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Drosophila and Vertebrate Casein Kinase Iδ Exhibits Evolutionary Conservation of Circadian Function

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ABSTRACT

Mutations lowering the kinase activity of Drosophila *Doubletime* (DBT) and vertebrate casein kinase Iε/δ (CKIε/δ) produce long-period, short-period, and arrhythmic circadian rhythms. Since most *ckI* short-period mutants have been isolated in mammals, while the long-period mutants have been found mostly in Drosophila, lowered kinase activity may have opposite consequences in flies and vertebrates, because of differences between the kinases or their circadian mechanisms. However, the results of this article establish that the Drosophila *dbt* mutations have similar effects on *period* (PER) protein phosphorylation by the fly and vertebrate enzymes *in vitro* and that Drosophila DBT has an inhibitory C-terminal domain and exhibits autophosphorylation, as does vertebrate CKIε/δ. Moreover, expression of either Drosophila DBT or the vertebrate CKIδ kinase carrying the Drosophila dbt^S or vertebrate *tau* mutations in all circadian cells leads to short-period circadian rhythms. By contrast, vertebrate CKIδ carrying the dbt^L mutation does not lengthen circadian rhythms, while Drosophila DBT^L does. Different effects of the dbt^S and *tau* mutations on the oscillations of PER phosphorylation suggest that the mutations shorten the circadian period differently. The results demonstrate a high degree of evolutionary conservation of fly and vertebrate CKIδ and of the functions affected by their period-shortening mutations.

ORGANISMS as phylogenetically diverse as cyanobacteria and man exhibit circadian rhythms, which produce cycles of behavior, physiology, and biochemistry that are synchronized (or entrained) by the environmental cycles. When organisms are maintained in the laboratory under constant conditions, these rhythms persist with a period length that is ~24 hr, thus demonstrating the existence of an endogenous biological clock underlying the overt rhythms. The endogenous nature of this circadian clock is thought to allow environmental changes to be anticipated by physiological alterations that would not occur soon enough if they were produced only after the environmental changes (Pittendrigh 1974).

The molecular mechanism for the circadian clock has yielded to genetic analyses in flies, mammals, bread mold, plants, and cyanobacteria (Sehgal 2004). While different gene products are used in different phyla, in eukaryotes a general property has been transcriptional feedback loops, wherein transcriptional regulators increase and decrease over the course of the day to regulate target genes. In Drosophila, the *period* (PER) protein negatively regulates a bHLH-PAS *clock/cycle* transcription factor (CLK/CYC). Since the *per* gene is a target of CLK/CYC, PER represses its own mRNA. When PER protein levels fall, *per* mRNA is transcribed again, leading to antiphase cycling of *per* mRNA and nuclear PER protein (Price 2004).

To generate ~24-hr oscillations, the timing of negative feedback must be regulated post-translationally by the activities of protein kinases and phosphatases (Harms *et al.* 2004). It was first shown that PER was rhythmically phosphorylated (Edery *et al.* 1994), and isolation and analysis of the *dbt* mutants in Drosophila then showed that casein kinase I (CKI) targets PER for phosphorylation and degradation (Kloss *et al.* 1998; Price *et al.* 1998; Rothenfluh *et al.* 2000a; Suri *et al.* 2000; Muskus *et al.* 2007). *Doubletime* (DBT) has also been proposed to regulate the nuclear localization (Bao *et al.* 2001; Cyran *et al.* 2005; Nawathean *et al.* 2007) and the repressor capacity of PER (Nawathean and Rosbash 2004; Kim *et al.* 2007; Nawathean *et al.* 2007), as well as the phosphorylation of CLK protein (Kim and Edery 2006; Yu *et al.* 2006). In addition, two other kinases [casein kinase II, CKII (Lin *et al.* 2002) and SGG (Martinek *et al.* 2001)] and two protein phosphatases [PP2A (Sathyanarayanan *et al.* 2004; Fang *et al.* 2007) and PP1(Fang *et al.* 2007)] contribute to rhythmic phosphorylation of clock proteins. Without the activities of these kinases and phosphatases, clock protein expression and clock-controlled processes are not rhythmic. DBT's mammalian orthologs CKIε (Lowrey

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et al. 2000; Vielhaber *et al.* 2000; Lee *et al.* 2001; Eide *et al.* 2005) and -δ (Lee *et al.* 2001; Xu *et al.* 2005), and PP1 (Gallego *et al.* 2006b), have also been shown to contribute to mammalian circadian rhythms, in part by regulating PER stability and nuclear localization.

The importance of *dbt* or vertebrate *ckI* protein kinases for the core time-keeping process is exemplified by mutants that can either shorten or lengthen the circadian period (Price *et al.* 1998; Lowrey *et al.* 2000; Rothenfluh *et al.* 2000a; Suri *et al.* 2000; Xu *et al.* 2005; Muskus *et al.* 2007). Intriguingly, all of the known mutant kinases exhibit lower kinase activity on complex substrates like casein and PER *in vitro* (Lowrey *et al.* 2000; Suri *et al.* 2000; Preuss *et al.* 2004; Xu *et al.* 2005), with all of the long-period mutants identified in fly DBT and all but one of the short-period mutants in vertebrate CKIε/δ, raising the possibility that there are differences between the mammalian and Drosophila circadian kinases and mechanisms (Xu *et al.* 2005; Sekine *et al.* 2008).

Several other findings are relevant to the evolutionary relationship between the roles of CKI in the Drosophila and vertebrate clocks. It is noteworthy that Drosophila DBT and vertebrate CKIε/δ are highly conserved in their N-terminal regions but diverge significantly in the C-terminal domains (Kloss *et al.* 1998). Moreover, their proteins differ biochemically, because the vertebrate CKIε/δ's are enzymatically active when purified from *Escherichia coli* (Graves and Roach 1995; Preuss *et al.* 2004) while Drosophila DBT is not (Suri *et al.* 2000; Kloss *et al.* 2001; Preuss *et al.* 2004). While a knock-out mutation of *ckI*ε in mice lengthens period, the effect is relatively minor [~20 min (Meng *et al.* 2008)]. Recently, it was shown that expression of mammalian CKIε in flies antagonized their circadian rhythms, suggesting that mammalian CKIε is not entirely evolutionarily conserved with fly DBT in its circadian function (Sekine *et al.* 2008). Finally, while both DBT and CKIε/δ target PER, additional circadian targets may differ, with mammalian CKIε/δ targeting BMALI and CRY (Eide *et al.* 2002) and DBT targeting CLK (Kim and Edery 2006; Yu *et al.* 2006).

In this article, we have examined the evolutionary conservation of DBT and CKIδ function by further biochemical characterization of DBT and by expression in Drosophila of various mutations in both the fly DBT and the vertebrate CKIδ. Surprisingly, the results suggest a high degree of evolutionary conservation for CKIδ and DBT and for the circadian functions affected by the short-period mutations of DBT and CKIε/δ.

MATERIALS AND METHODS

Expression of DBT-MYC and PER-HA in cell culture lines: Vectors expressing untagged DBT and MYC-epitope-tagged DBT^{WT}, DBT^{S}, DBT^{L} (Preuss *et al.* 2004), or the catalytically inactive $DBT^{K/R}$ protein (Muskus *et al.* 2007) under the control of the metallothionein promoter have been previously described. DBT^{WT}, DBT^{S}, DBT^{L}, the catalytically inactive $DBT^{K/R}$ protein, or truncated forms of DBT were used to produce stably transfected S2 cell lines as previously described (Muskus *et al.* 2007). To produce the truncated forms of DBT, PCR reactions were employed to amplify different extents of the DBT C terminus. Each reverse primer encoded an *Age*I site and the MYC epitope tag and therefore contained the following sequence at its 5′ end: GCGCACCGGTGAGGTCTTCCTCGCTGATCAGCTTCTGCTC. This was followed by the noncoding strand for DBT starting at amino acid 296 (GCCAAACTTAAGCAGGTTCCAGTCAAACAC), 332 (GGCGGCTGCCGCTGCTGCCACCGCCGCTGC), or 387 (ATCGTCCATGTTGAGTCCGTTGCCGCCGAT). Each of these primers was used with a forward primer that annealed upstream of a unique *Nsi*I site in the *dbt* cDNA. Digestion of the PCR product with *Nsi*I and *Age*I yielded a fragment that was ligated in place of the *Nsi*I/*Age*I fragment from pMT-DBT, yielding a plasmid that expressed an epitope-tagged truncation mutant. DBT-MYC-expressing constructs were transiently cotransfected into S2 cells with pAC-PER-HA, expression of DBT-MYC was induced with $CuSO_4$, and PER and DBT levels were detected by immunoblot analysis with anti-HA and anti-MYC antibodies as previously described (Muskus *et al.* 2007). For the experiments examining autophosphorylation of DBT (Figure 3), stably transfected lines expressing DBT^{WT}-MYC or $DBT^{K/R}$-MYC from the pMT vector were used. These were induced with $CuSO_4$ 41 hr before harvest, and okadaic acid or vehicle (DMSO) was added 17 hr before harvest. The extracts were than analyzed by immunoblot analysis with an anti-MYC antibody.

***In vitro* kinase assays with DBT^{S}, DBT^{L}, or C-terminally truncated forms of DBT:** DBT-MYC expression was induced in stably transfected cell lines with $CuSO_4$, the cell lysates were immunoprecipitated with anti-MYC antibody, the immunoprecipitates were incubated with PER expressed and purified from bacteria, and radiolabeled substrate was detected and quantified by phosphor-imager analysis of SDS–PAGE (Muskus *et al.* 2007). Aliquots of each reaction were also analyzed by immunoblot analysis with an antibody detecting the C terminus of DBT (Figure 1) or the MYC epitope (Figure 2), and the signal for ^{32}P incorporation was normalized to the signal for DBT, as previously described (Muskus *et al.* 2007). The individual kinase activities measured in each of the experiments tabulated in Figure 1 were subjected to a one-way ANOVA, with each genotype as a categorical predictor (three altogether). Any comparisons between the kinase activities of two DBT genotypes were accomplished by applying a *post hoc* Tukey's honestly significant differences (HSD) test to this ANOVA. Significant differences were defined as those with $P < 0.05$. Group values are presented as means ± SEM. Statistics were performed using Statistica (StatSoft, Tulsa, OK).

Site-directed mutagenesis to produce mutant CKI and DBT: Generation of the dbt^{S}, dbt^{L}, ckI^{S}, ckI^{L} (Preuss *et al.* 2004), and $dbt^{K/R}$ (Muskus *et al.* 2007) mutations has previously been described. The ckI^{Tau} and $ckI^{D/N}$ mutants were constructed by annealing two PCR fragments that each had the mutation at an end and then amplifying a larger fusion fragment with the mutation in the middle, as previously described (Preuss *et al.* 2004). The mutagenic primers were as follows: for $ckI\delta^{Tau}$, F5′-GGAACAGCT**TGC**TATGCATCTATTAACAAC and R5′-AGAT GCATA**GCA**AGCTGTTCCGGTTAGGTT; and for $ckI\delta^{D/N}$, R5′-CAGAAAATT**GTT**AGGCTTCACATCCCGATG and F5′-GTG AAGCCT**AAC**AATTTTCTGATGGGACTT. In each reaction either the mutation-containing R primer was combined with a primer flanking an upstream *Pin*AI site (5′-CACTTTTTTT GCTTCTTAGCGCCGGATGGA) or the mutation-containing F primer was combined with a primer flanking a downstream *Nsi*I site (5′-TGGCTTATCATCAAAACGTAAGGACCGACA). The two fragments produced for each mutation were mixed,

denatured, annealed through their complementary ends (including the mutation site), and extended by several rounds of PCR and then final PCR with both flanking primers, thereby amplifying a full-size fragment. Cloning of these fragments into a full-length Xenopus CKIδ cDNA has been previously described (Preuss *et al.* 2004).

The *dbt*^{Tau} mutation was generated by the Quik-Change procedure (Stratagene, La Jolla, CA), with a pMT-*dbt*^{WT} vector as template. The primers were DBTtauF, 5′-CTCACGGGC ACTGCC**TGC**TATGCCTCCATC-3′ and DBTtauR, 5′-GATGG AGGCATA**GCA**GGCAGTGCCCGTGAG-3′.

Generation of transgenic flies expressing CKIδ and DBT-MYC: The cloning of the *ckI*δ genes into the GAL4-inducible fly transformation vector *pUAST* was performed by using the restriction enzymes *Sal*I and *Xba*I and isolation of a 1.6-kb fragment from *pVAX-ckI*δ (Preuss *et al.* 2004), which was inserted into the opened vector plasmid after digestion of the vector with *Xho*I and *Xba*I. This procedure placed *ckI*δ in a 5′–3′ orientation downstream of the UAS promoter sequence in pUAST. The *dbt*-myc cDNAs were cloned into a pUAST vector modified with a *Pme*I site, as previously described (Muskus *et al.* 2007). Embryos from a w^{1118} line (Bloomington Drosophila Stock Center, stock no. 3605) were injected by Duke Model Systems Genomics, and lines containing different transgene insertion sites were generated by crossing the injected adults to a w; TM3 or a w; CyO balancer stock, selecting transgenic progeny by their w^+ phenotype, and establishing lines by at least two backcrosses to the *w*; TM3 or w; CyO balancer stock. The insertions were mapped to chromosome II or III on the basis of the segregation of the w^+ *P* element relative to the dominant markers on the balancer chromosomes, and the line was crossed to the other balancer stock if its insertion site was not on the homologous chromosome of the balancer stock used initially. Independent insertions were produced by ensuring that all lines tested herein were derived from different injected w^{1118} embryos.

Rearing of flies and behavioral analysis: Males from UAS-responder transgenic flies were mated to virgin females carrying a GAL4 driver transgene. Male progeny were collected and carried one copy of the driver and responder transgene. The *tim*-GAL4 driver transgene is expressed in all circadian cells [P(w[+mC]=GAL4-tim.E)62; Bloomington stock no. 7126 (Kaneko and Hall 2000)]. This driver produced expression of MYC-tagged DBTs in both photoreceptors and brain neurons, including the lateral neurons that secrete PDF and control locomotor behavior (data not shown). Progeny were reared for at least 3 days in a 12-hr:12-hr light:dark cycle. Locomotor activity was then monitored in constant darkness with Trikinetics activity monitors, and period length and rhythmicity were determined by ClockLab analysis software, as previously described (Muskus *et al.* 2007). The average periods of all the individual lines in Table 1 were subjected to a one-way ANOVA, with each genotype (nine altogether) as a categorical predictor. In addition, the average periods of the responder without driver controls (seven averages of this type in Table 1) composed a 10th categorical predictor in this ANOVA. Any comparisons between two genotypes were accomplished by applying a *post hoc* Tukey's HSD test. Significant differences were defined as those with $P < 0.05$. Group values are presented as means ± SEM. Statistics were performed using Statistica (StatSoft).

Immunoblot analysis of DBT, CKI, and PER levels in the heads of transgenic flies: *tim*-GAL4>UAS-DBT-MYC or -CKI flies were reared, entrained to light:dark (LD), and collected at various times in an LD cycle as described above. Heads were cut off by razor blade and analyzed by immunoblot analysis as previously described (Muskus *et al.* 2007). Depending on the experimental setup, immunoblot analysis was performed using a 1:5000 dilution of anti-MYC antibody (Invitrogen, Carlsbad, CA), a 1:2000 dilution of rabbit anti-DBT antibody, a 1:25,000 dilution of rabbit anti-PER antibody, a 1:2000 dilution of mouse monoclonal anti-CKIδ 128A (kindly provided by Anthony DiMaggio of ICOS), or a 1:5000 dilution of mouse monoclonal anti-tubulin (Developmental Studies Hybridoma Bank, University of Iowa), followed by a 1:5000 dilution of the appropriate horseradish peroxidase-labeled secondary antibody and visualization of the secondary antibody using Amersham (Piscataway, NJ) ECL Plus Western blotting detection reagents (cat. no. RPN2132).

RESULTS

DBT^S and DBT^L phosphorylate PER less actively *in vitro* than does DBT^{WT}: We have previously shown that Xenopus CKIδ carrying the Drosophila *dbt*^{S} or *dbt*^{L} mutations (CKI^S or CKI^L) phosphorylates casein and PER less actively than does wild-type CKIδ [CKI^{WT} (Preuss *et al.* 2004; Constance *et al.* 2005)], and both the short-period and long-period mutant forms of DBT (DBT^S and DBT^L) phosphorylate casein less readily than does wild-type DBT [DBT^{WT} (Preuss *et al.* 2004; Kivimae *et al.* 2008)]. Here, it is shown that DBT^S and DBT^L immunoprecipitated from Drosophila S2 cells also phosphorylated recombinant Drosophila PER less actively than wild-type DBT, although a comparison with a catalytically inactive form of DBT [DBT$^{K/R}$ (Muskus *et al.* 2007)] demonstrates that both mutants retained some kinase activity (Figure 1; two reaction aliquots are shown from two separate reactions for each, and the average relative activity of DBT^{WT} was set to 1 in all assays). An ANOVA indicated a highly significant effect of genotype on kinase activity [$F(2, 6) = 14.3$, $P < 0.01$]. Both the DBT^S and the DBT^L proteins produced significantly less phosphorylation of PER than did DBT^{WT} ($P < 0.015$ by ANOVA and *post hoc* Tukey's test), while the differences between DBT^S and DBT^L were not significant ($P > 0.800$). Therefore, in the context of either vertebrate CKIδ or Drosophila DBT, the *dbt*^{S} and *dbt*^{L} mutations lower general kinase activity toward both PER and casein *in vitro*.

DBT is inhibited by a C-terminal domain and has autokinase activity: Kinase activity of vertebrate CKIε/δ is inhibited by its C-terminal domain, as removal of this domain by proteolysis or expression of truncated proteins leads to elevated activity *in vitro* (Graves and Roach 1995; Cegielska *et al.* 1998). While the DBT C terminus does not exhibit any significant homology with vertebrate CKIε/δ, this domain differs significantly between vertebrate CKIε and CKIδ, which most likely were produced by a gene duplication event and have evolved from a common progenitor gene (Constance *et al.* 2005). Despite the relatively low sequence conservation, the C termini of CKIδ and CKIε seem to have similar properties, since both mediate autophosphorylation-dependent inhibition of CKI's kinase activity (Graves and Roach 1995; Cegielska *et al.* 1998)

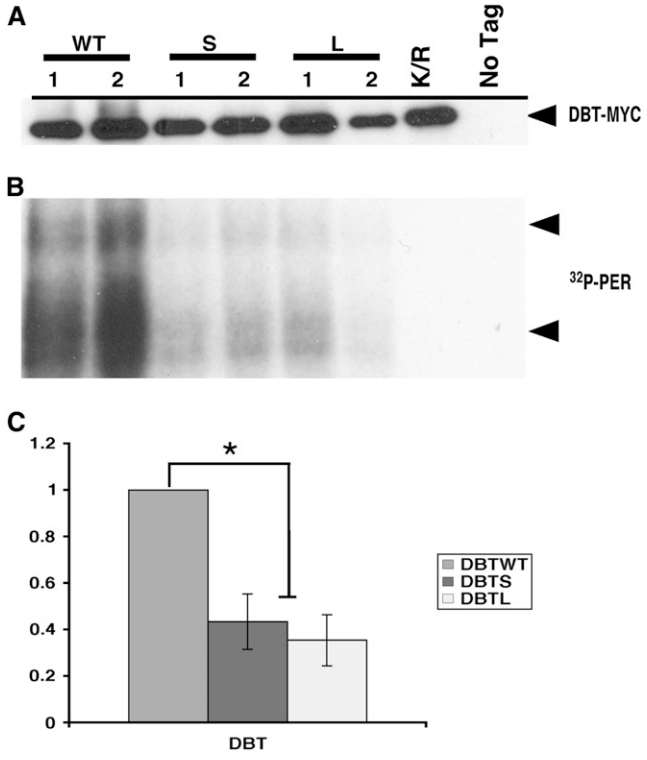

FIGURE 1.—DBT^S and DBT^L produce less phosphorylation of PER *in vitro* than does DBT^{WT}. pMT plasmids expressing MYC-tagged DBTs were transfected into S2 cells and selected for stable integration. After induction with $CuSO_4$, the MYC-tagged DBT was immunoprecipitated and incubated with [γ-^{32}P]ATP and recombinant PER isolated from bacteria. Reaction aliquots were subjected to SDS–PAGE. (A) Immunoblot analysis of reaction aliquots with an antibody to the C terminus of DBT. Aliquots of two replicate reactions ("1" and "2") containing immunoprecipitates of DBT^{WT}-MYC ("WT"), DBT^S-MYC ("S"), DBT^L-MYC ("L"), DBT$^{K/R}$-MYC ("K/R"), or S2 cells stably transfected with pMT-DBT (untagged DBT) were electrophoresed. (B) PER phosphorylation in another part of the same aliquots (1 and 2) analyzed in A was analyzed by autoradiography of SDS–PAGE of reaction aliquots. (C) The amount of radioactive signal in each aliquot was quantified by phosphorimager analysis and normalized to the amount of DBT detected by immunoblot, and the signals were further normalized in each experiment to the signal for DBT^{WT}, which was set to 1 (hence no variation in the DBT^{WT} signal). Three independent experiments were analyzed. Error bars, ±SEM. Both the DBT^S and the DBT^L proteins produced significantly less phosphorylation of PER than did DBT^{WT} (*$P < 0.015$ by ANOVA and *post hoc* Tukey's test), while the differences between DBT^S and DBT^L were not significant ($P > 0.800$).

Unlike the catalytic activity of the kinase, this function may not require much sequence conservation (perhaps just some serines or threonine targets). DBT's C-terminal domain has several serines and threonines that could mediate autophosphorylation-dependent inhibition, and it might serve the same function as the C terminus of CKIε/δ, despite the lack of apparent sequence conservation. Therefore, DBT truncation mutants were assayed to determine whether DBT's C terminus might inhibit DBT's kinase activity, as does the C-terminal domain in vertebrate CKIε/δ. The shortest of these terminated at amino acid 296 and was missing all of the evolutionarily divergent C-terminal domain, which extends from amino acid 296 to the C terminus at amino acid 440 (CONSTANCE *et al.* 2005). All of these were expressed as MYC-tagged proteins in Drosophila S2 cells, immunoprecipitated with an antibody to the MYC epitope, and assayed with PER *in vitro*. All of the truncated DBTs phosphorylated PER, and the DBT-296 produced increased phosphorylation of PER in comparison with full-length DBT^{WT} (Figure 2A). This qualitative result was reproduced in five experiments; although the low levels of DBT-296 recovery often precluded quantification of its chemifluorescent signal, the amount of PER phosphorylation was greater than or equal to that produced by higher levels of DBT-440.

When DBT is coexpressed with PER in S2 cells, PER is progressively phosphorylated by DBT, and this phosphorylation causes PER to exhibit reduced electrophoretic mobility and to be targeted for degradation by the proteasome (KO *et al.* 2002; MUSKUS *et al.* 2007). In these experiments, the amount of DBT can be altered by transfection of different amounts of *dbt* plasmid, and by the addition of different amounts of $CuSO_4$, which induces the metallothionein promoter in the pMT-*dbt* plasmid. This S2 cell phosphorylation and degradation program recapitulates the circadian phosphorylation and degradation program produced by DBT *in vivo*, and it has recently been employed to map DBT target sites in PER (CHIU *et al.* 2008). When expressed together with PER in S2 cells, all of the DBT truncation mutants targeted PER for phosphorylation and degradation (Figure 2B). The activity of the DBT-296 protein was particularly noteworthy, as it accumulated only to low levels but was more effective than DBT^{WT} in targeting PER. Hence, deletion of the C-terminal domain leads to hyperactivity for Drosophila DBT, as it does for vertebrate CKIε/δ.

In vertebrate CKIε/δ, the inhibitory effects of the C terminus are mediated by autophosphorylation of the C terminus (GRAVES and ROACH 1995; CEGIELSKA *et al.* 1998; RIVERS *et al.* 1998). To address whether DBT might be autophosphorylated, we expressed MYC-tagged DBT^{WT} or DBT$^{K/R}$ in Drosophila S2 cells in the presence of increasing amounts of the phosphatase inhibitor okadaic acid (nanomolar concentrations given) or vehicle alone (DMSO; 0 nM) and analyzed the tagged proteins by immunoblot analysis (Figure 3). Lower mobility forms were detected for DBT^{WT} but not for DBT$^{K/R}$. Because the novel forms in DBT^{WT} require the kinase activity of DBT and are enhanced by phosphatase inhibition, they are likely to be produced by autophosphorylation of DBT. Taken together with the data in Figure 2, they argue that the DBT C-terminal domain inhibits the kinase activity of DBT and that DBT is autophosphorylated, like vertebrate CKIε/δ.

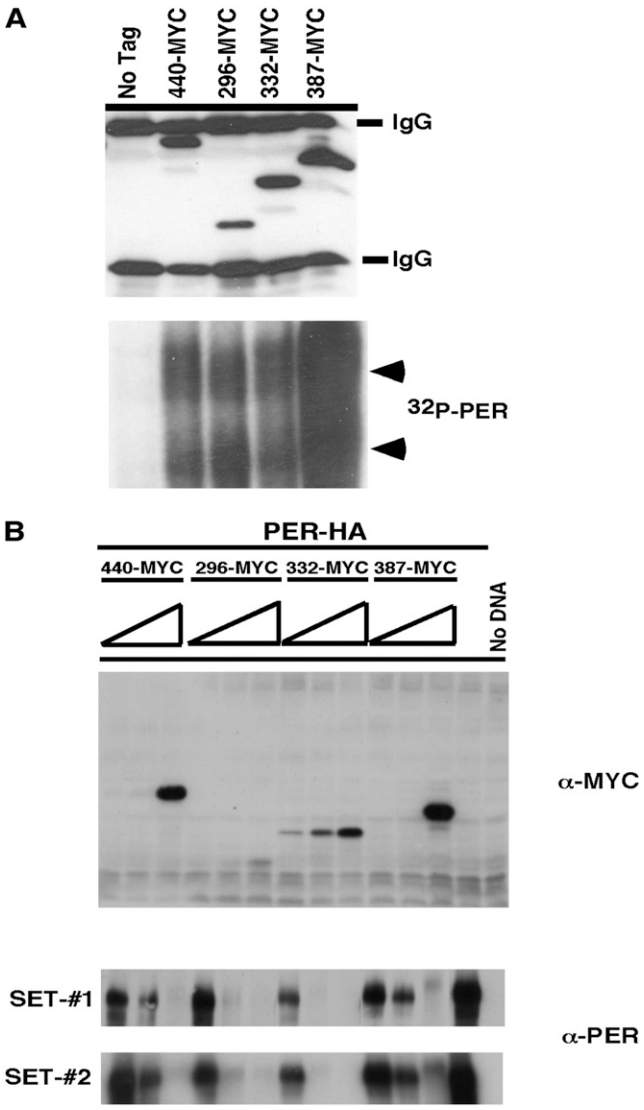

FIGURE 2.—Truncations that affect the DBT C terminus are more active kinases. Truncation mutants that end at the indicated amino acid number (untruncated wild-type DBT ends at amino acid 440) were cloned into the pMT vector with a MYC epitope at the C terminus and expressed in Drosophila S2 cells, with varying amounts of plasmid and induction by $CuSO_4$. (A) The kinases were immunoprecipitated from lysates of stably transfected cells. The amount of kinase was detected by immunoblot analysis of a reaction aliquot part with an anti-MYC antibody (top), and the amount of ^{32}P incorporation into PER was detected by autoradiography of another reaction aliquot part subjected to SDS–PAGE (bottom). The result is representative of those obtained in five experiments, which consistently showed equivalent or higher levels of PER phosphorylation produced by levels of DBT-296 protein lower than the levels of DBT-440 protein. The anti-MYC antibody heavy and light chains ("IgG") present in the immunoprecipitates are detected in the immunoblot because the secondary antibody reacts with them. (B) The indicated DBT truncation mutants were transiently cotransfected with pAc-PER-HA, and the amount of DBT-MYC expressed was detected by immunoblot (top). The two bottom panels are independent experiments detecting PER-HA by immunoblot. The penultimate lane on the right was transfected with a plasmid expressing PER-HA only (no DBT-MYC), and the last lane on the right contains extract from untransfected S2 cells ("no DNA"). Equal numbers of cells were plated for each condition, and equal volumes of extracts were loaded in each lane.

The *dbt*^{s} and *tau* mutations shorten the circadian period in both CKIδ and DBT: To compare the circadian phenotypes of various mutant vertebrate CKIδ proteins and Drosophila DBT, both were placed under the control of a yeast UAS promoter element, which allows these kinase "responder" genes to be expressed when a GAL4 transcription factor "driver" gene is also present. Expression of the kinases in progeny flies is produced by crossing parents that carry a driver with those with a responder. We did not attempt to rescue a *dbt* null mutant with this approach because prior work had shown incomplete rescue of the null phenotype even with widespread expression of Drosophila UAS-DBT, and vertebrate UAS-CKIε did not produce any viable adults whose circadian rhythms could be assayed (SEKINE *et al.* 2008). Instead, we overexpressed UAS-DBT and -CKIδ transgenes in a genotype that included *dbt*^{WT} at the endogenous locus. We had previously established that it is possible to produce fully penetrant *dbt* mutant phenotypes by overexpression of mutant DBT proteins that outcompetes endogenous DBT for interactions with clock components (MUSKUS *et al.* 2007). By restricting the expression with clock-cell-specific drivers, it is possible to avoid lethality and reduce the possibility of general developmental effects or activation of diverse signaling pathways (*e.g.*, *wingless* and *hedgehog* pathways) that could indirectly affect rhythms.

Locomotor activity was assayed for flies expressing the wild-type and various mutant forms of CKIδ and DBT with a *tim*-GAL4 driver, which produces expression in all circadian cells (KANEKO and HALL 2000). As with overexpression of DBT^{WT}, overexpression of CKIδ^{WT} examines whether the introduction of a vertebrate casein kinase into fly circadian cells causes lethality or disrupts the circadian system of the fly, and it serves as a baseline against which the effects of period-altering mutations are measured. There was no lethality or disruption of external morphology when CKIδ^{WT} was expressed with *tim*-GAL4 (data not shown). While ANOVA indicated a highly significant effect of genotype on circadian period [$F(9, 37) = 73.4$, $P < 0.0001$], the UAS-DBT^{WT} or -CKI^{WT} responders did not produce a statistically significant change in period with the *tim*-GAL4 driver relative to the average period of the responders without the driver (Table 1, $P > 0.05$ by ANOVA with *post hoc* Tukey's test; however, the average period of the *tim*-GAL4>UAS-DBT^{WT} or -CKI^{WT} lines was slightly longer than the average period of the UAS-DBT^{WT} or -CKI^{WT} responders without the driver). They did produce substantial arrhythmicity. Marginal period lengthening like that shown here has been previously observed for overexpression of both mammalian CKIδ and Drosophila

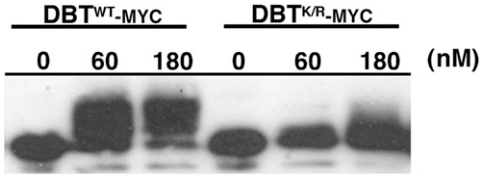

FIGURE 3.—When cellular phosphatases are inhibited, DBT^{WT} (but not a catalytically inactive $DBT^{K/R}$) accumulates in a low-mobility form. S2 cells stably transfected with pMT-DBT^{WT}-MYC or -$DBT^{K/R}$-MYC were incubated with increasing concentrations of the general phosphatase inhibitor okadaic acid or vehicle (DMSO; 0 nM) for the final 17 hr after induction with $CuSO_4$. Immunoblot analysis with an anti-MYC antibody detected the transgenic DBT protein. Note the novel slow-mobility forms appearing in DBT^{WT}. The result is representative of four independent experiments.

DBT in flies (XU *et al.* 2005; MUSKUS *et al.* 2007). The period lengthening caused by CKIδ in the previous analysis was somewhat more than shown here, while the arrhythmicity was significantly less, probably because of subtle differences in the transgenes or rearing conditions. Our molecular analyses of these lines show that the oscillations of PER are robustly rhythmic in all of our lines except the *tau* mutants (Figure 6), so the apparent arrhythmicity of locomotor activity is not likely to arise from a damping of the underlying circadian mechanism but rather from an effect of transgene overexpression on the locomotor output pathway.

Six different insertion lines of UAS-CKI^S responder transgenic lines were analyzed and showed a shortened average circadian period ranging from 20 to 22 hr when expressed with the *tim*-GAL4 driver (Table 1 and Figure 4; average period of lines = 20.8 ± 0.3 hr, with $P < 0.0002$ for the difference with *tim*-GAL4>UAS-CKI^{WT} by ANOVA and *post hoc* Tukey's test). This period shortening was similar to the one observed in flies expressing a copy of the UAS-DBT^S transgene with the *tim*-GAL4 driver (see Table 1, Figure 4, and MUSKUS *et al.* 2007; average period of lines = 18.3 ± 0.1 hr, with $P < 0.0002$ for the difference with *tim*-GAL4>UAS-DBT^{WT} by ANOVA with *post hoc* Tukey's test). Transgenic DBT^{WT}-MYC and DBT^S-MYC were more highly expressed than endogenous DBT in clock cells (Figure 5A), and they should therefore titrate endogenous DBT for stable interactions with PER to dictate an allele-specific set point for circadian period. Both DBT-MYC and CKIδ under the control of the *tim*-GAL4 driver are expressed at all times of day in the head, and the levels of both wild-type and short-period transgenic protein are comparable for both CKIδ and DBT (Figure 5, A and B). The periods for CKI^S are significantly longer (difference between *tim*-GAL4>UAS-DBT^S and *tim*-GAL4>UAS-CKI^S, $P < 0.0002$ by ANOVA with *post hoc* Tukey's test) and show a higher variance than those produced by DBT^S (Table 1), suggesting the CKIδ may not titrate endogenous DBT as effectively as transgenic DBT. However, it is clear that the period-shortening effect of the amino acid change in CKI^S is manifest and confers on the vertebrate protein the ability to act dominantly in the same manner as DBT^S. This finding strongly supports the proposed evolutionary conservation of function between CKIδ and DBT and further establishes the validity of assessing the biochemical effects of the dbt^S and dbt^L mutations in the context of the vertebrate gene.

Besides the dbt^S mutation from Drosophila (PRICE *et al.* 1998) and the CKIδ familial advanced sleep phase syndrome (FASPS) mutation in humans (XU *et al.* 2005), a mutation of a single amino acid in CKIε of Syrian hamsters has been identified that gives rise to a semidominant period shortening in hamsters (LOWREY *et al.* 2000). Similar to DBT^S, this $CKI\varepsilon^{Tau}$ exhibits a reduced kinase activity *in vitro* (LOWREY *et al.* 2000). We therefore sought to determine whether effects of the *tau* mutation, which maps to the highly identical N-terminal region of *ckI* [amino acid R178C (LOWREY *et al.* 2000)], would be functionally conserved if introduced into the vertebrate CKIδ (a paralog of CKIε) or Drosophila DBT and expressed in circadian clock cells in Drosophila. Expression of either UAS-$CKI\delta^{Tau}$ or UAS-DBT^{Tau} with the *tim*-GAL4 driver produced comparable period shortening of up to 3 hr in all responder lines tested (21- to 23-hr periods in Table 1 and Figure 4), consistent with the short period length described for a homozygous $ckI\varepsilon^{Tau}$ hamster [20 hr (LOWREY *et al.* 2000)]. The difference between the average periods of the *tim*-GAL4>UAS-DBT^{Tau} (21.6 ± 0.5 hr) or -UAS-CKI^{Tau} (21.1 ± 0.1 hr) lines and the average periods of the corresponding *tim*-GAL4>UAS-DBT^{WT} (24.7 ± 0.1 hr) or >UAS-CKI^{WT} (24.2 ± 0.1 hr) lines was significant ($P < 0.0002$ by ANOVA with *post hoc* Tukey's test), while the difference between the average periods of the *tim*-GAL4>UAS-DBT^{Tau} and >UAS-CKI^{Tau} lines was not ($P > 0.9000$). The level of expression of the *tau* mutant proteins was comparable to those of the wild-type and dbt^S forms of CKIδ and DBT, and there was again no evidence of oscillation (Figure 5, A and B).

The reproduction of the period alteration caused by a mutation in CKIε when introduced into CKIδ or Drosophila DBT and tested in a transgenic fly is very intriguing, since it is strong evidence for the high amount of functional conservation between *ckI*ε and *ckI*δ, both of which are proposed regulators of the vertebrate clocks (LOWREY *et al.* 2000; VIELHABER *et al.* 2000; LEE *et al.* 2001; CONSTANCE *et al.* 2005; EIDE *et al.* 2005; XU *et al.* 2005). In addition, these results show the mechanistic conservation of the clock between flies and vertebrates, as the combined analyses of the dbt^S and *tau* mutations show that mutations that appear hypomorphic when assessed *in vitro* but produce short periods in flies or vertebrates do so in both types of organisms and in the context of either the fly or the vertebrate enzyme, rather than producing opposite effects on period.

TABLE 1

Locomotor activity rhythms in flies expressing Drosophila DBT or vertebrate CKIδ

Line	Genotype	Mean period ± SEM (SD)	% rhythmic (n^a)
DBT^{WT}		**24.7 ± 0.1**	**46 ± 8**
21M1C[b]	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.7 ± 0.2 (0.7)	77 (22)
6M3B[b]	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.2 ± 0.2 (0.8)	36 (67)
6M3B[b]	UAS-DBT^{+}/+	24.1 ± 0.1 (0.4)	94 (16)
45F2B[b]	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.9 ± 0.2 (0.9)	49 (57)
12M1B[b]	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.8 ± 0.5 (1.0)	31 (13)
22M1A[b]	*tim*-GAL4/+>UAS-DBT^{WT}/+	24.9 ± 0.2 (0.5)	38 (16)
CKI^{WT}		**24.2 ± 0.1**	**58 ± 12**
9M3B	*tim*-GAL4/+>UAS-CKI^{WT}/+	24.0 ± 0.1 (0.6)	77 (30)
29M1B	*tim*-GAL4/+>UAS-CKI^{WT}/+	24.4 ± 0.1 (0.6)	60 (75)
29M1B	UAS-CKI^{WT}/+	23.6 ± 0.08 (0.3)	100 (16)
1M1A	*tim*-GAL4/+>UAS-CKI^{WT}/+	24.1 ± 0.1 (0.8)	36 (83)
DBT^{S}		**18.3 ± 0.1**[c]	**57 ± 9**
10F5A[b]	*tim*-GAL4/+>UAS-DBT^{S}/+	18.4 ± 0.1 (0.4)	74 (23)
10F5A[b]	UAS-DBT^{S}/+	23.9 ± 0.06 (0.2)	88 (16)
28M3A[b]	*tim*-GAL4/+>UAS-DBT^{S}/+	18.2 ± 0.07 (0.4)	50 (54)
13M1A[b]	*tim*GAL4/+>UAS-DBT^{S}/+	18.4 ± 0.09 (0.2)	47 (15)
CKI^{S}		**20.8 ± 0.3**[c]	**57 ± 9**
2.1	*tim*-GAL4/+>UAS-CKI^{S}/+	20.7 ± 0.5 (1.7)	34 (29)
1.2	*tim*-GAL4/+>UAS-CKI^{S}/+	22.2 ± 0.6 (1.8)	38 (26)
5.1	*tim*-GAL4/+>UAS-CKI^{S}/+	20.4 ± 0.1 (0.6)	77 (48)
7.2	*tim*-GAL4/+>UAS-CKI^{S}/+	20.4 ± 0.2 (0.8)	41 (32)
4.2	*tim*-GAL4/+>UAS-CKI^{S}/+	20.7 ± 0.3 (1.5)	66 (32)
13.2	*tim*-GAL4/+>UAS-CKI^{S}/+	20.3 ± 0.07 (0.2)	87 (15)
DBT^{Tau}		**21.6 ± 0.5**[c]	**54 ± 8**
2.1	*tim*-GAL4/+>UAS-DBT^{Tau}/+	20.8 ± 0.4 (0.9)	40 (15)
1.3	*tim*-GAL4/+>UAS-DBT^{Tau}/+	23.2 ± 1.1 (1.9)	60 (5)
15.4	*tim*-GAL4/+>UAS-DBT^{Tau}/+	21.9 ± 0.2 (1.0)	34 (59)
XL5	*tim*-GAL4>/UAS-DBT^{Tau}	20.6 ± 0.2 (0.6)	80 (10)
4.2	*tim*-GAL4/+>UAS-DBT^{Tau}/+	21.6 ± 0.5 (1.6)	56 (18)
CKI^{Tau}		**21.1 ± 0.1**[c]	**49 ± 7**
2F3B	*tim*-GAL4/+>UAS-CKI^{Tau}/+	21.3 ± 0.2 (1.1)	39 (62)
2F3B	UAS-CKI^{Tau}/+	23.9 ± 0.07 (0.3)	94 (16)
20M2B	*tim*-GAL4/+>UAS-CKI^{Tau}/+	21.0 ± 0.2 (0.9)	45 (44)
15M1B	*tim*-GAL4/+>UAS-CKI^{Tau}/+	21.1 ± 0.2 (1.0)	63 (40)
DBT^{L}		**27.3 ± 0.03**[c]	**32 ± 10**
22F1C[b]	*tim*-GAL4/+>UAS-DBT^{L}/+	27.3 ± 0.1 (0.8)	49 (69)
22F1C[b]	UAS-DBT^{L}/+	23.8 ± 0.09 (0.3)	100 (14)
38M3A[b]	*tim*-GAL4/+>UAS-DBT^{L}/+	27.2 ± 0.2 (0.5)	33 (18)
27M1C[b]	*tim*-GAL4/+>UAS-DBT^{L}/+	27.3 ± 1.1 (2.2)	13 (30)
CKI^{L}		**24.1 ± 0.1**	**56 ± 8**
4F2B	*tim*-GAL4/+>UAS-CKI^{L}/+	23.9 ± 0.2 (0.9)	46 (59)
19M1A	*tim*-GAL4/+>UAS-CKI^{L}/+	23.8 ± 0.06 (0.5)	66 (83)
19M1A	UAS-CKI^{L}/+	24.3 ± 0.1 (0.4)	90 (20)
24M1A	*tim*-GAL4/+>UAS-CKI^{L}/+	24.0 ± 0.2 (1.0)	75 (44)
29M1A	*tim*-GAL4/+>UAS-CKI^{L}/+	24.3 ± 0.7 (1.2)	21 (14)
7M1B	*tim*-GAL4/+>UAS-CKI^{L}/+	24.6 ± 0.3 (0.9)	70 (10)
23M1A	*tim*-GAL4/+>UAS-CKI^{L}/+	24.0 ± 0.3 (0.5)	60 (5)
$CKI^{D/N}$		**25.6 ± 0.4**[c]	**76 ± 6**
24M3A	*tim*-GAL4/+>UAS-$CKI^{D/N}$/+	24.6 ± 0.2 (1.0)	86 (21)
22F1B	*tim*-GAL4/+>UAS-$CKI^{D/N}$/+	27.0 ± 0.5 (1.2)	56 (9)
29M3B	*tim*-GAL4/+>UAS-$CKI^{D/N}$/+	25.3 ± 0.2 (0.7)	75 (16)
29M3B	UAS-$CKI^{D/N}$/+	24.0 ± 0.08 (0.3)	100 (15)
25F1A	*tim*-GAL4/+>UAS-$CKI^{D/N}$/+	26.8 ± 0.3 (1.3)	61 (28)

(*continued*)

TABLE 1

(Continued)

Line	Genotype	Mean period ± SEM (SD)	% rhythmic (n^a)
13M2A	*tim*-GAL4/+>UAS-CKI $^{D/N}$/+	25.3 ± 0.4 (1.2)	90 (10)
23M1A	*tim*-GAL4/+>UAS-CKI $^{D/N}$/+	24.7 ± 0.2 (0.6)	90 (10)
tim-GAL4[b]	*tim*-GAL4/+	24.4 ± 0.2 (0.7)	72 (18[d])

Mean periods and mean percentage of rhythmicity for each of the lines tested are shown. The flies were tested in the hemizygous condition for driver and responder [*tim*-GAL4/+; UAS-DBT (CKI)/+]. All responder insertions are on chromosome III except DBT^{Tau} XL5. The mean period ± SEM and the mean percentage of rhythmicity ± SEM for each genotype class are tabulated in boldface at the top of each class.

[a] Number of flies tested.

[b] These lines were reproduced from Muskus *et al.* (2007).

[c] Mean periods for the genotype classes that are different from the mean period of flies overexpressing the corresponding wild-type protein (DBT^{WT} or CKI^{WT}) with $P < 0.05$.

[d] Eighteen flies tested as progeny of one outcross of the *tim*-GAL4 driver line.

CKIδ proteins with the *dbt*^{L} mutation do not produce period lengthening when expressed in Drosophila: Six independent insertion lines of *ckIδ*^{L} were tested for alterations in their locomotor behavior when the transgene was expressed by the *tim*-GAL4 driver. None of the *ckIδ*^{L} transgenes gave rise to a clear change of period length of the transgenic flies, which had periods in the wild-type range (Table 1). The average period of the *tim*-GAL4>UAS-CKI^L lines (24.1 ± 0.1 hr) was not significantly different from the average period of the *tim*-

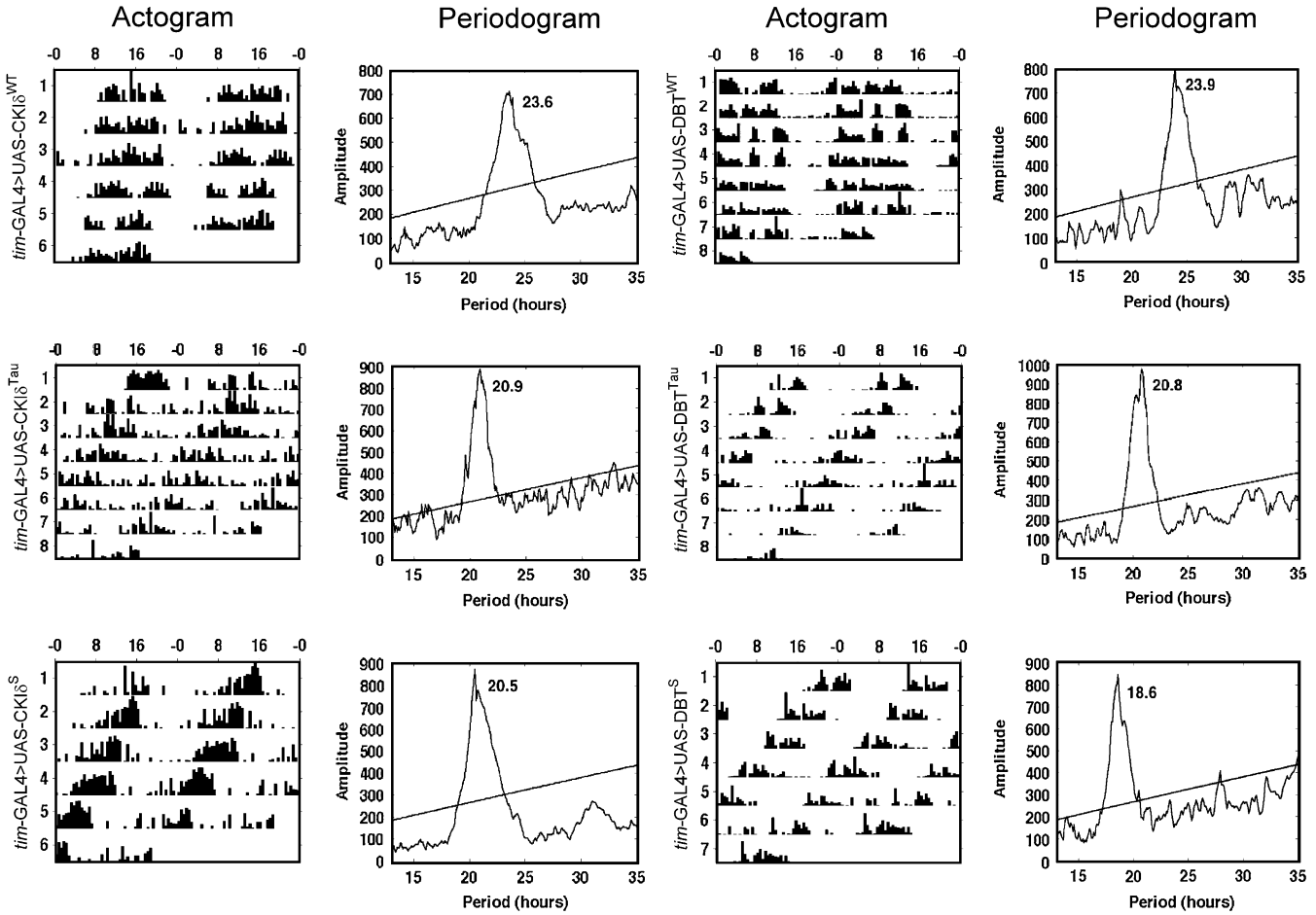

Figure 4.—Expression of the *dbt*^{S} or *tau* mutant proteins shortens circadian period in the context of either Drosophila DBT or Xenopus CKIδ. Representative actograms and periodograms are shown for each of the indicated genotypes. Each actogram is a double plot, with the amount of activity in consecutive half-hour bins plotted in 2-day intervals, and the day on the right plotted again on the left of the next line down.

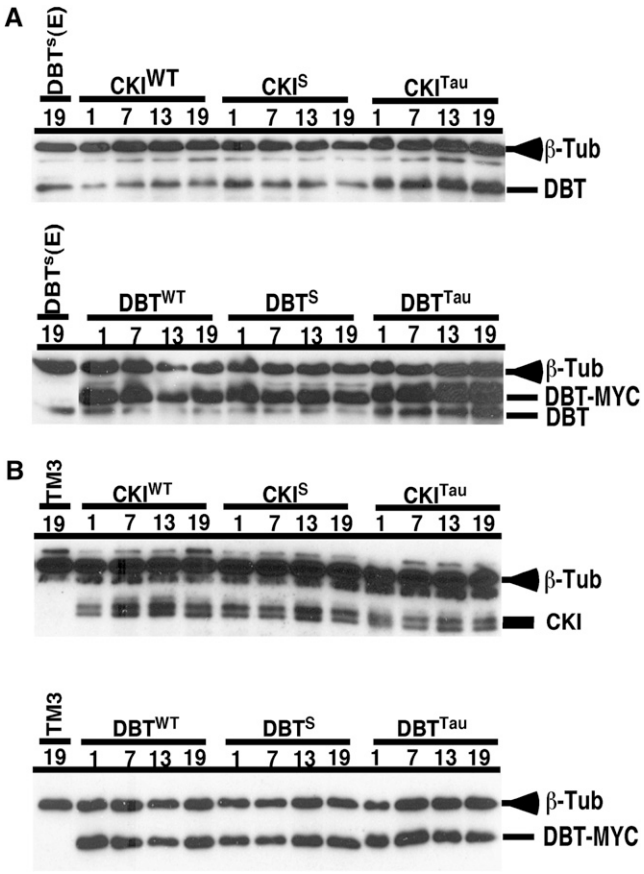

FIGURE 5.—Expression of DBT-MYC or CKI in the heads of transgenic flies. At the indicated ZT in a 12-hr:12-hr LD cycle (lights on from ZT 0–12 and off from ZT 12–24), heads were collected from flies expressing the indicated kinase under the control of the *tim*-GAL4 driver. The head extracts were analyzed by immunoblot analysis. Tubulin detection was used as a constitutive control in all lanes. The results are representative of three separate experiments. (A) DBT expressed from the endogenous gene and/or transgenic DBT-MYC were detected with an antibody that detects the C terminus of DBT. (E): flies expressing no transgene but carrying a dbt^S mutation in the endogenous *dbt* gene. (B) DBT-MYC or CKIδ were detected with anti-MYC or anti-CKIδ antibodies, respectively. TM3: flies that inherited the TM3 balancer chromosome rather than the UAS-DBT-MYC or the UAS-CKIδ responder.

GAL4>UAS-CKI^{WT} lines (24.2 ± 0.1 hr; $P > 0.9$ by ANOVA with *post hoc* Tukey's test). By contrast, all of the *tim*-GAL4>UAS-DBT^L flies exhibited ~27-hr periods, similar to the original dbt^L allele (PRICE *et al.* 1998) and significantly different from the *tim*-GAL4>UAS-DBT^{WT} lines (averages of 27.3 ± 0.1 and 24.7 ± 0.1 hr, respectively; $P < 0.0002$ by ANOVA with *post hoc* Tukey's test), as previously shown (MUSKUS *et al.* 2007). The expression levels of these UAS responders varied somewhat from line to line, and this was particularly true for the CKI^L lines (supplemental Figure 1). However, the lines that produced wild-type periods in combination with the *tim*-GAL4 driver included ones with high levels of CKI^L expression. For instance, line 4F2B did not lengthen period (Table 1), despite the fact that it expressed CKIδ at as high a level as did any of the lines expressing CKI^{WT} (supplemental Figure 1). Hence, the lack of period lengthening in *tim*-GAL4>UAS-CKI^L lines is not a consequence of low expression levels.

To address the possibility that long periods cannot be produced by lowered kinase activity of vertebrate CKIδ but can be in the context of the Drosophila DBT enzyme, another mutation that generated lower kinase activity (data not shown) was generated in vertebrate CKIδ by altering an amino acid (D132N) in the catalytic loop and introduced into transgenic flies. The effect on the circadian behavior is summarized in Table 1. The results show that the introduction of the hypomorphic form of the CKIδ protein leads to variable period lengthening in the fly in most tested lines. The average period of the *tim*-GAL4>UAS-$CKI^{D/N}$ lines (25.6 ± 0.4 hr) was significantly longer than the average for the *tim*-GAL4>UAS-CKI^{WT} lines (Table 1, 24.2 ± 0.1 hr; $P < 0.05$ by ANOVA with *post hoc* Tukey's test). However, while it is possible to lengthen period by expression of a CKIδ with reduced kinase activity, the effect is not as penetrant as with period shortening caused by the dbt^S and *tau* mutations (only two of six lines produced >1.5 hr period lengthening).

The dbt^S and *tau* mutations lead to distinct phosphorylation profiles for PER: Since DBT is known to affect circadian rhythms by phosphorylating PER and targeting it for degradation, the molecular cycles of PER were examined in CKIδ and DBT-overexpressing flies. Flies were collected throughout the day, and the level and electrophoretic mobility (altered by phosphorylation state) of PER were assessed by immunoblot analysis of head extracts. In wild-type flies, PER exhibits a high mobility as it accumulates during the day and early night [Zeitgeber time (ZT) 7–13; lights on from ZT 0–12 and off from ZT 12–24] but becomes progressively less mobile on SDS–PAGE as it becomes phosphorylated during the late night and early day [*e.g.*, ZT 19–1 (EDERY *et al.* 1994)]. In flies overexpressing CKI^{WT} or DBT^{WT}, this pattern of phosphorylation was observed (Figure 6A; compare with nontransgenic + flies in Figure 6A), as we have previously observed in *tim*-GAL4>UAS-DBT^{WT} flies (MUSKUS *et al.* 2007). Oscillations of PER were affected in *tim*-GAL4>UAS-DBT^S and -UAS-DBT^L flies as they are in the original EMS-induced mutations (PRICE *et al.* 1998) and are consistent with the more rapid and less rapid (respectively) phosphorylation kinetics observed in Drosophila S2 cells (KO *et al.* 2002). In *tim*-GAL4>UAS-DBT^S flies, PER's electrophoretic mobility retardation (caused by its progressive phosphorylation) was more striking at ZT 19 than in *tim*-GAL4>UAS-DBT^{WT} flies, and PER disappeared more rapidly in the short-period genotype (compare amounts at ZT 1). In *tim*-GAL4>UAS-DBT^L flies, this expedited phosphorylation of PER was not observed, and PER levels persisted at higher levels at ZT 1 than in *tim*-GAL4>UAS-DBT^S flies (Figure 6A). PER accumulation and mobility profiles in *tim*-GAL4>UAS-

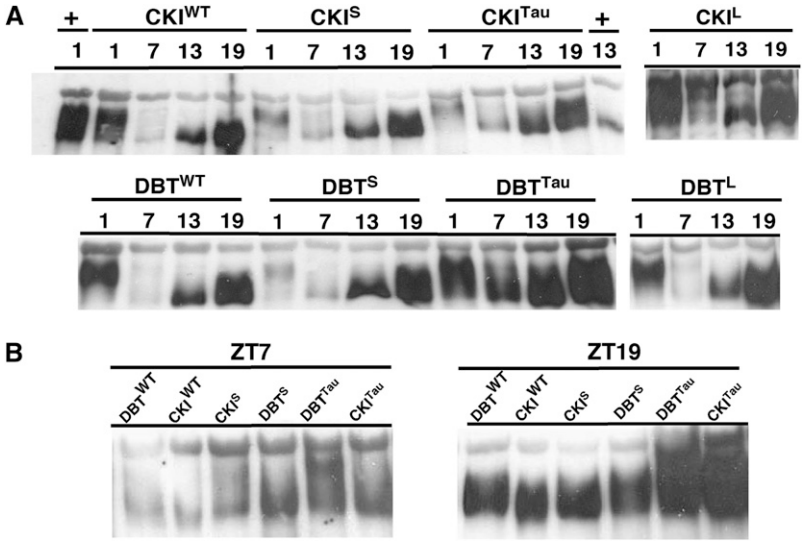

FIGURE 6.—PER expressed in the heads of transgenic flies exhibits distinct, genotype-specific abundance and mobility oscillations. At the indicated ZT in a 12-hr:12-hr LD cycle (lights on from ZT 0–12 and off from ZT 12–24), heads were collected from flies expressing the indicated kinase under the control of the *tim*-GAL4 driver. The head extracts were analyzed by immunoblot analysis for PER. The results are representative of three separate experiments. (A) Extracts from a particular genotype collected at different times were analyzed in adjacent lanes. +, wild-type progeny that carried the *tim*-GAL4 driver but no responder gene. (B) Extracts of different genotypes from a particular time point (ZT 7 or ZT 19) were analyzed in adjacent lanes. Note the hypershifted PER band at ZT 7 in *tim*-GAL4>UAS-DBT^{Tau}.

CKI^{L} flies were essentially wild type, as expected because behavioral rhythms were not altered in these flies (Table 1 and Figure 6A). The phase advance of PER cycling was not as dramatic in *tim*-GAL4>UAS-CKI^{S} flies as in *tim*-GAL4>UAS-DBT^{S} flies (note, however, the more rapid decline of PER levels at ZT 1 than in *tim*-GAL4>UAS-CKI^{WT}), but this is consistent with the somewhat reduced period shortening caused by the UAS-CKI^{S} transgene relative to the UAS-DBT^{S} transgene.

The pattern of PER phosphorylation was distinctly different in *tim*-GAL4>UAS-CKI^{Tau} and -UAS-DBT^{Tau} flies from other genotypes. PER exhibits more dispersed mobilities at ZT 13 and ZT 19 in both *tim*-GAL4>UAS-DBT^{Tau} and -UAS-CKI^{Tau} flies, as well as at ZT 1 and ZT 7 in *tim*-GAL4>UAS-DBT^{Tau} (Figure 6, A and B), suggesting a more heterogeneous phosphorylation state for PER and a damped oscillation of phosphorylation. In addition, the oscillation in level was less robust than in other genotypes. Note in particular the slow mobility form of PER at ZT 7 in *tim*-GAL4>UAS-DBT^{Tau} flies and the lack of this form in any of the other genotypes (Figure 6B). Thus, while both the *dbt^{s}* and *tau* mutations produce short circadian periods, their effects on the phosphorylation program of PER are different. Presumably, the sequence in which sites are phosphorylated, or their extent of phosphorylation, is different in the *dbt^{s}* and *tau* mutants.

DISCUSSION

DBT is essential for circadian molecular oscillations because it introduces time delays into the negative feedback exerted by PER (PRICE *et al.* 1998). These phosphorylation-dependent delays are thought to be mediated via several mechanisms. For instance, DBT is thought to destabilize PER in the cytoplasm, while TIM (PER's dimerization partner) is thought to prevent this destabilization and trigger the movement of both proteins to the nucleus, perhaps indirectly (VOSSHALL *et al.* 1994; PRICE *et al.* 1995; SHAFER *et al.* 2002; CYRAN *et al.* 2005; MEYER *et al.* 2006). The destabilization of PER in the cytoplasm delays its nuclear accumulation, while the stabilization of PER in the nucleus by TIM delays the DBT-dependent decrease of nuclear PER (PRICE *et al.* 1995; ROTHENFLUH *et al.* 2000b; KLOSS *et al.* 2001). Because DBT controls the timing of PER's nuclear accumulation so that it does not occur until after the *per/tim* mRNA levels have peaked, molecular rhythms of *per* and *tim* mRNA are possible (PRICE *et al.* 1998). Additional regulation has been proposed to occur at the level of PER's capacity to negatively regulate its transcription factor target (CLK/CYC) (NAWATHEAN and ROSBASH 2004; KIM and EDERY 2006; YU *et al.* 2006; KIM *et al.* 2007; NAWATHEAN *et al.* 2007).

Although DBT's orthologs CKIδ and CKIε are involved in the mammalian circadian clock, the extent of evolutionary conservation of their circadian mechanisms has not been clear, as discussed in the Introduction. Our cellular and biochemical analysis argues for a significant degree of conservation. We have previously shown that both the original long-period mutation (*dbt^{L}*) and the short-period *dbt* mutation (*dbt^{s}*) reduce the enzymatic activity of Drosophila DBT and a Xenopus CKI ortholog of DBT on casein (PREUSS *et al.* 2004), and here we showed that Drosophila DBT^{S} and DBT^{L} also exhibit reduced activity on PER. In our current work, deletion of the DBT C terminus was shown to increase the kinase activity of DBT and its capacity to target PER for degradation. The latter result is consistent with interaction studies, as it is the N-terminal catalytic domain rather than the C-terminal domain that interacts with PER (PREUSS *et al.* 2004). Finally, it was shown here that in the presence of a general phosphatase inhibitor DBT produces forms that migrate more slowly on SDS–PAGE, in a manner that requires its kinase

activity. These results suggest that DBT is autophosphorylated. Inhibitory autophosphorylation of the C-terminal domain is a common feature of both vertebrate CKIε and -δ (Graves and Roach 1995; Cegielska *et al.* 1998; Rivers *et al.* 1998). All of these biochemical results demonstrate a high degree of evolutionary conservation between Drosophila DBT and vertebrate CKIε/δ.

Our *in vivo* analysis herein of vertebrate CKIδ and Drosophila DBT further establishes the evolutionary conservation of these kinases and the clock mechanisms in which they participate. Mutations that shorten the circadian period in the context of DBT produced corresponding shortening in the context of CKI in flies, and the *tau* mutation produced almost identical period shortening in both the mammalian and the Drosophila clocks, in the context of either the fly or the vertebrate enzyme. The data for the *tau* mutation are particularly complete for analysis of evolutionary conservation, as this mutation has been tested in all possible combinations of organism (fly or mammal) and kinase (DBT or CKI) except one (DBT^{Tau} in mammals). These results argue against the possibility that reduced kinase activity produces only long periods in flies and short periods in mammals. On the other hand, the CKIδ FASPS mutation, which has a small effect on period, produces opposite effects on period in flies and mammals (Xu *et al.* 2005). The mutations analyzed herein have much stronger effects on period and clearly show similar effects in both vertebrates and flies. These findings strongly support the proposed evolutionary conservation of CKIδ and DBT protein kinases and of at least some of the circadian processes in which they are involved.

The reproduction of the period alteration caused by a mutation in CKIε when introduced into CKIδ or Drosophila DBT and tested in a transgenic fly is strong evidence for the high amount of functional conservation between *ckI*ε and *ckI*δ, both of which are proposed regulators of the vertebrate clocks (Lowrey *et al.* 2000; Vielhaber *et al.* 2000; Lee *et al.* 2001; Constance *et al.* 2005; Eide *et al.* 2005; Xu *et al.* 2005). However, CKIδ and -ε are not completely interchangeable, as similar overexpression experiments with CKIε in flies have produced a very different set of results from the ones presented here for CKIδ. A catalytically active CKIε produces a dominant negative effect on circadian rhythms in Drosophila, with relatively constant expression of hypophosphorylated PER at all times of day, while overexpression of a catalytically inactive form of CKIε produces only a mild lengthening of circadian period (Sekine *et al.* 2008). Taken together with our results, this result suggests that vertebrate CKIδ is better able to interact with fly clock proteins than is CKIε, and in a manner more comparable to Drosophila DBT in the same expression protocol. The difference between the finding of Sekine *et al.* (2008) and our results, as well as those of Xu *et al.* (2005) (who also employed overexpression of CKIδ in flies and did not observe dominant negative phenotypes), may be due to the divergent C-terminal domains of CKIδ and -ε.

We have shown that flies expressing the dominant negative form of DBT have very long periods or are arrhythmic, and this result argues that general reductions in DBT's kinase activity produce long-period rhythms that grade to arrhythmicity (Muskus *et al.* 2007). While the lower activity of DBT^{L} is predicted to (and in fact does) lengthen circadian period, the short-period PER oscillation produced by DBT^{s} and *tau* mutations is not readily explained by their lower kinase activity *in vitro*. One possible explanation is that the short-period mutants affect something besides kinase activity (Muskus *et al.* 2007)—an interaction with a regulator, for example.

Another possible explanation is that reduced activity of short-period CKI enzymes *in vitro* may not translate into lower phosphorylation of PER *in vivo* because other kinases provide compensatory phosphorylation. In fact, progressive phosphorylation of PER, at least as assessed by a reduction in PER's electrophoretic mobility, occurs more rapidly in dbt^{s} flies or cells than in wild-type or dbt^{L} flies or cells (Price *et al.* 1998; Ko *et al.* 2002), as is also shown herein for *tim*-GAL4>UAS-DBT^{s} flies, so the *in vivo* phosphorylation profile indicates more rapid phosphorylation in the dbt^{s} mutant. Likewise, in the *tau* mutant, the phosphorylation of PER is only slightly delayed and ultimately appears to be as complete as in wild-type hamsters (Lee *et al.* 2001), despite the strong reduction in kinase activity caused by the *tau* mutation *in vitro*. In mammals, at least two kinases (CKIε and CKIδ) (Lowrey *et al.* 2000; Vielhaber *et al.* 2000; Lee *et al.* 2001; Constance *et al.* 2005; Eide *et al.* 2005; Xu *et al.* 2005) associate with PER and phosphorylate it, while in flies casein kinase II phosphorylates PER together with DBT (Lin *et al.* 2002; Nawathean and Rosbash 2004).

While the phosphorylation profiles of PER in *tim*-GAL4>UAS-DBT^{s}, -CKI^{s}, and -DBT^{L} resemble the phosphorylation profiles of the original *dbt* mutants, the PER phosphorylation profiles of *tim*-GAL4>UAS-DBT^{Tau} and -CKI^{Tau} do not resemble the profile that has been previously reported for the endogenous mutant (Lee *et al.* 2001); in particular, the oscillation of PER phosphorylation is notably blunted by overexpression of the DBT^{Tau} mutant kinases. It is possible the overexpression of the DBT^{Tau} has a stronger effect on the phosphorylation profile of PER in flies than in mammals because the *tau* mutant effects are partially masked in mammals by compensatory activity of CKIδ, with which CKIε may be partially redundant (Meng *et al.* 2008). Nevertheless, the period of the circadian clock is dramatically shortened by overexpression of both DBT^{s} and DBT^{Tau} mutant kinases, and the circadian period of the *tau* mutation in flies is similar to that for the hamster *tau* mutant, suggesting that the alterations in the DBT/CKI kinase are still altering period as they do in the original mutants.

Another possible reason for the phenotypes of the short-period mutants is that phosphorylation of specific sites in PER affects multiple, specific aspects of its regulation with opposite effects on period length. Phosphorylation of Drosophila PER at multiple sites, only some of which affect stability, has recently been demonstrated (Chiu *et al.* 2008; Kivimae *et al.* 2008). Along this line of thinking, it has been proposed that the *ckIε*^{Tau} mutation is a gain-of-function mutation that enhances phosphorylation of PER at specific sites—an enhancement that is missed in global analysis of multisite substrates like PER (Gallego *et al.* 2006a; Meng *et al.* 2008). Others have offered explanations for the *tau* mutant that include lowered phosphorylation at all sites, but with cytoplasmic destabilization produced by phosphorylation at some sites and increased nuclear retention (and stabilization) produced by phosphorylation at other sites (Vanselow *et al.* 2006). If these hypotheses for site-specific effects are correct, the dispersed phosphorylation profile detected for Drosophila PER in our CKI^{Tau}- and DBT^{Tau}-expressing flies is indicative of general changes in phosphorylation, most of which are not relevant to the period shortening, gain-of-function phenotype produced at a subset of sites. While both the *dbt*^{S} and *tau* mutations produce short circadian periods, their effects on the phosphorylation program of PER are different, as reflected in their different effects on circadian changes in PER electrophoretic mobility.

Why does CKI^{L} overexpression not lengthen the period of the Drosophila rhythm, while DBT^{L} overexpression does? It is likely that the *dbt*^{L} mutation compromises CKIδ function and reduces its ability to compete with endogenous wild-type DBT for interactions with PER. In fact, CKIδ may have generally less ability to compete with endogenous DBT than transgenic DBT, as overexpression of CKI^{S} also has weaker effects than overexpression of the corresponding DBT^{S} protein. Not all mutations that reduce the kinase activity of vertebrate CKI produce short periods, or no effect like CKI^{L}. We show that the D/N mutation, which like the K/R mutation is predicted to have a very specific effect on the catalytic properties of the enzyme and not other aspects of its function, produced variable period lengthening. The variability is most likely a consequence of chromosomal position effects at the *P*-element insertion site on the transgene expression levels. The stronger effects of period-lengthening mutations in the context of fly DBT than in the vertebrate CKI suggest that there may be differences in the way they affect the fly and vertebrate CKI orthologs, with a consequence that fly DBT can be mutated more readily to produce a long period. These differences argue against the idea that lack of long periods is produced by a difference in circadian targets, as the lack of long periods correlates with the vertebrate enzyme rather than the species in which the enzyme is expressed (*i.e.*, DBT^{L} can produce long periods in flies, while CKI^{L} cannot).

The involvement of protein kinases with circadian clocks spans a phylogeny that is larger than the one separating vertebrates and fruit flies. Casein kinases I and II are also involved in the bread mold (Neurospora) clock and target both the FRQ transcriptional repressor and the WCC transcriptional activator in a mechanism reminiscent of the one involving DBT (Garceau *et al.* 1997; Schafmeier *et al.* 2005; He *et al.* 2006; Huang *et al.* 2007). Recently, a kinase involved in DNA replication control was also shown to target Neurospora FRQ, with implications for the interplay between circadian rhythms and cell cycle control in higher eukaryotes (Pregueiro *et al.* 2006). The core circadian oscillator mechanism in cyanobacteria involves rhythmic phosphorylation and dephosphorylation of the KaiC protein (Nakajima *et al.* 2005). The evolutionary conservation of kinase function has led to a synergy between research in different organisms—for instance, with work in Drosophila identifying a circadian role for CKI (Kloss *et al.* 1998; Price *et al.* 1998) that has now been shown in diverse phyla and work in Neurospora showing a kinase-targeting role for FRQ (Schafmeier *et al.* 2005; He *et al.* 2006) that was subsequently shown for PER (Kim and Edery 2006; Yu *et al.* 2006) as well. Further elucidation of the evolutionarily conserved processes regulated by phosphorylation will reveal general mechanisms at the core of the circadian mechanism.

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