

# The Double-Time Protein Kinase Regulates the Subcellular Localization of the *Drosophila* Clock Protein Period

Shawn A. Cyran,<sup>1</sup> Georgia Yiannoulos,<sup>1</sup> Anna M. Buchsbaum,<sup>1</sup> Lino Saez,<sup>2</sup> Michael W. Young,<sup>2</sup> and Justin Blau<sup>1</sup>

<sup>1</sup>Department of Biology, New York University, New York, New York 10003, and <sup>2</sup>The Rockefeller University, New York, New York 10021

The Period (PER), Timeless (TIM), and Double-Time (DBT) proteins are essential components of one feedback loop in the *Drosophila* circadian molecular clock. PER and TIM physically interact. Coexpression of PER and TIM promotes their nuclear accumulation and influences the activity of DBT: although DBT phosphorylates and destabilizes PER, this is suppressed by TIM. Experiments using *Drosophila* cells in culture have indicated that PER can translocate to the nucleus without TIM and will repress transcription in a DBT-potentiated manner. In this study, we examined the control of PER subcellular localization in *Drosophila* clock cells *in vivo*. We found that PER can translocate to the nucleus in *tim*<sup>01</sup> null mutants but only if DBT kinase activity is inhibited. We also found that nuclear PER is a potent transcriptional repressor in *dbt* mutants *in vivo* without TIM. Thus, *in vivo*, DBT regulates PER subcellular localization, in addition to its previously documented role as a mediator of PER stability. However, DBT does not seem essential for transcriptional repression by PER. It was reported previously that overexpression of a second kinase, Shaggy (SGG)/Glycogen Synthase Kinase 3, accelerates PER nuclear accumulation. Here, we show that these effects of SGG on PER nuclear accumulation require TIM. We propose a revised clock model that incorporates this tight kinase regulation of PER and TIM nuclear entry.

**Key words:** circadian; Period; Double-Time; Timeless; nuclear entry; *Drosophila*

## Introduction

Rhythms of gene expression and protein levels lie at the core of the molecular clocks that regulate circadian (~24 h) rhythms of behavior and physiology. The best-studied circadian feedback loop involves the *Drosophila* clock genes *period* (*per*) and *timeless* (*tim*). Many components of this negative feedback loop play similar roles in the mammalian clock (Panda et al., 2002; Stanewsky, 2003).

In *Drosophila*, *per* and *tim* transcription is activated by Clock (CLK)/Cycle (CYC) heterodimers binding to E-boxes in the *per* and *tim* promoters. PER and TIM proteins heterodimerize in the cytoplasm, and, until recently (see below), it was believed that they enter the nucleus together. PER is more stable than TIM in the nucleus and represses additional *per* and *tim* transcription by directly inhibiting CLK/CYC.

The PER/TIM loop is characterized by delays that separate transcription from repression, important for rhythmic, as op-

posed to continuous, negative feedback. Peak *per* RNA and protein levels are separated by ~6 h at least partly by Double-Time (DBT)/Casein Kinase 1 $\epsilon$  (CK1 $\epsilon$ ), a protein kinase that destabilizes cytoplasmic PER while TIM levels are low (Price et al., 1998; Suri et al., 2000). Thus, PER levels are constitutively low in *tim*<sup>01</sup> mutants (Price et al., 1995). DBT stably associates with PER (Kloss et al., 2001) and phosphorylates PER (Price et al., 1998; Ko et al., 2002) (S. Kivimae and M. W. Young, unpublished data), which leads to PER ubiquitination and degradation (Grima et al., 2002; Ko et al., 2002). These *in vivo* findings led to the model that rising TIM levels allow the formation of a DBT-resistant PER/TIM complex (Kloss et al., 1998, 2001; Price et al., 1998). DBT also regulates the stability of nuclear PER, consistent with the identification of stable PER/DBT complexes after TIM degradation in the early morning (Kloss et al., 2001) and by altered nuclear PER stability in *dbt* mutants (Price et al., 1998).

Stabilized PER fusion proteins are cytoplasmic in *tim*<sup>01</sup> mutants (Vosshall et al., 1994), and, reciprocally, TIM accumulates in the cytoplasm of *per*<sup>0</sup> mutants (Hunter-Ensor et al., 1996; Myers et al., 1996), leading to the model that PER and TIM enter the nucleus together. The timing of PER and TIM nuclear entry is tightly regulated, at least in the pacemaker lateral neurons (LNs), and is promoted by the Shaggy (SGG) and CK2 kinases (Martinek et al., 2001; Lin et al., 2002; Akten et al., 2003).

However, certain features of this *Drosophila* clock model have been questioned. Shafer et al. (2002, 2004) found PER in the nuclei of LNs before TIM, and, in some *Drosophila* S2 cell studies, transfected PER repressed CLK/CYC activity without cotransfected TIM (Chang and Reppert, 2003; Weber and Kay, 2003; Nawathean and Rosbash, 2004). Therefore, PER nuclear accumulation may not simply depend on heterodimerization with TIM.

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Correspondence should be addressed to Justin Blau, Department of Biology, New York University, 100 Washington Square East, New York, NY 10003. E-mail: justin.blau@nyu.edu.

G. Yiannoulos' present address: Veterans Affairs Medical Center, 130 West Kingsbridge Road, Bronx, NY 10468.  
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We therefore initiated an additional study of PER localization in clock cells *in vivo*. Here, we show that DBT kinase activity normally inhibits nuclear accumulation of PER in pacemaker neurons and adult photoreceptor cells: in the absence of both DBT kinase activity and TIM, PER enters the nucleus and represses transcription. We propose a revised *Drosophila* clock model based on properties of clock proteins established *in vivo*.

## Materials and Methods

**Fly strains and immunocytochemistry.** Larvae and flies were entrained in standard light/dark cycles or were mainly taken from unentrained conditions if in an arrhythmic *tim<sup>01</sup>* background. *yellow white (y w)* larvae and flies were used as wild-type controls. *Upstream activation sequence (uas)*–*sggB* flies were described by Bourouis (2002). SG3 flies were originally described by Vosshall et al. (1994). The SG3 transgene uses the *per* promoter to drive the expression of a fusion protein of the first 636 aa of PER fused to LacZ. Staining of whole brains and of adult head sections was as described previously (Myers et al., 1996; Price et al., 1998; Kloss et al., 2001). Antibodies to PER were provided by Ralf Stanewsky and Jeff Hall. Rat and guinea pig antibodies to Pigment-Dispersing Factor (PDF) were provided by Jae Park and Paul Taghert, respectively. Mouse anti-PDF antibodies were generated against amidated PDF peptide (NSELINSLLSLPKNMNDA-NH<sub>2</sub>) by PickCell Laboratories (Amsterdam, The Netherlands), and antibodies to LacZ were from Promega (Madison, WI). For adult head sections, PER antibody was first preabsorbed against *y w* embryos and then against acetone powder made from *per<sup>0</sup>* flies.

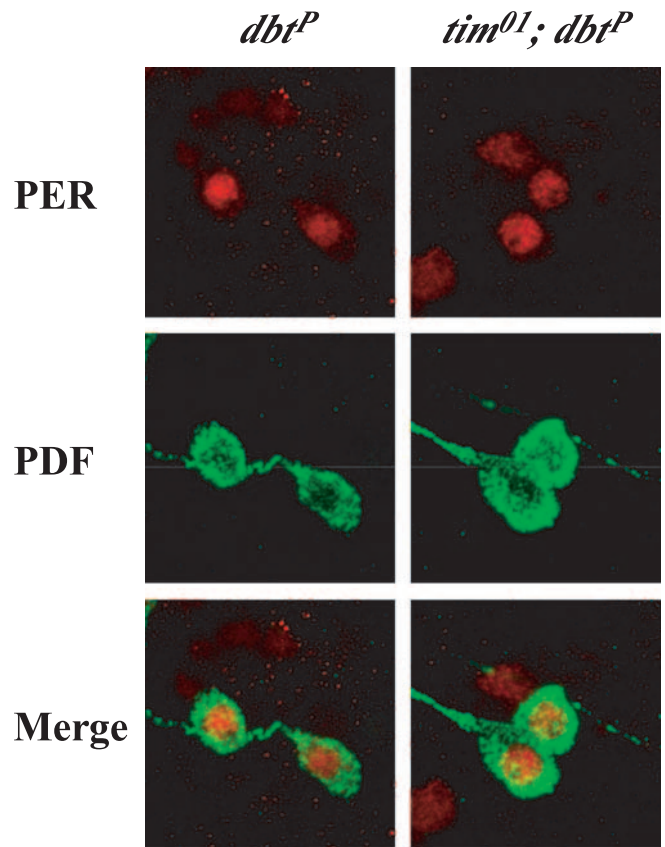
**Western blots, immunoprecipitation, and RNA analysis.** Western blots were as described previously (Cyran et al., 2003), except that only 2 μg of protein extracts were used to test SGG levels. Affinity-purified DBT antibodies were described by Kloss et al. (2001), and antibodies to SGG/Glycogen Synthase Kinase 3 (GSK3) were purchased from Upstate Biotechnology (Lake Placid, NY). Immunoprecipitations with anti-DBT were performed as described previously (Kloss et al., 2001).

RNA levels were analyzed by quantitative real-time PCR as in the study by Cyran et al. (2003), except that *tim<sup>01</sup>* and *tim<sup>01</sup>; dbt<sup>ar</sup>* flies were maintained in constant light. Primer combinations for *vri* and *Par domain protein 1ε (Pdp1ε)* were described previously by Cyran et al. (2003). Primers (TIB Molbiol, Adelpia, NJ) used were as follows: *tim* forward, ATGGTGGCTCTGATGAG; *tim* reverse, CCAAAGAGACAT-TGTCGC; *tim* Hybridization Probe 1 (HP1), AGTCCTCGTTC-GAGCGG–fluorescein; *tim* HP2, Red640–GGAGGTTAAACGGATCG-CACT; *cyc* forward, TTCGCAACTCCACAGTAC; *cyc* reverse, AGGGATTCTTGAAGGCC; *cyc* HP1, GGGCAAGTTCCTGTTCATA-GACC–fluorescein; *cyc* HP2, Red640–CGTGCCACCCTCGTGA.

## Results

### PER enters the nucleus without TIM in *dbt<sup>P</sup>* mutants

PER is constitutively located in the nucleus of the pacemaker LNs in *dbt<sup>P</sup>* single mutants, which produce very low levels of *dbt* RNA (Kloss et al., 1998; Price et al., 1998). Because DBT was proposed to destabilize cytoplasmic PER (Price et al., 1998), one prediction was that PER should accumulate to high levels in the cytoplasm of clock cells in the absence of both TIM and DBT. Because *dbt<sup>P</sup>* mutants die during pupation (Price et al., 1998), PER localization was tested in the pacemaker LNs of third instar larvae doubly mutant for *tim<sup>01</sup>* and *dbt<sup>P</sup>*. These cells are the precursors to the adult small-LN<sub>s</sub> (s-LN<sub>s</sub>), which are essential for adult circadian behavior. The LNs were identified with an antibody to the PDF neuropeptide. PER was located in the nucleus of the LNs in *dbt<sup>P</sup>* single mutants, as observed previously (Fig. 1) (Price et al., 1998). Surprisingly, we also observed that PER was located in the nucleus of the LNs in *tim<sup>01</sup>; dbt<sup>P</sup>* double mutants (Fig. 1). Therefore, PER can enter and accumulate in the nucleus in the absence of TIM when DBT protein levels are significantly lower than in wild type. These *in vivo* results are in contrast to the *in vitro* results of



**Figure 1.** PER enters the nucleus independently of TIM in a *dbt<sup>P</sup>* background. Third instar larval brains were stained with antibodies to PER (red; top panels) and PDF (green; middle panels). Red and green images are shown merged in the bottom panels. PDF marks the cell body and axons of pacemaker neurons, the LNs. PER was detected in the nuclei of larval LNs in *dbt<sup>P</sup>* single mutants (left) and *tim<sup>01</sup>; dbt<sup>P</sup>* double mutants (right). Essentially identical results were seen in at least 50 brain hemispheres for each genotype.

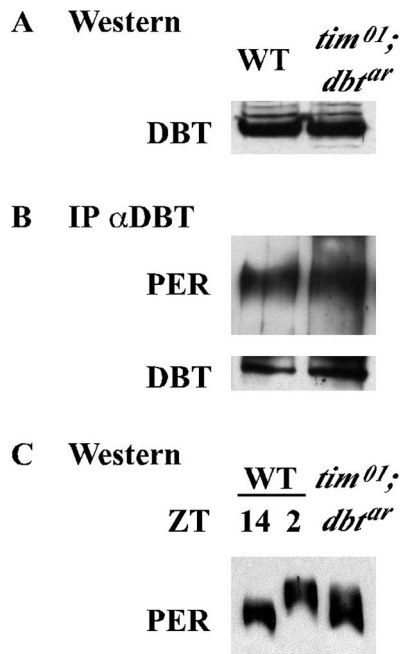
Nawatheg and Rosbash (2004), in which inactivation of *dbt* by RNA interference (RNAi) in *Drosophila* cultured cells did not increase nuclear accumulation of PER.

### DBT kinase activity regulates nuclear entry of PER

DBT has two previously established activities toward PER: it forms a stable complex with PER *in vivo* (Kloss et al., 2001), and it promotes PER phosphorylation in both the cytoplasm and the nucleus (Price et al., 1998; Ko et al., 2002). These two functions are effectively removed by the hypomorphic *dbt<sup>P</sup>* mutation. To test whether physical association between DBT and PER, or DBT kinase activity for PER, normally prevents PER from accumulating in the nucleus, we used a point mutation in *dbt* (*dbt<sup>ar</sup>*), originally identified and characterized by Rothenfluh et al. (2000c).

DBT<sup>AR</sup> protein was detected at levels indistinguishable from wild-type DBT (Fig. 2A) (Rothenfluh et al., 2000c), and affinity-purified antibodies to DBT coprecipitated similar amounts of PER in extracts isolated from the heads of either adult wild-type flies or *tim<sup>01</sup>; dbt<sup>ar</sup>* double-mutant flies (Fig. 2B). These results indicate that, like DBT<sup>+</sup> (Kloss et al., 2001), DBT<sup>AR</sup> can stably associate with PER.

The *dbt<sup>ar</sup>* mutation causes a histidine-to-tyrosine change in the highly conserved catalytic domain of the enzyme (Rothenfluh et al., 2000c), and this renders DBT<sup>AR</sup> a very poor kinase for PER *in vitro* (Ko et al., 2002) (Kivimae and Young, unpublished data). Consistent with this, most of the PER in extracts isolated from

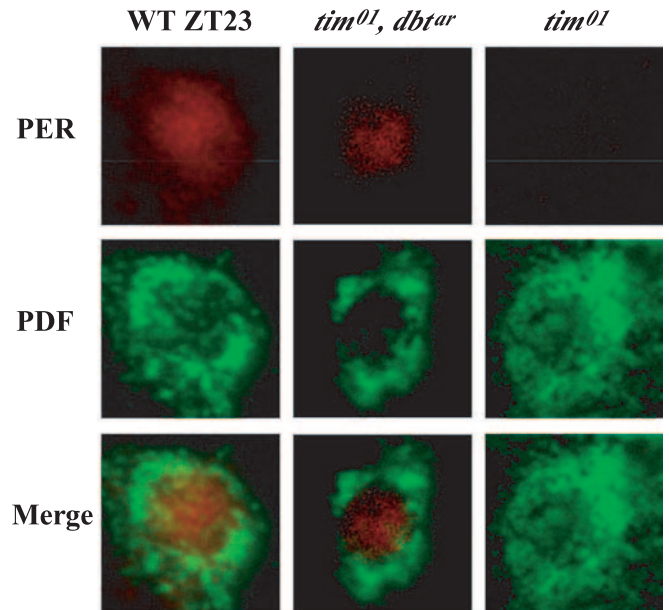


**Figure 2.** PER stably associates with DBT<sup>AR</sup> but is mostly hypophosphorylated. **A**, Western blots of DBT protein from adult head extracts of control wild-type flies (WT; ZT23; left) and *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies (right). DBT<sup>+</sup> and DBT<sup>AR</sup> proteins accumulated to similar levels. The result shown here is representative of three experiments and is consistent with the results of Rothenfluh et al. (2000c). **B**, An affinity-purified anti-DBT antibody was used to immunoprecipitate (IP) DBT from head extracts of wild-type flies (ZT23; left lane) and *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies (right lane). Immunoprecipitates were then subjected to Western blotting with anti-PER (top) and anti-DBT (bottom) antibodies. PER was immunoprecipitated by anti-DBT antibodies in both wild-type and *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies. The results shown are representative of three experiments. **C**, Head extracts of wild-type flies from ZT14 (left lane) and ZT2 (middle lane) were run alongside extracts from *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies (right lane) and blotted for PER protein. Because *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies are arrhythmic, they were not entrained. PER shows a characteristic difference in mobility in wild-type flies between ZT14 and ZT2, with the decreased mobility at ZT2 attributable to increased PER phosphorylation (Edery et al., 1994). Neither of these times correspond to peak PER levels in wild-type flies but were chosen as extremes in PER phosphorylation status. PER protein extracted from *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies was mostly hypophosphorylated, although some slower mobility PER was detected. The results shown are representative of three experiments.

*tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies had an electrophoretic mobility similar to the hypophosphorylated form of PER typically seen when PER first begins to accumulate in wild-type flies at Zeitgeber time (ZT) 14 (Zeitgeber time is the time in a 12 h light/dark cycle) (Fig. 2C), which is in contrast to the highly phosphorylated forms of PER seen in the early morning (ZT2). In conclusion, DBT<sup>AR</sup> apparently binds PER normally but is mostly defective in phosphorylating PER, in agreement with the previous genetic interactions of *dbt<sup>AR</sup>* with some *per* alleles (Rothenfluh et al., 2000c).

Subsequently, we used the *dbt<sup>AR</sup>* mutation to test whether DBT binding to PER or DBT phosphorylation of PER regulates PER subcellular localization. Because ~5% of *dbt<sup>AR</sup>* homozygotes survive to adulthood (Rothenfluh et al., 2000a), PER localization was tested in the adult s-LN<sub>s</sub>. Wild-type (ZT23), *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant, and *tim<sup>01</sup>* single-mutant brains were stained with antibodies to PER and PDF. The results in Figure 3 revealed that PER was detected in the nucleus of the s-LN<sub>s</sub> of wild-type and *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double mutants but was undetectable in *tim<sup>01</sup>* single mutants.

We also tested PER localization in peripheral clock cells of *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double mutants. We used photoreceptor cells, be-

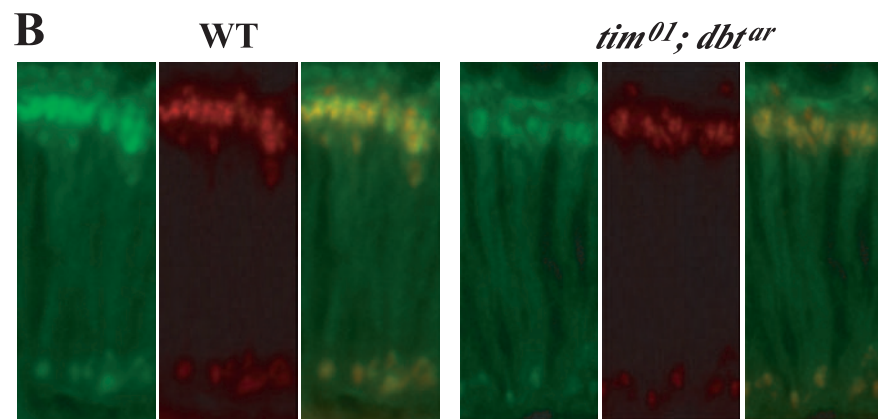
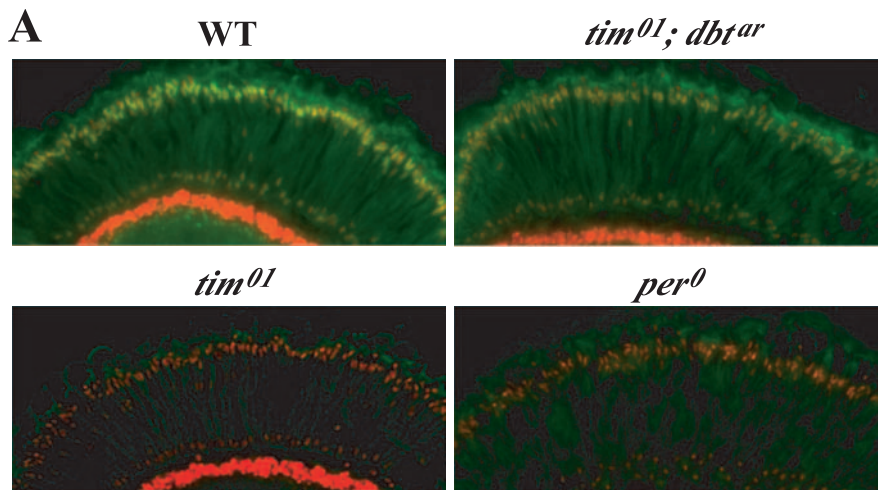


**Figure 3.** PER enters the nucleus of adult pacemaker neurons independently of TIM in a *dbt<sup>AR</sup>* background. Brains of adult wild-type flies (WT; ZT23; left panels), *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant flies (middle panels), and *tim<sup>01</sup>* single-mutant flies (right panels) were stained as in Figure 1 with antibodies to PER (red) and PDF (green). PER accumulated in nuclei of wild-type and *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant s-LN<sub>s</sub> but was undetectable in a *tim<sup>01</sup>* single-mutant background. Essentially identical results were seen in at least 20 brain hemispheres from each genotype.

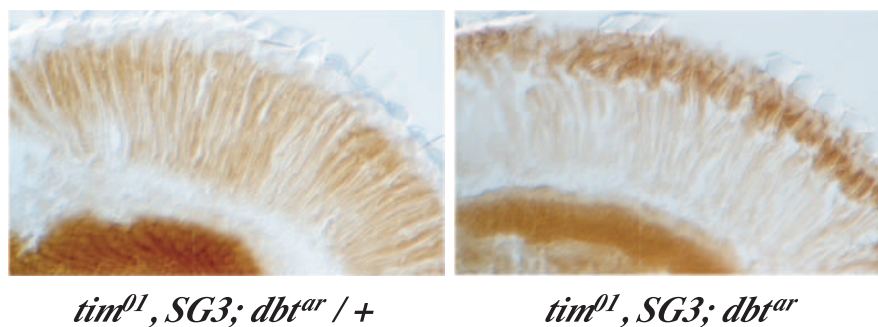
cause they contain functional molecular clocks. The cell nuclei of all neurons (including photoreceptors) were marked with an antibody that recognizes the Embryonic Lethal Abnormal Vision protein (ELAV) (Robinow and White, 1991). The results in Figure 4 show that PER was localized to the nuclei of photoreceptor cells in wild-type (ZT23) and in *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double mutants but was undetectable in *tim<sup>01</sup>* or *per<sup>0</sup>* single mutants (Fig. 4). Our results are consistent with previous data showing that PER is constitutively nuclear in the photoreceptor cells of *dbt<sup>AR</sup>* single mutants (Rothenfluh et al., 2000c), and our findings here reveal that nuclear PER accumulation in *dbt<sup>AR</sup>* mutants is independent of TIM. The presence of PER in the nuclei of central and peripheral clock cells that synthesize wild-type levels of a kinase-defective DBT protein indicates that DBT kinase activity, rather than a simple DBT–PER protein interaction, normally prevents PER from accumulating in the nucleus in the absence of TIM. Thus, PER, if hypophosphorylated and not degraded, translocates to the nucleus, indicative of its intrinsic properties.

#### A stable PER fusion protein associates with nuclei in the absence of *tim* and wild-type *dbt*

Nuclear PER in *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double mutants could simply reflect that high levels of PER in pacemaker neurons are sufficient to override the normal block to PER nuclear entry in the absence of TIM. However, the levels of PER detected in *tim<sup>01</sup>;* *dbt<sup>AR</sup>* double-mutant pacemaker cells are not as high as those in wild type, and yet, PER is nuclear (Figs. 3, 4). This observation suggests that nuclear PER can repress the transcription of clock genes in the absence of TIM (directly examined below). Furthermore, levels of PER in the Western blots of *tim<sup>01</sup>;* *dbt<sup>AR</sup>* head extracts are within the normal physiological range of PER from wild-type flies (Fig. 2C). Thus, it is unlikely that nuclear PER is simply a consequence of increased PER stability. Instead, PER nuclear accumulation in



**Figure 4.** PER enters photoreceptor cell nuclei independently of TIM in a *dbt<sup>ar</sup>* mutant background. **A**, Sections through adult heads from wild type (WT; ZT23; top left), *tim<sup>01</sup>; dbt<sup>ar</sup>* double mutants (top right), *tim<sup>01</sup>* single mutants (bottom left), and *per<sup>0</sup>* single mutants (bottom right) stained with antibodies to PER (green) and ELAV (red). PER localized to the nucleus in wild-type and *tim<sup>01</sup>; dbt<sup>ar</sup>* double-mutant flies (seen more clearly at higher magnification in **B**) but was undetectable in *tim<sup>01</sup>* and *per<sup>0</sup>* single mutants. Essentially identical results were seen in sections from at least 25 different adult heads from each genotype.



**Figure 5.** The subcellular distribution of a stable PER fusion protein, SG3, is also controlled by *dbt*. Sections through adult heads of *tim<sup>01</sup>, SG3; dbt<sup>ar</sup>/+* (left) and *tim<sup>01</sup>, SG3; dbt<sup>ar</sup>* (right) flies stained with antibodies to LacZ and visualized via a DAB color reaction. SG3 was detected throughout the long cell bodies of the photoreceptors in *tim<sup>01</sup>* flies heterozygous for *dbt<sup>ar</sup>*, as originally seen in *tim<sup>01</sup>* mutants (Vosshall et al., 1994). However, SG3 localizes to the periphery of the photoreceptor cells (where the nuclei are located) in *tim<sup>01</sup>; dbt<sup>ar</sup>* double mutants. Essentially identical results were seen in three independent experiments.

*tim<sup>01</sup>; dbt<sup>P</sup>* and *tim<sup>01</sup>; dbt<sup>ar</sup>* double-mutant clock cells presumably reflects the default state of PER in the absence of its regulator, DBT.

To test these ideas more extensively, we used the SG3 transgene in which the *per* promoter drives the expression of a cDNA encoding the first half of PER protein (amino acids 1–636) fused to LacZ (Vosshall et al., 1994). The SG3 protein is stable in a *tim<sup>01</sup>*

background, unlike full-length PER, and was used previously to demonstrate that PER nuclear accumulation depends on TIM, because SG3 was nuclear in an otherwise wild-type background but cytoplasmic in a *tim<sup>01</sup>* background (Vosshall et al., 1994). SG3 contains a sufficient amount of PER to interact stably with DBT, and this interaction is independent of TIM (Kloss et al., 2001).

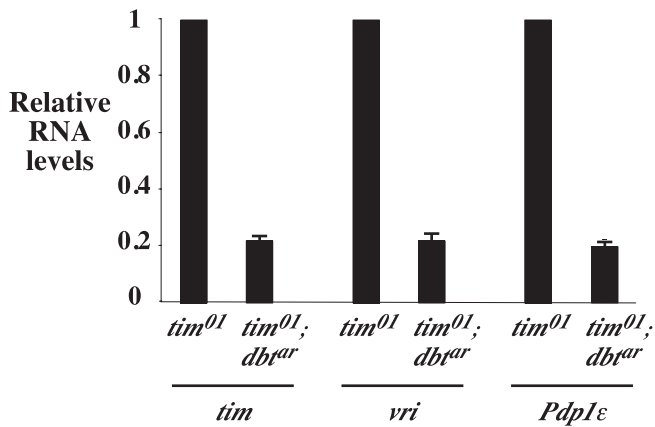
We used an antibody to LacZ to test the subcellular localization of SG3 in *tim<sup>01</sup>; dbt<sup>ar</sup>* double-mutant photoreceptor cells. The results in Figure 5 show a dramatic redistribution of SG3 between *tim<sup>01</sup>* single mutants and *tim<sup>01</sup>; dbt<sup>ar</sup>* double mutants. SG3 was widely distributed throughout the cytoplasm of the photoreceptor cells in *tim<sup>01</sup>* single mutants as originally observed (Vosshall et al., 1994). In contrast, SG3 was located at the periphery of the photoreceptor cells (where the nuclei are localized) in *tim<sup>01</sup>; dbt<sup>ar</sup>* double mutants. Thus, the subcellular localization of this stabilized PER fusion protein is controlled by DBT kinase activity, and this suggests that DBT kinase activity regulates wild-type PER subcellular localization, in addition to its effects on PER stability. We infer that the detection of PER in the nucleus of *tim<sup>01</sup>; dbt<sup>P</sup>* and *tim<sup>01</sup>; dbt<sup>ar</sup>* clock cells is not a simple consequence of increased PER stability but instead reflects that DBT kinase activity opposes the tendency of PER to enter the nucleus and normally prevents premature PER nuclear localization.

#### Nuclear PER represses CLK/CYC activity without TIM

Previous studies have suggested that PER rather than PER/TIM inhibits CLK/CYC-activated transcription (Rothenfluh et al., 2000a; Chang and Reppert, 2003; Weber and Kay, 2003; Nawathean and Rosbash, 2004). Constitutive nuclear PER in *tim<sup>01</sup>; dbt<sup>ar</sup>* mutant flies allowed CLK/CYC activity to be quantitatively measured *in vivo* in the complete absence of TIM. We assayed the levels of three genes directly activated by CLK/CYC: *tim*, *vri*, and *Pdp1ε* (Allada et al., 1998; Darlington et al., 1998; Rutilla et al., 1998a; Blau and Young, 1999; McDonald and Rosbash, 2001; Cyran et al., 2003). *tim*, *vri*, and *Pdp1ε* levels were compared in RNA isolated from *tim<sup>01</sup>* single mutants and *tim<sup>01</sup>; dbt<sup>ar</sup>* double-mutant adult fly heads. The results in Figure 6

show that steady-state RNA levels of all three genes were approximately fivefold lower in *tim<sup>01</sup>; dbt<sup>ar</sup>* than in *tim<sup>01</sup>* mutants.

These effects on PER target gene transcription probably indicate direct repression of CLK/CYC activity rather than an indirect effect of nuclear PER on CLK protein levels, because *Clk* RNA levels are approximately threefold higher in *tim<sup>01</sup>; dbt<sup>ar</sup>* than in



**Figure 6.** Nuclear PER represses CLK/CYC-activated transcription without TIM. RNA was extracted from *tim*<sup>01</sup> and *tim*<sup>01</sup>; *dbt*<sup>Δ</sup> adult fly heads, and levels of *tim*, *vri*, and *Pdp1ε* were compared with *cyc* RNA levels by quantitative PCR, as described previously (Cyran et al., 2003). RNA levels in *tim*<sup>01</sup> fly heads were set to 1 in each of two independent experiments and compared with RNA levels in *tim*<sup>01</sup>; *dbt*<sup>Δ</sup> flies. The data are shown as the average, with error bars indicating the SD. Levels of *tim*, *vri*, and *Pdp1ε* RNA were approximately fivefold lower in *tim*<sup>01</sup>; *dbt*<sup>Δ</sup> than in *tim*<sup>01</sup> fly heads. Similar results were also seen in one additional experiment analyzed by ribonuclease protection.

*tim*<sup>01</sup> mutants (J. Blau, unpublished data). Indeed, *tim* and *Pdp1ε* RNA levels were essentially identical to their levels in wild-type flies at ZT2, the trough of their normal RNA cycle, indicating that their transcription is maximally repressed (Blau, unpublished data). Thus, nuclear PER represses CLK/CYC-activated transcription in the absence of TIM.

These effects of PER on CLK/CYC activity, independently of TIM, are consistent with repression of *tim* promoter activity in *tim*<sup>01</sup>; *dbt*<sup>Δ</sup> double-mutant flies (Weber and Kay, 2003). Our results, and those of Weber and Kay (2003), also show that wild-type DBT activity is not required for transcriptional repression by PER. This conclusion is consistent with the constitutive repression of CLK/CYC activity (measured via *tim* RNA levels) in constant darkness *in vivo* in *dbt*<sup>P</sup> larval LNs (Price et al., 1998). However, these *in vivo* findings differ from those of Nawathean and Rosbash (2004), whose studies of *Drosophila* S2 cells *in vitro* (in which RNAi was used to remove DBT) indicated that DBT is required for repression by ectopically expressed PER. Evidently, S2 cells, which do not possess endogenous circadian clocks, do not provide an accurate model for certain steps of the *in vivo* mechanism, even when programmed to express clock genes and proteins.

#### Overexpressing SGG has no effect on PER nuclear localization or stability without TIM

Our results indicate that DBT kinase activity helps separate the phases of transcription and repression of CLK/CYC-activated genes by preventing premature PER accumulation in the nucleus. However, PER does enter the nucleus in wild-type clock cells around the middle of the night. This is generally considered to require TIM (Vosshall et al., 1994), and the precise timing of PER and TIM nuclear localization is affected by mutations in *per*, *tim*, *dbt*, *CK2α*, *CK2β*, and *sgg* (Curtin et al., 1995; Rothenfluh et al., 2000b; Bao et al., 2001; Martinek et al., 2001; Lin et al., 2002; Akten et al., 2003). Overexpression of *sgg* in clock cells shortens the behavioral period and advances the timing of PER and TIM nuclear localization, presumably opposing the effect of DBT-mediated phosphorylation. The period-altering effects of overexpressing *sgg* were correlated with increased TIM phosphorylation

*in vivo* (Martinek et al., 2001). However, because SGG/GSK3 and members of the Casein Kinase I family (including CKIε, mammalian DBT) have been shown to often phosphorylate the same substrate (Jia et al., 2002; Price and Kalderon, 2002; Okamura et al., 2004), we tested whether SGG could alter PER subcellular localization and/or stability independently of TIM.

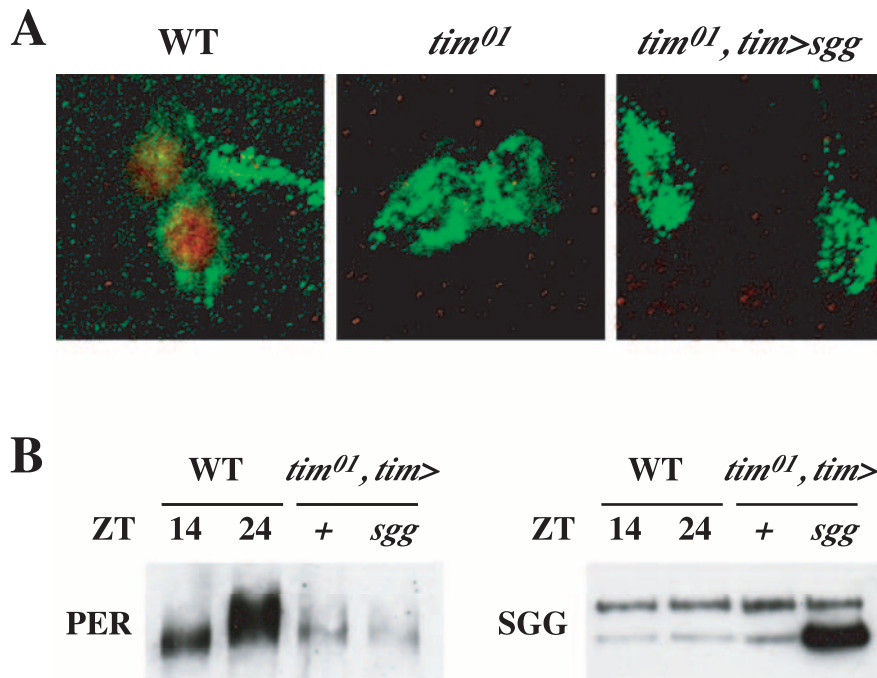
*sgg* was overexpressed specifically in clock cells using the *tim*(UAS)-*gal4* driver (Blau and Young, 1999) in a *tim*<sup>01</sup> background. We chose a *uas*-*sggB* transgene on the third chromosome (Bourouis, 2002), because it had the strongest effect on the period of adult locomotor rhythms of the *sgg* transgenes that we tested in a *tim*<sup>+</sup> background, shortening the period to 18.5 h (A. M. Buchsbaum and Blau, unpublished data). The results in Figure 7A show that PER was undetectable in the LNs of third instar larvae that overexpress *sgg* in a *tim*<sup>01</sup> background just as in *tim*<sup>01</sup> mutants without increased *sgg* expression (Fig. 7A). Western blots from fly head extracts isolated from *tim*<sup>01</sup> mutant flies overexpressing *sgg* also did not show altered PER levels or mobility compared with *tim*<sup>01</sup> mutants, indicating no change in PER stability or phosphorylation (Fig. 7B). In contrast, previous work has shown that TIM is hyperphosphorylated in response to overexpression of *sgg* in a *per*<sup>0</sup> background (Martinek et al., 2001). Therefore, we conclude that the effects of SGG on the *Drosophila* circadian clock require *tim*. Our results provide additional evidence for a functional interaction of SGG and TIM and are consistent with the finding that TIM can be directly phosphorylated by mammalian SGG (GSK3) *in vitro* (Martinek et al., 2001).

#### Discussion

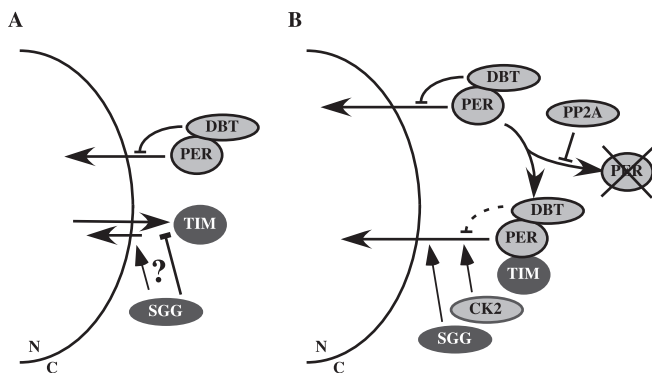
The results presented in this study reveal a novel function for DBT in the *Drosophila* circadian clock: DBT kinase activity controls the subcellular localization of PER. Phosphorylated PER is retained in the cytoplasm, whereas hypophosphorylated PER can accumulate in nuclei. Hypophosphorylated PER does not require TIM for access to the nucleus; however, as discussed below, TIM plays an important role in regulating DBT-dependent phosphorylation of PER. A model depicting our current view of the *Drosophila* clockworks is depicted in Figure 8.

Our results may provide a mechanistic explanation for the findings of Bao et al. (2001), who showed that the mutant DBT<sup>S</sup> protein delayed nuclear accumulation of PER in photoreceptor cells. Delayed PER nuclear entry would be expected if DBT<sup>S</sup> elevates PER phosphorylation, and *in vitro* studies by Ko et al. (2002) found that DBT<sup>S</sup> is a more active PER kinase than wild-type DBT. However, there may be a more important qualitative shift in kinase activity in DBT<sup>S</sup> that produces this PER response. An independent study (Preuss et al., 2004) found that engineering the DBT<sup>S</sup> mutation into *Xenopus* CK1δ reduced phosphorylation of another substrate, casein, and Bao et al. (2001) found that DBT<sup>S</sup> delayed nuclear accumulation of PER without affecting its stability; wild-type or higher levels of PER protein accumulation were observed in *dbt*<sup>S</sup> mutants. Thus, as originally suggested by Bao et al. (2001), DBT<sup>S</sup> might produce a specific change in the pattern of PER phosphorylation that promotes cytoplasmic retention of PER without affecting stability.

CK1ε, the vertebrate DBT ortholog, has been shown to phosphorylate mammalian PER (Lowrey et al., 2000; Toh et al., 2001). CK1ε also affects the subcellular localization of mammalian PER proteins. However, independent studies differ, finding that CK1ε alternatively promotes or retards PER nuclear accumulation, depending on the cell line used (Vielhaber et al., 2000; Akashi et al., 2002; Miyazaki et al., 2004; Takano et al., 2004). These studies all monitored PER transfected into mammalian cell lines, and the



**Figure 7.** Overexpression of *sgg* has no effect on PER localization in the absence of *tim*. **A**, LNs of third instar larval brains were stained for PER (red) and PDF (green), as described in Figure 1. Genotypes analyzed were wild type (WT) at ZT23 (left), *tim*<sup>01</sup> (middle), and *tim*<sup>01</sup>, *tim(UAS)-gal4/tim*<sup>01</sup>; +/*uas-sggB* (*tim*<sup>01</sup>, *tim>sgg*; right). Nuclear PER was only detected in wild-type LNs. The results are representative of >20 brain hemispheres per genotype analyzed in at least two independent experiments. **B**, Western blots of extracts of wild-type (ZT14 and ZT2), *tim*<sup>01</sup>, *tim(UAS)-gal4*; +/+ (*tim*<sup>01</sup>, *tim>+*), and *tim*<sup>01</sup>, *tim(UAS)-gal4/tim*<sup>01</sup>; *uas-sggB*/+ (*tim*<sup>01</sup>, *tim>sgg*) adult heads stained with antibodies to PER (left) and SGG (right). PER levels and mobility in the gel are similar in extracts of *tim*<sup>01</sup> flies regardless of whether *sgg* is overexpressed. The different bands in the SGG blot are different SGG isoforms. Levels of the upper band (SGG39) are unchanged, but the lower band (SGG10) is overproduced in flies in which *uas-sggB* is activated by the *tim(UAS)-gal4* driver line.



**Figure 8.** Model describing regulation of PER and TIM nuclear entry. **A**, PER and TIM have opposite intrinsic tendencies. Monomeric PER tends to enter the nucleus (N), but this is inhibited by DBT kinase activity. TIM can enter the nucleus in the absence of PER (Ashmore et al., 2003), but its default state is cytoplasmic (C). SGG phosphorylation of TIM is proposed either to inhibit TIM nuclear export or to promote TIM nuclear entry. **B**, Model for regulation of PER and TIM nuclear entry. PER protein is prevented from entering the nucleus prematurely by DBT, which targets PER for degradation in the absence of TIM. PER accumulates with delayed kinetics in protein phosphatase 2A (PP2A) regulatory subunit mutants, indicating that PP2A normally opposes DBT-mediated PER degradation by dephosphorylating PER (Sathyanarayanan et al., 2004). Formation of a PER/TIM complex prevents DBT from phosphorylating PER (Kloss et al., 2001; Ko et al., 2002), thus stabilizing PER. PER and TIM nuclear entry is promoted by SGG, which phosphorylates TIM (Martinek et al., 2001). CK2 activity also promotes PER and TIM nuclear entry, and CK2 can phosphorylate PER *in vitro* (Lin et al., 2002; Akten et al., 2003). Because PP2A can dephosphorylate PER, it may also play a role in the timing of nuclear entry of PER. DBT and PP2A also determine PER stability in the nucleus, but this is omitted from this model for simplicity.

varying levels of PER overexpression could explain the conflicting experimental results. Our demonstration of a central role for DBT in the nuclear localization of PER in the fly suggests that studies of endogenous PER proteins in naturally occurring clocks might confirm a similar function for CK1 $\epsilon$  in mammals.

PER has been detected in the nucleus without TIM before this study. Shafer et al. (2002, 2004) performed a careful analysis of the timing of PER and TIM nuclear entry in wild-type LNs that indicated that PER was nuclear at times when TIM was only detectable cytoplasmically. Although this suggests that PER can enter the nucleus independently of TIM, the results of Ashmore et al. (2003) permit another interpretation. Although TIM normally accumulates in the cytoplasm of *per*<sup>0</sup> mutant pacemaker cells (Hunter-Ensor et al., 1996; Myers et al., 1996), Ashmore et al. (2003) found TIM in the nucleus in *per*<sup>0</sup> mutants in the presence of nuclear export and proteasome inhibitors. It is therefore likely that TIM normally shuttles in and out of the nucleus and/or is unstable in the nucleus. Even if PER and TIM enter the nucleus as a complex, nuclear PER would accumulate more rapidly than TIM if some nuclear TIM disassociates from PER and is then exported (to recruit more PER) or degraded. Alternatively, given the results of the present study, if DBT activity

were to be transiently suppressed by factors other than TIM, PER could enter the nucleus without TIM.

Certainly *tim* must be included in any clock model given that a null *tim* mutation stops the molecular clock (Sehgal et al., 1994) and given the dramatic effects of other *tim* mutations on period length (Rutila et al., 1998b; Rothenfluh et al., 2000a,b). Our model (Fig. 8B) also incorporates data presented in this study, showing that the effects of SGG on the clock require *tim*. Given the recent report that TIM is required for the mammalian molecular clock (Barnes et al., 2003), it will be interesting to determine its precise function.

### Contrasting intrinsic properties of PER and TIM

Our results reveal that hypophosphorylated PER can enter the nucleus without TIM (Fig. 8A). In contrast, the intrinsic state of TIM appears to be cytoplasmic. Although it is free to enter the nucleus, TIM is rapidly exported and/or degraded in the nucleus, unless it interacts with PER (Fig. 8) (Ashmore et al., 2003). It seems likely that the phosphorylation of TIM by the SGG kinase shifts the balance of TIM localization in favor of the nucleus (Fig. 8A). Thus, the timing of nuclear localization of PER and TIM probably reflects the divergent effects of DBT and SGG on their substrates. PER has an intrinsic tendency to enter the nucleus but is prevented from doing so via DBT-mediated phosphorylation; TIM tends to stay in the cytoplasm, but SGG makes TIM more likely to enter the nucleus and/or remain there.

In a potentially analogous situation, the transcription of many genes is controlled by both activators and repressors. Such combinations of positive and negative factors have been proposed to

establish on/off switches in transcription rather than a range of levels of gene expression (Rossi et al., 2000). The opposing tendencies of unmodified PER to enter the nucleus and of unmodified TIM to accumulate in the cytoplasm might similarly help to turn PER and TIM nuclear entry into a switch rather than a gradual event, at least in pacemaker neurons. Indeed, PER and TIM nuclear entry in pacemaker neurons marks a major transition in the behavioral response of flies to light. A light pulse given in the early evening, before PER and TIM enter the nucleus, delays both the molecular clock and the resulting behavior. In contrast, a light pulse at the end of the night, when the proteins are nuclear, phase advances both the clock and behavior (Myers et al., 1996). It is also possible that tight regulation of PER and TIM nuclear entry could help the individual pacemaker neurons within one fly stay synchronized. The results of this study add to our understanding of the intricate regulation of this step in the molecular clock.

### Conclusions

DBT plays two important roles in the *Drosophila* circadian clock. First, DBT decreases the stability of PER proteins through phosphorylation in the absence of TIM and therefore regulates both the initial cytoplasmic accumulation of PER and the turnover of PER in the nucleus at the end of each cycle as TIM is lost. Second, DBT kinase activity prevents premature accumulation of PER in the nucleus. The function of DBT kinase activity in the cytoplasm establishes the first half of each circadian cycle, separating the phases of transcription and repression of CLK/CYC-activated genes.

TIM also has multiple roles in the *Drosophila* clock. In wild-type flies, TIM inhibits DBT-dependent phosphorylation of PER (Kloss et al., 2001), and this finding was replicated in S2 cells in culture (Ko et al., 2002). Coproduction of PER and TIM promotes nuclear accumulation of both proteins, supported by SGG-dependent phosphorylation of TIM. Thus, in each of its roles, TIM can be viewed as an important vehicle for controlling PER nuclear localization.

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