Two Novel *doubletime* Mutants Alter Circadian Properties and Eliminate the Delay between RNA and Protein in *Drosophila*

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Phosphorylation is an important feature of pacemaker organization in *Drosophila*. Genetic and biochemical evidence suggests involvement of the casein kinase I homolog *doubletime* (*dbt*) in the *Drosophila* circadian pacemaker. We have characterized two novel *dbt* mutants. Both cause a lengthening of behavioral period and profoundly alter *period* (*per*) and *timeless* (*tim*) transcript and protein profiles. The PER profile shows a major difference from the wild-type program only during the morning hours, consistent with a prominent role for DBT during the PER monomer degradation phase. The transcript profiles are delayed, but there is little effect on the protein accumulation profiles, resulting in the elimination of the characteristic lag between the mRNA and protein profiles. These results and others indicate that light and post-transcriptional regulation play major roles in defining the temporal properties of the protein curves and suggest that this lag is unnecessary for the feedback regulation of *per* and *tim* protein on *per* and *tim* transcription.

Key words: circadian; entrainment, Drosophila*; CKIε; PERIOD; phosphorylation*

The metabolic and behavioral physiology of living systems is intimately tied to the geophysical periodicity caused by the 24 hr rotational frequency of the earth. Most eukaryotes and some prokaryotes have evolved extensive mechanisms to adapt to and exploit the consequent diurnal changes in illumination and temperature. These circadian pacemakers maintain temporal harmony between the organism and its environment. They continue in the absence of any external environmental cue (a zeitgeber), can be entrained to follow imposed cycles of illumination or temperature, and can be phase-altered by a sharp, brief change in illumination or temperature (Saunders, 1982). Additionally, the periods of these pacemakers are remarkably temperature-insensitive. Extensive molecular details are now available because of some outstanding genetics, genomics, and biochemical investigations in *Neurospora*, *Drosophila*, *Cyanobacteria* and, more recently, mammals and zebra fish (Dunlap, 1999).

The key mechanistic feature of circadian pacemakers is the presence of autoregulatory feedback loops (Dunlap, 1999). In general, oscillations are generated by product feedback on synthesis reactions. For circadian clocks, oscillatory gene expression defines periodicity, and a delay between the synthesis and feedback steps prevents dampening of the oscillations to a steady-state. *period* (*per*) and *timeless* (*tim*) are two central components of the *Drosophila* circadian pacemaker (Young, 1998; Edery, 1999). Temporal expression of both genes is manifest as rhythmic transcription, rhythmic mRNA, and rhythmic protein accumulation (Hardin et al., 1990; Edery et al., 1994; Sehgal et al., 1995; Marrus et al., 1996; So and Rosbash, 1997). CLOCK (CLK) and CYCLE (CYC) form a heterodimer, which activates transcription at the *per* and *tim* loci (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998a). Both *per* and *tim* show highest rates of transcription at approximately zeitgeber time 12 (ZT12; ZT0 is defined as the time when lights come on, and ZT12 is the time when lights go off; So and Rosbash, 1997). Steady-state levels of *per* and *tim* mRNA show a robust cycle, with a peak at approximately ZT15 and trough at approximately ZT3 (Marrus et al., 1996). Temporal dynamics of *per* transcript are also influenced by post-transcriptional regulation (So and Rosbash, 1997; Suri et al., 1999). Assays of steady-state protein levels on Western blots show a robust diurnal cycle of accumulation and protein phosphorylation of both PER and TIM (Edery et al., 1994; Marrus et al., 1996; Myers et al., 1996; Zeng et al., 1996). PER protein peaks at approximately ZT20 and is least abundant at approximately ZT8. TIM is slightly phase-advanced, with the peak and trough at approximately ZT19 and ZT7, respectively. The striking temporal phosphorylation pattern of PER (and probably TIM) is responsible for, among other things, regulating protein half-life (Dembinska et al., 1997; Kloss et al., 1998; Price et al., 1998). DOUBLETIME (DBT), a casein kinase Iε homolog, is believed to be a PER kinase (Kloss et al., 1998; Price et al., 1998). Although it has not been directly shown to phosphorylate PER, DBT forms stable complexes with PER (Kloss et al., 1998). Additionally, the absence of DBT results in hyperaccumulation of under-phosphorylated PER in larvae, and the *dbt* tissue expression pattern overlaps with that of the *per* transcript (Kloss et al., 1998; Price et al., 1998). In the lateral neurons, the likely pacemaker cells of *Drosophila*, PER and TIM enter the nucleus at approximately ZT18 (Curtin et al., 1995; Lee et al., 1996). PER and TIM protein accumulation and nuclear entry correlate approximately with downregulation of *per* and *tim* transcripts, and this has been suggested to be caused by direct inhibition of CLK-CYC activation by the PER-TIM dimer (Darlington et al., 1998; Lee et al., 1999).

The molecular mechanisms and machinery involved in the generation of circadian rhythms are remarkably similar between *Drosophila* and mammalian systems. The mammalian homolog of CLK is mCLK, and that of CYC is bMAL1 (Gekakis et al., 1998). The CLK–bMAL1 complex is the transcriptional activator in mammals (Gekakis et al., 1998). There are three PER-like proteins: mPER1, mPER2, and mPER3 (Zylka et al., 1998a). All three are cyclically expressed in the suprachiasmatic nucleus, the anatomical location of the mammalian pacemaker, as well as in several other tissues (Zylka et al., 1998a). There is also a TIM-like protein, mTIM (Sangoram et al., 1998; Zylka et al., 1998b), and a DBT homolog, CKIε (Fish et al., 1995). Very recent genetic and biochemical

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evidence indicates that CKIε plays a prominent role in the mammalian pacemaker (Keesler et al., 2000; Lowrey et al., 2000).

We report here the characterization of Dbt^g and dbt^h, two novel long-period mutants of the *Drosophila dbt* kinase. Both mutations strongly reduce kinase activity, when tested in the context of a highly similar yeast HRR25 kinase (DeMaggio et al., 1992). Analysis of PER in the mutant flies shows defective PER degradation. Additionally, *per* and *tim* mRNA cycles are significantly delayed in the mutants. However, PER and TIM kinetics during the accumulation phase are identical to wild-type kinetics, eliminating the usual 4–6 hr delay between the protein and mRNA accumulation. In conditions of constant darkness, the protein and mRNA profiles are equally delayed from those of wild type, suggesting that light overrides the *dbt* defects through some post-translational mechanism. The mutants provide a unique opportunity to address the mechanisms underlying parametric entrainment of *Drosophila* circadian rhythms.

Table 1. dbt^h and Dbt^g lengthen the period of behavioral rhythms

Genotype	Period (SD)	No. of flies	Rhythmic (%)
CS	24.1 (0.3)	15	93
dbt^h	29.1 (0.6)	28	71
$dbt^h/+$	25.1 (0.3)	6	84
dbt^h/Df (*dbt*)	28.3 (0.3)	9	88
dbt^h/dbt^p	27.7 (0.3)	10	90
$per^s;;dbt^h$	22.9 (0.4)	14	64
$Dbt^g/+$	28.5 (0.4)	29	82
Dbt^g/Df (*dbt*)	AR	13	
Dbt^g/Dbt^P	AR	7	

Locomotor activity behavior of wild-type (canton-S, CS)and *dbt* mutant flies is shown. Flies were entrained for 3 d under 12 hr light/dark conditions and then allowed to free run under constant darkness condition. Behavior was monitored at 25°C. Locomotor activity periods were calculated as previously described (Wheeler et al. 1993). AR, Arrhythmic.

MATERIALS AND METHODS

Isolation and identification of dbt *mutants.* Both *dbt* mutants were isolated in the previously described third chromosome mutagenesis screen (Rutila et al., 1996; Allada et al., 1998; Rutila et al., 1998a). Complementation analysis indicated that the mutation mapped to the *dbt* locus. Total DNA was isolated from wild-type and dbt^h mutant flies, and the entire *dbt* open reading frame was amplified using 5′-CGAACTCTACCACACAGAAACC-3′ [*dbt1,* nucleotides (nts) 24–45 in GenBank (gb) accession number AF0055583] as the forward primer and 5′-CCGCATATATAAAGCAAGTAT-3′ (*dbt16*, nts 1500–1520 in gb AF0055583) as the reverse primer (numbering is based on GenBank sequence AF0055583). Both strands were sequenced using Applied Biosystems (Foster City, CA) PRISM sequencing. Total mRNA was isolated from $Dbt^g/+$ flies, and the *dbt* transcript was amplified using reverse transcription with *dbt16* primer and PCR with *dbt1* as the forward primer and AGAGCTCTCGAGTTTGGCGTTCCCCAC (nts 1372–1386 in gb AF0055583) as the reverse primer. The PCR product was cloned in pBluescript (Stratagene, La Jolla, CA), and several independent clones were sequenced.

Behavioral analysis. Flies were entrained for 3 d in 12 hr light/dark cycles; then lights were turned off for the next 4–7 d, and the activity of individual animals was recorded in every 30 min bin. Periods were obtained using χ^2 analysis (with $\alpha = 0.05$). Light/dark activity profiles were plotted as previously described (Wheeler et al., 1993).

Western blotting and RNase protection analysis. Flies were entrained for three 12 hr light/dark cycles and collected on dry ice at each time point. Western blotting was performed as previously described (Suri et al., 1999). Head extracts (10 heads per lane) were fractionated using 6% SDS-PAGE (29.6:0.4 acrylamide/bisacrylamide) and transferred to nitrocellulose membranes. The blots were stained with Ponceau-S to ensure equal loading. Rabbit α-PER and rat α-TIM were used to detect PER and TIM, respectively. Band intensity was calculated using an AGFA scanner and Molecular Analyst software. RNase protection assays were performed as previously described (Suri et al., 1999). Total mRNA was isolated from 30 fly heads and hybridized to ^{32}P-labeled *per* 2/3, *tim*, and *rp-49* probes, as previously described. The free probe was digested with RNASE-ONE, and the reactions were run on a 5% sequencing gel. Band intensity was calculated using a phosphoimager and Molecular Analyst software.

Cloning of HRR25, binding, and kinase assays. The entire HRR25 open reading frame was amplified by PCR from yeast genomic DNA using 5′-CTCATACATATGGACTTAAGAGTAGGAAGG-3′ (nts 1–21 in gb M68605) as the forward primer and 5′-CATTCACTCGAGCAACCAAATTGACTGGCC-3′ (nts 1465–1482 in gb M68605) as the reverse primer. The PCR product was directly cloned into *Nde*I–*Xho*I sites in the polylinker region of pET 23b (Novagen, Madison, WI) vector. Full-length DBT was also cloned into the same sites in the same vector. Proteins were expressed and purified according to the manufacturer's protocol. Glutathione *S*-transferase (GST)-PER 1–640 was expressed and purified as previously described (Saez and Young, 1996). The final product was ~60% pure. The GST-PER concentration was calculated using the Bradford assay for total protein and estimating protein amounts by running GST-PER with BSA standards on an SDS-PAGE gel. For binding assays, GST-PER 1–640 was bound to glutathione-agarose beads. ^{35}S-Labeled DBT and HRR25 were *in vitro*-transcribed and translated using the TnT-coupled transcription–translation system (Promega, Madison, WI), following the manufacturer's instructions. GST-PER-coupled beads were incubated with increasing volumes of the translation product in a final volume of 500 μl for 4 hr. The beads were thoroughly washed, boiled in SDS-PAGE sample buffer, and loaded on a 10% SDS-polyacrylamide gel. Kinase assays were done exactly as previously described (Fish et al., 1995). Briefly, GST-PER immobilized on glutathione beads was washed with kinase buffer (in mM: 20 Tris, pH 7.5, 100 NaCl, 10 $MgCl_2$, and 1 DTT). His_6-tagged HRR25 was purified according to the manufacturer's instructions (Novagen). Ten microliter beads were then incubated with 50 μl of kinase buffer containing [γ-^{32}P]ATP for 30 min and the purified kinase. The beads were rinsed once with kinase buffer containing 1 mM EDTA, boiled in SDS-sample loading buffer, and run on an 8% gel. The peptide phosphorylation assays used AHALS(P)VASLPGLKKK as substrate. The HPLC-purified peptide was purchased from Sigma-Genosys (St. Louis, MO). Wild-type and mutant HRR25 were expressed in *Escherichia coli*. Whole-cell extracts were used in the assay. The assay mixture contained 1 mM peptide, 100 μM ATP (5 μCi), 20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. One hundred micrograms of total protein were incubated with the assay mixture in a volume of 20 μl for 30 min. The mixture was then spotted on P81 phosphocellulose paper. The paper was washed several times with 75 mM phosphoric acid, dried, and counted. HRR25 mutants were made by using the QuikChange site-directed mutagenesis system and following the manufacturer's instructions. For the R124H mutation, the forward oligonucleotide was 5′-TCGTTCATTCATCACGATATCAAACCAGAC-3′ (nts 367–396 in gb M68605), and the reverse oligonucleotide was 5′-GTCTGGTTTGATATCGTGATGAATGAACGA-3′ (nts 367–396 in gb M68605). For the T44I mutation, the forward oligonucleotide was 5′-GAATCGATCAGGATCAGACATCCTCAATTG-3′ (nts 6118–147 in gb M68605), and the reverse oligonucleotide was 5′-CAATTGAGGATGTCTGATCCTGATCGATTC-3′ (nts 118–147 in gb M68605. For the K38R mutation, the forward oligonucleotide was 5′-GAAGTAGCCATCAGGCTGGAATCGATCAGG-3′ (nts 100–114 in gb M68605), and the reverse oligonucleotide was 5′-CCTGATCGATTCCAGCCTGATGGCTACTTC-3′ (nts 100–114 in gb M68605).

RESULTS

Dbt^g and dbt^h are two *dbt* alleles

While screening for third chromosome mutations affecting locomotor activity periods, we found two lines that caused a strong lengthening of the endogenous period. Complementation analysis indicated that the mutations were allelic to *dbt*. They were named Dbt^g and dbt^h. Dbt^g is the stronger of the two mutants and renders the flies behaviorally arrhythmic when combined with a chromosomal deficiency deleting *dbt* or with a *dbt* allele carrying a P-element insertion. It is also a stronger dominant mutant, which lengthens the locomotor activity period to 29 hr as a heterozygote. dbt^h lengthens the locomotor activity period to 29 hr as a homozygote and to 25.1 hr as a heterozygote (Table 1).

Figure 1, *A–D*, shows the light/dark (LD) activity profiles of wild-type and *dbt* mutant flies. As previously reported, wild-type flies show an anticipation of both the light-to-dark (evening) and the dark-to-light (morning) transitions by 2–3 hr. In *dbt* mutant flies, there is neither an anticipation of the morning nor an anticipation of the evening events. *dbt* flies can, however, still entrain to 24 hr cycles, and the peak of evening activity is delayed by several hours into the night. Homozygous dbt^h flies in a per^s background have a period of 22 hr, intermediate between the periods of the two mutants when assayed individually. The double mutant combination shows a normal anticipation of the LD transition, although the evening peak is advanced by 1–2 hr, consistent with the <24 hr period (Fig. 1*D*).

Sequencing of the two *dbt* mutants revealed amino acid changes at conserved locations. The dbt^h mutation is caused by a G→C change, causing a Thr^{44}→Ile change in the nucleotide binding

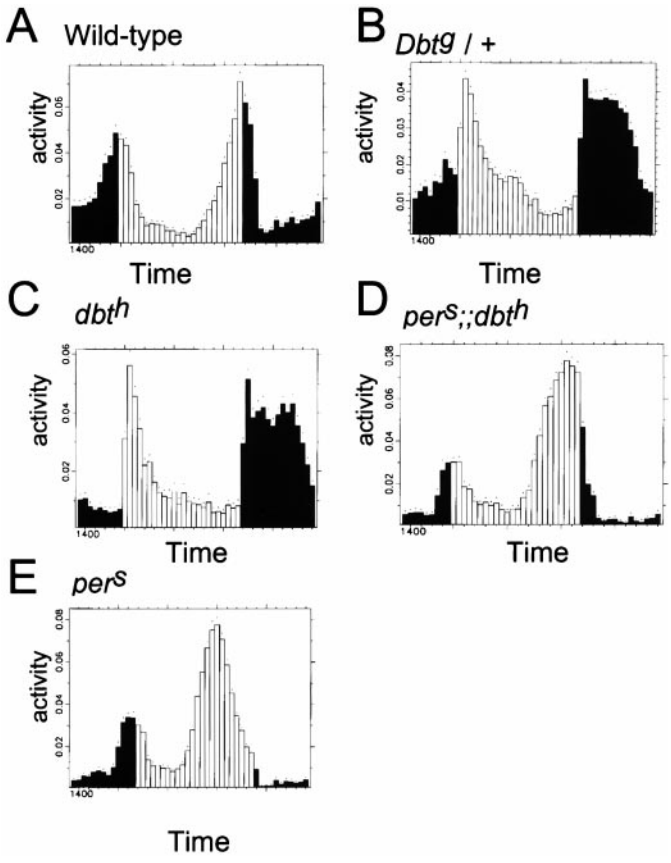

Figure 1. Light-dark activity profiles for wild-type and mutant flies. The genotype of the flies is given at the *top* of each plot. Flies were kept in light/dark cycles for 4 d. Data were pooled from 30–32 individual flies. Light/dark activity profiles were plotted as previously described (Wheeler et al.; 1993). The *shaded bars* indicate times when the lights were off, and the *open bars* indicate the times when the lights were on. The data are plotted starting at 2 P.M. (*1400*); lights came on at 8 P.M. and were turned off at 8 A.M. Light intensity used was ~2000 lux. All behavioral runs were at a constant temperature of 25°C.

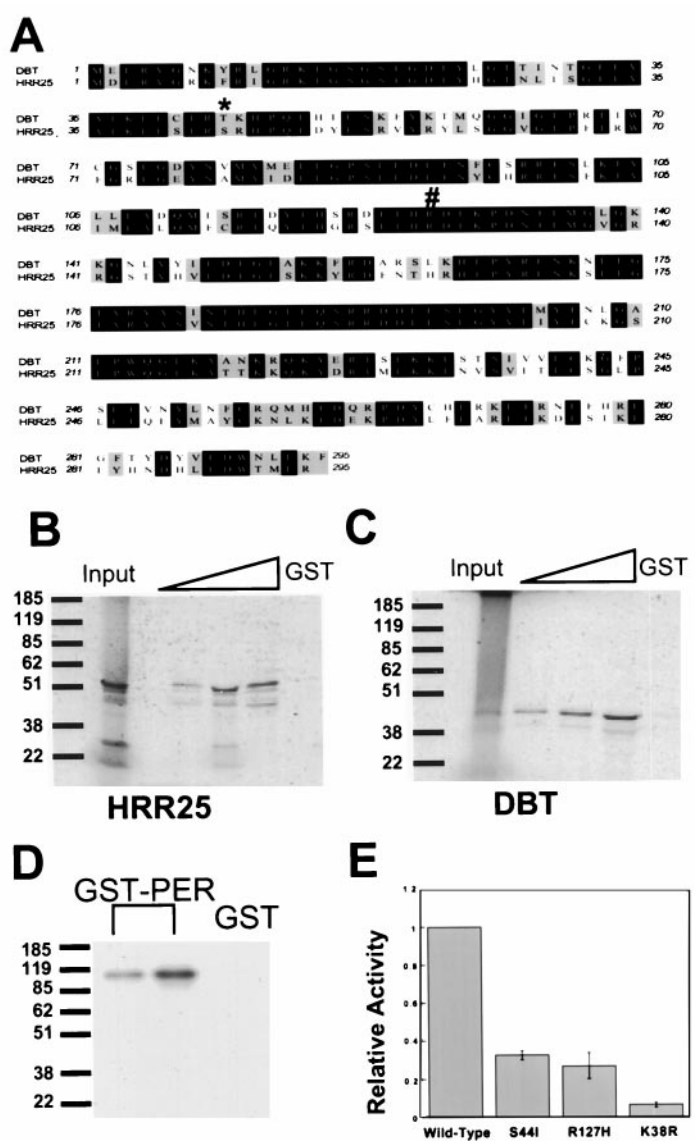

Figure 2. *dbt*h and *Dbt*g mutations alter conserved amino acids and strongly reduce kinase activity. *A*, DBT and HRR25 sequences were aligned using CLUSTAL W algorithm of the MacVector Package. *dbt*h mutation alters Thr44 to Ile (*), and *Dbt*g mutation alters Arg127 to His (#). Sequence numbering is based on GenBank accession numbers O76324 for DBT and AAA34687 for HRR25. Only the kinase domains are shown in the alignment. The homology outside of the kinase domain is not significant. The *darkly shaded areas* indicate identities, and the *lightly shaded areas* indicate similarities. *B, C*, ^{35}S-Labeled *in vitro*-translated HRR25 (*B*) or DBT (*C*) were incubated with increasing concentrations of GST-PER 1–640 immobilized on glutathione beads for 3 hr. GST-PER concentrations used were 10, 50, and 200 nM. The beads were pelleted, washed three times, loaded on 10% SDS-PAGE, and autoradiographed. GST was used as a negative control. *D*, Two different concentrations of GST-PER 1–640, 10 and 100 nM, were immobilized on glutathione beads and incubated with HRR25 and γ-^{32}P for 30 min. The beads were pelleted, washed, loaded on 10% SDS-PAGE, and autoradiographed. GST was used as a control. *E*, Kinase activity of wild-type and mutant HRR25 proteins was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into AHALS(P)VASLPGLKKK. Kinase activity relative to that of wild-type HRR25 (set to 1) is plotted. Error bars indicate SD.

domain of DBT (Fig. 2*A*). This position is occupied by a hydroxy-amino acid in most casein kinase I homologs. The *Dbt*g mutation alters Arg127→His in the catalytic domain of the enzyme (Fig. 2*A*). This position is always strongly basic (R/K) in the casein kinase family. In the structure of casein kinase Iδ, this arginine provides a contact to a regulatory phosphate (Xu et al., 1995).

There are no reports of active DBT expressed in a heterologous system. In addition, we made extensive efforts to express functional DBT, as assayed by transfer of labeled phosphate to either recombinant PER or to generic casein kinase I peptide substrates, with no success (data not shown). However, the highly homologous HRR25 yeast casein kinase I was easily expressed as an active kinase (DeMaggio et al., 1992). HRR25 binds to PER with affinities similar to DBT; it also efficiently phosphorylates PER (Fig. 2*B–D*). We studied the effects of altering the amino acids corresponding to those mutated in the *Dbt*g and *dbt*h alleles on HRR25 activity. This was assayed by following incorporation of ^{32}P into a peptide substrate optimized for casein kinase phosphorylation. A K38R mutant served as a negative control. In several kinases, this mutation within the ATP binding domain completely abolishes activity (Fish et al., 1995). Peptide phosphorylation was reduced to ~20–30% in the mutant versions of the enzyme, suggesting that both are strong loss-of-function mutations (Fig. 2*E*).

dbt mutants strongly alter *per* and *tim* transcript and protein accumulation profiles in light/dark conditions

During the late day and early night, much of PER appears as a fast-migrating species on Western blots (Fig. 3*A*). However, as the night progresses, several forms of PER appear as slower-migrating species. Phosphatase treatment converts them to faster-migrating forms, indicating that much of the temporal mobility shift is attributable to PER phosphorylation. A similar but less striking mobility shift is observed for TIM (Fig. 3*A*; Myers et al., 1996; Zeng et al., 1996). By comparison with this wild-type PER profile, the *dbt* profiles were dramatically altered (Fig. 3*A–C*). In the day (~ZT1–ZT11), PER levels in the mutant strains were significantly higher

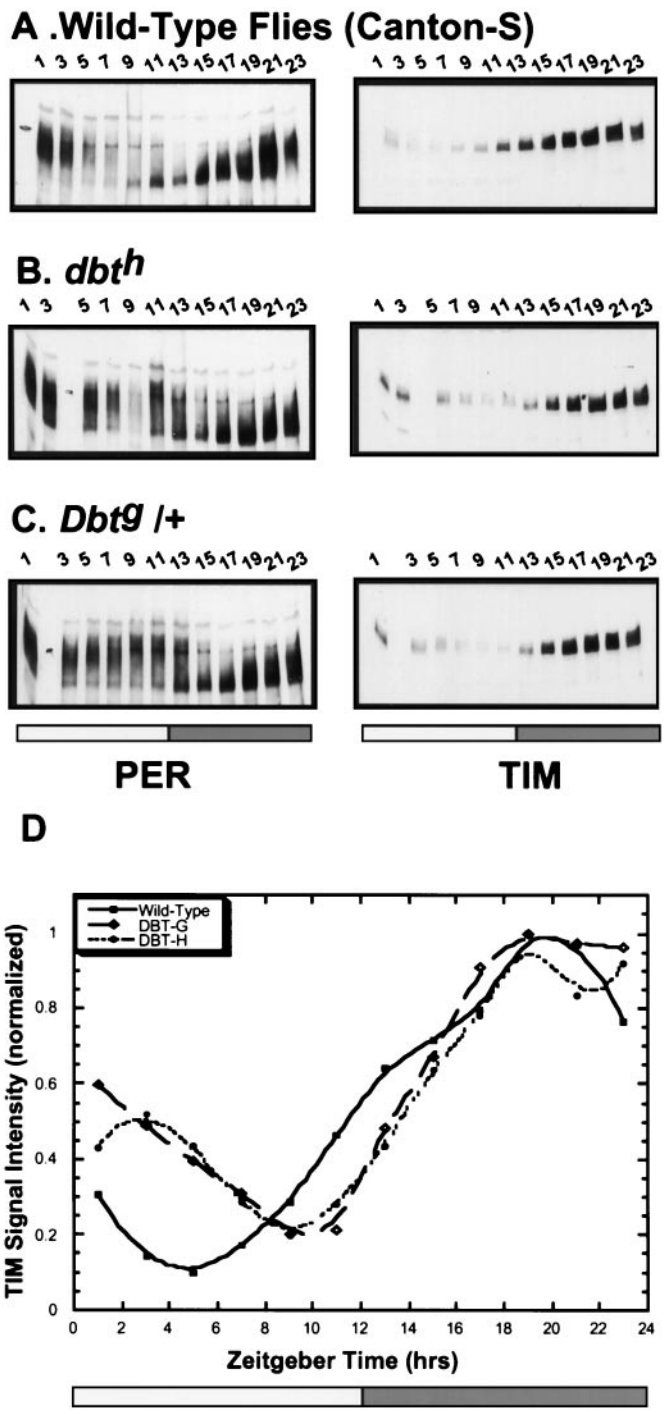

Figure 3. PER and TIM cycling is altered in *dbt* mutants under light/dark conditions. Shown are Western blots on fly heads collected from files maintained under 12 hr light/dark conditions with anti-PER (*left panels*) and anti-TIM (*right panels*) antibodies. The zeitgeber time of fly collections is mentioned at the *top* of each *lane*. Quantitation of the TIM blot is shown at the *bottom*. The blots were stained with Ponceau-S to ensure equal loading. TIM signal was quantitated using an AGFA scanner and Molecular Analyst software. The *penciled-in lines* (*left panels*, lanes 1, 3, and 2 in *A*, *B*, and *C*, respectively) are the molecular weight markers corresponding to 185 KDa. The experiment was repeated three times with similar results.

than in wild type, and PER levels decreased several hours after the time of the PER nadir in wild-type flies. This delayed disappearance suggests a role for *dbt*-catalyzed phosphorylation in targeting PER for degradation. This is consistent with the hyperaccumulation of hypophosphorylated PER in larvae, which carry a P-element-inactivated allele of *dbt* (Price et al., 1998). It is important to note, however, that the highest levels of PER in the mutants are only slightly higher than peak levels of PER in wild-type flies.

Interestingly, PER did not appear not to be underphosphorylated in the kinase mutants, at least by the one-dimensional SDS-PAGE mobility criterion. In fact, several slower-migrating forms that are hardly visible in wild-type flies were readily apparent in *dbt* mutant flies, suggesting that PER may even be hyperphosphorylated in the *dbt* mutant flies (Fig. 3*B,C*). Because the mutants probably have lower *dbt* enzymatic activity (see below), *dbt* might make only a minor contribution to the overall PER phosphorylation program, at least by this assay (see Discussion). Furthermore, PER accumulation profiles during the night (~ZT13–ZT23) were hardly affected, despite the strong effect on the earlier disappearance phase during the day. This suggests that there is only a weak relationship between the disappearance phase of the program during the day and the accumulation phase during the night. This is apparent in the mutant profiles, in which the high-mobility forms of PER begin accumulating well before the lower-mobility forms disappear at ZT11 and ZT13 (Fig. 3).

The effect of the *dbt* mutants on the TIM phosphorylation profile was much less striking. The TIM trough was delayed relative to that in wild-type flies (ZT9 in mutants vs ZT5 in wild-type flies). We suggest that this modest effect is attributable to delayed template disappearance (see below). Thereafter (ZT11–ZT19), the TIM accumulation in the mutant flies was even faster than in wild-type flies, so that the peak occurred at approximately the same time, namely ZT19. In summary, both *dbt* mutations had strong effects on the PER morning disappearance profile as well as the timing of the PER and TIM nadir, but there were only minor effects on the nighttime accumulation profiles. It is likely that the strong effects are directly attributable to reduced DBT activity on PER metabolism.

PER and TIM have been suggested to feedback inhibit directly their own transcription (Zeng et al., 1994; Darlington et al., 1998; Lee et al., 1999). It is therefore likely that the mutant alterations in the PER-TIM program affect *per* and *tim* steady-state mRNA levels. To address this possibility, we measured the steady-state *per* and *tim* mRNA levels across a circadian cycle in wild-type and mutant flies. As previously reported, *per* and *tim* mRNA peaked at approximately ZT13–ZT15 in wild-type flies (Fig. 4). In both long-period *dbt* mutants, the mRNA profiles were delayed by ~4–6 hr, with the peaks at ZT19–ZT21 (Fig. 4). This delay is consistent with the delay in the evening activity peak under these same conditions (Fig. 1). We surmise that the altered protein pattern during the day determines the timing of the subsequent mRNA rise. The same relationship has been previously described for the per^s mutant strain (Marrus et al., 1996). The amplitudes of *per* and *tim* oscillations were comparable in wild-type and *dbt* mutant flies.

Juxtaposition of the RNA and protein profiles, in wild-type and *dbt* mutant flies, highlighted a striking difference: the mRNA profile was advanced by 4–6 hr relative to the protein profile in wild-type flies as previously described, whereas there was almost no advance in both *dbt* mutant strains (Fig. 5*A,B*). This is because of the delayed RNA profiles with little or no effect on the protein accumulation profiles. The origins of the characteristic transcript–protein product "lag" are not very well understood, but it is believed to contribute to the "delay" for a limit cycle oscillator and considered an important feature of *Drosophila, Neurospora*, and mammalian pacemakers (Garceau et al., 1997; So and Rosbash, 1997; Hastings et al., 1999; Scheper et al., 1999). These data indicate that robust oscillations can persist without the mRNA–protein lag, consistent with previous observations of modest behavioral and molecular oscillations with strongly reduced *per* RNA oscillations (Ewer et al., 1988; Frisch et al., 1994; Cheng and Hardin, 1998). More importantly, the data indicate that robust transcriptional oscillations of *per* and *tim* RNA and presumably feedback by PER and/or TIM do not require the characteristic lag between protein and RNA. It is still possible that the lag plays an important part in period determination.

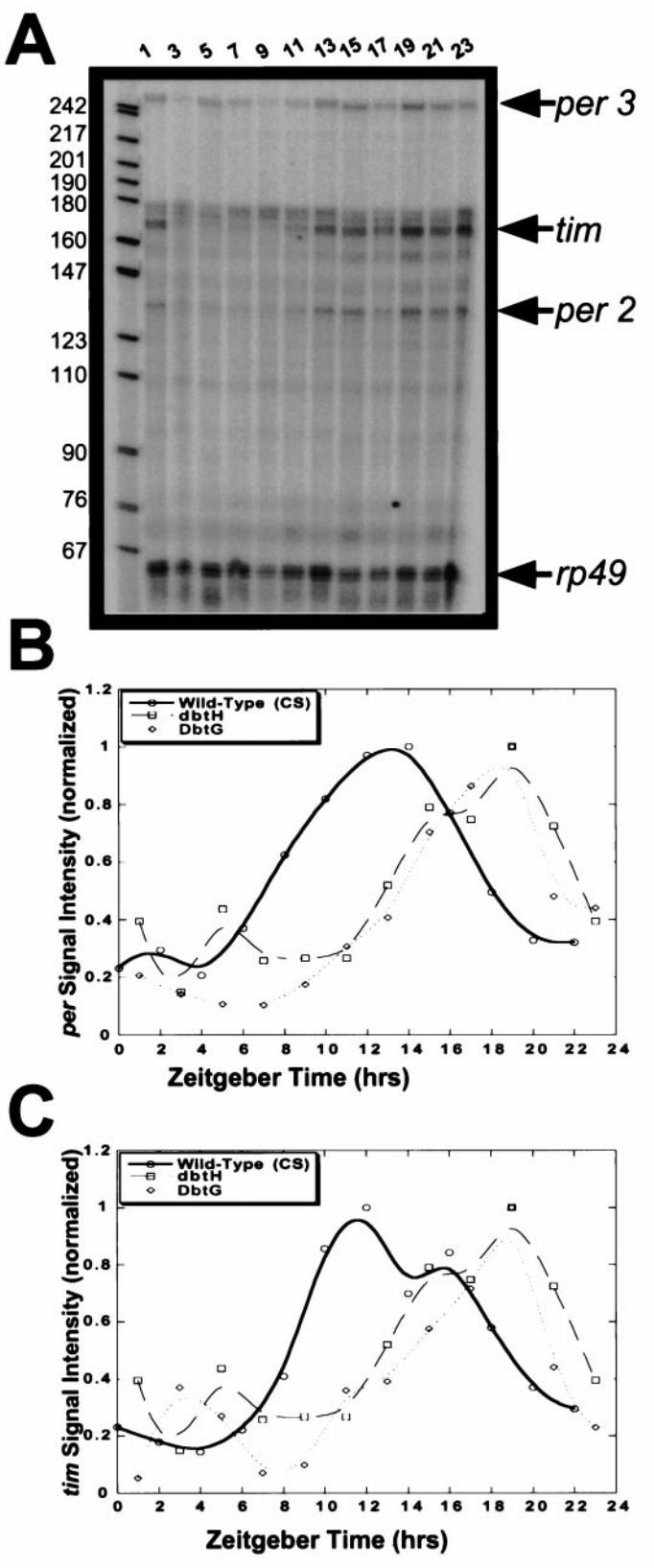

Figure 4. PER and TIM mRNA cycling is altered in the *dbt* mutants. mRNA amounts were quantitated from RNase protection assays using *rp49* as an internal control. *A*, Representative RNase protection assay showing *per*, *tim*, and *rp49* bands in heads from dbt^{h} flies collected at different zeitgeber times. The *first lane* contains the ladder, a *Hin*dIII–*Msp*I digest of pBR322, labeled with [^{32}P]dCTP. The circadian time of fly collections is at the *top* of each *lane*. *B*, *per* mRNA levels in wild-type (*CS*), dbt^{h}, and Dbt^{g} flies. *C*, *tim* mRNA levels in wild-type (*CS*), dbt^{h}, and Dbt^{g} flies. The experiment was repeated twice with similar results.

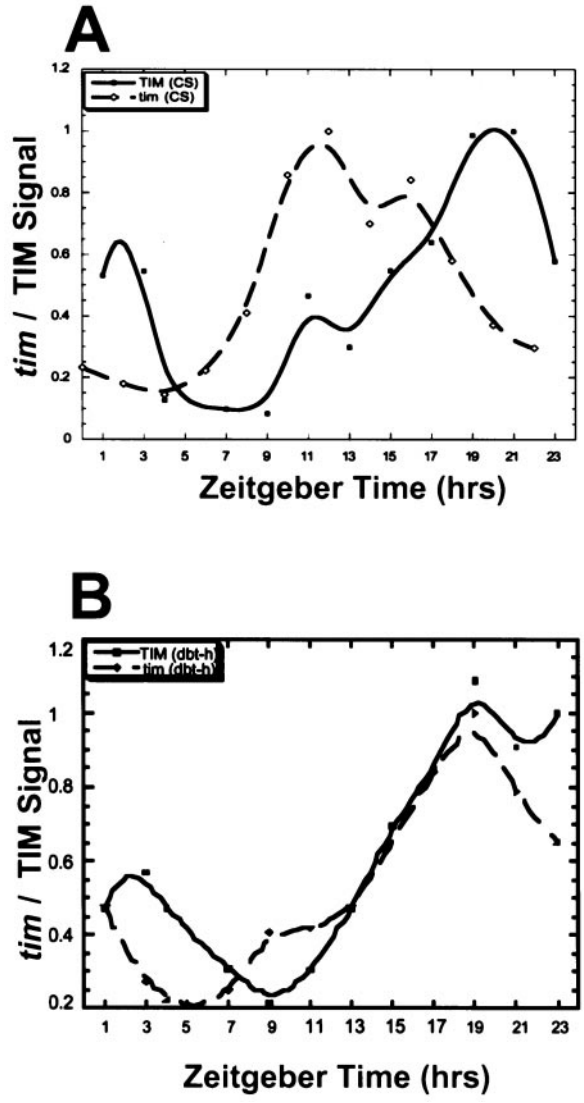

Figure 5. The mRNA–protein lag is much reduced in *dbt* mutant flies. Comparison of *tim* mRNA and protein profiles for wild-type flies (*A*) and dbt^{h} mutant flies (*B*) is shown.

Absence of the lights-on transition delays both transcript and protein accumulation programs in *dbt* mutants

We next assayed PER accumulation and phosphorylation profiles under conditions of constant darkness (DD), i.e., when the lights do not come on at ZT0. As previously reported, the DD PER profile in wild-type flies was similar to the LD profile, with somewhat reduced PER levels (Fig. 6*A*; Marrus et al., 1996). Importantly, in the absence of the dark-to-light transition, the phase of neither PER nor TIM was substantially altered in wild-type flies. On the other hand, both mutant lines showed significant differences from LD conditions and from wild-type flies (Fig. 6*B–D*). There was much more TIM in the morning when the lights did not come on, and the phase of the TIM cycle was delayed by several hours compared with wild-type flies. The mutant PER profile was also delayed by several hours relative to the wild-type profile; i.e., the appearance of more rapidly migrating, newly synthesized forms of PER was delayed by several hours compared with wild-type or with *dbt* mutant flies in LD conditions. When the lights failed to come on, the *dbt* mutants delayed the accumulation profiles as well as the disappearance profiles of PER and TIM.

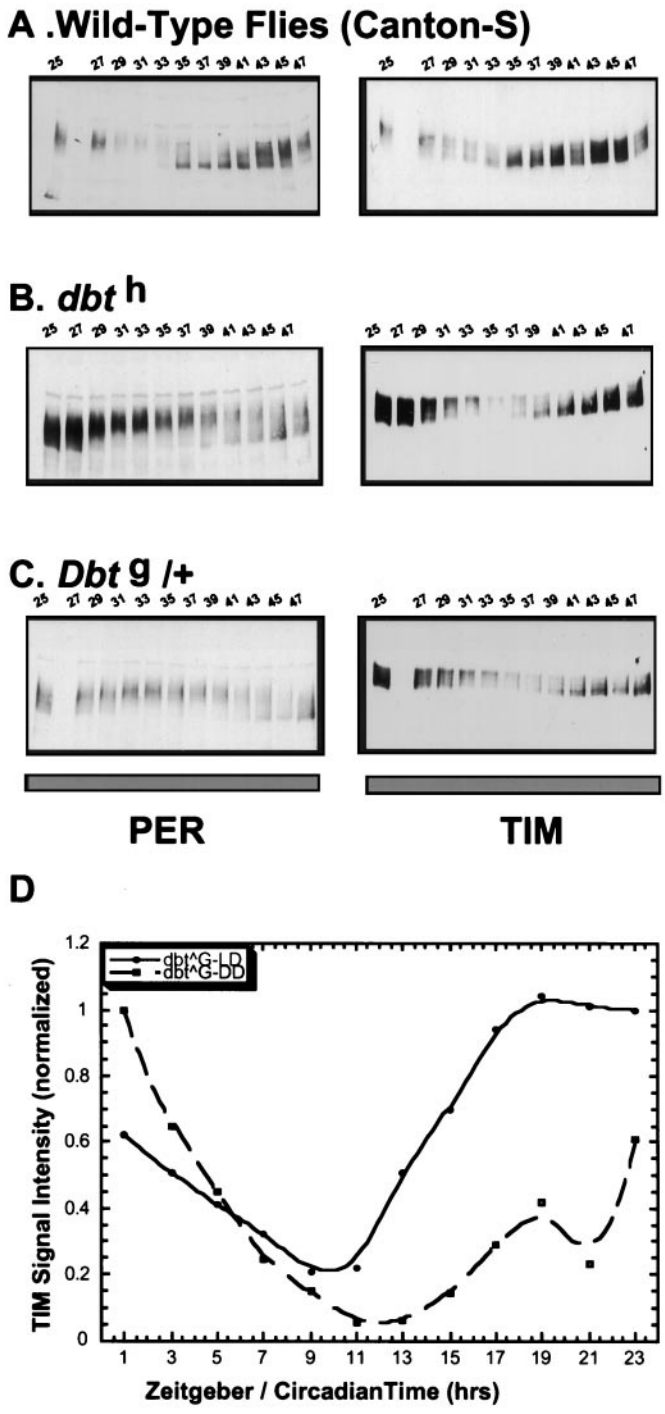

Figure 6. PER and TIM cycling is altered in *dbt* mutants under constant darkness conditions. Western blots on fly heads collected from files kept under LD for 3 d and then transferred to DD with anti-PER (*left panels*) and anti-TIM (*right panels*) antibodies are shown. The circadian time of fly collections is mentioned at the *top* of each *lane*. Quantitation of the TIM blot in DD versus that in LD conditions is shown. The experiment was repeated twice with similar results.

We also assayed the mRNA profiles under these conditions. As previously reported, the *per* and *tim* DD mRNA profiles in wild-type flies are very similar to those in LD (Fig. 7). Also in the mutants, the mRNA profiles were not significantly affected by the absence of the 12 hr of illumination or the lights-on transition (Fig. 7). This is surprising in view of the differences in the protein accumulation profiles between DD and LD and suggests that light affects protein accumulation without affecting the mRNA dynamics.

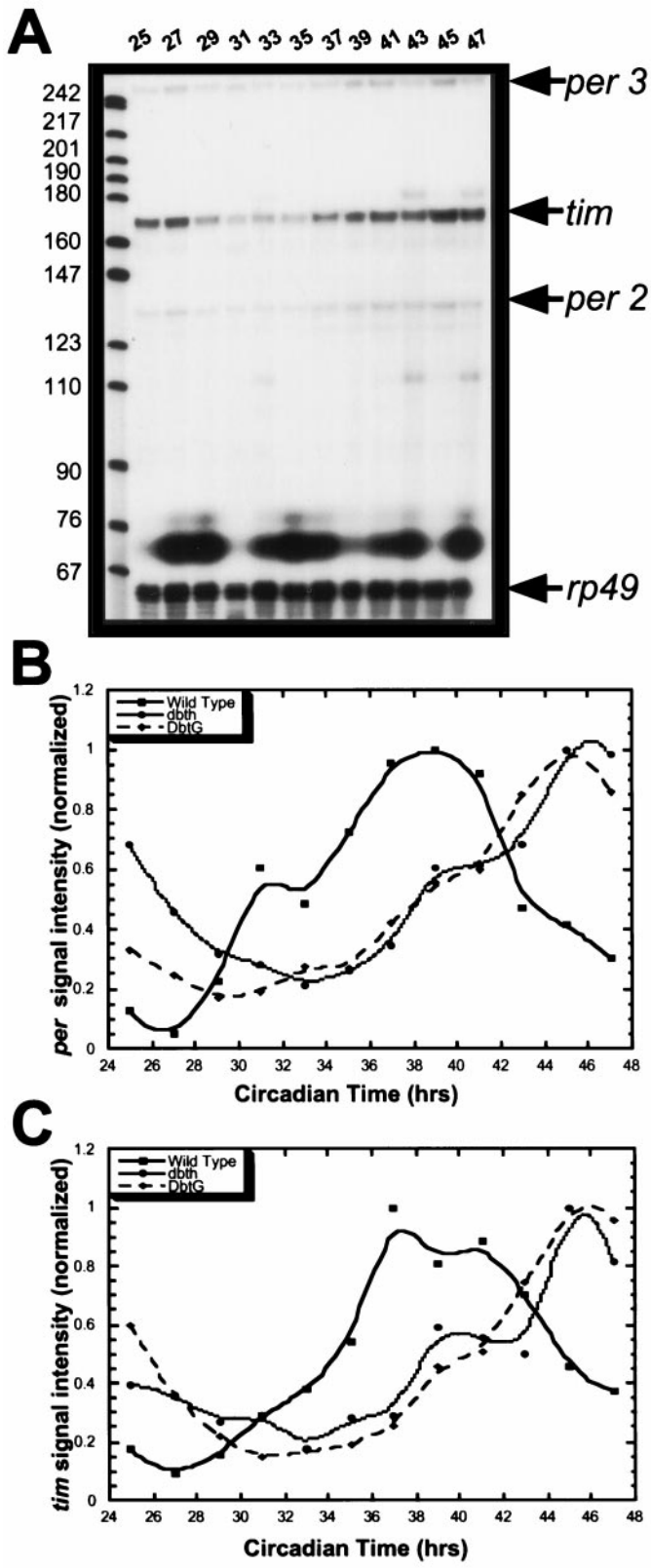

Figure 7. *per* and *tim* profiles in wild-type and *dbt* mutant flies under DD conditions. *A*, Representative RNase protection assay showing *per*, *tim*, and *rp49* bands in heads from *dbt*^{h} flies collected at different circadian times. The *first lane* contains a DNA ladder, a *Hin*dIII–*Msp*I digest of pBR322, labeled with [^{32}P]dCTP. The *numbers* at the *top* of the *lanes* indicate the times at which flies were collected. The last LD cycle ends at circadian time 24. Samples were collected every 2 hr starting at circadian time 25. *B*, *per* mRNA levels in wild-type (*CS*), *dbt*^{h}, and *Dbt*^{g} flies. *C*, *tim* mRNA levels in wild-type (*CS*), *dbt*^{h}, and *Dbt*^{g} flies. Two independent sets of RNase protection assays were performed, both with similar results.

DISCUSSION

Temporal phosphorylation of the clock proteins PER and TIM is dramatic and was suggested to be a major post-translational regulatory mechanism important for the *Drosophila* circadian pacemaker (Edery et al., 1994; Myers et al., 1996; Zeng et al., 1996; Edery, 1999). A major advance then came with the identification of DBT as a pacemaker kinase (Kloss et al., 1998; Price et al., 1998). The relationship between DBT activity and the pacemaker, however, is not clear. It is uncertain whether the long- and short-period mutations are a consequence of decreased and increased activity, respectively, or if one class of mutation is caused by aberrant DBT regulation (if any). Moreover, there is only indirect evidence that PER is a DBT substrate. Finally, the relationship between the PER phosphorylation program and other molecular and behavioral oscillatory phenomena is not understood.

To clarify the role of *dbt*-catalyzed phosphorylation in the pacemaker, we characterized two novel *dbt* mutants. Both mutations, when presented in the context of the highly similar yeast casein kinase I HRR25, severely reduce kinase activity on peptide substrates (Fig. 2). The long-period phenotypes are likely caused by insufficient DBT activity, so it takes longer to reach some required level of PER phosphorylation. We also assume that both mutants are expressed at levels similar to that of wild-type DBT.

Both dbt^{h} and $Dbt^{g}/+$ have ~29 hr periods and are similar in all other respects, suggesting that the phenotypes are not idiosyncratic features of the mutations but reflect the role of DBT in the pacemaker. Although the mutant flies entrain to imposed 24 hr photoperiods, the LD locomotor activity patterns indicate that there is no anticipation of the morning or evening light/dark transitions, and the evening activity peak is delayed by several hours into the night. The altered LD patterns are probably a consequence of the longer periods. Indeed, flies that carry per^{s} as well as dbt^{h} have a period of ~22.5 hr and manifest robust anticipation of both morning and evening transitions as well as an advanced evening activity peak. Both *dbt* mutant LD profiles resemble that of the 29 hr period per^{l} mutant strain, consistent with this altered period notion.

The molecular features of the per^{l} circadian program are difficult to compare with those of wild-type flies, because the mutant rhythms are weak and of low amplitude as well as long period even under 12 hr LD entraining conditions (Rutila et al., 1996, 1998b; Zeng et al., 1996). In contrast, PER and TIM cycling in the long-period *dbt* mutants is robust. Protein levels are comparable with those in wild-type flies during the night, and levels in the two mutant strains appear even higher than wild-type levels during the daytime (Fig. 3). Because previous work suggests a role for DBT-catalyzed phosphorylation in targeting PER for degradation, this probably reflects slower protein turnover during the morning in the *dbt* mutants. The TIM phosphorylation pattern in the mutants did not show any noticeable difference from the wild-type pattern. These observations suggest that the modest mutant effects on the TIM profiles are indirect, perhaps through a primary effect of the *dbt* mutants on PER.

PER phosphorylation was still readily observable in both mutant lines. In fact, there was a hint that PER was even hyperphosporylated in these strains. Although this might reflect phosphorylation events that never take place in a wild-type background, less active DBT mutants might be expected to depress the magnitude as well as the kinetics of the temporal phosphorylation program. This suggests that PER might not be a direct DBT substrate *in vivo* but is only influenced indirectly, through intermediates that are direct DBT targets. For example, DBT may phosphorylate and activate a direct PER kinase or a specific protease. In this context, PER has not yet been shown to be a direct DBT substrate. It is also possible that DBT is a functionally relevant but minor PER kinase. In this case, the bulk of the PER mobility shift on SDS-PAGE is a consequence of other kinases. Because PER persists for several hours longer in the mutants than in wild-type flies, the other kinases would continue to function and give rise to even more highly phosphorylated species than are usually observed. These would be an indirect consequence of weak *dbt* activity and delayed degradation. A final possibility is that the enhanced and delayed PER phosphorylation simply reflects some misregulation of DBT activity.

Careful analysis of the PER and TIM protein profiles in the long-period *dbt* mutants suggests that DBT acts in the late night and morning phase of the molecular cycle: the mutants leave the early evening protein profile almost unaltered. This indicates that *dbt* probably targets nuclear, monomeric PER, consistent with previous observations (Kloss et al., 1998; Price et al., 1998). It was also suggested that DBT acts in the early night to destabilize cytoplasmic PER, thus delaying nuclear entry and repression (Kloss et al., 1998; Price et al., 1998). The *dbt* mutants reported here do not significantly change this early night, presumptive cytoplasmic phase of accumulation. It is possible that DBT prefers free PER over PER complexed to TIM. If free PER is a better substrate, then DBT mutants should show a greater effect in the late night and early morning, after a large fraction of TIM has disappeared. Alternatively, DBT might influence only marginally the PER accumulation phase for some other reason. But *dbt* mutant larvae accumulate high levels of hypophosphorylated PER, which suggests that DBT is the major PER kinase and strongly influences PER accumulation as well as degradation (Fig. 8). There is evidence, however, that much of this PER accumulation occurs in cells and tissues where PER is not normally detectable, making the connection with the normal PER-TIM cycle uncertain.

To assess the effect of the *dbt* mutants on transcription, we assayed *per* and *tim* mRNA cycling in wild-type and *dbt* mutant flies. Both mutant profiles were delayed by 4–5 hr. This is presumably because of the delayed disappearance of PER as well as TIM, which has been suggested to repress *per* and *tim* transcription (Zeng et al., 1994; Darlington et al., 1998; Lee et al., 1999). This relationship is very similar to that previously reported for the per^{S} mutant strain; in this case, the clock proteins disappear more quickly, leading to an advance in the RNA profiles (Marrus et al., 1996; Rothenfluh et al., 2000). The per^{S} effect is more pronounced on PER than on TIM, consistent with the notion that monomeric PER might be the major transcriptional repressor (Marrus et al., 1996). In any case, comparable results in the three mutants indicate a solid relationship between the timing of the decline in protein levels and the timing of the subsequent increase in *per* and *tim* transcription (Fig. 8).

There is also an impressive relationship between the *per* and *tim* RNA profiles on the one hand and the evening locomotor activity peak on the other. In all cases, these begin to increase at approximately the same time, i.e., around ZT7 in the middle of the daytime. Mutants or physiological manipulations that affect the timing of the RNA profiles affect the timing of the evening activity peak in parallel (Marrus et al., 1996; Qiu and Hardin, 1996). This fits with the emerging view, from mammalian as well as *Drosophila* work, that cycling transcription plays an important role in circadian output as well as within the central pacemaker oscillator (Jin et al., 1999; Renn et al., 1999; Park et al., 2000; Ripperger et al., 2000; Sarov-Blat et al., 2000). A further implication of these relationships is that the protein oscillations from one day affect behavior as well as the RNA profiles on the next one: the morning decline and eventual disappearance of PER and TIM terminate a protein cycle from the previous day, which then causes the subsequent increases in both RNA levels and locomotor activity (Fig. 8).

In contrast, the delayed PER and TIM disappearance in the mutants had little if any effect on the subsequent protein accumulation phase (ZT13–ZT20) under these standard LD conditions; it was hardly affected, and both proteins peaked at approximately the same time as they do in the wild-type flies (ZT19–ZT21). Because of the delayed RNA rise in the mutants, the *per* and *tim* RNA accumulation profiles almost coincide with those of the proteins, between ZT15 and ZT21. This indicates that the timing of the RNA rise is insufficient to time the protein rise. The increase in protein levels may reflect protein half-life regulation, which is

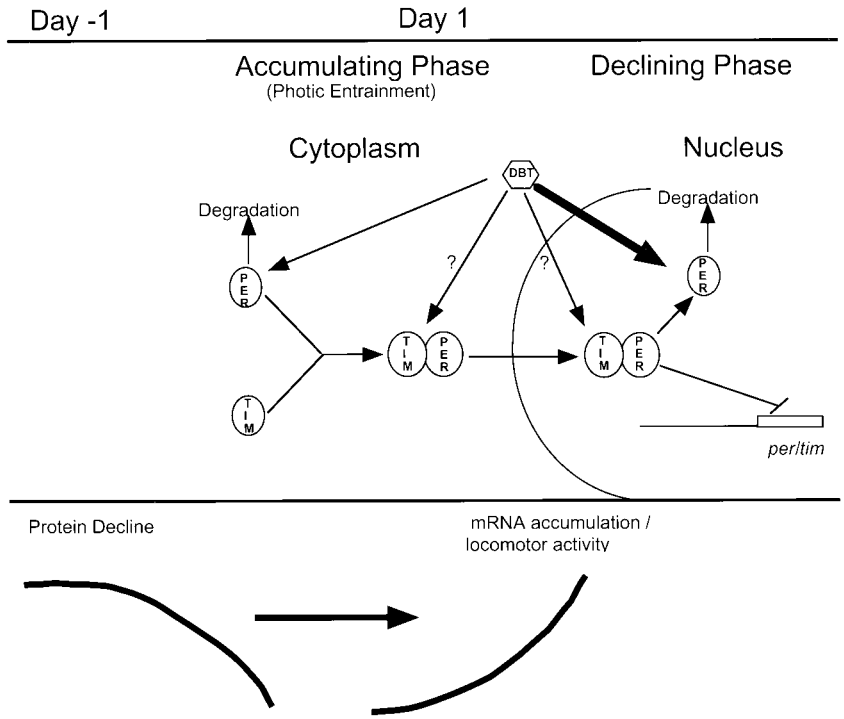

Figure 8. A possible model for DBT function in the *Drosophila* pacemaker. *Day −1* indicates the light/dark cycle before *Day 1*. In the cytoplasm, destabilization of PER delays substantial buildup of PER-TIM complexes. In the nucleus, PER destabilization relieves repression. In DBT mutants, PER degradation is much slower. This prolongs repression and delays the *per* and *tim* mRNA upswing in the next cycle. Because both mutants entrain well to an imposed 24 hr light/dark cycle, it is likely that photic entraining signals play a major role in the protein accumulation phase.

uncoupled from the underlying mRNA levels, at least under some circumstances.

The coincidence of the protein and RNA curves also raises doubts about the importance of the 4–6 hr lag between these two accumulation profiles. The data presented here indicate that it is dispensable for robust behavioral and molecular oscillations. This is especially relevant for the RNA fluctuations. Despite evidence that at least *per* mRNA fluctuations may not be necessary for core oscillator function (Frisch et al., 1994; Cheng and Hardin, 1998), they normally correlate with other molecular and behavioral circadian fluctuations. Moreover, there are substantial data indicating that PER and TIM feedback regulate these transcriptional oscillations (Hardin et al., 1990; Darlington et al., 1998). There are also considerable experimental evidence and theoretical models, suggesting that the normal 4–6 hr lag between the RNA and protein curves is essential for generating these robust, high-amplitude transcriptional oscillations (Zerr et al., 1990; Marrus et al., 1996; Garceau et al., 1997; Edery, 1999; Hastings et al., 1999; Scheper et al., 1999). The general view is that the protein accumulation delay gives enough time for transcription to increase substantially, before protein levels have increased sufficiently to inhibit transcription (Edery, 1999). The presence of robust transcriptional oscillations without the delayed protein accumulation makes this scheme less likely. It redirects focus toward some post-transcriptional delay (e.g., the timing of nuclear entry of the PER-TIM dimer), which we predict to be functional and important for transcriptional feedback regulation. It is important to note that our conclusions are based on biochemical experiments with whole-head extracts. It is still possible that the mRNA–protein lag may be important in the specific pacemaker neurons of *Drosophila* (Kaneko, 1998).

All of these experiments were performed under LD conditions. When the light comes on at ZT24, it causes a rapid decline in TIM levels. In DD conditions, therefore, TIM levels were much higher in the early subjective day as expected. But a major, unanticipated difference was that the PER and TIM profiles in the *dbt* mutant flies were profoundly delayed in DD, as evidenced by the late appearance of faster-migrating species. This occurred without a comparable change in the RNA profiles, giving rise to a quasi-normal lag between RNA and protein. The light-mediated advance of the protein curves and the absence of a comparable light reset of the RNA profile reinforce the independent regulation of the accumulation phase of the clock RNAs and proteins: only the RNA profiles are influenced by the declining phase of the protein cycle of the previous day, whereas only the protein profiles appear to be reset by the light entrainment stimulus. The data are therefore consistent with a post-translational route of light entrainment, perhaps mediated by some aspect of the normal light effect on TIM. This presumably contributes to the daily advance of the *dbt* mutant clock under LD conditions, which counteracts the 5 hr period-lengthening effect that would take place under DD conditions (Fig. 8).

Further understanding of the role of DBT in the clock will require experiments that directly address DBT function and regulation. For example, it is possible that temporal regulation of DBT activity makes a major contribution to the temporal phosphorylation profile and more generally to the normal timing of the circadian program. Additionally, the extent to which DBT modifies other pacemaker proteins is not clear. It is possible that these other putative DBT substrates may also be intimately connected to the pacemaker mechanism. Addressing these issues would provide us with a much deeper understanding of the role of phosphorylation in the pacemaker.

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