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The *Drosophila double-time*^{S} Mutation Delays the Nuclear Accumulation of *period* Protein and Affects the Feedback Regulation of *period* mRNA

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The *Drosophila double-time* (*dbt*) gene, which encodes a protein similar to vertebrate epsilon and delta isoforms of casein kinase I, is essential for circadian rhythmicity because it regulates the phosphorylation and stability of *period (per)* protein. Here, the circadian phenotype of a short-period *dbt* mutant allele (*dbt*^{S}) was examined. The circadian period of the *dbt*^{S} locomotor activity rhythm varied little when tested at constant temperatures ranging from 20 to 29°C. However, *per*^{L}*;dbt*^{S} flies exhibited a lack of temperature compensation like that of the long-period mutant (*per*^{L}) flies. Light-pulse phase–response curves were obtained for wild-type, the short-period (*per*^{S}), and *dbt*^{S} genotypes. For the *per*^{S} and *dbt*^{S} genotypes, phase changes were larger than those for wild-type flies, the transition period from delays to advances was shorter, and the light-insensitive period was shorter. Immunohistochemical analysis of *per* protein levels demonstrated that *per* protein accumulates in photoreceptor nuclei later in *dbt*^{S} than in wild-type and *per*^{S} flies, and that it declines to lower levels in nuclei of *dbt*^{S} flies than in nuclei of wild-type flies. Immunoblot analysis of *per* protein levels demonstrated that total *per* protein accumulation in *dbt*^{S} heads is neither delayed nor reduced, whereas RNase protection analysis demonstrated that *per* mRNA accumulates later and declines sooner in *dbt*^{S} heads than in wild-type heads. These results suggest that *dbt* can regulate the feedback of *per* protein on its mRNA by delaying the time at which it is translocated to nuclei and altering the level of nuclear PER during the declining phase of the cycle.

Key words: biological clocks; circadian rhythms; temperature compensation; phase–response curves; casein kinase I; phosphorylation; clock genes; protein stability; protein degradation; negative feedback

Circadian rhythms are daily behavioral, physiological, or biochemical cycles that persist with a precise period in the absence of cycling environmental cues. They arise from endogenous biological clocks driven by cycling proteins and mRNAs. Environmental cues, such as the daily light/dark (LD) or temperature cycle, normally adjust the phase and period of the clock to maintain precise synchrony with the rotation of the earth, a process termed "entrainment" (Pittendrigh, 1974). The mechanisms of these clocks share many features, and there is homology between the mammalian and fruit fly (*Drosophila melanogaster*) clock genes (Hall, 1998; Dunlap, 1999). In *Drosophila*, the protein products of the *period* and *timeless* genes (PER and TIM, respectively) accumulate during the night, become phosphorylated, and are transported as a PER/TIM complex to the nucleus, where they negatively regulate transcription of the *per* and *tim* mRNAs (Rosbash et al., 1996; Young, 1998) and positively regulate transcription of *dClk* mRNA (Bae et al., 1998; Glossop et al., 1999). The recent identification of additional clock genes has expanded our understanding of this core mechanism, as well as its entrainment by light and coupling with behavioral and physiological circadian rhythms (Cermakian and Sassone-Corsi, 2000).

One of these genes is the *Drosophila double-time* (*dbt*) gene (Kloss et al., 1998; Price et al., 1998; Rothenfluh et al., 2000b; Suri et al., 2000), which encodes a protein similar to casein kinase I isoforms that are involved in the mammalian clock (Lowery et al., 2000; Vielhaber et al., 2000; Toh et al., 2001). The *dbt* gene is essential for a lag between the mRNA levels and nuclear protein levels of both *per* and *tim*, thereby delaying the feedback of the PER/TIM complex on *per*/*tim* mRNA expression. Immediate negative feedback by PER/TIM would not result in molecular oscillations, but rather in an equilibrium level of expression, determined by the opposing forces of synthesis on the one hand, and degradation and negative feedback on the other hand (Sehgal et al., 1995; Leloup and Goldbeter, 1998). It has been proposed that *dbt* protein (DBT) causes this lag by binding to PER in the cytoplasm, causing it to become phosphorylated and thereby signaling its degradation (Kloss et al., 1998; Price et al., 1998). A role for *dbt* in the turnover of nuclear PER has also been proposed (Price et al., 1998; Rothenfluh et al., 2000b; Suri et al., 2000).

Here, we examine the effects of the *dbt*^{S} mutation, which shortens the period of circadian rhythms (Price et al., 1998), on temperature compensation and entrainment to light. The *dbt*^{S} mutation does not affect the relative constancy of circadian period length over an extended range of constant temperatures. By contrast, the *dbt*^{S} mutation does affect the response of the clock

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Table 1. Rhythmicities and mean periods of locomotor activity rhythms in constant darkness at different temperatures

Genotype	Temperature (°C)	Number arrhythmic	Number rhythmic	Mean period ± SEM (hr)
per^L	20	0	15	27.8 ± 0.3
	25	8	21	29.1 ± 0.2
	29	5	22	30.4 ± 0.2
WT	20	3	17	24.2 ± 0.2
	25	4	11	24.3 ± 0.1
	29	0	43	24.3 ± 0.06
$per^L;dbt^S$	20	6	20	21.0 ± 0.1
	25	6	24	20.9 ± 0.1
	29	0	14	23.3 ± 0.3
per^S	20	2	13	19.7 ± 0.2
	25	6	24	19.7 ± 0.1
	29	6	55	19.2 ± 0.04
dbt^S	20	4	13	19.0 ± 0.2
	25	12	16	18.7 ± 0.1
	29	7	60	18.3 ± 0.1

Number arrhythmic, the number of flies for which periodogram analysis did not extract a definitive period (see Materials and Methods for criteria); number rhythmic, the number of flies for which periodogram analysis did extract a definitive period.

Table 2. Relative average intensity of nuclear anti-PER immunoreactivity in eyes of adult flies

	Genotype		
Time (hr)	WT	dbt^S	per^S
ZT13	0.8 (N/W/S, 142)	0.5 (N/W, 145)	0.2 (N/W, 79)
ZT15	1.2 (W/S, 207)	0.4 (N/W, 241)	1.0 (W, 91)
ZT16	1.3 (W/S, 222)	0.7 (N/W, 124)	
ZT17	1.6 (W/S, 117)	0.8 (N/W, 75)	
ZT18	1.6 (W/S, 369)	1.3 (W/S, 435)	0.9 (N/W, 68)
ZT19	1.9 (S, 160)	1.2 (N/W/S, 77)	
ZT21	1.8 (W/S, 375)	1.7 (W/S, 223)	1.2 (W/S, 65)
ZT1	1.8 (W/S, 346)	1.6 (W/S, 174)	0.9 (N/W, 45)
ZT3	1.8 (W/S, 264)	1.7 (W/S, 144)	
ZT5	1.4 (W/S, 149)	1.2 (N/W/S, 143)	
ZT7	1.2 (W/S, 233)	0.9 (N/W/S, 145)	
ZT11	0.8 (N/W, 73)	0.5 (N/W, 50)	

Fly heads were collected, processed, and scored as described in Materials and Methods. Time is the time of collection in LD and is given in ZT, where ZT0 = lights on and ZT12 = lights off. A staining class present in 10–30% of the sections is indicated with a letter, whereas a staining class present in >30% of the sections is indicated with an underlined letter (N, no staining, score of 0; W, weak staining, score of 1; S, strong staining, score of 2). The number of sections scored is also indicated in parentheses. The average score (ranging from 0–2) is given for each condition. All 97 per^O sections that were scored exhibited no staining (average score of 0).

to light and reveals an effect of DBT on the nuclear entry or stability of PER, similar to what has been proposed for casein kinase I in the mammalian clock (Vielhaber et al., 2000). The results suggest that DBT contributes to the generation of circadian rhythmicity by regulating multiple steps of clock biochemistry.

MATERIALS AND METHODS

Fly stocks. The genotypes that were used were WT (wild-type Canton S), dbt^S (*ry*,dbt^S), per^L (*y*,per^{L1}, *w*), per^S, per^{L1};dbt^S, and per^o (*y*,per^o;tim^o; *ry*). The stocks were maintained on standard *Drosophila* medium in pint-size bottles at 25°C, unless a different temperature is noted in the text.

Analysis of locomotor activity rhythms. Newly eclosed flies were entrained to a 12 hr LD cycle at 25°C [for determination of the phase–response curve (PRC)] or the specified temperature (for analysis of temperature compensation) for at least 3 d. Then, individual male flies were placed in glass tubes, entrained to one more 12 hr LD cycle at the test temperature, and monitored for activity with infrared detectors and a computerized data collection system (Sehgal et al., 1992). This system allows temporal records of activity to be acquired in constant darkness (DD) for analysis of temperature compensation, or as outlined below for the phase resetting experiment. Periods for each record were determined by χ^2 periodogram analysis with the TAU analysis software (Minimitter Co., Sunriver, OR) as previously described (Sehgal et al., 1992). Each fly was analyzed for 6.5 d. Periodogram analysis of flies that were tabulated as rhythmic in Table 1 produced a single strong peak that was statistically significant with $p < 0.05$, or a single strong peak with weaker peaks that were harmonics of the strong peak. Periodogram analysis of flies that were tabulated as arrhythmic in Table 1 produced no strong peak that was statistically significant with $p < 0.05$, or multiple peaks that were statistically significant with $p < 0.05$ but were not harmonics. Average periods and SEM were calculated only from the flies that were scored as rhythmic.

To generate the phase–response curves (see Fig. 1) for wild-type, per^S, and dbt^S, flies of each genotype were divided into several groups and placed in constant darkness after entrainment (20 flies per group). Several groups from each genotype received a 2 hr light pulse (the intensity of which was equal to the intensity of light in the entrainment regime, i.e., 3000 lux) at various times after the termination of the last photophase of LD entrainment, while one group received no light pulse. All manipulations that were performed in the dark were done using a red safelight (Kodak GBX2 filter) that does not entrain or phase-shift the *Drosophila* rhythm. Then, individual locomotor activity rhythms were monitored in constant darkness for 6 d. Using this data for individual flies, periodogram and waveform analyses were performed using the TAU software program to determine the period and the median time of activity offset for each fly (Sehgal et al., 1992). Within each group of flies, the mean activity offset time and the strength of the phasing were determined as described (Sehgal et al., 1992). Phase shifts caused by exposure to light pulses were calculated by subtracting the mean activity offset for the light-pulsed groups from the mean activity offset for the non-pulsed control group. A phase–response curve was generated for each genotype by plotting the change in phase versus the time of the light pulse.

Immunoblot analysis. Extracts were made from adult heads, electrophoresed on a 5.7% SDS-polyacrylamide gel, blotted to nitrocellulose, and assayed for PER as described (Edery et al., 1994; Price et al., 1995), with the following modifications. The primary antibody (anti-PER, kindly provided by Jeff Hall, Brandeis University) (Stanewsky et al., 1997) was used at a 1:25,000 dilution. The secondary antibody, an affinity-purified goat anti-rabbit IgG conjugated with horseradish peroxidase (American Qualex, San Clemente, CA), was used at a 1:1000 dilution. Chemiluminescent detection was accomplished with the ECL plus system (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunohistochemistry. Newly eclosed fruit flies were entrained for at least 3 d at 25°C to a 12 hr LD cycle. Heads were cut off with a sharp razor blade under room light (if collected during the photophase) or a red light (Kodak filter GBX-2, if collected during the scotophase). dbt^S, per^S, and wild-type genotypes were collected by two people at exactly the same time to eliminate the effect of different collection times. The heads were processed for immunohistochemical detection of PER as described (Vosshall et al., 1994), with the following modifications. The primary antibody (anti-PER) (Stanewsky et al., 1997) was used at a 1:20,000 dilution. The secondary antibody (goat anti-rabbit IgG-horseradish peroxidase; American Qualex) was used at a 1:200 dilution. The chromogenic substrate was a liquid 3,3′-diaminobenzidine (DAB) solution supplied by BioGenex (San Ramon, CA).

For each batch of stained slides, a standard slide with sections from wild-type flies that were collected at zeitgeber time (ZT) 21 or ZT1 (the times of peak staining intensities) was observed under the microscope, and staining was stopped by dipping the slide in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) when strong nuclear staining was observable. Then, all of the slides in a batch were stained with the same amount of DAB staining solution for the same length of time as the wild-type standard, so that immunohistochemistry with different batches of slides always was done under conditions that produced indistinguishable levels of staining at the wild-type peak (ZT21–1). Moreover, either a complete night time course (ZT13–21; three separate experiments) or

day time course (ZT1–11; four separate experiments) was stained in every experiment, except for ZT15 and ZT18, which were analyzed in an additional two experiments. One experiment was analyzed blind, and the relative timing of nuclear PER in all three genotypes was the same as that shown for the total data set (Table 2). The total number of sections analyzed in all experiments is tabulated in Table 2. The slides were photographed with Nomarski optics at 200× on a Zeiss Axioplan microscope. Staining of individual eye sections in which most photoreceptors had no stained nuclei was scored as "none" and received a score of "0". Staining of individual eye sections in which most photoreceptors had stained nuclei that were still somewhat translucent was scored as "weak" and received a score of "1". Staining of individual eye sections in which most photoreceptors had dark, opaquely stained nuclei was scored as "strong" and received a score of "2." See Figures 2 and 3 for examples of typical eye sections scored in each category. The average score was calculated for each genotype–time condition, and these are tabulated in Table 2.

RNase protection analysis. Adult flies (1 to 7-d-old) of the dbt^S and wild-type genotypes were entrained to a 12 hr LD cycle for at least 3 d and flash-frozen at the indicated times in LD. RNA was isolated from the heads of these flies with Trizol reagent using the method specified by the supplier (Life Technologies, Gaithersburg, MD). The yields of RNA were quantitated by absorbance at 260 nm, and 20 μg of RNA were processed for each time point. The ^{32}P-labeled α-tubulin and *per* probes have been described previously (Sehgal et al., 1994), and the RNase protections were performed with the RPA III kit (Ambion, Austin, TX). Protected fragments were analyzed on a 6% polyacrylamide, 8 M urea gel. Visualization was achieved by exposure to Kodak XAR-5 film, and quantitation was achieved with a phosphorimager and Image-Quant software (Molecular Dynamics, Sunnyvale, CA). Background values were measured in each lane above the protected *per* fragment, and these values were subtracted from the *per* and tubulin signals in each lane. The corrected *per* signal was divided by the corrected tubulin signal for each lane, and these values are plotted in Figure 5*B*. Control hybridizations demonstrated that the *per* and tubulin signals were derived from the *per* and tubulin probes, respectively, and that the signal was linearly related to the amount of target RNA in the *Drosophila* total RNA (data not shown).

RESULTS

The dbt^S mutation does not affect the temperature compensation phenotypes of per^+ or per^L flies

To determine whether the dbt^S mutation alters temperature compensation of the circadian clock, circadian rhythms of wild-type and mutant flies were assayed at several constant temperatures in constant darkness. Although a temperature or LD cycle typically entrains circadian rhythms, constant temperature and constant darkness allow the circadian rhythm to oscillate with its own endogenous period. Typically, this period is quite similar at different temperatures within the physiological temperature range, as long as the temperature does not change while the rhythm is recorded. This phenomenon has been termed temperature compensation (Pittendrigh, 1974), and several clock mutations in *Drosophila* and *Neurospora* affect temperature compensation.

Accordingly, the temperature compensation of dbt^S flies was assessed by analyzing the period length of dbt^S as well as per^L, wild-type, per^L;dbt^S, and per^S locomotor activity rhythms at different temperatures in DD (Table 1). dbt^S rhythms are temperature compensated as well as those of wild-type and per^S flies (Table 1). The dbt^S period fluctuated between 19.0 and 18.3 hr in the temperature range tested (20–29°C). A slight shortening of period at higher temperatures has consistently been found in per^S flies (Konopka et al., 1989), and a similar degree of shortening was found in dbt^S flies. By contrast, the average period of the per^L genotype increased by 2.6 hr between 20 and 29°C, as previously reported (Konopka et al., 1989; Curtin et al., 1995). per^L;dbt^S double mutant flies also were not temperature compensated. Despite having a shorter circadian period than wild-type flies, there was a 2.3 hr difference in their average periods at 20 and 29°C, comparable with the difference in the average per^L period. Thus, dbt^S does not suppress the temperature compensation defect of the per^L mutation, although it does shorten the circadian period of per^L over the entire temperature range. However, the dependence of circadian period on temperature does differ somewhat in per^L and per^L;dbt^S flies, because the lengthening of period is only observed above 25°C and shows a stronger dependence on temperature above 25°C in the double mutant.

Light-pulse PRCs for dbt^S and per^S are similar and substantially different from the wild-type PRC

A phase–response curve for dbt^S was generated to determine whether *dbt* affects the clock-regulated response to light. Like other circadian rhythms, the fruit fly circadian rhythm is reset by short light pulses that are administered at different times after the termination of LD (Pittendrigh, 1974). These pulses elicit changes in the phase of subsequent rhythms in DD, with the magnitude of the change determined by both the light pulse and the phase of the clock. These average phase changes, plotted as a function of the time at which the pulse was administered, generate a PRC.

Figure 1 shows the PRCs that were generated by 2 hr light pulses for wild-type, per^S, and dbt^S flies. As has been demonstrated previously (Myers et al., 1996; Stanewsky et al., 1998), the wild-type PRC (Fig. 1*A*) is a weak curve (Winfree, 1973) with moderate phase delays (negative changes in phase) after early-night light pulses and moderate phase advances (positive changes in phase) after late-night light pulses. In contrast to the wild type, the PRCs for both short-period mutants (per^S and dbt^S) (Fig. 1*B*,*C*) are strong curves (Winfree, 1973) (see Hall and Rosbash, 1987; and Saunders et al., 1994, for per^S PRCs with varying light exposure). The very large phase delays after an early-night light pulse (up to 7 hr in dbt^S and 7.5 hr in per^S) and the very large phase advances after a late-night pulse (6.5 hr in dbt^S and 7 hr in per^S) are larger in magnitude than any observed in the wild-type PRC. Furthermore, there is a rapid transition from phase delay to phase advance (compare 5 hr after lights out with 6 hr in both dbt^S and per^S PRCs) without the transition zone observed in the wild-type PRC. During the subjective day dead zones when lights would have been illuminated if the LD cycle had continued, light pulses produced little change of phase. The light-insensitive subjective days are much shorter in both the dbt^S and the per^S mutants than in WT flies (Fig. 1, *hatched bars*). In all three genotypes, the subjective day dead zone begins 12 hr after lights out, before which phase shifts in response to light define subjective night. Therefore, it is only the length of the subjective day that is shortened in the mutant PRCs.

The PRCs have the same period length that is observed for the locomotor activity rhythms of the genotypes (Konopka and Benzer 1971; Price et al., 1998). For example, a light pulse given at 27 hr after lights out elicits a 4 hr phase delay in wild-type flies, which is comparable to the shift observed 24 hr earlier, at 3 hr after lights out (*arrows* connect the relevant time points in Fig. 1.). Although the dbt^S and per^S PRCs are very similar, they exhibit slightly different periods. At 24 hr after lights out, a light pulse of per^S flies elicits a very strong phase delay, like the one elicited 19 hr earlier (5 hr after lights out), whereas a light pulse of dbt^S flies elicits a very strong phase advance, like the one elicited 18 hr earlier (6 hr after lights out).

PER accumulates in photoreceptor nuclei later in dbt^S flies than in wild-type flies

A lag in negative feedback by PER/TIM protein on *per*/*tim* mRNA expression is required in a mathematical model to pro-

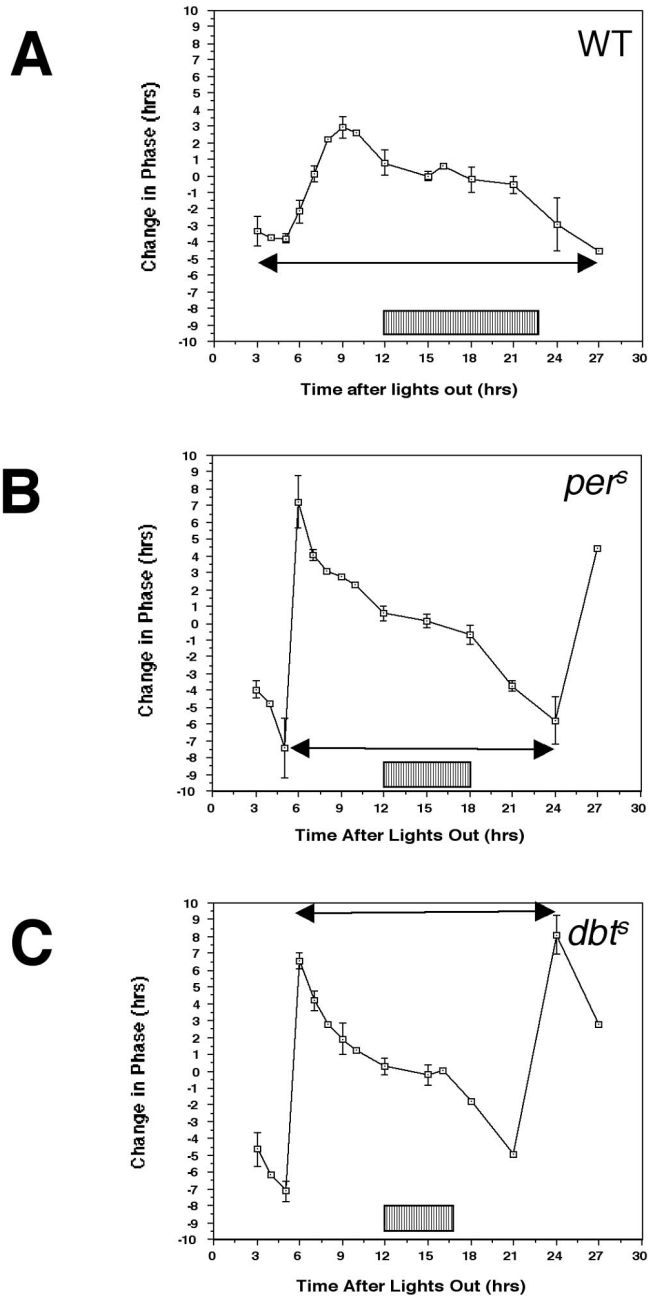

Figure 1. Both the per^S and dbt^S mutations increase the amplitude of the phase–response curve and shorten its period. After entrainment of flies with wild-type (*A*), per^S (*B*), or dbt^S (*C*) genotypes to at least three cycles of 12 hr LD, the LD cycle was terminated. A control group of flies with each genotype was left in constant darkness. Experimental groups were subjected to a 2 hr light pulse at the indicated time (*Time after lights out*) after termination of the last 12 hr photophase, but otherwise were treated the same as the control. The difference in the average activity offset time between experimental and control flies is plotted as a function of the time of the light pulse. A phase advance in the experimental group is plotted as a positive change in phase, whereas a phase delay is plotted as a negative change in phase. Error bars depict the SD for each point. The subjective day, or the interval during which light pulses do not reset the clock, is denoted by the *hatched box* under each PRC, whereas *double-headed arrows* link time points with comparable phase shifts that define the period of the PRC. See Results for a more extensive discussion of the differences in these phase–response curves.

duce the molecular oscillations of *per*/*tim* gene products (Leloupand Goldbeter, 1998). As outlined in the introductory remarks, it has been proposed that DBT acts in the cytoplasm to destabilize PER, thereby leading to a lag in the accumulation of PER protein relative to the accumulation of *per* mRNA (Kloss et al., 1998; Price et al., 1998). Besides destabilizing cytoplasmic PER, another possible way to delay negative feedback would be to delay translocation of the PER/TIM complex to the nucleus, in which negative feedback is effected. Here, immunohistochemical detection of PER shows that it accumulates in photoreceptor nuclei later in dbt^S flies than in wild-type flies, thereby providing evidence that DBT affects the nuclear accumulation as well as cytoplasmic stability of PER.

To determine the effect of the dbt^S mutation on the nuclear accumulation of PER, PER levels were assessed by immunohistochemistry in the eyes of wild-type, per^S, and dbt^S flies that were isolated at different times. The eye is the predominant site of PER expression in the head, and so immunohistochemical detection of nuclear PER in eyes reflects the localization of PER that is detected by immunoblot analysis of head extracts (Zeng et al., 1994; this reflection will be important for the arguments that follow). The immunohistochemical staining of photoreceptor nuclei in each section through the eye was scored as none (score of 0), weak (score of 1), or strong (score of 2) (see Figs. 2, 3 for examples of each and Table 2 for a tabulation of the average scores). The per^o mutation, which is a single nucleotide change producing a translational stop codon in the amino terminal part of the *per* reading frame (Baylies et al., 1987), provided a negative control for these experiments. Because PER protein is not present in this mutant (Zerr et al., 1990; Edery et al., 1994), any staining that is detected in per^o eyes is nonspecific or background in nature, whereas additional immunoreactivity in per^+ flies should result from PER protein expression. The only significant staining in per^o eyes was found in bands just under the lens (Fig. 2, *blue arrows*), and therefore, this was considered nonspecific in all sections.

No punctate nuclear staining was ever detected at the surface of the eye (photoreceptors 1–7) or the inside of the eye (photoreceptor 8) in per^o flies (Fig. 2, Table 2). By contrast, anti-PER immunoreactivity was detected in some eye sections at all times of day in per^+ (dbt^S and wild type) and per^S genotypes (Figs. 2, 3, *red arrows* for photoreceptors 1–7 and *yellow triangles* for photoreceptor 8; see also Table 2). The absence of such staining in the per^o controls demonstrates that the immunoreactivity derives from bona fide nuclear PER. At the time points showing the lowest levels of nuclear PER in wild type (e.g., ZT11–13), more wild-type eye sections showed weak nuclear staining than no staining, although almost none was strongly stained. At ZT13, most per^S eye sections showed no staining, although some exhibited weak staining. A significant number of strongly stained wild-type eye sections was detected by ZT15, and most per^S eye sections became weakly stained at this time. These results pinpoint an initial wave of nuclear accumulation occurring between ZT13 and ZT15 in wild-type and per^S eyes (Fig. 2, Table 2). A preponderance of strongly stained wild-type eye sections was observed by ZT18 (Table 2), whereas per^S staining increased only marginally after ZT15.

The accumulation of nuclear PER in dbt^S photoreceptors was significantly delayed relative to wild-type and per^S photoreceptors. Almost no strong nuclear staining was observed in dbt^S eye sections until ZT18, and a preponderance of strongly stained dbt^S

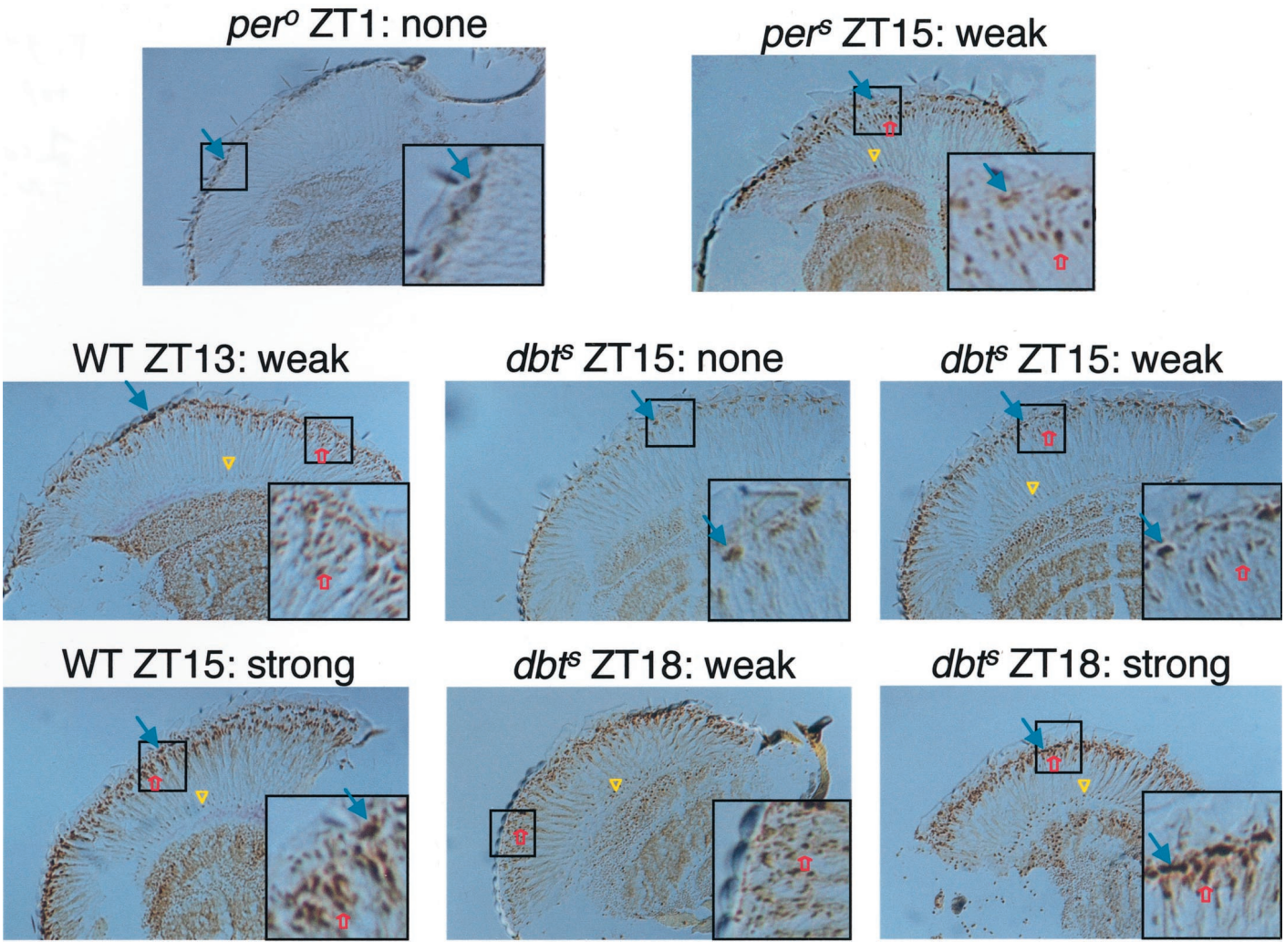

Figure 2. An increase in nuclear PER occurs later in the eyes of dbt^S flies than in wild-type and per^S flies. Heads from the per^o mutant, wild-type (*WT*), per^S, or dbt^S mutant flies that had been entrained to a 12 hr LD cycle were removed at the indicated times (*ZT*; ZT0 = lights on, ZT12 = lights off), sectioned, and processed for detection of PER as described in Materials and Methods. Each panel is a section of an eye visualized with Nomarski optics at 200×; in the *bottom right corner*, a small region of the field (outlined with a *black square* on the larger image) is magnified an additional 3×. The per^o flies make no detectable levels of PER, so the level of diaminobenzidine chromogen in these eye sections is indicative of nonspecific detection. Some background staining is seen in the optic lobes, and sometimes lines of nonspecific staining are seen immediately under the lens tissue (*blue arrow*), but no punctate nuclear staining. By contrast, dbt^S, per^S, and wild-type eye sections can exhibit punctate staining at the surface of the eye in the nuclei of photoreceptor types 1–7 (*red open arrows*) and at the inside of the eye in the nuclei of photoreceptor type 8 (*yellow triangles*). Staining of eye sections was scored as none, weak, and strong; the panels here, for which the scores, genotypes, and collection times are given, are indicative of the level of staining observed for each class. A significant increase in the staining of eye sections was first observed in wild-type and per^S eyes at ZT15. By contrast, most dbt^S eyes exhibited no staining at this time, with some showing weak staining. By ZT18, most wild-type eye sections were strongly stained (Table 2), whereas dbt^S eye sections exhibited a mixture of weak and strong staining. A preponderance of strongly stained eye sections was not obtained until ZT21 in dbt^S (Fig. 3, Table 2). The overall staining scores of many sections are tabulated in Table 2.

eye sections was not observed until ZT21 (Figs. 2, 3, Table 2). These results demonstrate a time course for nuclear accumulation of PER in eyes that is delayed by ~3 hr in dbt^S flies relative to wild-type flies. The delay in accumulation of nuclear PER in dbt^S eyes was not paralleled by a delayed disappearance of nuclear PER from dbt^S eyes. High levels of nuclear PER persisted from late night to well after lights were illuminated (ZT3) in both dbt^S and wild-type photoreceptors, but the levels of nuclear PER eventually declined at a faster rate to reach a lower level in dbt^S than in wild-type photoreceptors. More dbt^S than wild-type eye sections were observed with no staining at their troughs (ZT11–13 for wild-type flies, ZT7–17 for dbt^S; Table 2).

Immunoblot analysis of PER demonstrates that total PER levels in dbt^S heads are not lower than those in wild-type heads during the first half of the night

To determine whether the delayed nuclear accumulation of PER in dbt^S eyes derived from less total PER accumulation than in wild-type eyes, PER levels were analyzed in the heads of these two genotypes during the accumulation phase (i.e., the night). Most of the PER that is detected by immunoblot analysis of heads is produced in the eyes (Zeng et al., 1994), so immunoblot analysis of heads detects PER that is principally in the nuclei or cytoplasm of photoreceptors.

It has been shown previously that an important role of DBT is to signal degradation of PER by causing it to become phosphor-

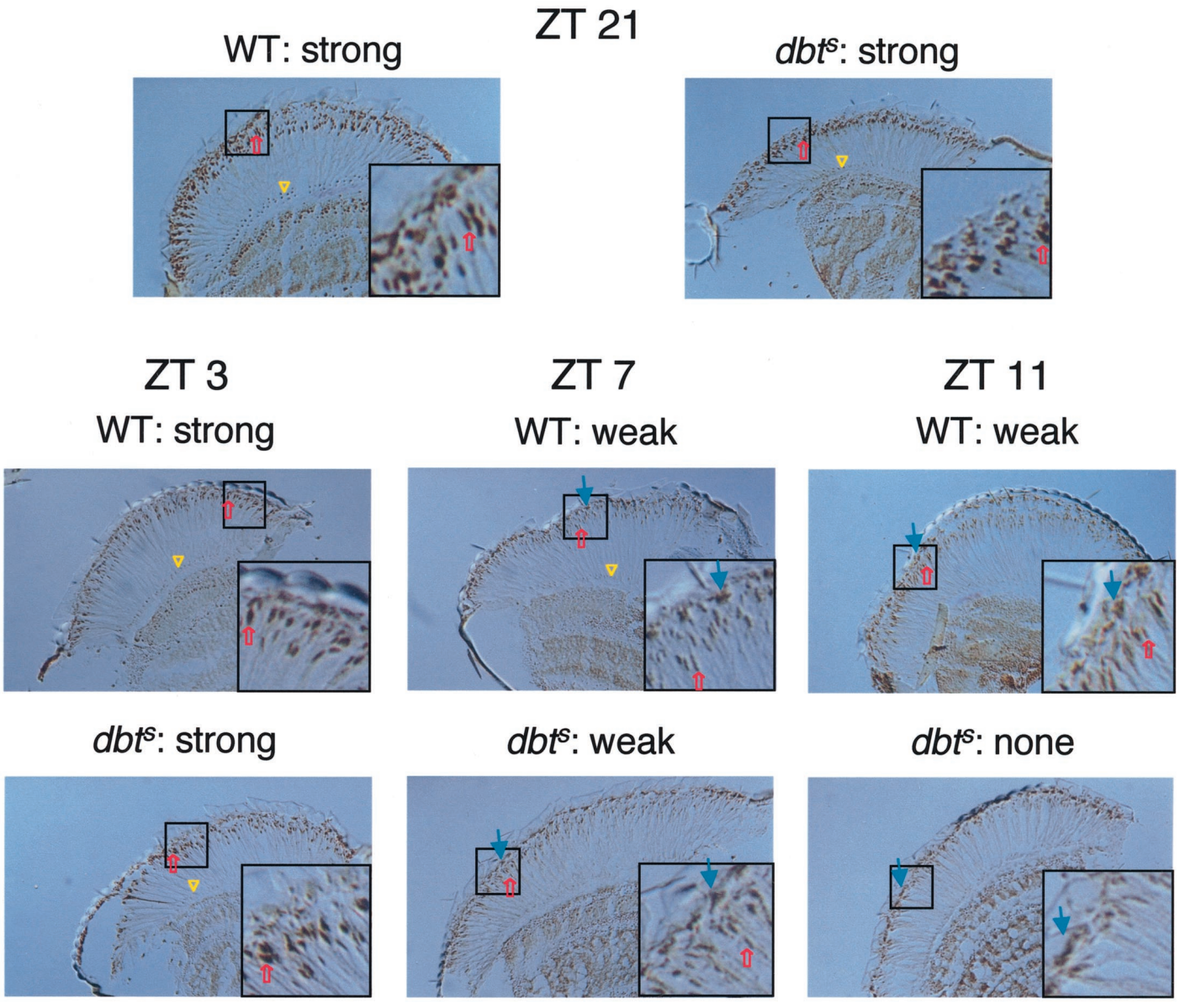

Figure 3. Comparable levels of nuclear PER are found from late night to early morning in dbt^S and wild-type eyes, with larger declines during the middle to the end of the day in dbt^S eyes. Fly heads were collected, processed, and scored as described in the legend to Figure 2. Refer to the Figure 2 legend for an explanation of the labels in this figure. High levels of nuclear PER staining were observed in both wild-type and dbt^S eyes from ZT21–3, with a mixture of weak and strong levels from ZT5–7 (see also Table 2). From ZT11–13 (ZT11 shown here; see also Table 2), weak or undetectable levels of PER immunoreactivity were obtained in both genotypes, with a higher proportion of unstained dbt^S eye sections than wild-type eye sections. The overall staining scores of many sections are tabulated in Table 2.

ylated (Kloss et al., 1998; Price et al., 1998). Consistent with this hypothesis, immunoblot analysis of head extracts has shown that dbt^S leads to both more rapid phosphorylation and disappearance of PER during its circadian cycle, suggesting that the increased rate of phosphorylation leads to an increased degradation of PER during the decline phase (Price et al., 1998). Also consistent with this hypothesis are the slower phosphorylation and delayed disappearance of PER in long-period *dbt* mutants and the high level and hypophosphorylated state of PER in the dbt^P *and* dbt^{AR} mutants, which are nulls or strong hypomorphs (Price et al., 1998; Rothenfluh et al., 2000b; Suri et al., 2000). Our finding that nuclear PER declines to generally lower levels in the photoreceptor nuclei of dbt^S flies than wild-type flies (Table 2) further suggests that the dbt^S mutation decreases the stability of nuclear PER.

A priori, a decreased overall stability of PER in dbt^S eyes could also cause the effect of the dbt^S mutation on the accumulation of nuclear PER. Overall PER levels may accumulate more slowly or peak at lower levels in dbt^S heads than in wild-type heads, thereby precluding detection of nuclear PER in the mutant until later in the night. A previous immunoblot analysis of PER demonstrated that PER accumulates somewhat sooner in dbt^S heads than in wild-type heads (Price et al., 1998). However, it is not clear from this previous analysis how the actual levels of PER compare in dbt^S and wild-type heads during the interval when PER is accumulating to higher levels in nuclei of wild-type photoreceptors than in nuclei of dbt^S photoreceptors (ZT15–19) (Fig. 2, Table 2). Here, our immunoblot analysis compares the levels of PER in wild-type and dbt^S head extracts on adjacent lanes of the same

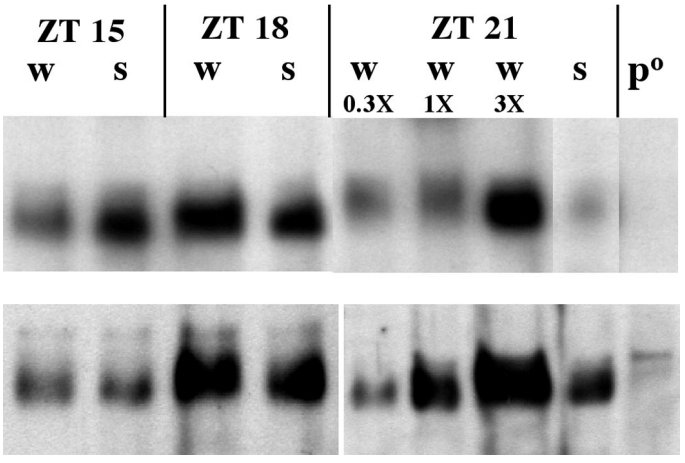

Figure 4. Immunoblot analysis of PER levels in head extracts demonstrates that the delay in accumulation of nuclear PER in dbt^S eyes is not the result of a delayed or reduced accumulation of total PER protein levels. Wild-type (*W*), dbt^S (*S*), or per^o (p^o) flies were frozen in liquid nitrogen at the indicated times (*ZT*; ZT0 = lights on, ZT12 = lights off). Extracts were prepared from the heads of these flies, electrophoresed on SDS-polyacrylamide gels, and subjected to immunoblot analysis with an antibody to PER (see Materials and Methods). Two representative experiments are shown. Sixty micrograms of extract were electrophoresed in each lane, except as indicated (*3X* = 180 μg, *0.3X* = 20 μg). During the time at which PER accumulates in wild-type photoreceptor nuclei more than in dbt^S photoreceptor nuclei (ZT15–18), the total amount of PER in the dbt^S heads is higher than or comparable to the amount in wild-type heads. Note in particular the higher level of PER at ZT15 in dbt^S, when immunohistochemical detection of nuclear PER is at its trough in dbt^S and significantly weaker than in wild-type eyes (Fig. 2, Table 2). By contrast, total PER levels are lower in dbt^S at ZT21 than in wild type, although the amount of nuclear PER is indistinguishable (Fig. 3, Table 2). The *gap* in the *bottom panel* indicates that the ZT21 and per^o samples were on a separate gel from the other samples (the relative amounts and mobilities of samples on different gels cannot be directly compared). The populations of flies that were processed for this immunoblot were also processed for immunohistochemical detection of PER at ZT15, and the immunohistochemical detection was weak in dbt^S and strong in wild type. (These sections are part of the data set tabulated in Table 2.)

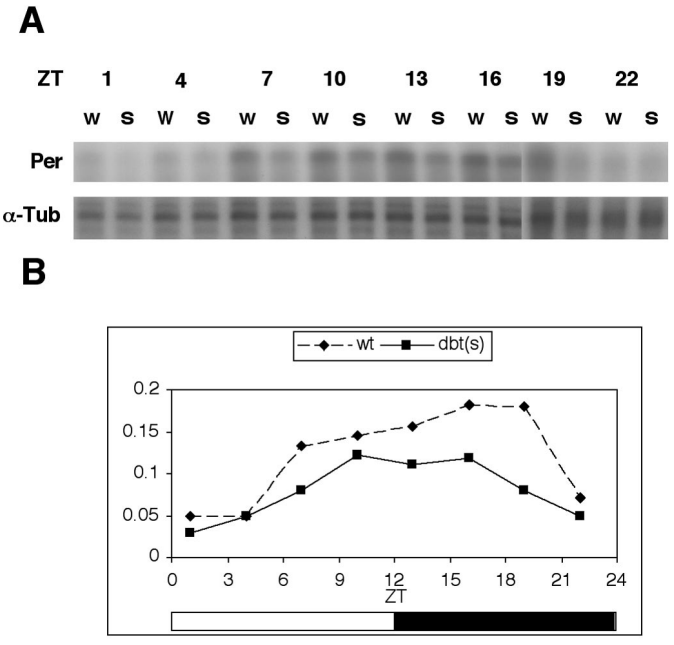

Figure 5. RNase protection analysis of *per* RNA in heads demonstrates that *per* RNA accumulates later and declines sooner in dbt^S heads than in wild-type heads. Wild-type (*W*) and dbt^S (*S*) flies were frozen in liquid nitrogen at the indicated times (*ZT*; ZT0 = lights on, ZT12 = lights off). RNA was isolated from the heads of these flies, and 20 μg of RNA for each time point were analyzed for expression of *per* RNA and α-tubulin RNA (*α-Tub*, a constitutive control). *A* shows a representative analysis that was visualized by exposure to film, and *B* shows the quantitation of these signals by a phosphorimager analysis. For each time point, the normalized signal (*per* signal/α-tubulin signal) is plotted.

blot to determine whether PER levels are generally lower in dbt^S heads than in wild-type heads from ZT15–18 (Fig. 4). This analysis demonstrates that the levels of PER expression are comparable in both genotypes, or perhaps higher at ZT15 in dbt^S heads than in wild-type heads, as predicted by the earlier accumulation in dbt^S heads (Price et al., 1998). By contrast, nuclear levels of PER are generally higher in wild-type eyes than in dbt^S eyes during this time period (Fig. 2, Table 2). Because expression of PER in the head comes principally from photoreceptors (Zeng et al., 1994), these immunoblot analyses argue that equivalent overall levels of PER are expressed in photoreceptors at times when higher levels of PER have accumulated in the nuclei of wild-type photoreceptors than dbt^S photoreceptors. It is also noteworthy that nuclear anti-PER immunoreactivity does eventually reach qualitatively similar levels in dbt^S and wild-type photoreceptors by ZT21 (Fig. 3, Table 2), when total PER levels are lower in dbt^S heads than wild-type heads (Price et al., 1998) (Fig. 4). Together, these results support the argument that dbt^S specifically delays accumulation of nuclear PER rather than downregulating the total amount of PER that accumulates during this interval. Although higher levels of PER accumulate sooner in dbt^S eyes than in wild-type eyes, the earlier increase in PER is not detectable immunohistochemically in the nuclei of dbt^S eyes, presumably because PER is diffusely localized in the cytoplasm at these times.

In wild-type eyes, peak levels of nuclear PER in photoreceptors also lag the peak in total PER (Zerr et al., 1990; Edery et al., 1994; Zeng et al., 1994). These results argue that even in wild-type flies there is a lag between accumulation of PER in the cytoplasm and accumulation of PER in nuclei. In small neurons (e.g., the lateral neurons of the central brain), previously published data have directly indicated that PER accumulates for some time in the cytoplasm of wild-type flies before moving to the nucleus. However, at the times during which differences are seen between wild-type, per^S, and dbt^S photoreceptors (ZT15–18), we have found that PER in the lateral neurons is difficult to detect and reproducibly quantitate with our reagents. Therefore, our analysis here is restricted to the photoreceptors, which are much more numerous than the lateral neurons and easy to identify by their distinctive morphology.

RNase protection analysis demonstrates that *per* RNA accumulates later and declines sooner in dbt^S heads than in wild-type heads

It has been proposed that one role for nuclear PER is negative regulation of the *per* and *tim* promoters (Zeng et al., 1994). Because PER accumulates later in nuclei of dbt^S flies than wild-type flies, this negative feedback should occur later in dbt^S flies than in wild-type flies. As a consequence, *per* mRNA might decline later in dbt^S flies than in wild-type flies. To test this prediction, we monitored *per* RNA and tubulin RNA levels in the heads of wild-type and dbt^S flies around the clock. Tubulin RNA, which is constitutively expressed, served as a control; the signal

for *per* was normalized to that of tubulin to correct for loading or quantitation errors (representative experiment shown in Fig. 5). Surprisingly, *per* RNA declined sooner in the heads of dbt^S flies than in the heads of wild-type flies, while the accumulation phase was delayed in dbt^S flies. Hence, dbt^S disrupts the wild-type phase relation between PER protein and *per* mRNA, which begins to decline only after PER becomes nuclear in wild-type flies. Moreover, the delayed increase in *per* mRNA in dbt^S results in *per* mRNA and PER protein accumulations (Fig. 4) (Price et al., 1998) that are not separated by as much lag as in wild-type flies. Similarly, two dbt^L mutations eliminate the lag between *per* mRNA and protein accumulation in LD (Suri et al., 2000). These results are consistent with modification of the feedback of PER on its mRNA in the dbt^S mutant. The implications are discussed further in the next section.

DISCUSSION

The long circadian period of per^L is not necessary for its defects in temperature compensation

In *Drosophila*, the strongest temperature compensation defects have previously been seen in long-period mutants, i.e., per^L (Konopka et al., 1989), tim^{rit} (Matsumoto et al., 1999), per^{SLIH} (Hamblen et al., 1998), and dbt^L (Rothenfluh et al., 2000a). Short-period mutations [per^S (Konopka et al., 1989), per^T (Konopka et al., 1994), per^{CLK} (Dushay et al., 1990), tim^S (Rothenfluh et al., 2000a), and dbt^S (this study)] have not affected temperature compensation as strongly. Moreover, genetic suppressors of long-period mutants have also suppressed the lack of temperature compensation (Rutila et al., 1996; Matsumoto et al., 1999). However, long-period length is not sufficient to produce lack of temperature compensation, because most long-period *tim* alleles have no effect on temperature compensation (Rothenfluh et al., 2000a). Our results in the present study demonstrate that a long period is also not necessary for strong temperature compensation defects, because a strong defect is manifest in per^L;dbt^S rhythms in which the period is shorter than in wild-type rhythms (Table 1). Therefore, there is not a clear correlation between period length and temperature compensation. Despite several intriguing correlations between temperature compensation and various alterations in PER structure (Price, 1997), no simple hypothesis for temperature compensation is consistent with all of the data. It seems increasingly possible that temperature compensation is produced by the composite functioning of the complete circadian system and that it is possible to disrupt it at many steps in the system.

dbt affects both the stability and the daily gating of nuclear accumulation of PER

As outlined previously, it has been shown that DBT can act in the cytoplasm to destabilize PER (Kloss et al., 1998; Price et al., 1998), thereby leading to a lag in both the accumulation of PER and its negative feedback that may be essential for rhythmic gene expression. DBT also regulates the stability of PER during its decline (Price et al., 1998; Rothenfluh et al., 2000b; Suri et al., 2000). Similarly, phosphorylation of mammalian PER by casein kinase I epsilon leads to more rapid turnover of PER (Keesler et al., 2000; Lowery et al., 2000), and inhibition of phosphorylation of the *Neurospora* clock protein FRQ leads to increased stability of FRQ and longer circadian periods (Liu et al., 2000).

However, the data presented here suggest that DBT is involved in other features of PER regulation as well. Immunohistochemical detection of PER shows that it accumulates in photoreceptor nuclei later in dbt^S eyes than in wild-type and per^S eyes. Previous work has shown that the amount of nuclear PER and TIM is regulated temporally and not merely dictated by the levels of either protein; both PER and TIM require a protein–protein interaction with each other for nuclear accumulation (Vosshall et al., 1994; Curtin et al., 1995; Hunter-Ensor et al., 1996; Myers et al., 1996; Saez and Young 1996), and the oscillation in the level of nuclear PER lags the oscillation of total protein even in wild-type flies (Zerr et al., 1990; Edery et al., 1994; Zeng et al., 1994). Because *dbt* can be mutated to affect this lag, DBT plays a role in generating it. The delayed accumulation of nuclear PER is not attributable to slower total accumulation or a lower peak level of total PER protein, because levels of PER are actually as high (or higher) in dbt^S heads than in wild-type heads at times when there is more nuclear PER in wild-type eyes than dbt^S eyes.

It is possible that dbt^S delays the nuclear accumulation of PER by specifically destabilizing nuclear PER more than does the wild-type *dbt* allele. There is evidence that nuclear PER is less stable in dbt^S flies, because it declines to lower levels in dbt^S photoreceptors than in wild-type photoreceptors (Fig. 3, Table 2). However, nuclear PER appears to accumulate to comparable levels in wild-type and dbt^S flies by ZT21, so it is possible that any effects of dbt^S on nuclear stability of PER are restricted to certain times in the cycle. Temporal gating of the nuclear stability of PER would be one mechanism for regulating its nuclear accumulation. Alternatively, the process of nuclear translocation may be temporally gated, and *dbt* may affect this process. PER may be held in the cytoplasm longer in dbt^S eyes than in wild-type eyes.

A similar role for mammalian casein kinase I epsilon has been proposed by Vielhaber et al. (2000), because co-expression of this isoform in cultured cells prevents the localization of mPER1 to nuclei. Our work extends this finding to a functioning circadian clock. In cultured mammalian cells, casein kinase I binds to mPER1 and phosphorylates a region that then masks the nuclear localization signal of PER (Vielhaber et al., 2000). In *Drosophila*, DBT may also serve to antagonize nuclear localization of PER, and DBT^S would then be a stronger antagonist than wild-type DBT. As in mammals, this antagonism could be mediated in *Drosophila* by phosphorylation and/or protein–protein interactions involving DBT.

However, in *Drosophila*, extensive phosphorylation of PER is neither necessary nor sufficient to keep it in the cytoplasm. For instance, in the tim^o and tim^{rit} mutants, PER is hypophosphorylated, and yet it is preferentially cytoplasmic in these mutants (Vosshall et al., 1994; Price et al., 1995; Matsumoto et al., 1999). Furthermore, in wild-type heads, PER is predominantly nuclear at times when it has the highest level of phosphorylation (Zerr et al., 1990; Edery et al., 1994). So an association with TIM may be required for nuclear localization of PER even when it is not phosphorylated. Because in the complete absence of TIM, PER does not accumulate to high levels (Price et al., 1995), it is possible that high levels of cytoplasmic PER are associated with TIM, and this association with TIM may eventually override the phosphorylation-dependent restraints on nuclear accumulation of PER. Alternatively, it is possible that the TIM/PER association alone is not enough for nuclear accumulation and that another factor is required. For instance, a specific phosphorylation profile (i.e., which amino acids are phosphorylated) rather than just the amount of phosphorylation may confer cytoplasmic or nuclear accumulation for PER, and both DBT and TIM might affect this profile.

Although nuclear entry of PER is delayed in dbt^S eyes during the LD cycle, the PER cycle is expedited by dbt^S in DD to produce a short circadian period, demonstrating that *dbt* regulates multiple features of the temporal program of PER

The circadian period of dbt^S behavior and molecular oscillations is 6 hr shorter than the wild-type period (Price et al., 1998). The mechanism of this period-shortening is likely to share features of the mechanism affected by short-period *per* mutations. The PRC analysis (Fig. 1) suggests that it is the subjective day (the period in DD which follows the first 12 hr after lights out) that is shortened by both the dbt^S mutation and the per^S mutation. The per^S mutation has been shown to cause a more rapid decline in the levels of nuclear PER during this time (Zerr et al., 1990; Curtin et al., 1995), and nuclear PER declines to lower levels in dbt^S flies during this time (Table 2). Other behavioral and molecular analyses of short-period *per*, dbt^S, and dbt^{AR} mutations have argued that all of these mutations affect turnover of nuclear PER (Rothenfluh et al., 2000b).

However, the later accumulation of *per* RNA and nuclear PER in dbt^S flies than in per^S flies supports the argument that dbt^S has additional effects that are not caused by short-period *per* mutations. Schotland et al. (2000) have shown that deletion of several putative phosphorylation sites in PER renders PER defective in negative feedback. It is possible that phosphorylation by DBT also regulates the capacity of PER for feedback. If the capacity of PER for negative feedback were reduced in dbt^S flies, the effect would be predicted to lead to an early accumulation in *per*/*tim*. And yet, *per* mRNA accumulates later in dbt^S flies (Fig. 5). Likewise, *per* mRNA declines sooner in dbt^S flies than in wild-type flies, despite a delay in accumulation of nuclear PER in dbt^S flies. So there is no evidence for a delay or reduction in the negative feedback loop of dbt^S flies. dbt^S might enhance the intrinsic capacity of PER to exert negative feedback, thereby counteracting the reduced negative feedback it causes by reducing the levels of nuclear PER at several times of day. However, the most parsimonious hypothesis accounting for all of our data is that dbt^S decreases the positive feedback loop in which PER participates (Bae et al., 1998; Glossop et al., 1999) by reducing nuclear PER levels during the late day and early evening. PER positively regulates the transcription of *dClk*, which elevates levels of *per* mRNA because dCLK is a transcription factor that binds to the *per* promoter. The delayed nuclear accumulation of PER in dbt^S and the reduced levels of nuclear PER from ZT7–15 would be predicted to produce less positive feedback during these times and therefore less *per* mRNA from the positive feedback loop. In DD, there is less decline in nuclear PER of dbt^S heads during the subjective day (E. Bjes and J. L. Price, unpublished data), which could conceivably produce more positive feedback during this time and accelerate the cycle.

The present study shows that *dbt* affects posttranscriptional regulation of PER at the level of nuclear accumulation, in addition to the previously demonstrated effects on cytoplasmic stability. *dbt* therefore affects multiple aspects of the PER temporal program, and it is possible that further analysis will reveal additional aspects of clock biochemistry that are regulated by *dbt*.

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