembinska, M.E., and Rosbash, M. (1994). Temporal phosphorylation of the *Drosophila* period protein. Proc. Natl. Acad. Sci. USA *91*, 2260–2264.

Emery, I.F., Noveral, J.M., Jamison, C.F., and Siwicki, K.K. (1997). Rhythms of *Drosophila* period gene expression in culture. Proc. Natl. Acad. Sci. USA *94*, 4092–4096.

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.

Feldman, J.F., and Hoyle, M.N. (1973). Isolation of circadian clock mutants of *Neurospora crassa*. Genetics *75*, 605–613.

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.

Gekakis, N., Saez, L., Delahaye-Brown, A.-M., Myers, M.P., Sehgal, A., Young, M.W., and Weitz, C.J. (1995). Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PER^{L}. Science *270*, 811–815.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

Giebultowicz, J.M., and Hege, D.M. (1997). Circadian clock in Malpighian tubules. Nature *386*, 664.

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.

Helfrich-Forster, C. (1995). The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *92*, 612–616.

Hogenesch, J.B., Gu, Y.-Z., Jain, S., and Bradfield, C.A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc. Natl. Acad. Sci. USA *95*, 5474–5479.

Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the *Drosophila* protein Timeless suggests a mechanism for resetting the circadian clock by light. Cell *84*, 677–685.

Ikeda, M., and Nomura, M. (1997). cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS protein (BMAL1) and identification of alternative translation initiation site usage. Biochem. Biophys. Res. Commun. *233*, 258–264.

Kaneko, M., Helfrich-Forster, C., and Hall, J.C. (1997). Spatial and temporal expression of the period and timeless genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. J. Neurosci. *17*, 6745–6760.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D.L., Hotz Vitaterna, M., Kornhauser, J.M., Lowrey, P.L., et al. (1997). Positional cloning of the mouse circadian *Clock* gene. Cell *89*, 641–653.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998). The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell, this issue, *94*, 97–107.

Kondo, T., Tsinoremas, N.F., Golden, S.S., Johnson, C.H., Kutsuna, S., and Ishiura, M. (1994). Circadian clock mutants of cyanobacteria. Science *266*, 1233–1236.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *68*, 2112–2116.

Konopka, R.J., Hamblen-Coyle, M.J., Jamison, C.F., and Hall, J.C. (1994). 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 *9*, 189–216.

Kunes, S., Wilson, C., and Steller, H. (1993). Independent guidance of retinal axons in the developing visual system of *Drosophila*. J. Neurosci. *13*, 752–767.

Lee, C., Parikh, V., Itsukaichi, T., Bae, K., and Edery, I. (1996). Resetting the *Drosophila* clock by photic regulation of PER and a PER–TIM complex. Science *271*, 1740–1744.

Leloup, J.-C., and Goldbeter, A. (1998). A model for circadian rhythms in *Drosophila* incorporating the formation of a complex between the PER and TIM proteins. J. Biol. Rhythms *13*, 70–87.

Lewis, E.B., and Bacher, F. (1968). Method for feeding ethyl methane-sulfonate (EMS) to *Drosophila* males. Dros. Inf. Serv. *43*, 193.

Millar, A.J., Carre, I.A., Strayer, C.A., Chua, N.-H., and Kay, S.A. (1995). Circadian clock mutants in *Arabidopsis* identified by luciferase imaging. Science *267*, 1161–1163.

Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A., and Young, M.W. (1996). Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science *271*, 1736–1740.

Myers, M.P., Rothenfluh, A., Chang, M., and Young, M.W. (1997). Comparison of chromosomal DNA composing *timeless* in *Drosophila melanogaster* and *D. virilis* suggests a new conserved structure for the TIMELESS protein. Nucleic Acids Res. *25*, 4710–4714.

Pittendrigh, C.S. (1967). Circadian Systems. I. The driving oscillation and its assay in *Drosophila* pseudoobscura. Proc. Natl. Acad. Sci. USA *58*, 1762–1767.

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

Plautz, J.D., Kaneko, M., Hall, J.C., and Kay, S.A. (1997). Independent photoreceptive circadian clocks throughout *Drosophila*. Science *278*, 1632–1635.

Price, J.L., Dembinska, M.E., Young, M.W., and Rosbash, M. (1995). Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*. EMBO J. *14*, 4044–4049.

Ralph, M.R., and Menaker, M. (1988). A mutation of the circadian system in golden hamsters. Science *241*, 1225–1227.

Rosbash, M., Allada, R., Dembinska, M., Guo, W.Q., Le, M., Marrus, S., Qian, Z., Rutila, J., Yaglom, J., and Zeng, H. (1996). A *Drosophila* circadian clock. Cold Spring Harb. Symp. Quant. Biol. *61*, 265–278.

Rutila, J.E., Zeng, H., Le, M., Curtin, K.D., Hall, J.C., and Rosbash, M. (1996). The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron *17*, 921–929.

Rutila, J.E., Suri, V., Le, M., So., W.V., Rosbash, M., and Hall, J.C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila* period and timeless. Cell *93*, 805–814.

Saez, L., and Young, M.W. (1996). Regulation of nuclear entry of the *Drosophila* clock proteins Period and Timeless. Neuron *17*, 911–920.

Sehgal, A., Price, J., and Young, M.W. (1992). Ontogeny of a biological clock in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *89*, 1423–1427.

Sehgal, A., Price, J.L., Man, B., and Young, M.W. (1994). Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. Science *263*, 1603–1606.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P., and Young, M.W. (1995). Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. Science *270*, 808–810.

Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F.J., and Reppert, S.M. (1997). Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron *19*, 1261–1269.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J.J., Dunlap, J.C., and Okamura, H. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. Cell *91*, 1043–1053.

Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M.J., Rosbash, M., and Hall, J.C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the *Drosophila* clock gene *period* and a *lacZ* reporter: mapping elements of the PER protein involved in circadian cycling. J. Neurosci. *17*, 676–696.

Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., and Lee, C.C. (1997). *RIGUI*, a putative mammalian ortholog of the *Drosophila period* gene. Cell *90*, 1003–1011.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. Chromosoma *98*, 81–85.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M., and Sakaki, Y. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. Nature *389*, 512–516.

Vitaterna, M.H., King, D.P., Chang, A.-M., Kornhauser, J.M., Lowrey, P.L., McDonald, J.D., Dove, W.F., Pinto, L.H., Turek, F.W., and Takahashi, J.S. (1994). Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. Science *264*, 719–725.

Vosshall, L.B., Price, J.L., Sehgal, A., Saez, L., and Young, M.W. (1994). Block in nuclear localization of *period* protein by a second clock mutation, *timeless*. Science *263*, 1606–1609.

Vosshall, L.B., and Young, M.W. (1995). Circadian rhythms in *Drosophila* can be driven by *period* expression in a restricted group of central brain cells. Neuron *15*, 345–360.

Young, M.W., Wager-Smith, K., Vosshall, L., Saez, L., and Myers, M.P. (1996). Molecular anatomy of a light-sensitive circadian pacemaker in *Drosophila*. Cold Spring Harb. Symp. Quant. Biol. *61,* 279–284.

Zeng, H., Qian, Z., Myers, M.P., and Rosbash, M. (1996). A light-entrainment mechanism for the *Drosophila* circadian clock. Nature *380*, 129–135.