

# Phosphorylation of PERIOD Is Influenced by Cycling Physical Associations of DOUBLE-TIME, PERIOD, and TIMELESS in the *Drosophila* Clock

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## Summary

The clock gene *double-time* (*dbt*) encodes an ortholog of casein kinase I $\epsilon$  that promotes phosphorylation and turnover of the PERIOD protein. Whereas the *period* (*per*), *timeless* (*tim*), and *dClock* (*dClk*) genes of *Drosophila* each contribute cycling mRNA and protein to a circadian clock, *dbt* RNA and DBT protein are constitutively expressed. Robust circadian changes in DBT subcellular localization are nevertheless observed in clock-containing cells of the fly head. These localization rhythms accompany formation of protein complexes that include PER, TIM, and DBT, and reflect periodic redistribution between the nucleus and the cytoplasm. Nuclear phosphorylation of PER is strongly enhanced when TIM is removed from PER/TIM/DBT complexes. The varying associations of PER, DBT and TIM appear to determine the onset and duration of nuclear PER function within the *Drosophila* clock.

## Introduction

In *Drosophila*, circadian behavioral rhythms are controlled by “clock” genes that interact to form autoregulatory loops (Dunlap, 1999; Scully and Kay, 2000; Young, 2000). Activation of the *period* (*per*) and *timeless* (*tim*) genes depends on two transcription factors, dCLOCK (CLK) and CYCLE (CYC), which bind to the *per* and *tim* promoters. PER and TIM proteins suppress the activities of dCLK and CYC, but only after PER and TIM heterodimerize and translocate to nuclei. The PER and TIM proteins also positively regulate levels of *dClk* RNA (Dunlap, 1999; Scully and Kay, 2000; Young, 2000). Formation of PER/TIM complexes is delayed by the action of DOUBLE-TIME (DBT), a kinase that promotes PER phosphorylation and turnover in the absence of high levels of TIM (further reviewed later). These regulatory interactions lead to cycling expression of *per*, *tim*, and *dClk* (Scully and Kay, 2000; Young, 2000).

Mutant alleles of *dbt* have been identified that shorten, lengthen, or abolish circadian behavioral rhythms. Such mutations similarly affect *per* and *tim* mRNA and protein oscillations (Price et al., 1998; Suri et al., 2000; Rothen-

fluh et al., 2000c). Strong hypomorphic mutations of *dbt* also influence cell proliferation and cell survival (Kloss et al., 1998; Zilian et al., 1999). *dbt* encodes a protein similar to human casein kinase I $\epsilon$  (CKI $\epsilon$ ; Kloss et al., 1998). Because PER and DBT form a complex both in vitro and in cultured *Drosophila* S2 cells (Kloss et al., 1998), PER may be a direct substrate of DBT. Such a role for DBT is also suggested by recent findings in mammals. The *tau* mutation, which causes short-period (20 hr) circadian behavior in the hamster, involves an amino acid change in CKI $\epsilon$  (Lowrey et al., 2000). The wild-type hamster kinase binds and phosphorylates mammalian PER proteins in vitro and in cultured cells, but PER phosphorylation is defective when the *tau* mutant kinase is employed in such assays (Lowrey et al., 2000). In humans, Familial Advance Sleep-Phase Syndrome (FASPS) is caused by a missense mutation in hPer2. The mutation maps to a region of hPER2 that binds to CKI $\epsilon$ , and changes a consensus CKI $\epsilon$  target site. This mutation shortens the period of human circadian rhythms and causes hypophosphorylation of hPER2 by CKI $\epsilon$  in vitro (Toh et al., 2001).

In contrast to its mammalian orthologs, bacterially produced, recombinant DBT has shown no enzymatic activity in vitro, suggesting a requirement for additional factors, or presence of an activating modification of DBT or its substrate(s) in vivo (Suri et al., 2000; S. Kivimäe, L.S., and M.W.Y., unpublished observation). Nevertheless, it has been demonstrated that PER phosphorylation is dependent on DBT in pacemaker cells of the organism, properties not yet demonstrated for mammalian PER and casein kinase I $\epsilon$  (Price et al., 1998; Kloss et al., 1998; Lowrey et al., 2000; Vielhaber et al., 2000; Toh et al., 2001).

In this report, we show that the level of DBT protein in flies is not under circadian control. However, DBT shows striking circadian changes in subcellular localization in photoreceptor cells and in the brain’s pacemaker cells, the lateral neurons. The localization rhythms coincide with changes in the subcellular distribution of PER proteins. We demonstrate that DBT is found in complexes with PER at all times in vivo. Association of DBT with TIM occurs only in the presence of PER. We also show that dissociation of PER from TIM proteins coincides with increasing phosphorylation of nuclear PER. Because elimination of TIM does not stimulate PER phosphorylation in the absence of DBT (Price et al., 1998), we suggest that TIM excludes an activity of DBT when the latter protein is associated with a PER/TIM complex. These protein interactions should help determine the timing of PER’s phosphorylation and turnover in the nucleus.

## Results

### DBT Accumulation Is Not under Circadian Control

To determine the pattern of expression of DBT in *Drosophila*, two antisera were independently raised against the unique, carboxy-terminal region of the DBT protein

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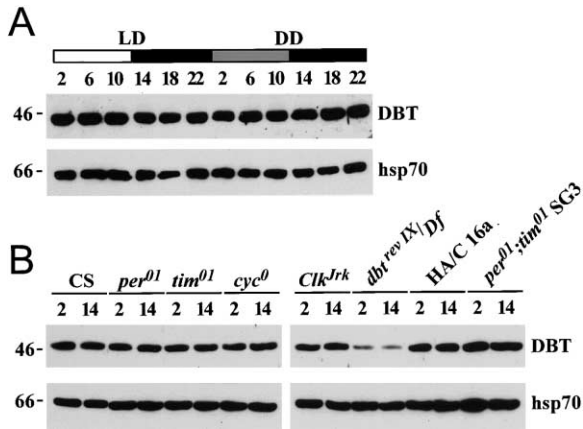


Figure 1. DBT Protein Levels Are Not under Circadian Control

(A) DBT protein levels are constant during the course of a daily cycle. Wild-type fly heads were collected during one day in LD (12h/12h light/dark cycle) and a subsequent day in DD (constant darkness), as reflected by the bar atop, with time (hours) shown in 24 hr cycles underneath. Blotted protein extracts were probed with a rabbit anti-DBT antibody (top panel; see also Experimental Procedures), and with anti-Hsp70 (lower panel) to ensure equal loading. DBT levels were constant during both days assayed. Identical results were obtained when we used a rat anti-DBT antibody (data not shown).

(B) DBT levels are unaffected in various clock mutants. Anti-DBT probed Western blots from various genetic backgrounds (top) are shown. For each genotype, two time points were assayed from an LD cycle. Two wild-type strains were also assayed: CS = Canton S, HA/C 16a = HA-tagged PER transformed into a *per*<sup>01</sup> background. SG3 is a transgenic line with a PER-β-Gal fusion protein. The only genotype that affected DBT levels was the hypomorphic allele *dbt*<sup>revIX</sup> (see text).

(Experimental Procedures; Kloss et al., 1998). Anti-DBT antibodies were affinity purified from both antisera. Equivalent results were obtained with both antibodies. In a first set of experiments, the levels of DBT protein accumulating during a circadian cycle were examined by Western blot analysis (Figure 1A). Protein extracts were prepared from heads of flies collected over two days, one in a light/dark cycle (LD), and one in subsequent constant darkness (DD). The molecular weight of DBT is predicted to be 48 kDa (Kloss et al., 1998), and a strongly immunoreacting protein that comigrated with the 46 kDa molecular weight marker was detected. Levels of this protein were strongly reduced in *dbt*<sup>revIX</sup> flies (Figure 1B, see later), indicating that the detected band corresponds to DBT protein. Similar levels of DBT were detected in extracts from each of the time points examined, showing that DBT levels are not under circadian control.

The electrophoretic mobilities of PER, TIM, and dCLK oscillate in a circadian fashion, reflecting progressive phosphorylations of the proteins during each molecular cycle (Edery et al., 1994; Zeng et al., 1996; Lee et al., 1998; Martinek et al., 2001). However, no change in DBT electrophoretic mobility was observed using methods that readily detected phosphorylation-induced changes in PER, TIM and dCLK (Figure 1A) (Edery et al., 1994; Martinek et al., 2001; B.K. and M.W.Y., unpublished data).

Alleles of *dbt*, *per*, *tim*, and *dClk* that abolish or alter

the period of *Drosophila*'s behavioral rhythms affect levels of *per*, *tim*, and *dClk* expression (Bae et al., 1998; Price et al., 1998). To test whether any clock mutation would similarly alter the levels of DBT protein, we assayed DBT levels in *per*<sup>01</sup>, *tim*<sup>01</sup>, *cyc*<sup>0</sup>, *dClk*<sup>Jrk</sup>, and *dbt*<sup>revIX</sup> mutant flies (Figure 1B). The only effect observed was a reduction of DBT levels in *dbt*<sup>revIX</sup> hemizygous flies. *dbt*<sup>revIX</sup> is a partial revertant of the lethal *dbt*<sup>P</sup> transposon-insertion allele and shows a high incidence of behavioral arrhythmicity. *dbt* RNA is reduced to ~20% of the wild-type level in *dbt*<sup>revIX</sup> hemizygous flies (Kloss et al., 1998). Therefore, in contrast to most genes involved in the regulation of the *Drosophila* clock, levels of DBT protein are neither under circadian control, nor are they regulated by the remaining elements of the clock.

### Subcellular Localization of DBT Cycles with a Circadian Rhythm

Heads of wild-type flies, collected throughout a circadian cycle, were sectioned and stained for DBT protein. DBT was expressed ubiquitously in the head (Figure 2A). In the photoreceptor cells, which contain an endogenous circadian clock (Plautz et al., 1997), as well as in the lateral neurons, the subcellular localization of DBT changed with a daily rhythm. Nuclear localization of DBT was observed throughout the circadian cycle; however accumulation in the cytoplasm varied with time. At the beginning of the day (ZT 2, corresponding to 2 hr after lights on in a 12hr/12hr light/dark, LD, cycle), DBT was readily detected in the nuclei but not cytoplasm of photoreceptor cells (Figure 2A). Subsequently, the localization of DBT became diffuse, with DBT still clearly detected in nuclei, but also at a low level in the cytoplasm (see Figure 2A, ZT 6 and ZT 10). By the beginning of the night (ZT 14, 2 hr after lights off in LD), uniform cellular staining was observed. Prominent nuclear staining of DBT returned later at night (at ZT 18 and ZT 22), which was consistent with the subcellular distribution observed at ZT2 (Figure 2A).

A similar pattern of DBT localization was observed in the lateral neurons of adult heads (Figure 2B) and in larval brains (data not shown). During the light phase of the LD cycle, a progressive redistribution of DBT between the nucleus and cytoplasm was observed. Shortly after lights off cytoplasmic staining became most prominent, reaching maximal levels at ~ZT 14. This timing of maximal cytoplasmic staining corresponds to the timing of cytoplasmic accumulation of PER and TIM, which translocate into the nucleus in the middle of the night (ZT 18–22) (Curtin et al., 1995; Rothenfluh et al., 2000b). These observations, together with the physical interaction of DBT and PER detected by immunoprecipitation (Kloss et al., 1998; see later), suggest that PER and/or TIM may influence the subcellular distribution of DBT.

To further examine the role of PER and TIM in the localization of DBT, we stained *per*<sup>01</sup> and *tim*<sup>01</sup> heads with antibody to DBT. In *per*<sup>01</sup> flies, which produce no PER protein, immunoreactivity was found predominantly in photoreceptor nuclei (Figure 3D), even though high levels of cytoplasmic TIM accumulate in these mutants (Myers et al., 1996). In *tim*<sup>01</sup> fly heads, which form no TIM and extremely low levels of PER (Price et al., 1998), primarily nuclear staining was again observed

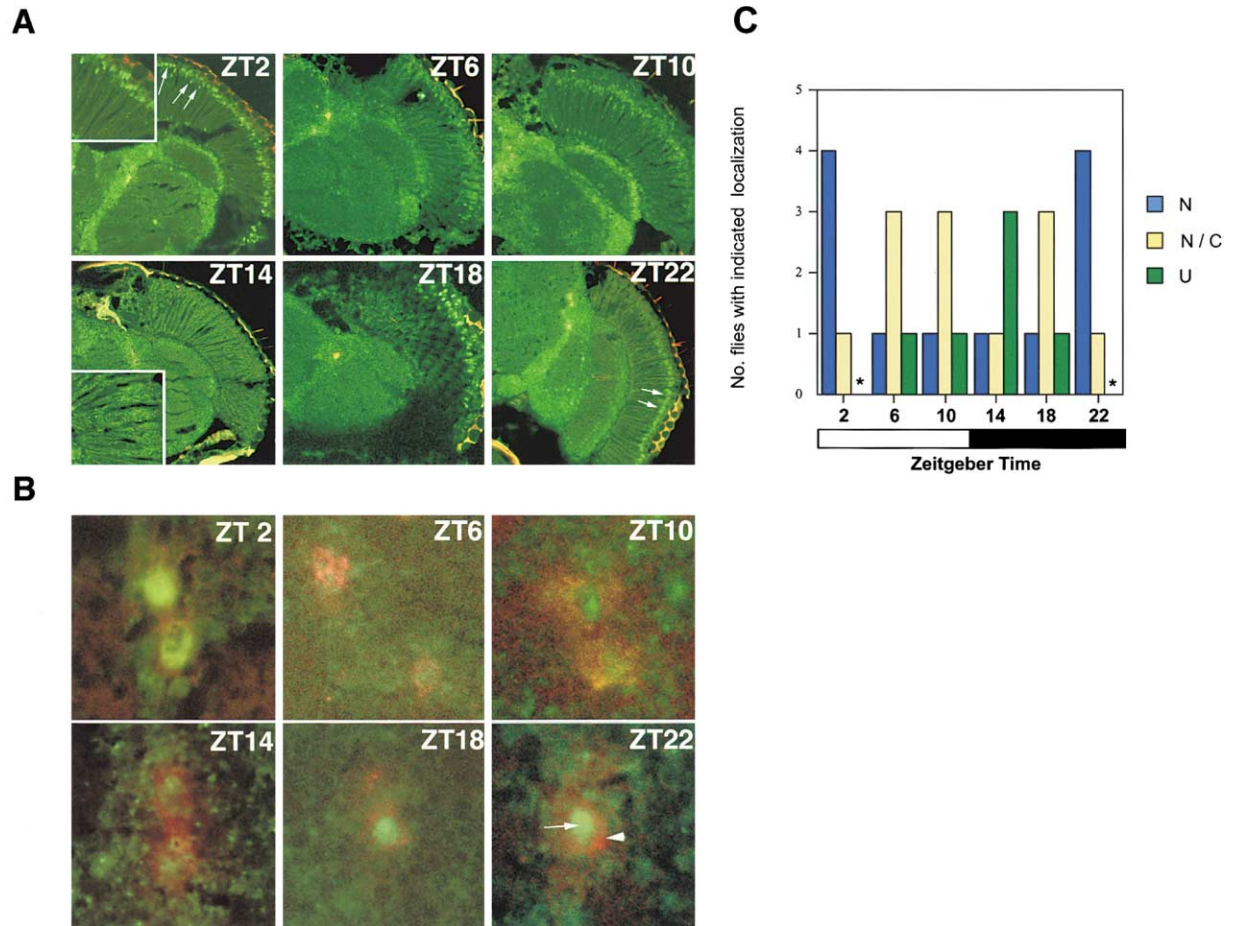


Figure 2. Daily Changes in Subcellular Localization of DBT in Photoreceptor Cells and Lateral Neurons (LNs)

Confocal images of anti-DBT (Green), and anti-PDF (Red)-stained cryosections of wild-type heads collected at 4 hr intervals in an LD cycle. In the photoreceptor cells, visible in (A), and lateral neurons (B), DBT staining shows a circadian oscillation ranging from uniform cell staining (ZT 14, ZT 0 = lights on and ZT 12 = lights off in LD cycles) to predominantly nuclear labeling (e.g., ZT 22 and ZT 2). Magnified insets in (A) show representative photoreceptor labeling at ZT 2 and ZT 14. Arrows show nuclear DBT staining in (A) and (B); arrowhead shows cytoplasmic PDF staining in (B).

(C) Histogram showing subcellular distribution of DBT staining in the lateral neurons at different times of the LD cycle. The subcellular distribution of DBT at each time point was determined by subjective, but blind, scoring of LNs from five individual animals. Numbers of animals with N (nuclear labeling without evidence of cytoplasmic staining), N/C (nuclear labeling with low but detectable cytoplasmic accumulation), and U (uniform cellular staining-label evenly distributed over cytoplasm and nucleus), are plotted. \*Indicates that a score of 0 was assigned (i.e., no uniform cellular staining was observed for any of the flies examined at ZT2 and ZT22).

(Figure 3C). These results indicate that in the absence of PER, DBT accumulates in the nucleus, that a substantial cytoplasmic accumulation of PER is required to promote cytoplasmic DBT localization, and that TIM alone has no effect on the subcellular distribution of DBT. Thus, when cytoplasmic PER is lacking, the default localization pathway for DBT in the fly head involves nuclear accumulation.

#### DBT Associates with PER and PER/TIM Complexes, but Not with TIM In Vivo

The timing of nuclear translocation of the PER/TIM complex is associated with predominantly nuclear DBT staining. This is preceded by an interval of highest cytoplasmic DBT localization that is correlated with cytoplasmic accumulation and turnover of PER. These observations suggested that cytoplasmic PER might

associate with newly formed DBT and carry the kinase to the nucleus in association with the PER/TIM complex. In the nucleus, DBT might remain associated with the PER/TIM complex, allowing nuclear PER phosphorylation when TIM is degraded at sunrise (Kloss et al., 1998; Price et al., 1998). The rhythmic subcellular localization of DBT would in this case result from varying in vivo associations of DBT, PER, and TIM. To test this hypothesis, coimmunoprecipitation experiments were performed (Figures 4A and 4B). Protein extracts were prepared from heads of flies carrying an HA-tagged PER transgene in a *per<sup>01</sup>* background (HA/C flies) (Rutila et al., 1992) at the indicated time points, and total PER protein was recovered by immunoprecipitation with monoclonal anti-HA antibody 12CA5 (Kolodziej and Young, 1991). DBT and any associated proteins were recovered by immunoprecipitation with affinity purified

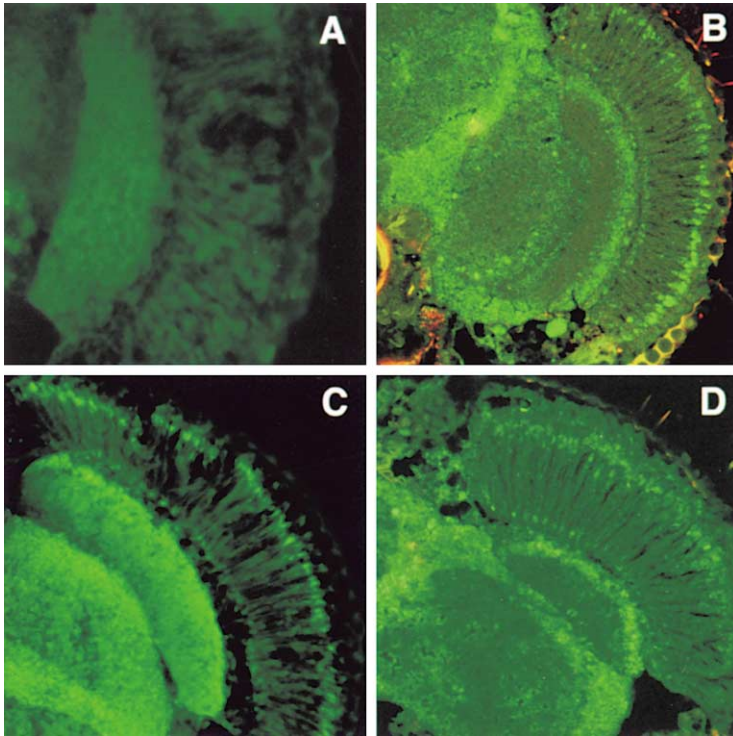


Figure 3. Confocal Images of Wild-Type and Clock-Deficient *per<sup>01</sup>* and *tim<sup>01</sup>* Strains

Specificity of the anti-DBT antibody was established by comparison of wild-type heads (collected at ZT 2) stained with DBT pre-absorbed anti-DBT antibody (A) and anti-DBT that had not been pre-absorbed (B). DBT staining is predominantly nuclear in photoreceptors of heads collected at ZT 14 from the clock deficient strains *tim<sup>01</sup>* (C) and *per<sup>01</sup>* (D).

DBT antibody. At all three times examined (ZT 2, 14 and 20; Figure 4A, lanes 3–8), immunoprecipitations with anti DBT recovered HA-tagged PER, indicating that PER and DBT are physically associated in vivo. Immunoprecipitations with anti-HA also recovered PER proteins at these time points. The same blot was probed with anti-TIM antibody. TIM coimmunoprecipitated with both PER and DBT protein at ZT 14 and ZT 20 (Figure 4B, lanes 5–8), suggesting that PER, TIM, and DBT form a ternary complex at night.

In *tim<sup>01</sup>* flies, the absence of TIM protein suppresses PER accumulation (Price et al., 1995). Nevertheless, as shown in Figure 4A, lane 2, anti DBT still coimmunoprecipitates residual PER proteins. Because TIM is required for nuclear localization of PER (Vosshall et al., 1994; Saez and Young, 1996), these DBT-associated PER proteins are localized in the cytoplasm and may reflect newly translated PER destined for turnover following DBT-dependent phosphorylation. In *per<sup>01</sup>* flies, TIM constitutively accumulates to high levels in the dark (Myers et al., 1996; Zeng et al., 1996). However, the anti-DBT antibody fails to coimmunoprecipitate these TIM proteins (Figure 4B, lanes 1 and 10), despite the abundance of TIM protein in the extract (Figure 4B, lane 9). These results suggest that DBT and TIM are physically associated in vivo only if PER is included in the protein complex.

By early morning (ZT 2), most TIM proteins have been degraded in response to light, and PER proteins derived from nuclear PER/TIM complexes persist in nuclei (Zerr et al., 1990; Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996; Price et al., 1998; Rothenfluh et al., 2000a). At ZT 2, TIM is only weakly coprecipitated with PER, and even lower levels of TIM are recovered in association with DBT (Figure 4B, lanes 3–4). However,

under these conditions PER is abundantly recovered with DBT (Figure 4A, lanes 3–4). Thus PER and DBT associate in the nucleus in vivo.

As shown previously, some, presumably cytoplasmic, PER proteins can be recovered in association with DBT in *tim<sup>01</sup>* flies. Additional evidence that PER and DBT form a complex in the cytoplasm in the absence of TIM comes from coimmunoprecipitation experiments using *per<sup>01</sup>;tim<sup>01</sup>* SG3 flies. These lack endogenous PER and TIM proteins, but express PER-βGal fusion proteins that are very stable and accumulate abundantly in the cytoplasm (Vosshall et al., 1994). Immunoprecipitations with anti-DBT antibody using extracts of *per<sup>01</sup>;tim<sup>01</sup>* SG3 flies recovered PER-βGal protein at all times analyzed (Figure 4C). We conclude that PER and DBT are capable of forming in vivo complexes that do not include TIM in both the cytoplasm and the nucleus.

#### PER Phosphorylation and Dissociation from TIM

TIM protein is degraded by light at dawn, and light pulses at the end of the night advance the molecular and behavioral cycle (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Although an effect of light on PER phosphorylation was noticed in earlier studies (Zeng et al., 1996; Lee et al., 1996), these investigations focused on wild-type flies, which also showed circadian, light-independent phosphorylation of PER. The phases of these phosphorylations overlap in wild-type flies, such that phosphorylation precedes lights-on in an LD cycle. In an effort to dissociate the mechanisms directing light-dependent and light-independent phosphorylation, we investigated patterns of PER phosphorylation in the long-period (33 hr) mutant *tim<sup>UL</sup>*. In *tim<sup>UL</sup>* flies, PER and TIM proteins associate for a prolonged

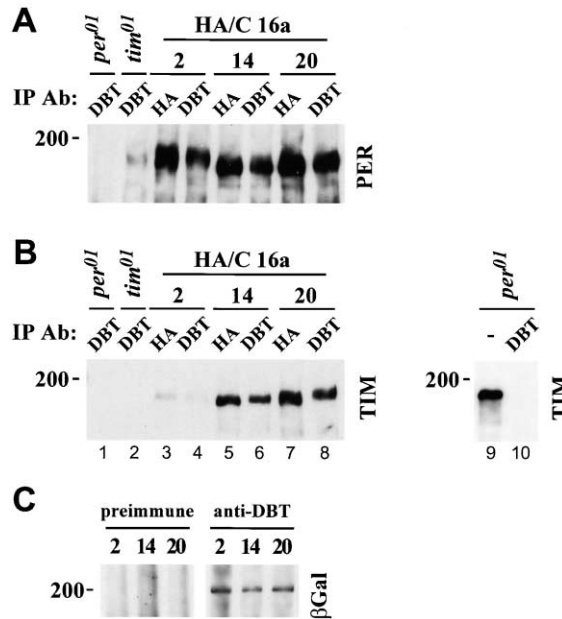


Figure 4. PER, TIM, and DBT Are Found in a Ternary Complex In Vivo

(A and B) Coimmunoprecipitation experiments from LD-derived, wild-type (HA/C 16a), and control (*per<sup>01</sup>* or *tim<sup>01</sup>*) head extracts. The immunoprecipitation antibody is indicated on top of the lane (anti-HA was used to precipitate the HA-tagged PER fusion from transgenic HA/C 16a flies). Precipitates were run on SDS-PAGE, blotted, and probed with anti-PER (A), and anti-TIM (B) antibody. At all three time points tested (ZT 2, 14, 20), anti-HA and anti-DBT recovered PER from wild-type heads. DBT antibody also coprecipitated some PER protein from *tim<sup>01</sup>* extracts (A), but no TIM protein from *per<sup>01</sup>* extracts (B).

(C) Immunoprecipitations with preimmune, and anti-DBT antibody from *per<sup>01</sup>*; *tim<sup>01</sup>* flies carrying a PER-β-Gal fusion protein (SG3). This cytoplasmic fusion protein was detected with anti-β-Gal antibody, and was recovered at all times with anti DBT-antibody.

interval in the nucleus, and light-independent phosphorylation is retarded by ~8–10 hr (Rothenfluh et al., 2000a).

Late night transfer of *tim<sup>UL</sup>* flies to light (or continued darkness) allows the state of PER phosphorylation to be assessed in the presence or absence of TIM by a mobility shift during gel electrophoresis (Edery et al., 1994; Price et al., 1998). Figure 5 shows a side by side comparison of extracts prepared from *tim<sup>UL</sup>* flies at various times following transfer to light or darkness from an LD 12:12 cycle. Starting with time points taken at the end of the final dark period of the LD cycle (ZT0), flies were collected and their PER proteins examined by electrophoresis. Flies transferred to light were collected after 2 hr or 6 hr (Figure 5, ZT 2 and ZT 6, respectively). Flies retained in constant darkness were also collected after 2 hr (CT 2) or 6 hr (CT 6).

The mobilities of PER were indistinguishable and indicated hypophosphorylation for all flies collected in continued darkness (Figure 5, ZT 0, CT 2, and CT 6). PER proteins remain complexed with TIM at these time points in *tim<sup>UL</sup>* flies (Rothenfluh et al., 2000a). In contrast to the results obtained in darkness, PER proteins derived from each population of light-treated flies showed substantially decreased PER mobility, reflecting phosphoryla-

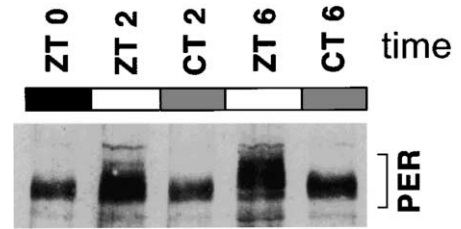


Figure 5. PER Phosphorylation Is Correlated with Dissociation of PER/TIM<sup>UL</sup> Complexes

Anti-PER Western blots from *tim<sup>UL</sup>* head extracts collected in the morning of an LD cycle (ZT 2, 6) or the first subjective morning in DD (CT 2, 6). PER from flies kept in DD runs with higher mobility following gel electrophoresis, while flies transferred into light show lower mobility, hyperphosphorylated forms of PER. Note that to obtain a qualitative result, roughly equal amounts of PER immunoreactivity and not equal amounts of total protein, were loaded (i.e., samples for ZT 2 and especially ZT 6 contain more total protein). The effects of light and dark on TIM accumulation in each of the samples shown was previously reported by Rothenfluh et al. (2000).

tion (Figure 5, ZT 2 and ZT 6). Thus, TIM<sup>UL</sup> degradation and release of “TIM-free” PER proteins was correlated with increased phosphorylation of PER. Because *tim<sup>UL</sup>* prolongs the physical association of PER and TIM and retards the light-independent, but not light-dependent, phosphorylation of PER, the results of Figure 5 suggest that cycles of PER phosphorylation are affected by rhythmically changing associations of DBT, PER, and TIM.

## Discussion

### Subcellular Localization Rhythms of DBT

We have previously shown that *dbt* RNA levels are constant throughout the day (Kloss et al., 1998). In this respect, we found that the same is true for DBT protein levels, as there was no detectable circadian oscillation of DBT accumulation in timed head extracts. Furthermore, we found that a variety of mutations disrupting the circadian clock and molecular oscillations had no effect on the level of DBT protein. Thus, production of DBT protein is not under the control of clock genes. In contrast, in the present study, we show that the subcellular localization of DBT in the lateral neurons and photoreceptor cells changes over the course of a daily cycle. DBT was consistently detected in the nucleus. However, at the end of the day and in the early part of the night, we found a substantial increase in cytoplasmic DBT, coincident with the cytoplasmic accumulation of PER proteins and PER/TIM complexes. Furthermore, when PER/TIM complexes translocate to the nucleus at ~ZT18, and early during the day when PER remains in the nucleus in absence of TIM, a substantial nuclear accumulation of DBT was observed. These changes in subcellular location of DBT appear to be influenced exclusively by the locus of PER accumulation (in the presence or absence of TIM). TIM protein was found to have little or no effect on the localization of DBT because DBT is always detected in the nucleus in *per<sup>01</sup>* flies, which lack PER and have a substantial amount of TIM in the cytoplasm. Consequently, there is circadian regulation of DBT proteins, in the form of a changing subcel-

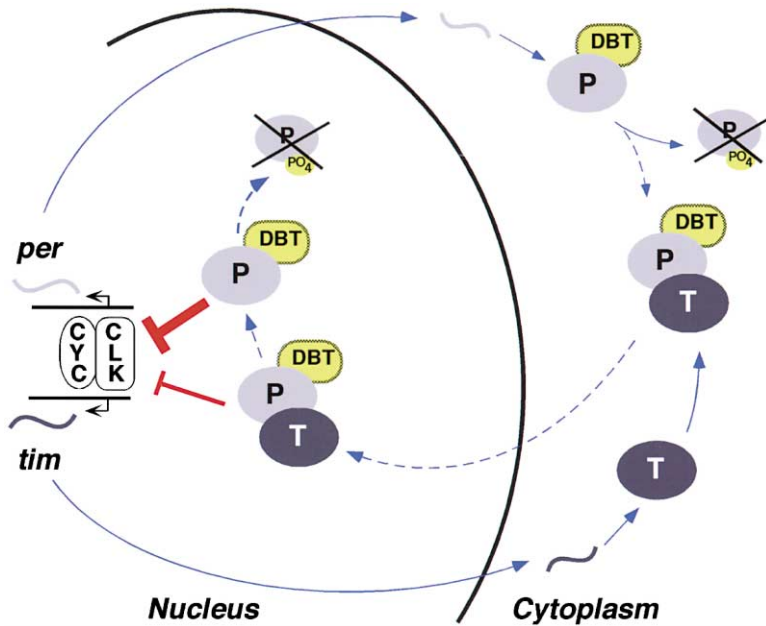


Figure 6. Model of Daily Interactions among PER, TIM, and DBT

In the absence of TIM, newly translated cytoplasmic PER protein binds to DBT. PER is subsequently phosphorylated and degraded, contributing to an approximately 4 hr delay between peak accumulation of *per* mRNA and PER protein. As TIM accumulates, it binds to PER, inhibiting its DBT-dependent phosphorylation and allowing formation of DBT/PER/TIM complexes. The complexes then translocate to the nucleus, where PER and TIM modestly suppress dCLK/CYC-dependent transcriptional activation of *per* and *tim*. TIM is slowly lost from the complexes, intensifying inhibition of *per* and *tim* transcription (Rothenfluh et al., 2000a), and accelerating phosphorylation of the released nuclear PER proteins. As PER becomes increasingly phosphorylated, it is marked for degradation. The loss of nuclear PER and TIM allows a resumption of *per* and *tim* transcription. Dashed lines indicate delays thought to promote molecular oscillations.

lular distribution. The fact that the movement of PER and TIM from the cytoplasm to the nucleus predicts the distribution of DBT implies a close correspondence between maximum levels of PER/TIM complex and cytoplasmic levels of DBT. Such a relationship could indicate that TIM associates with cytoplasmic PER once the latter protein has effected cytoplasmic localization of most cellular DBT.

Because DBT preferentially accumulates in nuclei in the absence of PER, cytoplasmic PER proteins must affect this default localization at certain times of day in wild-type flies. Although the half-life of DBT has not been determined, we have shown that *dbt* RNA and proteins are constantly synthesized. Therefore, the subcellular fate of newly translated DBT may simply depend on whether cytoplasmic PER is available to associate with DBT and retard its nuclear translocation. Alternatively, accumulation of DBT may involve mechanisms promoting both nuclear import and export, with the predominant localization of DBT governed by the presence or absence of cytoplasmic PER. Regardless of the specific mechanism, as DBT has also been implicated in vital developmental and cellular functions that are not mediated through PER (Kloss et al., 1998; Price et al., 1998; Zilian et al., 1999), an important product of any device generating cycling subcellular localization of this kinase could be temporal regulation of its access to alternative substrates.

Previously, we showed that DBT is a component of the cytoplasmic activity that destabilizes PER (Price et al., 1998). In that study, we also found evidence that DBT influences the stability of nuclear PER proteins. However, it was unclear whether DBT acts in both subcellular compartments, or whether nuclear stability of PER is affected by a DBT-dependent phosphorylation in the cytoplasm, with delayed effects once PER translocates into the nucleus. Here, we show that DBT proteins are found both in the cytoplasm and in the nucleus. Coupled with the finding that PER proteins are always found associated with DBT (see later), this suggests that

DBT is required both in the nucleus and in the cytoplasm for PER phosphorylations.

#### In Vivo Association of DBT and PER

The simultaneous changes in subcellular localization of the PER, TIM, and DBT make it likely that direct physical associations among these proteins cause the cycling DBT localizations. We had previously shown that PER and DBT proteins can associate in vitro and in cultured cells (Kloss et al., 1998). In the present study, using coimmunoprecipitation assays, we found that PER/DBT complexes can be recovered at all times during the day from head extracts, regardless of whether the majority of these proteins are localized in the cytoplasm or in the nucleus. Thus, PER proteins are associated with DBT proteins in vivo when PER is in a PER/TIM complex and when PER proteins are free from TIM.

Conversely, while DBT binds to PER and PER/TIM complexes, we could not find evidence that TIM protein free from PER associates with DBT in vivo. This finding is in line with our previous conclusion that DBT's effects on the circadian clock are primarily mediated through PER (Kloss et al., 1998; Price et al., 1998).

#### Regulation of DBT Activity

Extensive efforts by our laboratory and others (Suri et al., 2000) have failed to obtain a functional assay for bacterially produced, recombinant DBT in vitro. The putative kinase domains of DBT and its mammalian ortholog CKI $\epsilon$  are very closely related (86% aa identity), so it was surprising to find that recombinant, mammalian CKI $\epsilon$  readily phosphorylates *Drosophila* PER and human PER in vitro (L.S., data not shown; Toh et al., 2001). These observations suggest that DBT function might be tightly regulated in the fly. It has been established that truncation of mammalian CKI $\epsilon$  substantially increases its activity in vitro (Graves and Roach, 1995; Cegliska et al., 1998), and truncated forms of the enzyme were used in the above mentioned PER and hPER assays. Although a corresponding truncation of DBT failed to

generate activity, such studies of mammalian CKI $\epsilon$  also indicate more complex regulation for this kinase *in vivo*.

Without direct kinetic measurements of the activity of DBT at different times of day, we cannot determine whether DBT function is under circadian control. However, we can ask whether PER phosphorylation *in vivo* is (1) dependent upon the presence of DBT and (2) influenced by TIM. We found that in *tim<sup>UL</sup>* flies entrained to LD 12:12, where PER remains complexed with TIM<sup>UL</sup> for a prolonged interval in the nucleus (Rothenfluh et al., 2000a), PER remains hypophosphorylated during the dark phase. Because wild-type flies begin to phosphorylate their PER proteins during the dark phase of such LD cycles, the results with *tim<sup>UL</sup>* suggest that TIM influences the timing of light-independent PER phosphorylation.

Light-triggered removal of TIM<sup>UL</sup> protein was correlated with a rapid and progressive increase in the level of PER phosphorylation. Because a similar, cytoplasmic association of PER and DBT in *tim<sup>01</sup>* flies results in cytoplasmic PER degradation, and such PER degradation requires DBT (Price et al., 1998; Kloss et al., 1998), the most parsimonious explanation of our results should be that nuclear association of PER with TIM<sup>UL</sup> protects PER from phosphorylation and, secondarily, from turnover. In prior work, we have shown that light eliminates TIM, but will not promote PER phosphorylation in a hypomorphic mutant of DBT (*dbt<sup>P</sup>*; see Figure 5C, Price et al. 1998). Thus, PER phosphorylation appears to be influenced by the formation of PER/TIM complexes, and only when PER is free from TIM is it subject to phosphorylation by a DBT-dependent mechanism. While we favor this view, it is also possible that light directly activates elements of a DBT-dependent mechanism to promote some PER phosphorylations, or that additional factors associate with PER (or DBT) after TIM is removed by light. Such factors would then be essential for DBT-regulated phosphorylation of PER.

Our model for the accumulation, phosphorylation, and degradation of PER is summarized in Figure 6: DBT-dependent phosphorylation of PER in the cytoplasm is thought to delay the accumulation of PER proteins until lights off. Increasing TIM levels result in stable PER/TIM/DBT complexes containing hypophosphorylated PER. These complexes are translocated to nuclei, where continued physical association of TIM with PER prolongs the cycle. Subsequently, the formation of PER free from TIM allows the clock to advance by DBT-dependent phosphorylation of nuclear PER. This phosphorylation could be indirectly controlled by DBT. The cycle restarts after degradation of phosphorylated nuclear PER proteins. According to this model, DBT would have opposing effects on the cycle at different times of day and in different subcellular compartments. This regulation would determine the onset and duration of PER's activity in the nucleus, and should therefore be required to establish rhythmicity and set the period of *Drosophila*'s circadian clock.

#### Experimental Procedures

##### Antibody Preparation and Purification

The region of the *dbt* cDNA encoding the unique, carboxy-terminus of the DBT protein was amplified by PCR, ligated into pGEX4T (Pharmacia), and used to transform *E. coli*. GST-DBT fusion protein

was induced with IPTG and purified according to manufacturer's recommendations (Pharmacia). Affinity purified fusion protein was used to raise antibodies in rabbits (Covance, Inc.). Anti-DBT antibodies were affinity purified. Briefly, to remove antibodies recognizing the GST portion of the fusion protein used for antibody production, rabbit serum was first passed over a column containing GST alone. The flowthrough was collected and passed over a column containing the fusion protein used for antibody production. Bound antibodies were eluted with 7.5 M MgCl<sub>2</sub> dialyzed against PBS and concentrated by ultrafiltration (Amicon). A second, independent anti-DBT antibody was generated in rats using the above described GST-DBT protein.

##### Immunoprecipitation

Extracts for immunoprecipitation and Western analysis were prepared by homogenizing frozen heads in HE buffer (Edery et al., 1994), from which glycerol had been omitted. For immunoprecipitations, 1mg total head protein, 1  $\mu$ l antibody (or preimmune serum) and 30  $\mu$ l of an ~50% slurry of Gammabind beads (Pharmacia) were combined in a final volume of 500  $\mu$ l and incubated overnight at 4 $^{\circ}$ . Immune complexes were washed several times with immunoprecipitation buffer, resuspended in electrophoresis sample buffer, and boiled. Western analysis was performed as described previously (Myers et al., 1996; Price et al., 1998). The efficiency of all immunoprecipitations was such that most (>90%) of the target protein (HA-PER for anti-HA, DBT for anti-DBT) remained in the supernatant.

##### Immunocytochemistry

Immunohistochemical data were collected from fly heads collected at 4 hr intervals in an LD cycle. Cryosections were incubated with affinity purified anti-DBT antibody (1:750) using the Tyramide Signal Amplification Fluorescence System (NEN). PDF was detected using anti-PDF (1:500) and a secondary antibody conjugated with rhodamine. Times and washes were performed as described by the manufacturer. To determine the specificity of the affinity purified anti-DBT antibody in ICC, the antibody was preincubated with GST-DBT fusion protein attached to beads before incubation with sections of the fly head in some controls. This step completely eliminated the staining signal.

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