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Drosophila DBT Lacking Protein Kinase Activity Produces Long-Period and Arrhythmic Circadian Behavioral and Molecular Rhythms▽†

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A mutation (K38R) which specifically eliminates kinase activity was created in the *Drosophila melanogaster ckI* gene (*doubletime* [*dbt*]). In vitro, DBT protein carrying the K38R mutation ($DBT^{K/R}$) interacted with Period protein (PER) but lacked kinase activity. In cell culture and in flies, $DBT^{K/R}$ antagonized the phosphorylation and degradation of PER, and it damped the oscillation of PER in vivo. Overexpression of short-period, long-period, or wild-type DBT in flies produced the same circadian periods produced by the corresponding alleles of the endogenous gene. These mutations therefore dictate an altered "set point" for period length that is not altered by overexpression. Overexpression of the $DBT^{K/R}$ produced effects proportional to the titration of endogenous DBT, with long circadian periods at lower expression levels and arrhythmicity at higher levels. This first analysis of adult flies with a virtual lack of DBT activity demonstrates that DBT's kinase activity is necessary for normal circadian rhythms and that a general reduction of DBT kinase activity does not produce short periods.

Circadian rhythms are molecular, physiological, or behavioral processes which occur with a periodic oscillation of approximately 24 h. These rhythms can be entrained by environmental cues such as light/dark cycles, but they persist in the absence of such cues, thus demonstrating the presence of an endogenous clock (reviewed in reference 40). *Drosophila melanogaster* has been widely used as a model organism for a genetic analysis of circadian rhythms, and many of the gene products comprising the endogenous clock mechanism were identified in this organism, including the first discovered clock gene, *period* (*per*) (reviewed in reference 42). Molecular genetic analysis of these rhythms has elucidated a basic clock mechanism consisting of oscillating clock gene products, which regulate their own expression through positive and negative feedback loops. During the night, PER and Timeless protein (TIM) levels rise in the cytoplasm, where the proteins heterodimerize, and then translocate into the nucleus to negatively regulate the transcription of their own genes and other genes. Negative regulation is effected by interactions with the transcription factors dClock (dCLK) and Cycle (CYC), which activate transcription of the *per* and *tim* genes and indirectly repress transcription of *dClk* in the absence of PER and TIM. Throughout the day, TIM levels do not accumulate in the nucleus because of TIM's degradation via a light- and cryptochrome-mediated degradation pathway (reviewed in reference 42).

DBT, an ortholog of mammalian casein kinase Iε and casein kinase Iδ (CKIε/δ), regulates PER cytoplasmic and nuclear accumulation by triggering PER's degradation and regulating the timing of its nuclear accumulation (3, 8, 9, 22, 23, 43, 46, 49). DBT's activity on PER is supplemented by the activity of CKII (2, 27, 28) and SGG (31) and is antagonized by a rhythmically expressed protein phosphatase (47). DBT may regulate other aspects of PER function (35) and other circadian proteins (e.g., dCLK) as well (21, 58). At present, a comprehensive understanding of DBT's effects on PER and other clock proteins is lacking.

Intriguingly, a *dbt* mutation conferring a short-period phenotype (dbt^S) and *dbt* mutations conferring a long-period phenotype (dbt^L, dbt^G, and dbt^H) all produce lowered kinase activity in vitro when introduced into orthologous CKIs (41, 49). This suggests that both short and long periods can be produced by lowered kinase activity. Analysis of phosphorylation by mammalian CKI has also suggested this idea. A mutation in CKIε in hamsters (the *tau* mutation [30]) and a mutation in CKIδ causing a sleep disorder in humans (55) both produce short periods and have been shown to hypophosphorylate their substrates in vitro. By contrast, it has been shown that CKI inhibition produces long periods in mammalian cell culture rhythms (11). These results may point to complexity of kinase target sites, with phosphorylation at some sites lengthening the period and at other sites shortening it. Differential effects on various target sites in PER may explain the different effects of the *Drosophila* dbt^S and dbt^L mutations on the circadian period (41, 43). Alternatively, the dbt^S and dbt^L mutations may produce their period effects through some means other than by altering kinase activity. Since DBT forms a complex with PER and other clock proteins, such as dCLK (21, 58), some of its functions may be independent of its kinase activity and be attributable to associations that it forms with other proteins. It is not certain that the effects of the dbt^S and the dbt^L mutations are limited to effects on kinase activity.

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It would therefore be interesting to know if short and long periods can be produced by mutations which only produce lower kinase activity. In this paper, we investigate the effect on the clock of a mutant form of DBT which is normal except that it lacks any detectable kinase activity. A mutation which specifically eliminates DBT kinase activity and not other aspects of its function has never been analyzed in the adult fly. To avoid lethality, our kinase-inactive transgenic protein was expressed only in circadian cells in flies that also expressed wild-type DBT (DBT^{WT}) from the endogenous gene, and therefore the flies (and even the cells that expressed the kinase-inactive DBT) were viable. In addition to addressing the relationship between kinase activity and the circadian period, this mutant has allowed us to address more generally whether kinase activity is required for DBT's role in the circadian mechanism. If kinase activity is necessary for clock function of DBT and the kinase-inactive form of DBT could be expressed at high enough levels to effectively out-compete DBT^{WT} for stable interactions with clock protein substrates, it should effectively block DBT^{WT} activity in the clock mechanism and act as a dominant negative mutant. Alternatively, overexpression of this mutant DBT protein should produce the same effect as overexpression of DBT^{WT} for any DBT function that does not require its kinase activity. The results presented here demonstrate that we have created a dominant negative *dbt* mutation and that its overexpression reduces endogenous DBT-dependent PER phosphorylation and degradation, with consequences for both molecular and behavioral circadian rhythms.

MATERIALS AND METHODS

Site-directed mutagenesis. A cDNA clone carrying the *dbt* gene (*dbt*-pBS plasmid [22]) was used as a template in two separate PCRs in which complementary oligonucleotides GTGGCCATCAGGCTGGAGTGCATCCGCACC ($DBT^{K/R}$ F primer) and GCACTCCAGCCTGATGGCCACCTCCTCGCC ($DBT^{K/R}$ R primer), each carrying the codon for the single amino acid change (Lys38Arg), were used as primers. In the first reactions the $DBT^{K/R}$ R primer was used with an upstream DBT 5′F primer (GCGCGAGCTCGGCAACGCA AATCCGAGGAAC) and the $DBT^{K/R}$ F primer was used with a downstream DBT 5′R primer (GGAAGTTATCCGGCTTGATGT). Each of these reactions produced a $DBT^{K/R}$ subfragment with the mutation at one end, and these subfragments were used with the flanking primers in PCR to generate a longer fragment with the mutation in the center of the amplified fragment, as previously described (41). This product was then purified, digested with restriction enzymes SacI and ClaI, purified again, and ligated into the previously described pMT-DBT-MYC plasmid (41) that had been digested with SacI and ClaI, thereby replacing the wild-type SacI/ClaI partial fragment of the *dbt* coding region.

GST pull-down assays. The $DBT^{K/R}$-coding region was amplified by PCR from the pMT-$DBT^{K/R}$-MYC plasmid described above, with the forward primer DBT 1f (GCGCGAATTCATGGAGCTGCGCGTGGGTAAC, containing an EcoRI site) and the reverse primer DBT 5′R (described above). A fragment resulting from digestion with ClaI and EcoRI was purified and then ligated into the ClaI- and EcoRI-digested pGEX-GST-DBT^{WT} plasmid, which was previously described (41).

Bacteria expressing glutathione *S*-transferase (GST), GST-DBT^{WT}, or GST-$DBT^{K/R}$ were grown and induced with isopropyl-β-D-thiogalactoside (IPTG). GST pull-down assays and analysis were performed as previously described (41).

Expression of proteins in S2 cells. Generation of the *per* expression plasmid (pAC-PER-HA) and the wild-type pMT-DBT-MYC and pAC-LacZ-V5 plasmids was previously described (7, 41), and generation of the pMT-$DBT^{K/R}$-MYC expression plasmid is described above. The plasmids expressing the MYC-tagged DBTs, V5-tagged LacZ, or hemagglutinin (HA)-tagged PER were transfected into *Drosophila* S2 cells with Cellfectin, as described by the supplier of Cellfectin (Invitrogen, Carlsbad, CA). A total of 1.9 μg of the pAC-PER-HA-expressing plasmid and 0.7 μg of the pAC-LacZ-V5-expressing plasmid were used for each transfection. Low levels of DBT-MYC were produced with 0.2 μg of plasmid, 5 ml of cells in the transfection, and 0.05 mM $CuSO_4$ in the growth medium. Medium levels were produced with 0.2 μg of plasmid and 0.5 mM $CuSO_4$, and high levels were produced with 1.9 μg of plasmid and 0.5 mM $CuSO_4$. When the DBT protein carrying the K38R mutation ($DBT^{K/R}$) was coexpressed with DBT^{WT} in the same cells, the amounts of DBT^{WT} plasmid and $CuSO_4$ were the same in the three treatments just described, but the amount of $DBT^{K/R}$ plasmid was increased to 7.6 μg. An empty pMT vector containing no *dbt* gene was also used in the transfections in various amounts so that the total amount of plasmid transfected in all experiments equaled 4.5 μg (or 12.1 μg for DBT^{WT} and $DBT^{K/R}$ cotransfections). At 42 h after the addition of $CuSO_4$, the cells were collected and further processed for immunoprecipitation of DBT-MYC or $DBT^{K/R}$-MYC or for immunoblot analyses (see below).

Drosophila S2 cells were also stably transfected with pMT-DBT^{WT}-MYC or pMT-$DBT^{K/R}$-MYC and pBS-PURO as described above. Selection was accomplished in Schneider's *Drosophila* medium plus L-glutamine (Invitrogen) supplemented with 10% fetal bovine serum and 10 μg/ml puromycin.

Kinase assays. Immunoprecipitation of DBT-MYC or $DBT^{K/R}$-MYC from S2 cells was done as previously described (41), except that the transfected cells were homogenized in the lysis buffer with 5 μg of pepstatin/ml and 20 μg of aprotinin/ml, and the lysate was immunoprecipitated with a monoclonal anti-MYC antibody (MMS-150R from Covance Research Products, Berkeley, CA) at a concentration of 1:150. Immunoprecipitated DBT^{WT}-MYC or $DBT^{K/R}$-MYC was detected with a rabbit anti-DBT antibody generated to a GST-DBT C-terminal immunogen containing amino acids 293 to 440. The amounts of DBT used in the assays were detected by immunoblot analysis using the anti-DBT C terminus antibody at a concentration of 1:2,000. Kinase assays were done as previously described (41). For purification of the PER substrate, bacteria harboring PQE-PER (27) (generously provided by Ravi Allada) were induced with 1 mM IPTG, and PER was purified by Ni affinity chromatography as described by the supplier of the beads (Ni-nitrilotriacetic acid agarose from Invitrogen, Carlsbad, CA). Casein and PER phosphorylation were detected by phosphorimager analysis (Molecular Dynamics-GE Healthcare, Sunnyvale, CA) of gels containing ^{32}P-labeled substrates, and the activities were normalized to the amount of DBT-MYC detected by chemifluorescence detection of the MYC epitope (ECL Plus Western blotting detection reagents; Amersham-GE Healthcare, Piscataway, NJ). Both signals were corrected for background by subtracting the signal from the same region of the "no-DNA" lane.

Quantification of protein expression in S2 cells. After 42 h of induction with $CuSO_4$, the cells were harvested and immunoblotted as previously described (7). Individual bands were quantified by a quantitative line analysis of the scans, using a chemifluorescence imager and the ImagQuant 5.0 software program (Molecular Dynamics-GE Healthcare, Sunnyvale, CA). Briefly, the line analysis was performed by drawing a line through the entire length of the lane, followed by configuration of the width attributes to include the width of the lane. A graph representing the pixels within the defined area of the lane was created by the peak finder analysis. The peak area, corresponding to a protein signal, was defined by manually inserting two lines flanking the peak at the inflection points for both the ascending and descending lines. The value of the area in an equivalent length of a control lane expressing no protein was subtracted from this peak area to generate a background-corrected area value. These values representing the pixel areas beneath the peak for the PER signal were then normalized to the LacZ-V5 signal for the respective sample, which was quantified in a similar way. The final normalized PER/LacZ values for each lane were then normalized to the level of PER/LacZ without any DBT expression, which was defined as 100%. Three separate transfection experiments were done for Fig. 3B and three for Fig. 3D, and each experiment was analyzed two to four times by immunoblotting to generate average PER/LacZ percentages for each experiment. The percentages from the individual experiments were averaged to generate values from which the graphical representations of protein levels were generated (see Fig. 3B and D).

Generation of transgenic flies. In order to clone the *dbt* constructs (dbt^{+}, dbt^{S}, dbt^{L}, and $dbt^{K/R}$) into the P element transformation vector pUAST, we first modified the pUAST polylinker by inserting a PmeI restriction site. This site was introduced by digesting the plasmid with NotI and XhoI, followed by ligation of a double-stranded oligonucleotide produced by annealing two single-stranded oligonucleotides, 5′GGCCGCCCAAGGTTGTTTAAACC3′ and 5′TCGAGGT TTAAACAACCTTGGGC3′. Successful insertion of the oligonucleotide created a PmeI site in the pUAST polylinker, which was consecutively digested with PmeI and EcoRI to allow oriented insertion of PmeI-EcoRI-digested fragments obtained from our various pMT-DBT-MYC constructs. This cloning placed the gene under the control of the upstream activating sequence (UAS) promoter.

P-element transformants were produced by Model System Genomics of Duke University (Durham, NC), and lines were generated and mapped to chromosomes by standard procedures. Only third chromosome lines were used for the analyses.

For our analyses, three different GAL4 driver lines were employed to express the UAS-DBT transgenes. One line expresses the GAL4 transcription factor in all cell types because it expresses *gal4* under the control of the *actin* promoter {*yw*;P(w[+mC] = Act5C-GAL4)25F01/CyO, y^+} (from the Bloomington Stock Center, Indiana University; stock number 4414). In one line, expression was limited to all circadian clock cells, because GAL4 expression was dependent on the activity of the *timeless* promoter {*yw*;P(w[+mC] = GAL4-tim.E)62} (from the Bloomington Stock Center; stock number 7126) (20). The third line (kindly provided by Justin Blau, New York University) also contained a UAS, so that GAL4 expressed in *tim*-expressing cells would then autoactivate its own expression (4).

Entrainment conditions, fly collection procedures, and locomotor activity assays. All flies were raised at 23.5°C with 12-h:12-h light-dark (LD) cycles provided by cool white fluorescent bulbs (ca.3,000 lx during the photophase) for at least 3 days. For analysis of PER and DBT, progeny were placed in vials containing 15 to 30 flies each, followed by entrainment for at least 72 h to a 12-h:12-h LD cycle at a constant temperature of 23.5°C (All flies for immunoblot analysis of whole bodies were males, while both females and males were selected for immunoblot or immunohistochemical analysis of heads.) Depending on the experimental conditions, the vials were placed in LD or constant-darkness (DD) conditions and collected at the indicated time point (zeitgeber time [ZT], indicating the presence of the light cue [LD], or circadian time [CT], indicating the absence of any entrainment cue [DD]). Flies collected during DD were collected during the first day of DD, starting at CT1. Flies either were quickly frozen using liquid nitrogen and stored at −80°C for immunoblot analysis of whole bodies or were directly processed for analysis of photoreceptors, heads, or larval brains. For locomotor activity analysis, individual flies were loaded into glass cuvettes, placed in monitoring devices linked to a computer recording the movement of each individual (Trikinetics, Inc., Waltham, Mass.), and monitored as previously described (3). Using analytic software (ClockLab, Inc., Evanston, IL), the average period length of each fly was determined by chi-square periodogram analysis, and the overall activity pattern across the recorded time period (actogram) was used to confirm rhythmic or arrhythmic behavior of each tested individual. Rhythmic flies produced a single major periodogram peak with a P value of <0.01 and produced discernibly rhythmic actograms.

In vivo immunoblot analysis. After completion of the collection series, frozen bodies were homogenized in 7.5 μl 2× sodium dodecyl sulfate (SDS) Laemmli gel loading buffer per fly with a Kontes pellet pestle homogenizer, incubated at 95°C for 5 min, and diluted by addition of 75 μl 1× SDS buffer. For quantification of PER and DBT-MYC in head extracts, heads were removed with a razor blade, homogenized with 3.2 μl of 1.1× SDS Laemmli gel loading buffer per head, and heat treated; 3 to 5 μl of extract was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting. Depending on the experimental setup immunoblot analysis was performed using a 1:5,000 dilution of anti-MYC antibody (Invitrogen, Carlsbad, CA), a 1:2,000 dilution of rabbit anti-DBT antibody (see above), or a 1:25,000 dilution of a rabbit anti-PER antibody generated to the PQE-PER antigen described above. A 1:5,000 dilution of mouse monoclonal antitubulin antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) was included with the anti-MYC and anti-DBT antibodies. Incubation with primary antibodies was followed by incubation with a 1:5,000 dilution of the appropriate horseradish peroxidase-labeled secondary antibodies and visualization of the signal with the ECL Plus Western blotting detection reagents (Amersham-GE Healthcare, Piscataway, NJ). Experiments were repeated two or three times, with multiple immunoblots for each.

Immunohistology. The heads of the flies were collected and processed as previously described (3). Scores between 0 and 2 (indicating the relative intensity of nuclear localization of PER) were assigned for each photoreceptor section examined. Larval brains were collected from *tim*-GAL4>UAS-DBT larvae after entrainment of the larvae and were processed as previously described for detection of PER and PDF (43). Our rabbit anti-PER was used at a dilution of 1/5,000, while anti-PDF (kindly deposited by Justin Blau in the Developmental Studies Hybridoma Bank, University of Iowa) was used at 1/1,000. Secondary antibodies were Alexa-Fluor 488 anti-rabbit immunoglobulin G and Alexa-Fluor 568 anti-mouse immunoglobulin G (Invitrogen, Carlsbad, CA). Z-stacks were acquired on a Zeiss confocal LSM 410 with a 40× water immersion lens and a 4× zoom.

RESULTS

DBT K38R mutant protein retains the capacity of DBT^{WT} to bind PER in vitro but lacks the protein kinase activity of DBT^{WT}. We sought to create a catalytically inactive form of DBT that retained other functions, in order to determine if the DBT K38R mutant protein would sustain any clock function or block clock function (i.e., act as a dominant negative protein). The work of Peters et al. (39), in which a mutant CKIε carrying the K38R mutation had been shown to antagonize the Wnt pathway in *Xenopus*, guided this effort. Mutation of this site was chosen for the creation of a DBT dominant negative mutant because (i) this amino acid is modified by a reagent which inactivates several kinases (19, 61), (ii) the amino acid contacts the phosphates of ATP in the crystal structure of casein kinase I (54), (iii) numerous amino acid changes (including K to R) at this site have previously been shown to produce dominant negative mutants in other kinases (18, 39), and (iv) we had previously analyzed the *Xenopus* $CKI\varepsilon^{K/R}$ mutant form of this protein employed by Peters et al. (39) in vitro and showed that it lacked detectable kinase activity on PER or casein substrates (7). Therefore, it is likely that K38 makes direct contact with ATP and is involved in the phosphotransferase reaction (18). K38 and the surrounding region are conserved in *Drosophila* DBT and vertebrate CKIε/δ (Fig. 1A), and a K/R mutation was introduced into the corresponding site of DBT by site-directed mutagenesis. Mutation of K to R was chosen because it is a very conservative change and would not be predicted to have global effects on protein structure but rather to impair the specific catalytic steps involving ATP.

Previous studies using fly head extracts, *Drosophila* S2 cells, and in vitro GST pull-down assays have demonstrated that DBT associates with PER (22, 23, 41). In particular, GST pull-down assays demonstrate a direct protein-protein interaction between DBT and PER, without the possibility of an indirect interaction mediated by other *Drosophila* clock components. To address whether $DBT^{K/R}$ could interact directly with PER, the mutation was introduced into a construct producing a GST-DBT fusion protein that is otherwise identical to the GST-DBT^{WT} we had previously analyzed (41). GST-DBT^{WT} and GST-$DBT^{K/R}$ fusion proteins produced in bacteria were immobilized on glutathione-agarose beads and incubated with ^{35}S-labeled PER using two different incubation regimens (a larger amount of PER for 5 min and a smaller amount of PER for 2 h). PER-DBT binding was assessed by SDS-PAGE and autoradiography. The Coomassie blue-stained gel in Fig. 1B shows that the relative amounts of GST fusion proteins used in each reaction were comparable, except for unfused GST, which was substantially higher in level. Figure 1C shows the results of autoradiography to detect the amounts of labeled proteins bound under the two conditions, as indicated above the lanes. GST-DBT^{WT} and GST-$DBT^{K/R}$ proteins produced equivalent binding with PER under both conditions, while nonspecific binding between PER and GST or between luciferase and GST-DBT was negligible. These results (reproduced in three experiments) showed that GST-$DBT^{K/R}$ and GST-DBT^{WT} interact with ^{35}S-PER and that this association is the product of specific protein-protein interactions. There may be subtle differences in the association constants of wild-type and mutant DBT not detected by this

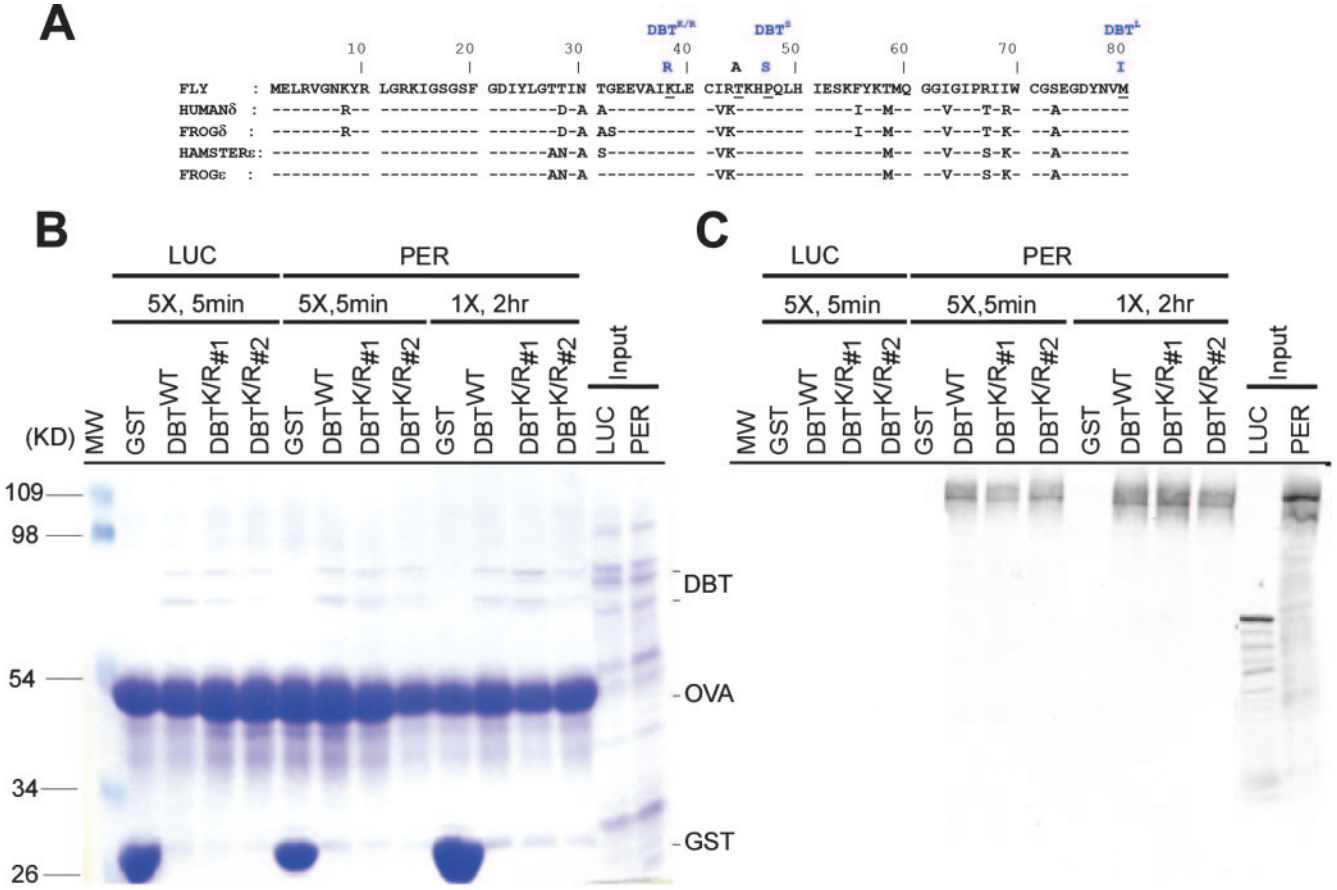

FIG. 1. GST pull-down assays demonstrate that DBT^{WT} and $DBT^{K/R}$ can both interact with PER. (A) An alignment of the amino terminus of *Drosophila* DBT with various vertebrate CKIε/δs is shown, and the conserved Lys38 which has been mutated to Arg to produce a dominant negative mutation in the Wnt pathway (39) is indicated, as well as the conserved amino acids which are mutated in the dbt^S and dbt^L mutants (22) and in a human sleep disorder (T44A) (55). The mutations marked with blue are analyzed in this paper. (B) GST alone or GST fusion proteins with DBT^{WT} and $DBT^{K/R}$ were expressed in *E. coli* and retained on glutathione-agarose beads, followed by a 5-min or 2-h incubation with [^{35}S]methionine-labeled PER or luciferase (LUC) produced by in vitro-coupled transcription-translation. The 5× amounts used 5 times the amount of labeled protein as the 1× amounts (amount of input in 1× amount electrophoresed directly on the right side of the gel). After the unbound material was washed off, the remaining bound material was analyzed by SDS-PAGE. Total amounts of proteins were visualized by Coomassie brilliant blue staining of the gel. Duplicate binding experiments used two confirmed mutant constructs for GST-$DBT^{K/R}$ (#1 and #2). DBT, full-length GST-DBT fusion protein; GST, GST alone or non-full-length GST-DBT fusion protein; OVA, ovalbumin used to prevent nonspecific binding to the beads. (C) ^{35}S-labeled proteins were detected by autoradiography.

assay, but clearly $DBT^{K/R}$ has the capacity to interact directly with PER.

To address whether the K38R mutation affected DBT's intrinsic kinase activity, DBT^{WT}-MYC and $DBT^{K/R}$-MYC immunoprecipitated via their MYC epitopes from S2 cells were assayed in vitro with casein and PER as substrates. (Purification of recombinant DBT from *Escherichia coli* would not have been informative, since we and others have previously shown that DBT^{WT} expressed in bacteria is not enzymatically active [23, 41, 49].) The MYC-tagged DBT was expressed in S2 cells from transiently transfected plasmids (Fig. 2A) or in S2 cells which had been stably transformed with these plasmids and therefore produced higher transgene-specific kinase activity relative to the background kinase activity in cells which did not carry the *dbt* transgene (Compare Fig. 2A with Fig. 2B and C). Following immunoprecipitation, immunoblot analysis showed successful recovery of DBT proteins (top panels in Fig. 2A to C) except in cells that did not carry the transgene. Endogenous DBT, which produces a faster-migrating band with the antibody used for the immunoblot (e.g., see Fig. S1A in the supplemental material), is not detected in any of the immunoprecipitates and therefore did not contribute significantly to the kinase activity. As previously shown (41), DBT^{WT}-MYC readily phosphorylates casein, the nominative substrate for casein kinases (Fig. 2A and B). While not as good a substrate, recombinant PER purified from bacteria by affinity chromatography was also phosphorylated by DBT^{WT}-MYC, by comparison with the activity exhibited with immunoprecipitates from no-DNA controls (Fig. 2C). In contrast to DBT^{WT}, immunoprecipitates containing mutant $DBT^{K/R}$-MYC protein produced no increased phosphorylation of casein or PER relative to the negative controls, even when present at comparable or even larger amounts than DBT^{WT}-MYC (as detected by immunoblot analysis of reaction aliquots [Fig. 2A to C]). The

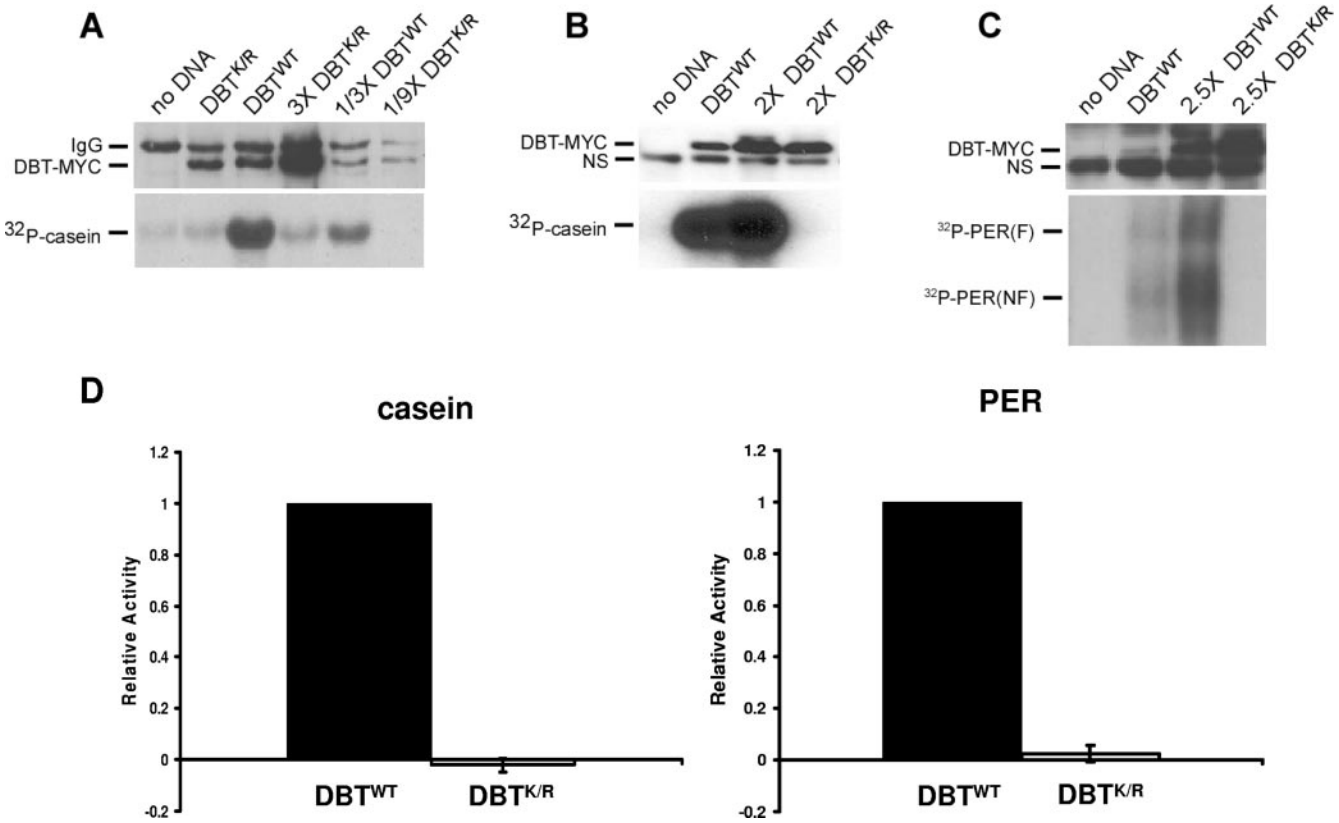

FIG. 2. In vitro kinase assays show that the $DBT^{K/R}$ mutation eliminates DBT's intrinsic kinase activity. (A to C) $DBT^{K/R}$-MYC and DBT^{WT}-MYC expression was induced by addition of $CuSO_4$ to transiently transfected S2 cells (A) or to stably transfected S2 cell lines (B and C) and immunoprecipitated using an antibody against the MYC epitope tag. The DBT proteins were then incubated with either casein (A and B) or PER (C) and [γ-^{32}P]ATP. Reaction aliquots were analyzed by SDS-PAGE, followed by detection of DBT with immunoblot analysis using an antibody against the C terminus of DBT (top panels) and detection of phosphorylated casein (A and B, bottom panels) or PER (C, bottom panel) by autoradiography. The band that migrates just ahead of DBT-MYC in some reactions is nonspecific (NS) rather than endogenous DBT because it was detected in lanes which contained only the substrate and no material immunoprecipitated from S2 cells (not shown). Increases or decreases in the volume of solution containing the kinase are indicated above the lanes (no label denotes a 1× volume). PER(F), full-length PER; PER(NF), PER protein that is not full length. Phosphorylation of casein or PER is readily detected for all DBT^{WT} reactions (DBT^{WT} lanes) and is not detected when using the $DBT^{K/R}$ or immunoprecipitates of cells that did not receive the DBT transgene ("no DNA"). (D) For lines permanently transformed with pMT-DBT-MYC, the amount of signal in the casein or PER mobility range was quantified by phosphorimager analysis and normalized to the amount of DBT detected by immunoblotting; both the ^{32}P and DBT signals were corrected by subtracting the background from an equivalent region of the "no-DNA" lane, resulting in negative values for some of the $DBT^{K/R}$ normalizations. These values were then plotted relative to the amount of normalized DBT^{WT} activity, which was set to 1. The results of three experiments for casein and six experiments for PER showed no evidence of any residual kinase activity for $DBT^{K/R}$ (representative autoradiographs are shown in panels B and C).

absence of detectable kinase activity for $DBT^{K/R}$ was confirmed by quantification of multiple independent assays comparing DBT^{WT} and $DBT^{K/R}$, with activities normalized to the amount of DBT-MYC in the assays (Fig. 2D) (note that subtraction of background signal from "no-DNA" lanes produced negative values for some $DBT^{K/R}$ assays). These results demonstrate that the K38R mutation in DBT eliminated the intrinsic casein kinase activity of DBT (Fig. 2) without affecting its capacity to interact with its substrate PER (Fig. 1).

$DBT^{K/R}$ does not trigger the degradation of PER in S2 cells, and it antagonizes the degradation of PER triggered by DBT^{WT}. We and others (7, 24, 35) have used expression of DBT and PER in *Drosophila* S2 cells to analyze the effects of DBT on PER's phosphorylation and stability. In S2 cells, DBT triggers progressive phosphorylation of PER, culminating in its degradation by ubiquitin-mediated proteolysis (14, 24). Constructs for both *dbt* and *per* were transiently transfected into S2 cells, and different amounts of *dbt* expression were achieved by varying the amounts of *dbt* DNA and the amounts of $CuSO_4$ used to induce DBT from a metallothionein promoter. When DBT^{WT}-MYC is coexpressed with PER produced constitutively from an actin promoter, the levels and electrophoretic mobility of PER decrease with higher levels of DBT (Fig. 3A, short and long exposures). While DBT is expressed endogenously in this cell line, evidently its levels are not high enough to form stable complexes with all of the PER-HA expressed, and therefore hypophosphorylated PER-HA is able to accumulate in the absence of transgenic DBT (24). The progressive mobility shift of PER in this experimental approach results from phosphorylation of PER, because it is eliminated if PER is treated with phosphatase before electrophoresis (24). By contrast, progressive induction of the $DBT^{K/R}$-MYC mutant protein does not produce mobility shifts in PER, and PER levels do not decrease substantially (Fig. 3A). The PER signal

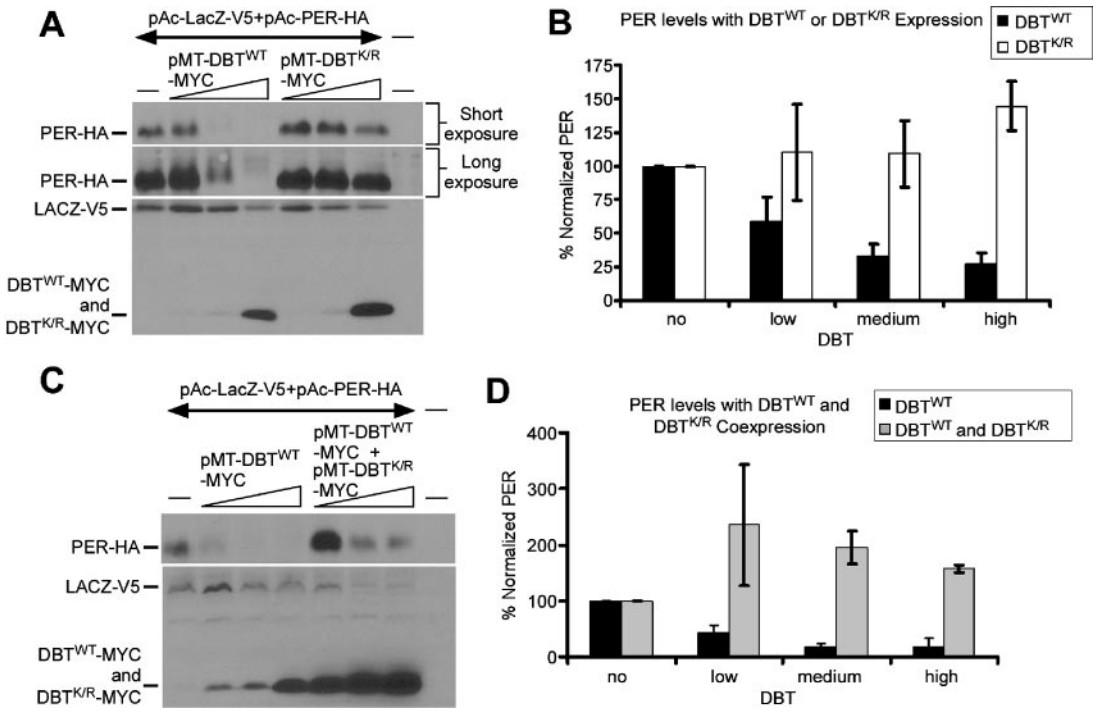

FIG. 3. $DBT^{K/R}$ does not phosphorylate and destabilize PER in transiently transfected S2 cells, and it antagonizes the phosphorylation and destabilization of PER by DBT^{WT}. (A) *Drosophila* S2 cells were transiently transfected with pMT-DBT^{WT}-MYC or pMT-$DBT^{K/R}$-MYC, pAC-PER-HA, and pAC-LacZ-V5. DBT expression was under the control of a metallothionein promoter, so that various amounts of DBT could be induced by altering the amounts of plasmid and $CuSO_4$. PER-HA and LacZ-V5 expression was placed under the control of the constitutively active actin promoter. Cells were lysed 42 h after induction, and the lysates were analyzed by immunoblot analysis. The different proteins were detected with antibodies specific to their epitope tags. The long exposure shows a PER mobility shift produced by DBT^{WT} (indicative of phosphorylation of PER by DBT^{WT}) but not by $DBT^{K/R}$. (B) Quantitative analysis of PER and LacZ from four immunoblots analyzed from each of three independent transfections shows that $DBT^{K/R}$ does not target PER for degradation. PER signals were normalized to LacZ signals, and PER expression without any DBT expression was set to 100% for each experiment. Error bars are standard errors of the means. (C) Experiments were conducted as for panel A, except that pMT-$DBT^{K/R}$-MYC plasmid was coexpressed in excess with pMT-DBT-MYC as indicated. $DBT^{K/R}$ antagonizes the degradation and mobility shift of PER in response to DBT^{WT} expression. (D) Two immunoblots for each of three experiments were quantified as for panel B.

was compared to the signal of bacterial LacZ, which was constitutively expressed from a cotransfected plasmid and was not expected to be affected in level or mobility by either DBT^{WT}-MYC or $DBT^{K/R}$-MYC. However, levels of LacZ declined somewhat at the highest levels of DBT^{WT}-MYC and $DBT^{K/R}$-MYC induction, possibly because of competition for general transcription and translation factors resulting from high levels of transgene expression (see especially Fig. 3C, with combined DBT^{WT} and $DBT^{K/R}$ expression). To correct for any general depression of protein synthesis, the level of PER-HA was normalized to the level of LacZ. We have quantified in this manner three such experiments, each of which was analyzed two to four times by immunoblot analysis. Our results show that the $DBT^{K/R}$ mutant protein does not target PER for degradation while the DBT^{WT} does, and in fact normalized PER-HA levels actually increase slightly with expression of $DBT^{K/R}$, perhaps due to antagonism of endogenous DBT (Fig. 3B). Therefore, DBT kinase activity and phosphorylation of PER resulting from DBT coexpression are necessary for degradation of PER in this cell culture model.

In order to function as a dominant negative mutant in this assay, $DBT^{K/R}$ must antagonize the phosphorylation and degradation of PER by DBT^{WT}. Since our GST pull-down assays showed that $DBT^{K/R}$ can associate stably with PER, it seemed likely that an excess of $DBT^{K/R}$ in S2 cells would antagonize DBT^{WT} by titrating PER and preventing its interaction with DBT^{WT}. Therefore, the possibility that $DBT^{K/R}$ might function as a dominant negative mutant was assessed in S2 cells by coexpressing DBT^{WT} with an excess of $DBT^{K/R}$. As in the experiments shown in the previous section, PER levels decrease when DBT^{WT} levels increase from low to high (Fig. 3C, lanes 1 to 4). To address whether the K38R mutation could antagonize DBT^{WT}-mediated degradation of PER, we cotransfected S2 cells with both pMT-dbt^{WT} and an excess of pMT-$dbt^{K/R}$ plasmids. Results from immunoblot analyses of S2 cell lysates show that the low, medium, and high levels of DBT^{WT} coexpressed with higher levels of $DBT^{K/R}$ sustain high levels of hypophosphorylated (i.e., high-mobility) PER compared to DBT^{WT} expressed alone (Fig. 3C). There is no substantial decline in PER levels normalized to LacZ under these conditions (Fig. 3D; results are for three experiments, each assayed two times). Taken together, the results from these experiments (Fig. 1 to 3) establish $DBT^{K/R}$ as a dominant negative mutant in S2 cells. Not only does it lack kinase activity and the capacity to target PER for degradation, but it also prevents DBT^{WT} from phosphorylating and degrading PER,

TABLE 1. Activity rhythms of flies expressing wild-type or mutant DBT in circadian cells[a]

DBT or GAL4	Line	Genotype	Avg period (h) ± SEM (SD)	% Rhythmic flies (no. of progeny tested)
DBT^{WT}	6M3B	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.2 ± 0.2 (0.8)	36 (67)
		UAS-DBT^{WT}/+	24.1 ± 0.1 (0.4)	94 (16)
	21M1C	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.7 ± 0.2 (0.7)	77 (22)
	12M1B	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.8 ± 0.5 (1.0)	31 (13)
	22M1A	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.9 ± 0.2 (0.5)	38 (16)
	45F2B	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.9 ± 0.2 (0.9)	49 (57)
		tim-UAS-GAL4/+>UAS-DBT^{WT}/+	24.3 ± 0.2 (0.8)	94 (31)
DBT^{S}	10F5A	*tim*-GAL4/+>UAS-DBT^{S}/+	18.4 ± 0.1 (0.4)	74 (23)
		UAS-DBT^{S}/+	23.9 ± 0.06 (0.2)	88 (16)
	13M1A	*tim*-GAL4/+>UAS-DBT^{S}/+	18.4 ± 0.09 (0.2)	47 (15)
	28M3A	*tim*-GAL4/+>UAS-DBT^{S}/+	18.2 ± 0.07 (0.4)	50 (54)
		tim-UAS-GAL4/+>UAS-DBT^{S}/+	18.0 ± 0.1 (0.4)	65 (26)
DBT^{L}	22F1C	*tim*-GAL4/+>UAS-DBT^{L}/+	27.3 ± 0.1 (0.8)	49 (69)
		tim-UAS-GAL4/+>UAS-DBT^{L}/+	27.0 ± 0.08 (0.3)	81 (16)
		UAS-DBT^{L}/+	23.8 ± 0.09 (0.3)	100 (14)
	38M3A	*tim*-GAL4/+>UAS-DBT^{L}/+	27.2 ± 0.2 (0.5)	33 (18)
		tim-UAS-GAL4/+>UAS-DBT^{L}/+	26.8 ± 0.5 (1.6)	59 (17)
	27M1C	*tim*-GAL4/+>UAS-DBT^{L}/+	27.3 ± 1.1 (2.2)	13 (30)
		tim-UAS-GAL4/+>UAS-DBT^{L}/+	27.9 ± 0.3 (0.7)	44 (16)
$DBT^{K/R}$	13F1A	*tim*-GAL4/+>UAS-$DBT^{K/R}$/+	28.0 ± 0.2 (1.3)	69 (48)
		UAS-$DBT^{K/R}$/+	24.1 ± 0.1 (0.4)	100 (9)
	1M1C	*tim*-GAL4/+>UAS-$DBT^{K/R}$/+	33.2 ± 0.8 (2.9)	38 (39)
	12F1A	*tim*-GAL4/+>UAS-$DBT^{K/R}$/+	33.0 ± 1.9 (6.2)	16 (70)
		UAS-$DBT^{K/R}$/+	24.2 ± 0.09 (0.4)	84 (19)
	17M1B[b]	*tim*-GAL4/+>UAS-$DBT^{K/R}$/+	34.9 ± 1.0 (2.6)	9 (81)
GAL4	*tim*-GAL4	*tim*-GAL4/+	24.4 ± 0.2 (0.7)	72 (18)
	tim-UAS-GAL4	*tim*-UAS-GAL4/+	24.1 ± 0.08 (0.3)	100 (20)

[a] Lines containing the indicated type of UAS-DBT insertion were crossed to flies containing the indicated GAL4 driver (*tim*-GAL4 or *tim*-UAS-GAL4), and progeny hemizygous for both the driver and responder were assayed in DD for locomotor activity. Each line contained an independent insertion of the responder UAS-DBT gene. The circadian period was determined by chi-square periodogram analysis. Rhythmic flies produced single strong peaks in the periodogram analysis and rhythmicity that was obvious by inspection of actograms. Control progeny contained only the responder (UAS-DBT/+) or the driver (e.g., *tim*-GAL4/+).

[b] More than one UAS-DBT insert was present.

most likely by competing with DBT^{WT} for access to the PER substrate.

Overexpression of DBT^{WT}, DBT^{S}, or DBT^{L} phenocopies the corresponding alleles of the endogenous gene. To overexpress various DBT proteins in vivo, our mutant *dbt* genes were inserted into the genomes of flies under control of a yeast UAS, which activates expression of the linked *dbt* gene (the responder) when a separate transgene expressing the yeast GAL4 transcription factor (the driver) is also present (5). Transformant flies were crossed to GAL4 driver lines to generate flies with both a driver and a *uas-dbt* responder. A priori, expression of mutant and DBT^{WT} with this approach might have caused apoptosis, necrosis, or developmental abnormalities due to high levels of expression, improper localization, or nonspecific targeting of substrates. However, expression of DBT^{WT} in neurons and circadian cells did not produce lethality or any obvious external defects, and expression of DBT^{WT} with an actin-GAL4 driver produced normal-looking flies, albeit at lower than expected Mendelian ratios (J. L. Price, M. J. Muskus, and A. Venkatesan, unpublished data).

Locomotor activity, which is similar to human sleep/wake cycles and is controlled by the circadian clock, was assayed as a behavioral test for effects on the circadian clock. Table 1 summarizes the locomotor activity results with flies carrying

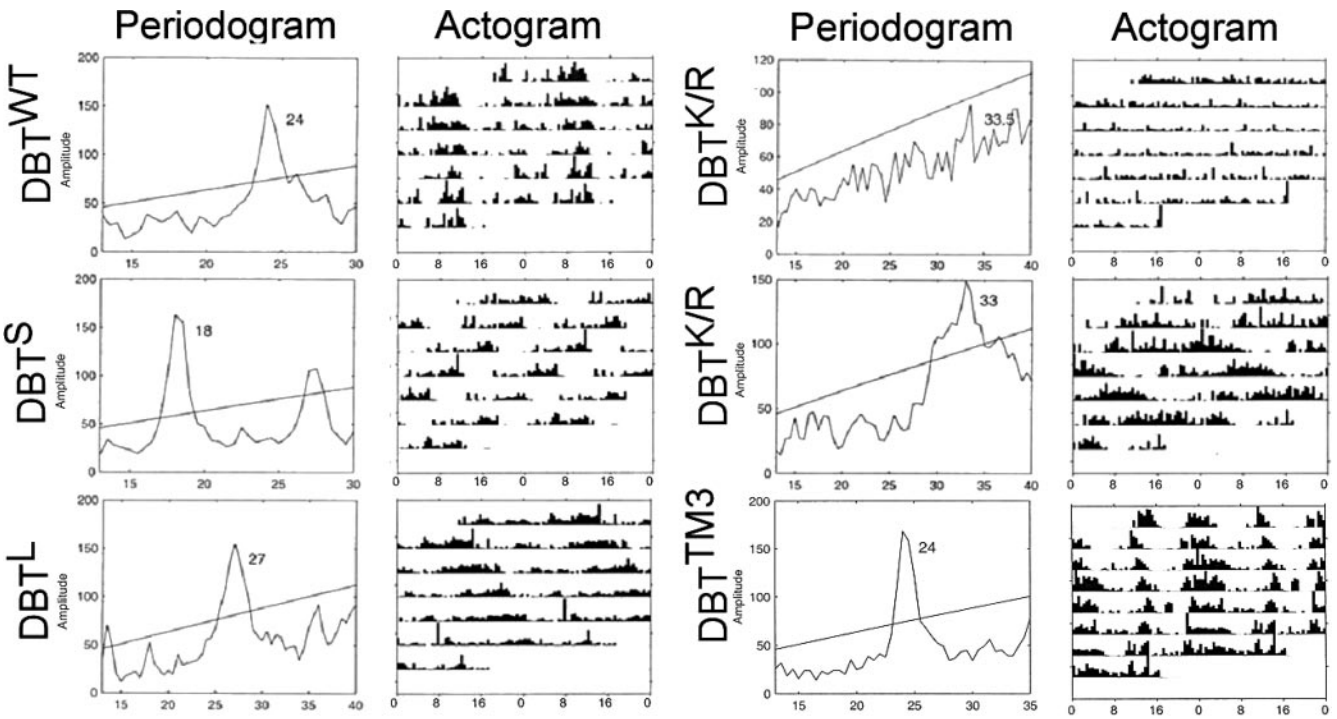

FIG. 4. Overexpression of DBT^S and DBT^L proteins in otherwise wild-type flies produces the same circadian period for locomotor activity as in the original mutant flies, while overexpression of $DBT^{K/R}$ produces high levels of arrhythmicity and some long periods. Shown are representative actograms and periodograms of *tim*-GAL4>UAS-DBT-MYC flies (the DBT genotype is indicated to the left of the periodogram) as well as those of a sibling control fly that carried the balancer rather than the responder gene (*tim*-GAL4>TM3; denoted "DBT^{TM3}"). The actogram plots the magnitudes of successive 0.5-h activity bouts. There are two 24-h intervals shown on each line, and each day plotted on the right is plotted again on the left side of the line underneath (a double plot). The periodogram to the left of each actogram plots the amplitude of the oscillation for each test period applied to the activity record. The period which produces the highest amplitude is taken as the best period for the data set. Periods for which the amplitude lies above the sloping line are statistically significant with a P value of <0.01. The responder inserts were tested in the hemizygous condition with the *tim*-GAL4 driver (*tim*-GAL4/+; UAS-DBT/+), as were all the flies listed in Table 1. Representative actograms and periodograms are shown for both an arrhythmic and a long-period *tim*-GAL4>UAS-$DBT^{K/R}$ fly.

two different GAL4 drivers expressing DBT^{WT} in all known circadian cells, along with the results from control flies carrying only the GAL4 driver or the UAS-DBT responder. A representative activity record (actogram) and period analysis (periodogram) are shown in Fig. 4 for each fly genotype discussed here. *tim*-GAL4>UAS-DBT flies from five different wild-type lines with different UAS-DBT transgene insertion sites exhibit almost-wild-type periods ranging from 24.2 to 24.9 h (Table 1). There is a tendency for the periods to be longer than those of nonexpressing controls (e.g., UAS-DBT^{WT}/+ flies or *tim*-GAL4/+ flies; see also reference 55), but the period is at most under an hour longer. The *tim*-UAS-GAL4 is a driver construct in which expression of GAL4 is controlled both by the *timeless* and the UAS promoter (4). This allows *timeless*-independent expression once GAL4 has been activated, since the GAL4 will feed back on its own promoter, keeping the UAS promoter active even if the circadianly regulated *timeless* promoter shuts off or oscillates in its expression (although we did not detect any variation in transgenic *tim*-GAL4>DBT-MYC levels throughout the day [see Fig. S1A and S1B in the supplemental material]). However, even with this self-maintaining enhancer, the *tim*-UAS-GAL4>UAS-DBT^{WT} flies tested (with transgene 45F2B) did not show any major effect of DBT on their locomotor behavior (Table 1). Many of the *tim*-GAL4>UAS-DBT flies showed reduced rhythmicity compared with the nonexpressing controls, but the reduction is variable over a wide range, and therefore it may result from fairly indirect effects of DBT overexpression.

To determine if high levels of DBT were expressed from the UAS-DBT transgene and if these levels oscillated, flies were collected at selected time points (ZT1, -7, -13, and -19) in an LD cycle. By convention, lights are on from ZT0 to -12 in an LD cycle and are off from ZT12 to -24. Figures S1A and S1B in the supplemental material show that no oscillation in the levels of transgenic DBT^{WT}-MYC protein is detected in heads in LD, even though it is expressed from a *tim*-GAL4 driver whose promoter contains circadian transcriptional regulatory elements (20). A minimum estimate of the ratio between transgenic and endogenous DBT protein levels comes from immunoblots with the antibody directed against the C terminus, which should detect both the transgenic and endogenous proteins with the same efficiency. These blots show that the levels of transgenic DBT^{WT}-MYC (or $DBT^{K/R}$-MYC) are higher than endogenous DBT levels in head extracts (see Fig. S1A in the supplemental material). Transgenic DBT-MYC expression was confirmed by utilizing the MYC epitope tag (see Fig. S1B in the supplemental material) (detected with the anti-MYC antibody). In situ, anti-MYC antibody detected transgenic DBT in cells where *tim* is known to be expressed, including the photoreceptors, optic lobe lamina and cells of the central

brain. DBT-MYC was observed in both the nucleus and cytoplasm at all times of day (not shown). The greater abundance of the transgenic DBT^{WT} (and $DBT^{K/R}$; see below) should allow them to titrate the endogenous DBT for substrate. Presumably, the effects of overexpressing DBT^{WT}-MYC are not dramatic, because it merely replaces wild-type endogenous DBT with wild-type transgenic DBT.

Both dbt^S and dbt^L have been shown to act as semidominant mutations in flies (43), but their mechanisms of action are unclear. One possibility was that the dbt^S mutation produced an excess of otherwise DBT^{WT} protein, while the dbt^L mutation produced a deficit. However, this possibility was not supported by standard complementation tests, which instead suggested that these mutations altered some aspect of DBT protein function (43). In the dbt^S/dbt^{WT} and dbt^L/dbt^{WT} genotypes, it was proposed that the mutant protein was competing with the wild-type protein for access to substrate, thereby producing a period phenotype intermediate between those of wild-type and *dbt* mutant flies.

We tested the dominance of the dbt^S-*myc* and dbt^L-*myc* transgenic constructs in flies by driving expression with *tim*-GAL4. If the transgenic DBT mutant protein were expressed at a high enough level, the transgenic DBT mutant protein should have been able to titrate DBT^{WT} expressed from the endogenous gene and tilt the balance towards the mutant phenotype. In fact, the ectopically expressed mutant DBT proteins did precisely phenocopy the endogenous alleles of dbt^S and dbt^L. The dbt^S and dbt^L data in Table 1 and Fig. 4 support the hypothesis that DBT^S and DBT^L can completely replace endogenous DBT^{WT} in complexes with PER and other clock proteins, if they are expressed at a high enough level. Locomotor assays of flies with the *tim*-GAL4>UAS-DBT or *tim*-UAS-GAL4>UAS-DBT genotype demonstrate that both dbt^S and dbt^L transgenes generate a dominant period alteration of the same magnitude as the one generated by the original homozygous mutants: ~18 h for dbt^S/dbt^S and ~27 h for dbt^L/dbt^L, respectively (Fig. 4 and Table 1) (43). Furthermore, there is little intra- or interline variation in circadian period (note the small standard deviations and similar average periods in different lines). This intriguing finding again suggests that the period changes are caused by effects of the mutations on specific processes or functions of the protein rather than by alterations in the levels of kinase available. With high levels of hypomorphic transgenic kinase present, both DBT^L and DBT^S titrate the endogenous enzyme in clock protein complexes and change the period length of the fly by a set amount of hours equal to the period changes observed in homozygous genetic mutants, rather than exceed the changes of the original mutants. The production of higher levels of transgenic DBT than endogenous DBT and the fully penetrant dbt^S and dbt^L mutant phenotypes validate this binary expression approach for the analysis of the $dbt^{K/R}$ mutant phenotype.

Expression of a catalytically inactive DBT protein produces dominant negative effects in adult flies: arrhythmicity and very long periods. Since *Drosophila* $DBT^{K/R}$ does not phosphorylate PER in S2 cells and antagonizes the action of DBT^{WT} in triggering PER degradation (Fig. 3), the possibility that $DBT^{K/R}$ might exert a general dominant negative effect on the clock in adult flies was examined with the same binary expression approach just described for DBT^{WT}, DBT^S, and DBT^L. Ubiquitous expression of $DBT^{K/R}$-MYC using an actin-GAL4 driver conferred high lethality when using a strong responder line (e.g., 1M1C, 12F1A, or 17M1B [data not shown]; there are a few escapers in flies with the weak responder 13F1A). Since strong loss-of-function *dbt* alleles produce embryonic lethality (60), this finding indicates that the $DBT^{K/R}$ can be a strong general dominant negative mutant in vivo and can therefore produce a null phenotype in an otherwise wild-type genotype.

To determine whether $DBT^{K/R}$ expression in clock cells would produce lethality or circadian phenotypes, we generated flies with both the UAS-$DBT^{K/R}$ transgene responder and the *tim*-GAL4 transgene driver, which would ultimately overexpress $DBT^{K/R}$ specifically in clock cells. Successful recovery of many flies with both transgenes (Table 1) indicates this combination is nonlethal. Furthermore, the viability of the clock cells, which include the eyes, is apparently normal, based on the normal external morphology of the eye (data not shown) and immunohistological analysis confirming the presence of *tim*-GAL4-expressing brain neurons and photoreceptors (see Fig. 6 and 7). The effects of *tim*-GAL4-mediated $DBT^{K/R}$ expression on circadian periodicity are shown in Table 1, with representative actograms and periodograms shown in Fig. 4. The data indicate that $DBT^{K/R}$-MYC is capable of interfering with endogenous DBT and preventing proper clock function. Depending on the responder transgenic insertion site (four different lines with independent insertions), flies exhibit higher levels of arrhythmicity with the *tim*-GAL4 driver than do any of the other types of DBT responders (fewer than 20% rhythmic in two of the four lines tested) and/or variably long periods (28 to 35 h). In controls bearing only UAS-$DBT^{K/R}$/+, the periods of the progeny were within the wild-type range (average, ~24 h) and locomotor activity was highly rhythmic, indicating that *tim*-GAL4-dependent expression of UAS-$DBT^{K/R}$ is required for the effects on rhythms. All of the UAS-DBT lines (wild type, dbt^S, dbt^L, and $dbt^{K/R}$) tabulated here were tested without outcross to a driver line, and all rhythmic flies had periods within the wild-type range (23 to 25 h), so the mutant periods require the presence of the driver and the responder in all lines (data not shown). As further evidence for this conclusion, no progeny which acquired the TM3 chromosome rather than the UAS-DBT chromosome (from UAS-DBT/TM3 parents) had a circadian period outside the normal circadian range, although in our hands the TM3 balancer is often associated with a high degree of arrhythmicity in most genetic backgrounds (not shown).

The standard deviations of the periods within a line and the variations in the average periods between lines for these *tim*-GAL4> UAS-$DBT^{K/R}$ flies are very high compared to those for our other *tim*-GAL4 flies with dbt^{WT}, dbt^S, and dbt^L responders (Table 1), suggesting that the greater period length produced by $DBT^{K/R}$ is sensitive to differences in levels of transgenic DBT expression. Unlike the cases of UAS-DBT^S and the UAS-DBT^L overexpression, period length does not appear to reach a specific value (or set point) for $DBT^{K/R}$ overexpression, but instead long periodicity seems to grade to arrhythmicity. Consistent with the notion of a graded effect, the one $DBT^{K/R}$ line with a high proportion of rhythmic flies (13F1A, with 69% rhythmic [Table 1]) exhibited the shortest period lengths when expressed with the *tim*-GAL4 driver (Table 1). While immunoblot analysis of most *tim*-GAL4>UAS-

$DBT^{K/R}$ lines showed higher levels of $DBT^{K/R}$ than of endogenous DBT (see Fig. S1A in the supplemental material), immunoblot analysis of *tim*-GAL4>UAS-$DBT^{K/R}$ flies from line 13F1A detected lower levels of transgene expression than from other $DBT^{K/R}$ transgenic lines with longer periods and more arrhythmicity (see Fig. S1C in the supplemental material). Nevertheless, even the relatively small amount of $DBT^{K/R}$ expression in 13F1A strongly lengthens the circadian period, suggesting that DBT^{WT} is substantially antagonized in this line. The higher levels of $DBT^{K/R}$ expression in other lines could further antagonize DBT^{WT} activity and produce additional period lengthening and increases in arrhythmicity.

$DBT^{K/R}$-expressing flies have high levels of hypophosphorylated and nuclear PER throughout the day. The behavioral results strongly argue that DBT kinase activity is required for circadian rhythmicity, and they establish DBT $^{K/R}$ as a dominant negative mutant in vivo. Recently, the effects of a similar transgenic DBT on inhibition of the *hedgehog* pathway in *Drosophila* have also been interpreted as evidence of a dominant negative effect in this pathway (17). Since $DBT^{K/R}$ successfully protects PER from phosphorylation and degradation in S2 cells, the in vivo behavioral data are consistent with the possibility that arrhythmicity and long periods are caused by stabilization of PER, thereby freezing the cells in a continuous transcriptionally repressed state or prolonging this transcriptional repression. This possibility was investigated by addressing PER's phosphorylation state, stability, and cellular localization in $DBT^{K/R}$ flies.

To analyze the effects of DBT or $DBT^{K/R}$ on PER expression in clock cells, flies with a *tim*-GAL4>UAS-DBT^{WT} or *tim*-GAL4>UAS-$DBT^{K/R}$ genotype were collected at four time points in a 12-h:12-h LD cycle or in DD. PER levels both in extracts from entire male bodies (Fig. 5A and B) and in extracts from heads (Fig. 5C and D) were analyzed. For the analysis of whole-body extracts, we collected only males because wild-type females express constitutively high levels of PER throughout the day in the ovarian tissues, and this PER masks the detection of PER oscillations in other tissues (15). As expected, PER levels oscillate over the course of the day in flies with no responder transgene (TM3) or in flies expressing DBT^{WT}-MYC from the *tim*-GAL4 driver. Both of these genotypes express high levels of PER at ZT19, when PER is mostly nuclear (9, 59) (see Fig. 6); the wild-type TM3 control flies also express high levels at ZT1, while the flies expressing DBT^{WT}-MYC express somewhat lower levels of PER at ZT1. Furthermore, at ZT1 and ZT7 PER levels exhibit a slower electrophoretic mobility on SDS-PAGE, indicative of a highly phosphorylated state (Fig. 5A, C, and D) (9). At ZT7 most of the highly phosphorylated PER has been degraded, and there is little or no newly synthesized and unphosphorylated PER. One hour after the light is out at ZT13, newly formed, unphosphorylated PER begins to accumulate, producing a fast-migrating PER on SDS-PAGE. Levels of largely unphosphorylated PER reach a high point at ZT19 (Fig. 5A, C, and D), when PER enters the nucleus and begins to inhibit the CLK/CYC transcription factor (3, 9, 21, 58, 59). In DD (CT) the wild-type oscillation in PER level and electrophoretic mobility persists in whole-body extracts for the first day, with changes in level and mobility equivalent to those seen in LD (Fig. 5B, TM3).

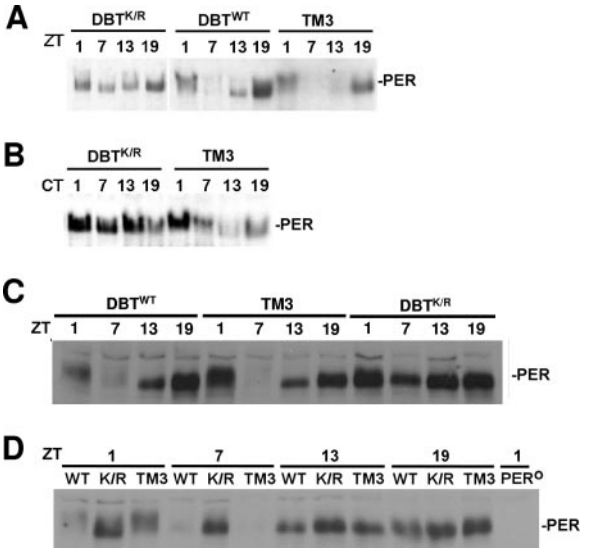

FIG. 5. Immunoblot analysis of PER in flies overexpressing DBT shows that $DBT^{K/R}$ damps the circadian oscillations of PER levels and phosphorylation, while DBT^{WT} overexpression does not affect the oscillation of PER. (A) Shown are the overall PER levels in male fly bodies collected during entrainment to LD at the indicated times ("ZT," with lights on from ZT0 to -12 and off from ZT12 to -24). Samples were homogenized and immunoblotted with an anti-PER antibody. *tim*-GAL4>UAS-DBT flies with the indicated DBT genotypes were tested in the hemizygous condition, or *tim*-GAL4>TM3 flies carrying the balancer chromosome instead of the responder. Robust oscillation in overall levels and mobility of PER can be seen in DBT^{WT}-expressing flies and the TM3 controls, whereas the overall level of PER appears to be higher and of constant mobility in flies expressing $DBT^{K/R}$. A damped oscillation in PER levels is detected in *tim*-GAL4>UAS-$DBT^{K/R}$ flies, with lower levels from ZT7 to -13. (B) A direct comparison of PER levels and mobilities in male bodies of *tim*-GAL4>UAS-$DBT^{K/R}$ flies and a balancer sibling control (*tim*-GAL4>TM3), both collected during the first day of DD. CT indicates the time of collection, with the first collection performed at CT1, 13 h after termination of the LD cycle. There are lower levels of PER and more electrophoretic mobility retardation in wild-type flies than in $DBT^{K/R}$ flies at several time points. There is a lack of discernible oscillation in PER levels and mobility in the *tim*-GAL4>UAS-$DBT^{K/R}$ flies. (C) An analysis of fly head extracts from LD-reared flies similar to the analysis of whole bodies shown in panel A. The DBT^{WT} line was 6M3B, the $DBT^{K/R}$ line was 17M1B, and the TM3 sibling progeny came from the cross of 6M3B to *tim*-GAL4 flies. (D) A side-by-side comparison of equivalent amounts of head extracts from the same *tim*-GAL4>UAS-DBT genotypes analyzed in panel C at various ZTs. PER from *tim*-GAL4>UAS-$DBT^{K/R}$ fly heads clearly is expressed at equal or higher levels and has equal or higher mobility than PER from *tim*-GAL4>UAS-DBT^{WT} fly heads at all times of day. The DBT^{WT} line was 45F2B, the $DBT^{K/R}$ line was 1M1C, and the TM3 sibling progeny came from the cross of 1M1C to *tim*-GAL4 flies. PER^o, extracts from the heads of *per^o* flies, which do not express PER.

Transgenic flies expressing the $DBT^{K/R}$ construct do not show a strong oscillation of PER protein levels. In LD, PER levels are somewhat depressed in *tim*-GAL4>UAS-$DBT^{K/R}$ at ZT7 and ZT13 relative to ZT1 and ZT19 in both whole-body extracts (Fig. 5A) and head extracts (Fig. 5C), but in repeated experiments the depression was never as strong as in wild-type or DBT^{WT}-expressing controls (Fig. 5A to D and data not shown). No obvious circadian change in PER mobility can be observed in the presence of $DBT^{K/R}$. High levels of PER with fast mobility are seen at all time points in *tim*-GAL4>UAS-

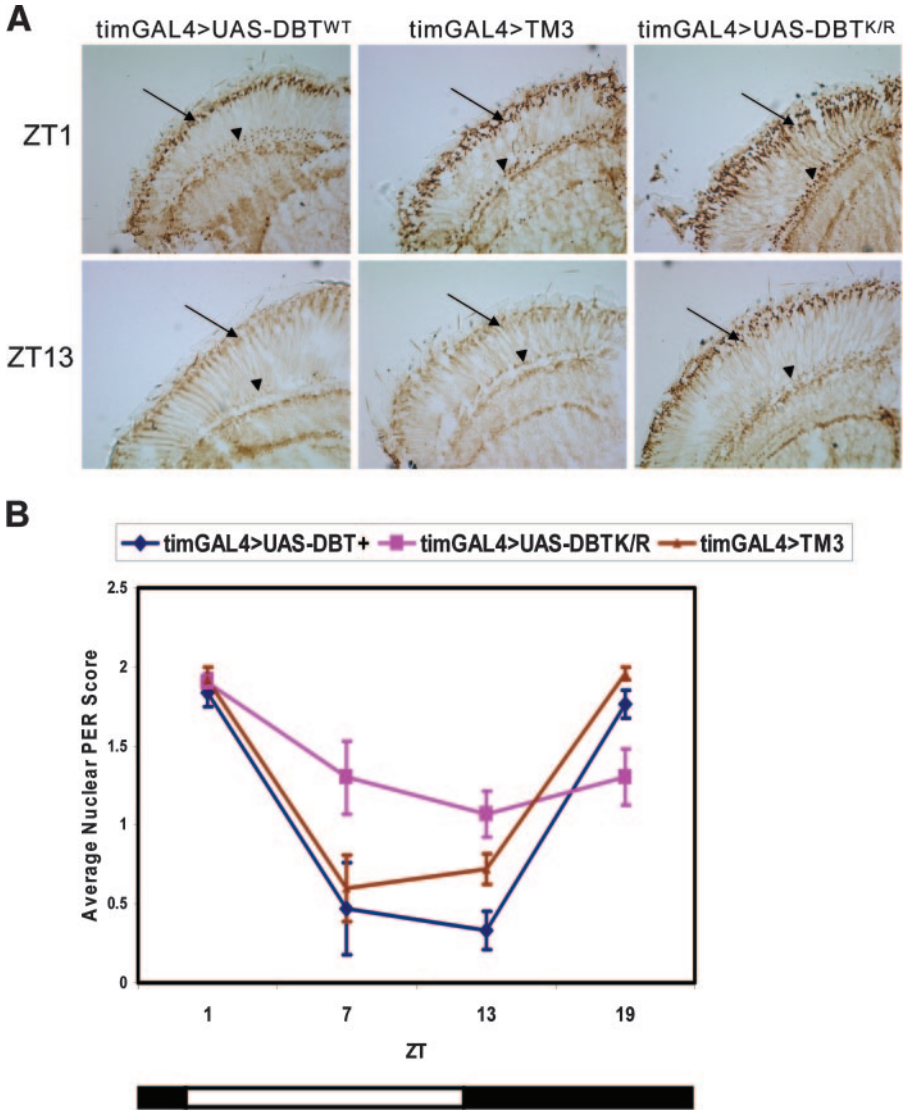

FIG. 6. A damped oscillation of nuclear PER is detected in the photoreceptors of flies overexpressing the $DBT^{K/R}$ transgene by immunocytochemistry. (A) Fly heads from flies expressing transgenic $DBT^{K/R}$ or DBT^{WT} with the *tim*-GAL4 driver or from wild-type sibling controls carrying the *tim*-GAL4 driver and the TM3 balancer chromosome rather than a DBT responder were collected at the indicated time points in a light/dark cycle (ZT). Head sections were immunostained using a rabbit anti-PER and diaminobenzidine. The arrows denote the locations of nuclei from photoreceptors 1 to 7, while the arrowheads denote the locations of nuclei from photoreceptor 8. (B) Multiple sections were scored on a scale ranging from 0 to 2, and the average scores for the indicated genotypes are plotted as a function of ZT (mean ± standard error of the mean). Each point is the product of three to five separate analyses scored with multiple sections, each of which was scored blind by two observers. The TM3 ZT13 section from panel A is typical of a score of 0, the $DBT^{K/R}$ section at ZT13 is typical of a score of 1, and the $DBT^{K/R}$ section from ZT1 is typical of a score of 2. The $DBT^{K/R}$ line is 1M1C, and the DBT^{WT} line is 6M3B.

$DBT^{K/R}$ flies, both in LD (Fig. 5A, C, and D) and in DD (Fig. 5B), as a side-by side comparison of PER from the different genotypes demonstrates most compellingly (Fig. 5D). These results are consistent with the results from the S2 cell competition experiments (Fig. 3C), in which $DBT^{K/R}$ appears to be capable of blocking phosphorylation of PER by endogenous DBT and therefore prevents rapid degradation of the protein in vivo. In DD this effect is even more prominent, as there are no evident circadian changes in mobility or levels (Fig. 5B). All of these immunoblot analyses of PER were repeated in two or three experiments, most of which were blotted multiple times.

The effect of $DBT^{K/R}$ expression on the circadian oscillation of PER nuclear accumulation in the eye and in brain neurons was also examined. The fly eye consists of a regular array of ommatidia, each containing eight photoreceptor neurons. The nuclei of photoreceptors 1 to 7 lie on the outside of the retina, while the nuclei for photoreceptor 8 lies on the inside of the retina (Fig. 6A). When fly head sections from wild-type control flies carrying the balancer chromosome (TM3) instead of a DBT responder or from flies overexpressing DBT^{WT}-MYC were collected in LD and stained for PER, strong nuclear localization of PER protein in photoreceptor cells could be detected on the outside and inside of the retina during the early day and the late night (ZT1 and ZT19) (Fig. 6). Unde-

tectable or weak nuclear staining was seen during the late day and early night (ZT7 and ZT13) (Fig. 6) in these wild-type flies. The circadian timing of PER nuclear localization in these wild-type controls is consistent with multiple published reports for the wild-type circadian oscillation (59). Blind scoring results of multiple sections, ranging from 0 (no nuclear staining) to 2 (strong nuclear staining), are presented in Fig. 6B and confirm this circadian oscillation in nuclear accumulation, which is unaffected by overexpression of DBT^{WT}-MYC, consistent with the lack of a strong effect of overexpression of DBT^{WT} on circadian behavior and total PER levels (Fig. 4 and 5; Table 1).

By contrast, immunocytochemical analysis of fly eyes from *tim*-GAL4>UAS-$DBT^{K/R}$ flies showed generally high nuclear staining for PER at all time points (Fig. 6). In particular, nuclear PER was consistently detected at ZT7 and ZT13, while PER was undetectable in many wild-type photoreceptors at these time points. Nevertheless, there was a persistent (albeit damped) oscillation of nuclear PER accumulation, with peak levels at ZT1 (Fig. 6B).

Expression of PER in the brain neurons which express the neuropeptide PDF (and control locomotor activity rhythms in adult flies [12, 25, 29, 32, 36–38, 45]) was examined in whole mounts of larval brains overexpressing DBT^{WT} or $DBT^{K/R}$ specifically in these neurons (Fig. 7). Control larvae which overexpress DBT^{WT} in all clock cells with the *tim*-GAL4 driver showed wild-type rhythmic nuclear accumulation of PER in the PDF-positive (PDF^+) neurons, with high nuclear levels at ZT 1 (Fig. 7A) and undetectable or cytoplasmic levels at ZT13 (Fig. 7B). By contrast, PER levels were high in PDF^+ neurons at both ZT1 (Fig. 7A) and ZT13 (Fig. 7B) when $DBT^{K/R}$ was overexpressed in these larval brains with the *tim*-GAL4 driver. PER localization was somewhat variable in PDF^+ brain neurons from *tim*-GAL4>UAS-$DBT^{K/R}$ larvae but was most commonly observed in the nucleus or both the nucleus and the cytoplasm, as judged by PER fluorescence which could overlap that of PDF but also was found more centrally (Fig. 7A and B). The robust oscillation of nuclear accumulation observed for *tim*-GAL4>UAS-DBT^{WT} brains was lacking in *tim*-GAL4>UAS-$DBT^{K/R}$ brains. The fast electrophoretic mobility of PER with expression of the dominant negative DBT (Fig. 5) suggests a very low DBT-dependent phosphorylation state for PER at all times, and the in situ analyses show high levels of nuclear PER at all times (Fig. 6 and 7). These results suggest that high levels of PER phosphorylation are not absolutely required for nuclear localization of PER, and they would be consistent with antagonism of PER nuclear localization by DBT in the wild-type circadian cycle (8). This possibility is discussed in more detail below.

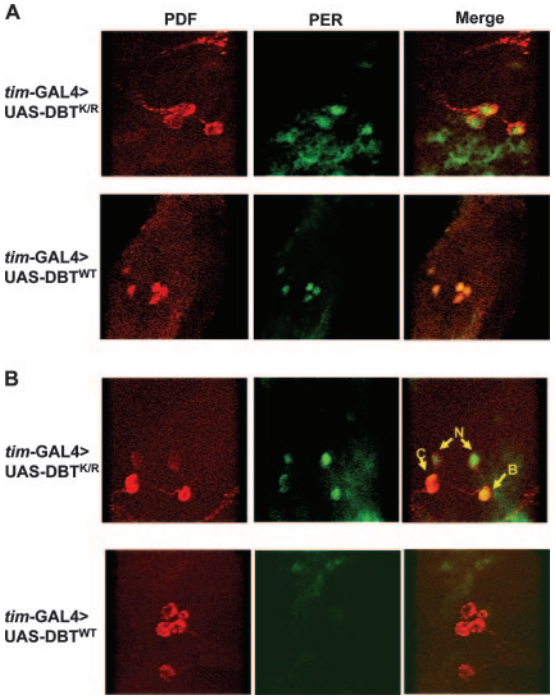

FIG. 7. High levels of nuclear PER are detected even during the early night by confocal microscopy in PDF^+ neurons of larval brains expressing $DBT^{K/R}$. Brains from larvae of two genotypes (*tim*-GAL4>UAS-DBT^{WT} or *tim*-GAL4>UAS-$DBT^{K/R}$) were dissected at ZT1 (A) or ZT13 (B) and incubated with a rabbit anti-PER antibody and a mouse anti-PDF antibody. PER immunofluorescence was visualized with Alexa-Fluor 488, while cytoplasmic PDF was detected with Alexa-Fluor 568. Confocal sections were obtained for both emissions, and Z stacks are shown. Immunofluorescence for PER in *tim*-GAL4>UAS-DBT^{WT} flies was strong in nuclei at ZT1 (A) and either colocalized with PDF or was undetectable at ZT13 (B). Immunofluorescence for PER in *tim*-GAL4>UAS-$DBT^{K/R}$-MYC was typically found in the nucleus or both the nucleus and the cytoplasm at both times, although a predominant cytoplasmic localization was sometimes observed in individual cells (never in the whole PDF^+ cluster [e.g., panel B]). For the representative *tim*-GAL4>UAS-$DBT^{K/R}$ brain shown in panel B, examples of nuclear (N), cytoplasmic (C), and both nuclear and cytoplasmic (B) localization are shown.

DISCUSSION

Initial studies of a strongly hypomorphic *dbt* mutant (dbt^P), resulting from the insertion of a transposable P element in an intron of the *dbt* gene and consequent reduction in *dbt* mRNA levels to ~10% of wild-type levels, showed that DBT is essential for oscillations of *per* and *tim* gene expression (43). This mutant produces a constitutively high level of PER, which constitutively represses *per* and *tim* mRNAs to low levels just sufficient to maintain repressing levels of PER and TIM proteins. This constitutively high level of PER can persist with greatly reduced levels of TIM (43). By contrast, in the tim^o mutant, in which TIM is absent but DBT is present, PER is cytoplasmic (8, 53) and is expressed at very low levels (44). It was therefore proposed that DBT^{WT} functions to destabilize PER in the cytoplasm during the day and early evening, while TIM accumulation during the night antagonizes DBT and allows PER to accumulate (22, 43). Because DBT delays the accumulation of PER in the cytoplasm, *per* and *tim* mRNA levels rise to their peak levels, before their transcription is repressed by nuclear PER (43). In this model, the delay in PER accumulation effected by DBT is essential for molecular rhythms of *per* and *tim* mRNA, which otherwise would be constitutively repressed by immediate accumulation of PER.

Kinases have multiple targets and complex effects on circadian clock components. In addition to the effects of DBT on PER cytoplasmic stability, it has become clear from additional work that DBT and its vertebrate orthologs (CKIε and CKIδ) cause additional effects on PER and other clock components.

A number of studies have suggested that CKI regulates nuclear localization and nuclear stability of PER (1, 3, 8, 33, 35, 50–52). As well, DBT or CKI has been proposed to activate the repressor function of PER (35). Other clock components have also been proposed as targets of DBT, including *Drosophila* dCLK (21, 58), which is also a target of PER's repression. *Drosophila* DBT destabilizes dCLK and may also repress its transcriptional activator function, suggesting that DBT may mediate PER's repressor function (21, 58). A similar function has been proposed for kinases in the bread mold *Neurospora crassa*, with FRQ mediating the targeting of the kinases to the white-collar transcription factor (16, 48), and other vertebrate transcription factors besides PER are also targets of CKI (10).

In both flies and mammals, DBT is part of a multiprotein complex (23, 26) that is likely to include other kinases, and it is not clear how much phosphorylation in this complex requires DBT. Reductions in DBT kinase activity do not necessarily lead to equivalent reductions in the phosphorylation of PER and other clock components in vivo. For instance, although normal oscillations of dCLK phosphorylation state are dependent on DBT, CLK is nevertheless phosphorylated to some extent in a dbt^{AR} mutant suggested by other work to have greatly reduced kinase activity. Hence, other kinases besides DBT may directly phosphorylate dCLK (21, 58). Because all of the short-period and long-period mutant alleles of *dbt* in both flies and vertebrates have lowered kinase activity on PER and casein in vitro (30, 41, 49, 55) but the short-period alleles apparently produce faster circadian cycles of phosphorylation in vivo, a faster clock is not the consequence of a simple increase in DBT's general kinase activity.

Antagonism of DBT^{WT} by $DBT^{K/R}$ produces long circadian periods but not short circadian periods. These results suggest two possible means by which DBT can regulate period length. The first possibility is that some of the period-altering mutants of *dbt* alter some other aspect of its function in addition to kinase activity and that these alterations differ in short- and long-period mutants. A second possibility is that the dbt^{S} and dbt^{L} mutations affect the rate of phosphorylation at different sites in PER in different ways or to different extents. Recently, two studies have provided evidence that the phenotype of the *tau* mutation, originally identified in CKIε of hamsters, may be explained by the latter possibility. One of these studies proposed that the *tau* mutant form of the kinase phosphorylates some of the sites more rapidly than wild-type kinase, while phosphorylating other sites less rapidly (13). The other study proposed less rapid phosphorylation at all sites but with different consequences of the phosphorylation events for PER stability (51). Reduced phosphorylation of one cluster of sites was proposed to reduce nuclear retention of PER and thereby destabilize PER, because it is still phosphorylated in the cytoplasm at other sites which target PER for degradation (51). Both of these studies postulated that the *tau* mutation affected the site preference of the kinase, with different consequences mediated at different sites. Analysis of mice carrying a mutation in a PER target site for CKI has also shown that different phosphorylation sites in PER produce different effects on clock biochemistry (56).

In order to address how a general reduction (rather than a site-specific reduction) in kinase activity would affect the circadian period, we constructed a very conservative mutation (K38R) that is predicted from prior work only to eliminate catalytic activity without affecting the general protein folding or interactions with other proteins. In fact, this protein, when immunoprecipitated from *Drosophila* S2 cells, completely lacks kinase activity. Our analysis of the $DBT^{K/R}$ protein in vitro and in cell culture has shown that while this mutation eliminates the kinase activity of DBT, it leaves its interactions with PER in tact. For this reason, $DBT^{K/R}$ can antagonize the action of DBT in S2 cells and in vivo, with the level of antagonism proportional to the amount of $DBT^{K/R}$ expression.

Since flies expressing $DBT^{K/R}$ can exhibit long circadian periods, reduced kinase activity is shown to produce long-period rhythms. Even the most weakly expressing $DBT^{K/R}$ line, which is mostly rhythmic and has the shortest periods, still exhibits periods longer than wild type. This finding demonstrates that period becomes progressively longer as DBT^{WT} is progressively antagonized by higher levels of $DBT^{K/R}$. While long-period mutants have not been identified in mammalian CKIε/δ, it is possible that redundancy between CKIε and CKIδ may mask the phenotypic effect of generally lowered kinase activity for either isoform. Importantly, no level of antagonism by $DBT^{K/R}$ produced short periods in our study, so short periods are not the consequence of a general antagonism of DBT^{WT} kinase activity in flies. The general reduction in the kinase activity of the DBT^{S} protein detected in vitro cannot fully explain the reason for its short-period phenotype.

Since the transgenic lines overexpressing DBT^{S} and DBT^{L} lines analyzed here have essentially the same periods as those resulting from the original mutations in the endogenous genes (43), both the dbt^{S} and dbt^{L} mutations produce an altered set point for the period length, which is not altered by overexpression of the proteins. Because the polypeptide chain folds back on itself to form a β-sheet in the ATP-binding lobe of CKI, the amino acid affected by the dbt^{L} mutation lies close to K38 in the ATP-binding domain of the X-ray crystal structure of CKI (54), and thus the dbt^{L} mutation (like the $dbt^{K/R}$ mutation) may reduce general catalytic activity. In contrast to $dbt^{K/R}$, the dbt^{L} mutation does not eliminate this activity, since the dbt^{L} period length is shorter than the maximum period lengths produced by $DBT^{K/R}$ in a wild-type genetic background, and DBT^{L} does exhibit detectable kinase activity (41). The dbt^{S} mutation affects an amino acid that lies on a surface-exposed loop of the ATP-binding lobe (54), and so it might affect an interaction with another protein, in addition to catalytic activity. Intriguingly, a human CKIδ mutation leading to short periods and an advanced sleep phase syndrome lies very close to the amino acid affected by the dbt^{S} mutation (55) (Fig. 1A). Like the dbt^{S} mutation, this mutation also leads to generally lower kinase activity in vitro. An intriguing possibility is that both of these mutations shorten period by the same mechanism, although in flies this mutation exhibits a modest lengthening of the circadian period (55).

DBT kinase activity is necessary for circadian rhythms of behavior and PER cycling. In addition to their relevance to the role of kinase activity in setting circadian period length, our results with $DBT^{K/R}$-expressing flies have broader relevance to the role of DBT's kinase activity in the circadian clock. To begin with, the results establish that DBT does have kinase activity in the fly. While this conclusion has been clear for the vertebrate CKIs, it has not been clear for the fly DBT protein.

Wild-type *Drosophila* DBT is not enzymatically active when expressed in *E. coli* (41). While DBT immunoprecipitated from S2 cells has kinase activity associated with it (41), it was formally possible that some other associating protein provided the kinase activity. However, the fact that the $DBT^{K/R}$ mutant protein immunoprecipitated from S2 cells completely lacks kinase activity in vitro while DBT^{WT} exhibits kinase activity establishes that DBT^{WT} has an intrinsic kinase activity, rather than a kinase activity resulting from an associated protein; the kinase activity does not derive from an associated kinase, or it would not be eliminated by the K38R mutation. The vertebrate CKIε/δs are known to autophosphorylate their C-terminal domains, and this phosphorylation autoinhibits their kinase activity (54). Absence of kinase activity in $DBT^{K/R}$ is predicted to eliminate this inhibition, and yet there is no detectable kinase activity in vitro, thereby reinforcing the notion that the primary effect of the mutation is on the catalytic mechanism of DBT.

Our further analysis of the $DBT^{K/R}$ phenotype in S2 cells and flies establishes the requirement for this kinase activity in all the circadian phenomena that were assessed here. $DBT^{K/R}$ acts as a dominant negative mutant when expressed in flies, resulting in arrhythmic and long-period phenotypes. At the molecular level in flies expressing $DBT^{K/R}$, PER levels are high at all times of day, and the oscillation in level is damped in LD and DD conditions, especially in the PDF^+ cells, which are essential for rhythmic locomotor activity. Because the oscillation in electrophoretic mobility shifts of PER is eliminated in $DBT^{K/R}$ flies and PER always has a high mobility, phosphorylation of PER that produces electrophoretic mobility shifts is shown to be dependent on DBT's kinase activity.

The phenotype of the $DBT^{K/R}$ mutant is the first which can be said to arise specifically from strongly reduced DBT catalytic activity and not some other aspect of its function or regulation. The original strong loss-of-function *dbt* mutation that was analyzed for effects on PER and TIM cycling resulted from the insertion of a P element in an intron of the gene (dbt^P); it depresses the expression level of the gene greatly but has not been shown to affect the nature of the encoded protein (22, 43). In any event, homozygous larvae do not survive to the adult stage. Likewise, the effects of existing strong loss-of-function *dbt* mutations (*dco* mutations [60]) on catalytic activity are unknown, and most produce embryonic lethality, so their effects on the adult circadian clock cannot be addressed.

The $DBT^{K/R}$ phenotype is similar to that of dbt^{AR} flies (8, 46), with constitutively high levels of nuclear PER and arrhythmicity or long periods produced by both mutants (8, 24, 46). Thus, the findings with both mutants are consistent with the notion that DBT kinase activity is needed for PER's temporal degradation but not for its nuclear localization. However, the interpretation is clearer for the $dbt^{K/R}$ mutant, because we have demonstrated here that it lacks kinase activity, and prior biochemical and structural studies point to a specific disruption of the catalytic site (18, 19, 54, 61). Although DBT^{AR} is considered to be strongly deficient in kinase activity, analysis of its kinase activity has never been reported in detail, and Rothenfluh et al. (46) have pointed out that the amino acid produced by the dbt^{AR} mutation (His126Tyr) is found in wild-type CKIγ, suggesting that the amino acid change may not eliminate kinase activity but may alter target specificity (by contrast, K38 is highly conserved in all kinases). In addition, the observations that the dbt^{AR} mutant is not lethal and exhibits constitutively repressed CLK/CYC-dependent transcripts (8), while DBT activity is associated with this repression in S2 cells (21, 35), suggest that DBT^{AR} retains some kinase activity. In this light, the dispersed PER (46) and CLK (21, 58) mobilities that are observed in the dbt^{AR} mutant but not for PER in the $dbt^{K/R}$ mutant (Fig. 5) suggest that the residual phosphorylation may be due to residual DBT activity in the dbt^{AR} mutant. Our binary expression approach has allowed us to demonstrate that period length is progressively increased by increasing levels of antagonism by $DBT^{K/R}$ and will allow us to antagonize DBT function in specific subsets of circadian cells with more restrictive GAL4 drivers. Our results clearly show that kinase activity is required for normal circadian locomotor rhythms, as well as for normal circadian cycling of PER levels, phosphorylation state, degradation, and nuclear accumulation. As strong loss-of-function and null *dbt* alleles are lethal (43, 60), the $DBT^{K/R}$ phenotypes are the most complete loss-of-function phenotypes yet analyzed for the adult clock.

Wild-type levels of DBT activity are likely not to be absolutely required for nuclear localization of PER, since high levels of nuclear PER are detected in photoreceptors and PDF^+ brain neurons at all times of day in the $DBT^{K/R}$ mutant. These results, together with those of earlier studies (3, 8), suggest that DBT^{WT} may antagonize nuclear accumulation of PER. However, the possibility that phosphorylation normally promotes PER nuclear localization or affects it only indirectly (35) cannot be excluded. On the one hand, PER might still become phosphorylated by other kinases or by incompletely inhibited endogenous DBT in *tim*-GAL4>UAS-$DBT^{K/R}$ flies. This limited phosphorylation might still promote PER nuclear accumulation, with the strong inhibition on further nuclear phosphorylation stabilizing this nuclear accumulation and leading to high nuclear PER accumulation over time. Alternatively, the nuclear localization of PER could be significantly compromised due to the insufficient phosphorylation state. However, in this case the constitutively high level of PER accumulation in the cytoplasm, due to lack of protein turnover caused by DBT, could cause leakage of PER protein into the nucleus or saturation of mechanisms that normally serve to retain PER in the cytoplasm at certain times of day. A final possibility, which we prefer, is that phosphorylation at some sites stimulates PER nuclear accumulation while phosphorylation at other sites represses its nuclear accumulation. In this scenario, the effect of any specific experiment on subcellular localization would then be determined by the specific alteration in the balance of phosphorylation events. Experiments in both mammals and flies have produced a conflicting set of results which could indicate an underlying complexity to control of nuclear accumulation by phosphorylation. Our analysis of PER localization in PDF^+ larval neurons suggests that PER can be found in both the cytoplasm and the nucleus in some individual neurons of *tim*-GAL4>UAS-$DBT^{K/R}$ larvae, suggesting that exclusive localization to the nucleus or cytoplasm is not dictated by greatly reduced phosphorylation of PER.

It is intriguing that continued oscillations of PER levels and nuclear accumulation, although strongly damped, are observed in LD in the heads and bodies of *tim*-GAL4>UAS-$DBT^{K/R}$ flies (Fig. 5 and 6). It is possible that these oscillations are the consequence of incomplete inhibition of DBT activity by

$DBT^{K/R}$; endogenous DBT^{WT} may displace the $DBT^{K/R}$ with enough frequency to produce residual phosphorylation of PER, and light cycles may facilitate this exchange. However, it is also possible that DBT-independent light-input pathways consisting of other kinases or clock proteins can support oscillations of PER in the absence of DBT kinase activity. Elimination of these residual oscillations should be produced by mutations that affect these pathways.

The $DBT^{K/R}$ responder transgenes are likely to affect other aspects of the clock cycling as well. If phosphorylation of PER is required for its repressor function in a cell culture assay (35), *per* and *tim* mRNA levels are predicted to be higher in these flies and *dClk* mRNA levels lower, due to lack of PER repressor function. This in turn could contribute to the higher levels of PER that are detected from ZT7 to -13. In addition, phosphorylation of dCLK or other clock components may be affected. The activities of CKII (2, 27) and Shaggy (31), which target PER and TIM, respectively, may be altered as a consequence of DBT inactivation. The analysis of the diverse effects and targets of DBT kinase activity will be facilitated by continued analysis of the $DBT^{K/R}$ flies.

Recently, it was shown that a circadian oscillation of cyanobacterial proteins could be produced in a test tube with purified components (34). These oscillations involved no feedback on transcription and no oscillating transcripts or protein levels; rather, the protein levels were constant, and the phosphorylation state changed. While eukaryotic circadian oscillations have not been produced in the absence of changes in protein levels, *per* and *tim* transgenes that express mRNA constitutively support circadian rhythms in vivo (6, 57). It is possible that circadian control of kinase activity is the most essential element of clock function and perhaps the first to evolve. A thorough understanding of its mechanisms and consequences is essential to understanding the molecular mechanism of the clock.

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