Wild-Type Circadian Rhythmicity Is Dependent on Closely Spaced E Boxes in the *Drosophila timeless* Promoter

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Transcriptional regulation plays an important role in *Drosophila melanogaster* circadian rhythms. The *period* promoter has been well studied, but the *timeless* promoter has not been analyzed in detail. Mutagenesis of the canonical E box in the *timeless* promoter reduces but does not eliminate *timeless* mRNA cycling or locomotor activity rhythms. This is because there are at least two other *cis*-acting elements close to the canonical E box, which can also be transactivated by the circadian transcription factor dCLOCK. These E-box-like sequences cooperate with the canonical E-box element to promote high-amplitude transcription, which is necessary for wild-type rhythmicity.

Many eukaryotic and some prokaryotic organisms regulate their metabolisms, physiology, and behavior with a circadian (~24-h) period. These rhythms have been shown to involve complex feedback circuits involving transcriptional regulation in a diverse set of organisms, including fungi, plants, cyanobacteria, insects, and higher mammals such as mice and humans. These feedback loops appear inextricably linked to the oscillation of central pacemaker components with circadian periodicity.

The current model for the circadian pacemaker of *Drosophila melanogaster* (10, 11, 44) posits that two basic helix-loop-helix PAS (for Period-Arnt-Sim) domain-containing transcription factors, CLOCK (dCLK) and CYCLE (CYC), activate transcription of the *period* (*per*) and *timeless* (*tim*) genes by binding to E-box elements (CACGTG) in the promoter regions of these genes (1, 3, 9, 42). Levels of *per* and *tim* mRNA increase throughout the day and peak in the early evening (19, 45). PER and TIM proteins reach their peak some 4 to 6 h later (12, 56). PER levels accumulate slowly, due to destabilization through phosphorylation by the *doubletime* protein kinase (DBT), a *D. melanogaster* homolog of mammalian casein kinase 1 ϵ (25, 31, 38). Increasing amounts of TIM lead to PER stabilization through dimerization (39). PER-TIM dimers then translocate to the nucleus (43, 53), where they then interact with dCLK and CYC (2, 28). Formation of this complex decreases dCLK-CYC-mediated transcription and invokes the negative limb of the pacemaker feedback loop by preventing dCLK-CYC binding to the E box (9, 29). This negative feedback is abrogated by light-mediated TIM degradation, which restarts the next cycle (22, 30, 34, 50, 55, 56). There might also be positive feedback, in which PER and TIM lead to an increase in transcription factor synthesis (16a, 28).

Strong genetic evidence from mammals supports the notion that the mCLOCK (dCLK orthologue) and BMAL1 (CYCLE orthologue) family of transcription factors is central to pace-

maker transcriptional regulation. mCLOCK-BMAL1-dependent transcriptional cycling has been shown previously for *mper1*, *mper2*, *mper3*, *mcry1*, *mcry2*, *dbp*, and *vasopressin* transcripts, which fail to oscillate in mCLOCK mutant mice (23, 24, 26, 35, 40). In cell culture reporter assays, mCLOCK-BMAL1 has been shown to activate transcription from E boxes in the *mper1*, *vasopressin*, and *dbp* promoters (16, 23, 26, 40). In *Drosophila*, the importance of dCLK-CYC-mediated transcription is readily observable in the clock mutants *jrk* and *cyc*⁰, which are arrhythmic and lack oscillations in both *per* and *tim* mRNA (1, 42). Furthermore, S2 cell transfection assays demonstrate that dCLK-CYC activates transcription through E boxes in the *period*, *timeless*, and *vri* promoters (7, 9).

The most extensive transcriptional studies in *Drosophila* circadian biology have centered on the *period* gene promoter. A promoterless *per* transgene, 7.2, was found to partially rescue arrhythmic *per*⁰ flies (15). The weak molecular cycling of the 7.2 transcript was attributed to posttranscriptional mechanisms (46). It was also demonstrated previously that *per* requires at least two sequences, one in the promoter and one in the 5' coding sequence, to fully rescue wild-type *per*-like cycling and PER expression (48). Hao et al. (18) discovered that a *per* promoter 69-bp clock regulatory sequence (CRS) containing an E box was sufficient to generate 5- to 10-fold oscillations of a *lacZ* reporter RNA. Deletions in either the 5' or 3' half site of the *period* E box along with surrounding nucleotides led to decreases in reporter levels and lower amplitudes of cycling, indicating the importance of the E-box region but also suggesting that sequences other than the E box within the 69-bp CRS contribute to cycling. These sequences have yet to be identified. In S2 cells, dCLK and CYC were shown to activate transcription from a luciferase reporter fused to a tetramerized 14-mer centered around the *per* E box. The activation was blocked by a central 2-bp inversion of the E-box sequence (9). Subsequent experiments have shown that dCLK and CYC physically bind this E-box sequence and that the binding is abrogated by the same central 2-bp inversion in the E-box core sequence (29). These experiments firmly establish the *per* E

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box as a central component of circadian transcriptional regulation.

In contrast, there are no published reports investigating the importance of the *timeless* promoter to the circadian pacemaker. The following study presents a detailed analysis of the elements controlling *tim* transcription. We distinguish between the contributions of two canonical E boxes and two noncanonical E boxes within the *tim* promoter. In addition, we describe a role for a novel sequence element that might contribute to *Drosophila* circadian transcription.

MATERIALS AND METHODS

Constructs. All constructs involving PCR-amplified fragments were confirmed by sequencing. All *tim* promoter-*tim* cDNA constructs originated from a *tim*⁺ rescue transgene (see Fig. 1 in reference 41) referred to here as 4.5-kb *tim*. The 4.5-kb *tim* construct was truncated to a 2.5-kb construct using the *Xba*I site at position 2512. The 1.5-kb construct was made by truncating the construct to the *Aat*II site at position 1587. The constructs that contain 1,082 bp or less of promoter were made by PCR, introducing a *Not*I site at the 5' end (except for *tim*^{DEAD}, in which a *Nsi*I site was used). These fragments were ligated back into the 4.5-kb *tim* parent construct utilizing the 5' *Not*I site (see Fig. 1 in reference 41). The truncation constructs used as reporters in S2 cell transfection experiments were created by PCR using *tim*^{FL} as a template and introducing a *Sac*II site at the 5' end and a *Xho*I site at +26. Three-way ligations were performed among these fragments, a *Sal*I-*Bam*HI luciferase cDNA fragment (pJD261 plasmid [32]), and the pMECA vector (a kind gift from Wayne Parrot, University of Georgia) cut with *Sac*II-*Bam*HI. Substitution constructs were created using PCR, and mainly transversion mutations were created. All mutated DNA elements contain a *Bgl*II site. To make the transformation vectors for luciferase-bearing flies, the S2 cell constructs were digested with *Sac*II-*Bam*HI and cloned into those sites of the pCaSpeR 4 transformation vector (52). The *per* constructs were made using PCR with PLO-LUC (8) as a template. These PCR products contain *Sac*II sites at their 5' end (−713) and *Sal*I sites at their 3' end (+9) and were cloned with luciferase into pMECA as described above. In S2 cell transfections, the pAC-dCLK construct used was created by introducing a *Not*I site at position 3029 in pBS-SK(−) clock by site-directed mutagenesis. An *Eag*I digest of this cDNA clone was then ligated into the *Not*I site of Invitrogen's pAc5.1-V5/HIS vector.

Transgenic flies. *y w* virgin females (luciferase constructs) and *y w;tim*⁰ virgin females (rescue constructs) were crossed to *y w*⁰; *KiP*^P Δ(2–3) and *y w;tim*⁰; *KiP*^P Δ(2–3) males, respectively. Flies were kept in bottles for 2 days and then transferred to a collecting cage for 2 days. Embryos were collected every hour and dechorionated with forceps. Embryos were fixed to double-sided tape on a glass slide and grouped by 2-min intervals. After being dried for about 10 min, embryos were covered with a thin layer of halocarbon oil (series 700; Halocarbon Production Corp.). Embryos were injected with Qiagen Midiprep DNA at a concentration of 0.5 μg/μl in 5 mM KCl–0.1 mM PO₄ (pH 7.8) buffer containing 10% green food coloring. Injected embryos were kept in a humidified chamber in a 25°C incubator overnight, and surviving larvae were picked into fresh vials the next day. Adults were crossed to *y w* virgin females or *y w;tim*⁰ virgins (rescue constructs), and transformants were judged by eye color. Transformants were balanced, and then lines were established. The lines analyzed biochemically and behaviorally are as follows: *tim*^{FL.1} (756–43.1), *tim*^{FLmut.1} (756–15.1), *tim*^{FLmut.2} (756–23.1), *tim*^{MIN.1} (624–39.1), and *tim*^{DEAD.1} (522/Y). When requesting lines, please request the numerical identifier for the line.

Transfection assays. S2 cells were maintained at high density in HyQ-SFX insect medium (HyClone Laboratories, Inc.) supplemented with 10% fetal bovine serum (GIBCO) and 10 mg of penicillin-streptomycin (Sigma) per ml. S2 cells at a density of 10⁶ per ml in HyQ-SFX-lacking serum were plated in six-well tissue culture plates (Costar Corp.). Twenty-four hours later, the medium was aspirated and replaced with 1 ml of transfection mix. This mix consists of HyQ-SFX-lacking serum containing 20 μl of Geneporter reagent (Gene Therapy Systems), 500 ng of reporter DNA, 25 ng of *Renilla reniformis* luciferase reporter under the control of the *copia* promoter, and either 100 ng of pAC-clockV5 or empty vector incubated at room temperature for 45 min. Transfection was stopped by adding 1 ml of serum containing HyQ-SFX medium. Cell lysates were prepared with Promega's dual luciferase reporter assay system per the manufacturer's instructions. *Photinus pyralis* luciferase activity was measured with a Wallace, Inc., TD20/20 luminometer and normalized to the *Renilla* luciferase activity.

Fly head Western blots. Flies were collected at the appropriate time point, and protein extracts were prepared from the homogenization of 30 heads in 30 μl of extraction buffer (12). PER and TIM Western blots were performed as described in reference 56. Western blots were quantified using the Molecular Analyst program (Bio-Rad).

RNAse protection assays (RPAs). Fifty heads were homogenized with a motorized pestle in 500 μl of TRIZOL reagent (GIBCO). RNA was precipitated according to the manufacturer's suggestions. RNA was resuspended in 50 μl of diethyl pyrocarbonate-treated water–50 μl of DNA digestion mix (40 mM Tris [pH 8], 6 mM MgCl₂, 5 mM NaCl, 5 mM dithiothreitol, 0.05 mg of bovine serum albumin per ml, 0.4 U of RNasin [Promega] per μl, and 0.05 U of RQ1 DNase [Promega] per μl). The mixture was incubated at 37°C for 1 h and then phenol-chloroform extracted twice and precipitated overnight at −80°C. The mRNA was resuspended in 50 μl of hybridization buffer (13). The *period* 2–3, *tim*, and *rp49* probes have been described previously (33, 49). Probes (1 × 10⁶ cpm of *per*2/3 and *tim* probes and 2.5 × 10⁵ cpm of *rp49* probe) were added to each reaction mixture and heated to 95°C for 5 min and then at 50°C overnight. RNA was digested with 15 U of RNase I (Promega) for 1 h at 37°C and then phenol-chloroform extracted twice and precipitated for 1 h at −80°C. The samples were resuspended in 10 μl of loading dye (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue) and resolved on a 5% sequencing gel. Quantification was carried out using a Bio-Rad GS-363 phosphorimager and Molecular Analyst software.

In vivo luciferase monitoring. The assays were performed essentially as described in reference 48. Monitoring of luciferase activity was performed every half hour for 7 days. The first 2 days' data were discarded, and the remaining data were analyzed using the I-and-A program (36). Lines *tim*^{FL-LUC.1} and *tim*^{FL-LUC/PERR.3} each displayed levels that were about twofold higher than those of their respective sister lines and were reproducible in all flies tested in those lines. The enhanced levels in these lines are most likely due to position effects. In our analysis, these were the only cases of significant variations between different lines in the same genotype.

Behavioral analysis. Fly circadian behavior was analyzed as described previously (41).

RESULTS

The *tim* E-box region is dispensable for rhythmic locomotor activity. To begin a study of *tim* transcriptional regulation, we generated several different *tim* promoter-*tim* cDNA transgenic lines in a *tim*⁰ background. In the initial behavioral characterization of these lines, constructs harboring 756 bp or more of *tim* promoter displayed only small differences from a construct bearing 2.5 kb of *tim* promoter (Table 1). Within this 756-bp region of the *tim* promoter, there is a 9-bp canonical E-box sequence, CACGTGAGC, which is conserved in the 69-bp CRS of the *period* gene promoter. Flies with the 756-bp promoter construct had 26-h periods and were named *tim*^{FL}, for *tim* promoter full-length. Flies that carry a *tim*^{FL} transgene with a mutated E box, CTCGAG, termed *tim*^{FLmut} (Fig. 1), displayed substantially longer periods, from 28 to 29.5 h for the three lines tested (Table 1). Lines that contain the minimal amount of *tim* promoter rescuing behavior, *tim*^{MIN}, lack this E box and surrounding region but still manifest periods of ~29 to 30 h, similar to the three *tim*^{FLmut} lines (Table 1). Lines possessing a further truncated promoter, *tim*^{DEAD}, are arrhythmic (Fig. 1). These data allow us to conclude that sequences other than this E box can contribute to *tim* transcription and that there is some important promoter element necessary to achieve behavioral rhythmicity in the region present in *tim*^{MIN} but absent in *tim*^{DEAD}, between −624 and −522 (Fig. 1).

***tim* mRNA levels strongly influence TIM levels and the strength of behavioral rescue.** To assay *tim* mRNA levels in these transgenic flies, heads were collected from entrained flies, sacrificed at peak and trough times, and subjected to mRNA analysis by RPA (Fig. 2). These times were selected

TABLE 1. Rescue of circadian behavior in *tim*⁰ flies with truncated and mutated *tim* promoters^a

Genetic background	Transgenic line	n	% AR	τ
y w		39	18	23.9 $\pm$ 0.3
y w; <i>tim</i> ⁰		49	96	23.2 $\pm$ 1.0
y w; <i>tim</i> ⁰	2512.1	11	0	25.3 $\pm$ 0.4
y w; <i>tim</i> ⁰	2512.2	18	17	26.1 $\pm$ 0.5
y w; <i>tim</i> ⁰	2512.3	13	0	25.2 $\pm$ 0.4
y w; <i>tim</i> ⁰	2512.4	19	0	25.9 $\pm$ 0.5
y w; <i>tim</i> ⁰	1587.1	12	8	26.5 $\pm$ 0.5
y w; <i>tim</i> ⁰	1587.2	11	18	25.9 $\pm$ 0.7
y w; <i>tim</i> ⁰	1587.3	14	0	26.2 $\pm$ 0.5
y w; <i>tim</i> ⁰	1587.4	16	0	26.6 $\pm$ 0.3
y w; <i>tim</i> ⁰	1082.1	13	0	26.3 $\pm$ 0.3
y w; <i>tim</i> ⁰	1082.2	13	15	25.8 $\pm$ 0.4
y w; <i>tim</i> ⁰	<i>tim</i> ^{FL.1} (756)	12	8	26.6 $\pm$ 0.5
y w; <i>tim</i> ⁰	<i>tim</i> ^{FL.2}	11	27	26.6 $\pm$ 0.5
y w; <i>tim</i> ⁰	<i>tim</i> ^{FLmut.1} /Y	29	3	28.2 $\pm$ 0.6
y w; <i>tim</i> ⁰	<i>tim</i> ^{FLmut.2}	8	12	29.0 $\pm$ 0.8
y w; <i>tim</i> ⁰	<i>tim</i> ^{FLmut.3}	8	0	29.5 $\pm$ 0.5
y w; <i>tim</i> ⁰	<i>tim</i> ^{MIN.1} (624)	25	20	29.3 $\pm$ 1.0
y w; <i>tim</i> ⁰	<i>tim</i> ^{MIN.2} /Y	12	50	29.9 $\pm$ 1.3
y w; <i>tim</i> ⁰	<i>tim</i> ^{MIN.3} /+	5	0	29.5 $\pm$ 1.6
y w; <i>tim</i> ⁰	<i>tim</i> ^{DEAD.1} /Y (522)	40	83	26.9 $\pm$ 1.2
y w; <i>tim</i> ⁰	<i>tim</i> ^{DEAD.2} /+	31	100	
y w; <i>tim</i> ⁰	<i>tim</i> ^{DEAD.3} /+	35	100	
y w; <i>tim</i> ⁰	<i>tim</i> ^{DEAD.4}	35	100	

^a n represents the number of flies analyzed, % AR represents the percentage of arrhythmic flies (cutoff: power, <10; width, <2; see reference 14 for power and width definitions), and τ represents the period of the behavioral circadian rhythms. The names of the lines 2512, 1587, 1082, 756, 624, and 522 correspond to the length (in base pairs) of the *tim* promoter present in the transgene used to create these lines. “/+” indicates lines tested as heterozygotes (transgene over wild type; “/Y” indicates lines tested as males and containing transgene insertions on the X chromosome. All other lines were tested as homozygotes.

based on an extended time point analysis for each strain (data not shown). Biochemical analysis was performed on a representative line from each genotype, but two *tim*^{FLmut} lines were analyzed because they showed a 1-h period difference.

In wild-type flies, *tim* mRNA cycles 5.5-fold in 12-h-light–12-h-dark conditions (LD) and 7.25-fold in constant-dark conditions (DD) (Fig. 2). These values are lower than previously

published data (20, 45, 46), probably due to relatively high background levels and consequent difficulties in accurately quantifying mRNA at trough time points. Cycling amplitudes and peak *tim* mRNA levels progressively diminish from line *tim*^{FL.1} to *tim*^{FLmut.1}, *tim*^{FLmut.2}, *tim*^{MIN.1}, and *tim*^{DEAD.1}, in both LD and DD. The residual *tim* mRNA cycling in lines *tim*^{FLmut.1} and *tim*^{FLmut.2} (Fig. 2) indicates that sequences other than this E box contribute to circadian transcription. Line *tim*^{MIN.1} has barely detectable *tim* mRNA levels (visible on the autoradiograms but not in Fig. 2) that do not appear to cycle, suggesting that low levels of noncycling *tim* mRNA are adequate to rescue behavior (Fig. 2). *tim* mRNA in line *tim*^{DEAD.1} was not detectable, indicating that a critical region of the promoter is present in *tim*^{MIN.1} but absent in *tim*^{DEAD.1} (Fig. 2A and C), i.e., between –624 and –522.

We also assayed cycling from the *per* and *tim*⁰ genes in these transgenic strains. Both mRNAs cycle with similar amplitudes in CS, *tim*^{FL.1}, *tim*^{FLmut.1}, and *tim*^{FLmut.2} (except *tim*⁰ in CS, because there is no *tim*⁰ gene in this strain) (Fig. 2). The amplitude is reduced in lines *tim*^{MIN.1} and *tim*^{DEAD.1}, principally due to an increase in mRNA levels at trough time points (Fig. 2A and C). This is probably a consequence of decreased negative feedback on these promoters due to low TIM levels (Fig. 3A and B). Western blots show that TIM oscillations closely follow the *tim* mRNA profiles, both in cycling amplitude and in levels (Fig. 2A and C, 3A and B, and Fig. 4). The transgenic lines show progressively lower amounts of TIM, from *tim*^{FL.1} through *tim*^{MIN.1}. This correlates well with progressively weaker behavioral rescue, both in period length and in percentage of arrhythmic flies. Line *tim*^{DEAD.1}, which is arrhythmic, has barely discernible levels of TIM (Fig. 3A and B, Fig. 4, and Table 1).

The cycling amplitude and levels of PER were moderately affected in lines *tim*^{FL.1}, *tim*^{FLmut.1}, and *tim*^{FLmut.2} compared to CS (Fig. 3C and D). In line *tim*^{MIN.1}, PER does not cycle in LD but does cycle in DD (Fig. 3C and D). An extended time point analysis in this strain confirmed that PER levels in LD are moderately high and stable and do not undergo cyclic

FIG. 1. Diagram of *tim* promoter-*tim* transgenes. Shown are the *tim* promoter-*tim* constructs (right) used to obtain the lines analyzed (left). The genomic sequence reaches 3' to the *SalI* site at +2206 and then is fused to the *tim* cDNA. *tim*^{FL} and *tim*^{FLmut} begin at –756, *tim*^{MIN} begins at –624, and *tim*^{DEAD} begins at –522. The E-box and mutant E-box sequences are shown. The transcription start site is marked by the arrow.

FIG. 2. LD and DD *tim* and *per* mRNA profiles of *tim* promoter-*tim* transgenic lines. The mRNA levels for *per*, *tim*, *tim⁰*, and *rp49* were measured in *tim* promoter-*tim* transgenic lines and Canton S flies by RPA in LD (12-h light; 12-h dark) and DD (constant darkness) conditions. “*tim*” denotes the transgene mRNA, “*per*” and “*tim⁰*” are endogenous *per* and *tim⁰* mRNAs, and “*rp49*” is the normalization control. The genotypes are indicated. (A) A representative RPA of fly heads from flies sacrificed at high (ZT15) and low (ZT3) zeitgeber time points in LD cycles. (B) Quantitation of *per* and transgenic *tim* mRNAs from four LD experiments. (C) A representative RPA of fly heads from flies sacrificed at high (CT15) and low (CT3) circadian time points in the first day of DD after entraining for 3 days of 12:12 LD cycles. (D) Quantitation of *per* and transgenic *tim* mRNAs from two DD experiments.

phosphorylation, whereas PER levels in DD cycle both in amplitude and in phosphorylation state and have a lengthened period as expected (data not shown). In summary, *tim* mRNA levels largely determine TIM levels, and these determine the strength of behavioral rescue and period length.

The intronic E box does not significantly contribute to *tim* transcription in S2 cells. The murine *dbp* and *per1* promoters, as well as the human *per1* promoter, have multiple E boxes that contribute to transcriptional activation (16, 40, 51, 54). In *Drosophila*, *period* and *vri* also contain multiple E boxes in their

promoters, although functional roles for all of these E boxes have not been confirmed (7; M. J. McDonald and M. Rosbash, unpublished observations). Through sequence searching, we found an additional canonical E box in the first intron of *timeless* at bp +1810. To assess whether this E box also contributes to *tim* transcription, we generated *tim* promoter-luciferase fusions with the upstream and intronic E-box regions mutated in various combinations (Fig. 5A) and compared the transcriptional activities of the constructs with an S2 cell-dCLK cotransfection assay (9).

FIG. 3. PER and TIM protein levels in *tim* promoter-*tim* transgenic lines. Representative LD and DD Western blots for PER and TIM in the *tim* promoter-*tim* transgenic lines. Protein extracts were made from heads of flies collected at high (ZT19 and CT19) and low (ZT7 and CT7) time points. Arrows denote the band of interest. Western blots from LD experiments were probed for TIM (A) and then stripped and reprobed for PER (C). Western blots from flies sacrificed on the first day of DD entrainment were probed for TIM (B) and then stripped and reprobed for PER (D).

In these S2 cell experiments, an intronic E-box mutation had little effect on dCLK-mediated transcriptional activation of constructs with either an intact or mutated upstream E box. In fact, addition of the intronic E-box region decreased transcription twofold (Fig. 5A, compare timp^{FL-LUC} to timp^{FL-LUC/wt}), suggesting that the intronic E box does not play an important role in *tim* transcription. This is consistent with the fact that timp^{DEAD.1} flies do not produce detectable levels of *tim* mRNA or rescue behavior and is consistent with the very weak activity of the timp^{DEAD-LUC} construct in S2 cells (Table 1, Fig. 2A and C, and Fig. 5B).

Although consistent with previously published observations (7, 9), the very weak activation of the timp^{FLmut-LUC} reporter by dCLK is misleading; luciferase activity from this construct in dCLK-transfected cells is about one-half to two-thirds of the activity of the intact E box, the timp^{FL-LUC} construct (Fig. 5A

and data not shown). This parallels the data for flies, in which peak *tim* mRNA levels in timp^{FLmut} flies are 50 to 60% of the levels in timp^{FL} flies (Fig. 2 and data not shown). The weak fold activation in S2 cells is due to the baseline luciferase activity of the timp^{FLmut-LUC} being ~24-fold higher than that of the timp^{FL-LUC} reporter in non-dCLK-transfected cells (Fig. 5A, far right panel). All constructs tested with a mutated upstream E box exhibit dramatically enhanced reporter activity in non-dCLK-transfected cells (Fig. 5A, far right panel). This might reflect a transcriptional repressor that recognizes an intact E box in S2 cells. In contrast, there are not large increases in *tim* mRNA levels at the trough time point ZT3 or CT3 in timp^{FLmut.1} or timp^{FLmut.2} flies (Fig. 2A and C) compared to levels for timp^{FL} flies. This suggests that the elevated baseline transcription is an S2 cell phenomenon and that other mechanisms exist in flies to suppress *tim* transcription in the

FIG. 4. Quantification of TIM Western blots. Shown are the results of quantitation of TIM protein levels from the different *tim* promoter-*tim* transgenic lines under LD and DD conditions (three experiments). The graph represents the amplitude of TIM cycling in head extracts between high (ZT19 and CT19) and low (ZT7 and CT7) time points.

FIG. 5. Truncation and substitutional mutagenesis of *tim* promoter. Black bars represent the induction of luciferase activity from *tim*-luciferase transgenes in S2 cells cotransfected with 100 ng of pAC-dCLK (see Materials and Methods). Standard deviations are indicated. (A) The contributions of the upstream and intronic E boxes were measured by mutating the E boxes (mutated E boxes are crossed out). In the far right panel, white bars represent the values of luciferase activity measured from reporters in non-dCLK-cotransfected cells (baseline values). The baseline value of the *tim*^{FL}-LUC construct was normalized to the baseline value obtained from the *tim*^{FL}-LUC construct. The baseline values for all intron-containing constructs were normalized to the baseline value obtained for the *tim*^{FL}-LUC/wt intron construct. (B) Sequence of the *tim* promoter region -756 to -522. The sequence elements that were mutated are boxed, and their names as referred to in the text are shown above the boxes. (C) Truncation constructs of the *tim* promoter. The elements are outlined by boxes with the name of the element above the box. (D) Substitutional mutagenesis of the elements within the *tim* promoter, as shown in panel C. The *tim*^{FL}-LUC ΔE-box construct contains an AGATCT substitution for the E box (CACGTG), whereas *tim*^{FL}-LUC contains the sequence CTCGAG.

the W box or another nearby sequence element plays a small role in *tim* transcription (Fig. 5C). Truncating the promoter to $\text{tim}^{\text{687-LUC}}$ causes a dramatic 20-fold decrease in reporter activity compared to that of $\text{tim}^{\text{FL-LUC}}$ (Fig. 5C). This decrease is most likely due to deletion of TER1 and not to a disruption of the E box itself (see below). Removal of six additional nucleotides up to the 5' border of the E box, $\text{tim}^{\text{681-LUC}}$, causes a further decrease in transcriptional activation, suggesting that some of these six upstream nucleotides are also important for dCLK-mediated transcription (Fig. 5C). This construct still shows an eightfold level of activation. Construct $\text{tim}^{\text{672-LUC}}$, which completely removes the E-box sequence, eliminates transcriptional activation by dCLK, indicating that the E box itself is still functional in the absence of any upstream *tim* sequence (Fig. 5C). Further truncations have smaller effects, suggesting that most of the regulation goes through the E box and TER1.

TER1 and PERR play major roles in *tim* transcription in S2 cells. Because results from deletions can be incomplete or misleading, we eliminated these putative sequence elements primarily with transversion mutations that did not create known transcription factor binding sites. Similar sequences were used in the mutagenesis of each element in order to minimize artifacts. These mutated *tim* promoter constructs were cotransfected with dCLK into S2 cells.

Mutating the W-box element demonstrated that it plays little if any role in *tim* transcription. However, transcriptional activation decreased about eightfold with a mutation in either TER1 or PERR (Fig. 5D). For TER1, the results correlate well with the truncation of that region, which caused a 20-fold decrease (Fig. 5C, compare constructs $\text{tim}^{\text{701-LUC}}$ and $\text{tim}^{\text{687-LUC}}$). Mutating the TER2 element had little or no effect on dCLK-mediated transcription (Fig. 5D). The double mutant TER1/TER2 led to a further threefold decrease, indicating that weak effects of TER2 may be masked by TER1 (Fig. 5D).

The PERR box plays a role in transcriptional activation of the *period* promoter in S2 cells. We also assayed the role of the PERR box in transcriptional activation by substitutional mutagenesis in the *period* promoter. The PERR box is upstream of the E box in *per* and downstream of the E box in *tim* but nearly the same distance from both E boxes (Fig. 5B and 6A). We examined four constructs in cotransfection assays with dCLK in S2 cells. A full-length construct, perp^{FL} , gives rise to a 5.8-fold activation when cotransfected with dCLK (Fig. 6B). perp^{DEL} , created by deleting the region -137 bp to -32 bp in perp^{FL} , increased activation to 26-fold. A similar deletion enhanced transcriptional activity from a *period*-LacZ fusion protein in flies (18) and presumably reflects the absence of an inhibitory sequence element. Mutation of the PERR box in the context of perp^{DEL} decreased transcriptional activation 10-fold (Fig. 6B), similar to the approximately eightfold decrease in reporter activity observed for the *tim* promoter containing a mutated PERR box (Fig. 5D). Mutation of the E box in perp^{DEL} caused a nearly identical 10-fold decrease in transcriptional activation of the *per* promoter (Fig. 6B).

In vivo promoter element analysis indicates that TER1 and TER2 play a major role in *tim* transcription. To assay the in vivo role of the PERR and TER elements, we analyzed four $\text{tim}^{\text{FL-LUC}}$ transgenic lines and TER1 (four lines), PERR

FIG. 6. Mutagenesis of the PERR box in the *per* promoter in S2 cell assays. The role of the PERR box in the context of the *period* promoter in the S2 cell transfection assay was analyzed. (A) Sequence map of the *per* promoter. Elements are outlined by boxes with names above the boxes. (B) Within these constructs, the region between -137 and -32 (bounded by half arrows) was deleted in the constructs indicated (denoted by the single whole arrow). Mutant E boxes are crossed out and have the sequence CTCGAG. The PERR box, E box, and W box are shown. Black bars represent the induction of luciferase activity from *tim*-luciferase transgenes in S2 cells cotransfected with 100 ng of pAC-dCLK.

(three lines), TER1/PER (three lines), and TER1/TER2 (three lines) mutants. Figure 7 shows the average luciferase activity for each genotype from a group of flies within a representative line. In all luciferase recordings, there is a second peak. This peak has been previously described by others (4, 8, 48) and is a function of the assay conditions (8, 48).

In the $\text{tim}^{\text{FL-LUC}}$ lines, luciferase activity cycles robustly with a periodicity of 24 h. The TER1 mutants manifest about 50% of the total luciferase activity of $\text{tim}^{\text{FL-LUC}}$ flies (Fig. 7A and B). In TER1/TER2 double mutants, luciferase activity is even lower, approximately 20 to 30% of that of $\text{tim}^{\text{FL-LUC}}$ flies (Fig. 7D). In these strains, cycling is nearly abolished, indicating that TER2 makes significant contributions to cycling and transcriptional activation in the absence of TER1. The PERR mutant lines were almost indistinguishable from $\text{tim}^{\text{FL-LUC}}$ flies (Fig. 7C). The TER1/PERR double mutants exhibit a slight reduction in luciferase activity levels compared to those for TER1 mutants, but cycling amplitude appears unaffected, suggesting that PERR functions differently in the adult fly than in S2 cells (Fig. 7E; also see the Discussion). The in vivo analysis validates the importance of the TER1 and TER2 boxes as well as the canonical E box in *tim* transcriptional regulation.

FIG. 7. In vivo functional analysis of *tim* promoter elements. Flies contained *tim* promoter-luciferase fusions. Each graph shows the average luciferase activity (y axis) of flies ($n = 12$) from a representative line for the genotype indicated. All experiments were done under LD conditions (12 h of light [white boxes]; 12 h of dark [black boxes]); zeitgeber times are denoted on the x axis. Mutations in the *tim* promoter are the same as those used in S2 cell assays. (A) Line containing a normal 756-bp *tim* promoter, designated *tim*^{FL-LUC}. (B) Line containing a mutagenized TER1 box. (C) Line containing a mutagenized PERR box. (D) TER1/TER2 double mutant line. (E) TER1/PERR double mutant line.

DISCUSSION

Noncanonical E-box-dependent transcription has been described extensively for the Myc family of binding proteins, whose canonical high-affinity binding site has been determined as CACGTG by sequential selection and amplification of binding sites (5, 6, 37). These studies led to the identification of lower-affinity, noncanonical MYC-MAX binding sites, such as

CATGTG, CACGCG, CATGCG, CACGAC, and CAACG TG. Grandori et al. (17) also found that MYC-MAX dimers were able to bind a similar set of sequences in vivo, in a tumorigenic cell line. A similar observation was recently reported for mCLOCK/CYC (40). Four E boxes were identified in the first and second introns of the mammalian *dbp* gene, whose gene product is important in generating the cycling

A. General mechanism

B. Developmental Tissues or Cell Specific mechanism

FIG. 8. Molecular model of *per* and *tim* transcription. (A) It may be in vivo that the majority of cells undergo a general process in which the TERs and E-box elements serve as the primary loading sites for dCLK and CYC and the PERR box plays a minor role in transcription. (B) If expression of the protein or protein complex that binds the PERR box were tissue specific or developmentally restricted, then that protein would interact with the proteins binding to the TERs and the E box, probably dCLK and CYC, and increase transcription of *per* and *tim*.

transcription of several circadian genes in the liver (27). All four E boxes were shown to activate a luciferase reporter in cell culture assays upon transfection with mCLOCK and BMAL1, but only two of the E-box regions showed circadian differences in DNase I-hypersensitive sites: one with a canonical CACGTG motif and the other with a noncanonical CAC ATG motif. Our study provides definitive evidence that a non-canonical E box contributes to circadian transcription in *Drosophila*.

We have also reaffirmed the idea that multiple E boxes contribute to circadian transcription. Previously, the study of the *mper1* promoter had demonstrated a role for multiple canonical E boxes (16, 54). However, the *tim* promoter is unique, as it possesses three functional E boxes within a short distance, about 150 bp. This combination of multiple elements likely allows important protein-protein interactions, which contribute to the stronger amplitude of *tim* than of *per* transcriptional cycling (46). The observed direct correlation between behavioral period length and *tim* mRNA levels suggests that this more robust *tim* transcription amplitude is crucial for proper 24-h behavioral rhythmicity. These interactions could also accelerate the kinetics of transcription factors binding to the *tim* promoter and be responsible for *tim*'s earlier peak of transcription (46). This also has important implications for the phase of PER accumulation, as TIM counteracts the effects of DBT on PER stability (V. Suri and M. Rosbash, unpublished results).

The TER boxes each have the identical sequence GCGGC ACGTTG. In S2 cells, the TER1 mutations, CAAGTTG and CTCGAAG, cause the same eightfold deficit in transcription as does deletion of the entire sequence by transversion mutations (data not shown), demonstrating the necessity of an intact noncanonical E box for proper function. These data suggest that TER1 and probably TER2 serve as additional binding sites for the dCLK-CYC transcription factor complex. It has been shown previously that the mammalian homolog of CYC

(BMAL1 or MOP3) can bind multiple members of the PAS domain family (21). Therefore, dCLK or CYC could also dimerize with another partner to bind the TER1 element with high affinity. Alternatively or in addition, another protein could bind to the 5' side of the TER motif (GCGG) and stabilize the dCLK-CYC heterodimer on the 3' adjacent noncanonical E box.

We also identified an E box in the first intron of the *tim* gene. We were unable to detect any activity of this sequence in S2 cells, and a transgenic construct lacking the whole TER E-box region but containing this intronic E box failed to generate detectable *tim* mRNA levels. This indicates that the intronic E box is probably nonfunctional, for unknown reasons. Nevertheless, we still cannot rule out the possibility that the TERs and the E box collaborate with other sequence elements. However, transgenic flies with only the region of the *tim* promoter containing TER1 and the E box fused to luciferase exhibited robust transcriptional cycling (data not shown). These two elements can therefore work independently of any other *tim* promoter sequence. TER1 alone may even be able to drive transcriptional cycling, as a mutation in the E box did not completely eliminate circadian oscillations of luciferase activity (data not shown).

Do the two TERs have different functions? TER1 appears to be more important than TER2. Both in vivo and in S2 cells, disrupting TER1 leads to strong effects on the transcriptional activity of the *tim* promoter. In addition, examination of the TER1/TER2 double mutant suggests that TER2 is somewhat redundant in regard to TER1. This could be due to a distance effect: TER1 is closer to the E box, and factors bound to these motifs could be interacting more strongly. Disruption of TER1 has a substantial effect on expression levels (decrease of ~50%) but little or no effect on cycling amplitude. In contrast, mutagenizing both TERs has a detrimental effect on cycling amplitude as well as expression levels. We therefore propose that both TERs are important determinants of *tim* mRNA

levels and their oscillations and thus contribute to proper rhythmicity. The observation that transgenic flies lacking an E box but containing TER1 have less arrhythmicity than do flies without both elements further supports this notion (Table 1).

We noticed another element conserved within the *timeless* and *period* promoters. This PERR box has the sequence GTT CGCAGAA, which does not correspond to any known transcription factor binding site described in the literature. Mutating this element in the context of the *tim* promoter-S2 cell assay led to decreases in transcription comparable to those caused by a mutation in the TER1 box. Activation of the double TER1/PERR mutant was equal to that of the TER1 and PERR single mutants, suggesting that these two elements might collaborate to enhance *tim* transcription in S2 cells. Importantly, mutation of the PERR box led to a similar ~10-fold decrease in transcription in the context of the *period* promoter.

These data strongly suggest that the PERR box plays a role in *per* as well as *tim* transcription. However, PERR box mutant flies have at most subtle differences in luciferase activity compared to wild-type, *tim*^{FL-LUC} flies. As S2 cells are derived from embryos, the PERR box might play a role during development rather than in adult flies (Fig. 8). Alternatively, PERR could contribute to tissue-specific transcriptional regulation of *per* and *tim* (Fig. 8). If this element were active in only a small subset of cells, it would explain why mutations have no observable effect in the luciferase assays.

Our study therefore shows the importance of *tim* regulation for proper 24-h rhythmicity. Circadian period is very sensitive to *tim* mRNA levels, with more *tim* mRNA generating periods closer to 24 h. *per* mRNA cycling follows the same trend, as *per* mRNA trough levels are progressively lowered with increasing *tim* mRNA. Near-wild-type TIM levels are therefore required for accurate negative feedback on PER transcription. Lower TIM levels probably delay PER stabilization and nuclear entry and therefore lengthen the period. However, the amount of *tim* mRNA required for rhythmicity is rather low, and the levels generated by the minimal promoter are probably very close to this threshold. It is somewhat surprising that these periods are not longer than ~29 to 30 h, suggesting that *tim* levels can affect period only within a narrow range. *tim* mis-sense mutations can have a much larger effect on period (40a). In the *tim*^{MIN} line, it is also surprising that PER is not cycling under LD, whereas it cycles in DD. Perhaps TIM levels fall just below threshold in LD, because TIM is degraded by light. PER cycling in *tim*^{MIN.1} would therefore be due to the increased amount of TIM in DD (data not shown), which is sufficient to generate PER stabilization and accumulation and yield a quasi-normal cycling profile.

It will be of considerable interest to monitor precisely the kinetics of PER and TIM nuclear entry in lines with very limited amounts of TIM, both in peripheral oscillators and in the circadian pacemaker cells (the ventral lateral neurons). This could solidify the relationship between PER and TIM relocalization on the one hand and period length on the other. It will also be important to verify that expression levels and cycling of PER and TIM in the lateral neurons, the cells responsible for behavioral rhythmicity, parallel what is observed in the biochemical assays.

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