Novel Insights into the Regulation of the Timeless Protein

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In the *Drosophila* circadian clock, *period* (*per*) and its partner, *timeless* (*tim*), play a central role in the negative limb of an autoregulatory feedback loop. Unlike *per*, the dosage of which affects the frequency (τ) of the circadian cycle, we found that increasing copies of the *tim* gene has no effect on clock period length. The use of the *tim* promoter to express *per* results in a shortening of circadian period, also indicating that the regulation of *tim* is different from that of *per*. *Drosophila* TIM is similar to the mammalian circadian protein mPER2 in that it shuttles independently between the nucleus and cytoplasm both *in vivo* and *in vitro*. Contrary to the current model that PER and TIM heterodimerization is a prerequisite for their nuclear entry, PER is not required to transport TIM into nuclei, although it influences TIM localization and vice versa. Blocking nuclear export led to increased nuclear expression of TIM in S2 cells and in wild-type and *per*⁰¹ larvae, suggesting that PER may be required for nuclear retention of TIM. Unlike PER, nuclear TIM alone has no ability to repress transcription. We propose that TIM drives cycles of PER expression by regulating its stability, and in turn, PER retains TIM in the nucleus, either for the regulation of its own stability or for a novel nuclear role of TIM.

Key words: period; timeless; rhythms; cycle; biological clocks; transcription factors; nuclear export; LMB

Introduction

Nearly all organisms have an endogenous 24 hr circadian clock. This clock is normally entrained to the organism's environment by light or other environmental cues and controls daily hormonal, behavioral, and social rhythms. In *Drosophila melanogaster*, the clock that controls rest–activity rhythms is located in the brain, predominantly in the small ventrolateral neurons (LN_s). Within these LN_s, *period* (*per*) and *timeless* (*tim*) transcript and protein levels oscillate with a daily rhythm (for review, see Williams and Sehgal, 2001; Stanewsky, 2003). PER and TIM form a heterodimer (PER–TIM) that enters the nucleus midway through the night and negatively regulates *per* and *tim* transcription by blocking the activity of transcriptional activators *Clock* (*dClk*) and *cycle* (*cyc*). The inhibition is released in the morning when the levels of TIM and PER decrease.

Between these two proteins, the main focus of the field has been on PER regulation and function. Early on, Smith and Konopka (1982) found that changing the gene dosage of *per* altered the period of the clock in an inverse manner; decreasing *per* copy number lengthened the period of the clock, and vice versa. This led to the hypothesis that PER is a state variable of this system, a timekeeper molecule with levels that indicate to the organism what time of day it is. The partner of PER, TIM, is important both for PER function [PER is unstable when not bound to TIM and requires TIM for nuclear expression (Vosshall

et al., 1994; Price et al., 1995; Saez and Young, 1996)] and as a point of photic input to the clock. TIM is actively degraded at dawn through light-independent as well as light-triggered mechanisms (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996; Naidoo et al., 1999). However, other than its effects on PER, the role of TIM in the actual clock mechanism has not been addressed. Is TIM a state variable of the clock? Can TIM repress transcription in the absence of PER? These are the questions we initially sought to answer in this study.

We examined the role of TIM in the circadian clock in two ways. First we addressed whether changes in TIM levels and TIM–PER levels, or both, affect the length of the circadian period and found that the clock is not sensitive to *tim* dosage. Second, to determine the role of TIM in feedback regulation, we assayed the ability of nuclear TIM to affect activity of dCLK and CYC in the absence of PER. Without PER, TIM showed no ability to repress transcription. We also discovered that TIM, like the mammalian clock protein mPER2 (Yagita et al., 2002), is regulated via active CRM1/Exportin 1-dependent nuclear export mechanisms. PER does not appear to be regulated by this pathway. Dimerization with PER is not a prerequisite for nuclear entry; however, PER is necessary for nuclear retention of TIM. Although PER is also affected by TIM localization, PER is probably the main target of mechanisms that control the timing of nuclear entry.

Materials and Methods

Generation of transgenes. Tim 4 and Tim 7 constructs were described in Ousley et al. (1998). Tim 4 contains 4.3 kb of the upstream sequence of the *tim* gene fused to a *tim* cDNA. Tim 7 contains the same promoter region as Tim 4, but most of the coding portion has been replaced with genomic sequence. To generate Per 1, we first cloned an *EcoRI* fragment containing full-length *per* cDNA from pEMBL into BC1. BC1 was constructed by cloning the *EcoRI*–*PstI* fragment (563 bp) of pCaSpeR-hs (Thummel and Pirrotta, 1992) into pBluescript. This allowed us to excise *per* from BC1 with *StuI* (originally from pCaSpeR-hs) and *KpnI* (origi-

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nally from pBluescript) and clone it into the unique sites *Swa*I and *Kpn*I behind the *tim* promoter in pCaSpeR4. Thus, Per 1 contains the identical *tim* upstream promoter region as Tim 4 and Tim 7, fused to *per* cDNA (see Fig. 2).

To allow nuclear expression of TIM in the absence of PER, two different constructs were generated using a *tim* cDNA capable of rescuing behavioral rhythms subcloned into the *hsp70* promoter-driven expression vector pCaSpeR-hs, described in Naidoo et al. (1999). In the first modified construct, we added a nuclear localization signal (NLS) to the C-terminal end of full-length *tim*. A PCR fragment containing the sequence for the SV40 Large T-antigen NLS (PKKKRKV) was amplified from a *tim* cDNA using the following primers: 3'-(5'-ATGCGATATCTCATACCTTACGCTTCTCTTAGGGTGATAGTGGGGCACCCGGATCTCGGTTTCGCTCAAGTC-3') and 5'=(5'-GGCCGTGGAGAAGGCACACG-3'). The 3' primer contained the NLS and an *Eco*RV restriction site. The resulting fragment was cloned into the *Eag*I and *Eco*RV restriction sites of *tim* and named *hs-tim*^{NLS}. The second nuclear *tim* construct, *hs-tim*ΔCLD, had the C-terminal 150 amino acids, identified as the cytoplasmic localization domain (CLD) (Saez and Young, 1996) removed. The *tim* *Eag*I–*Eco*RV fragment was replaced with a PCR product that lacked the CLD (codon 1228 was replaced by a stop codon). The primers 5'=(5'-GGCCGTGGAGAAGGCACACG-3') and 3'=(5'-CTATAGTCGACGAGGAGCCAAACGCA-3') were used to generate this PCR fragment. These two modified constructs were then cloned into the multiple cloning site of a P(pCasPer-hs) vector and injected into *yw*, *KiΔ2–3* flies for germline transformation.

pAct-*dClock*, pAct-*dper*, and *perE-hsp-luc* were provided by Steve Kay and the *ie* β-galactosidase (β-gal) reporter was provided by K. P. Gopinathan.

Preparation of transgenic strains. Tim 4, Tim 7, and Per 1 constructs were inserted into the *Drosophila* genome through P element-mediated transformation. *Yw/yw*; *KiΔ2–3* eggs were injected with the constructs. Multiple independent insertion lines containing each transgene were created from surviving injected eggs. The Tim 4–7 and Per 1-A and 1-B lines were crossed to create fly lines containing multiple copies of *per* and *tim* transgenes in wild-type backgrounds.

Locomotor assays. Flies between 1 and 14 d old were isolated from stocks and entrained in a 12 hr light/dark (L/D) incubator at 25°C for at least 3 d. Each fly was then inserted into individual locomotor tubes supplemented with 5% sucrose, 1% agar. The flies were transferred to dark/dark, and their locomotor activity was monitored using the Trikinetics system. Actogram and periodogram analyses of Tim 4, Tim 7, Per 1, and Per 1 × Tim 4 locomotor data were performed using the Tau program.

Antibodies and Western analysis. Antibodies used in this study were UPR 8 (Hunter-Ensor et al., 1996), UPR 34, and TIM 991. UPR 34 was raised in rats using full-length PER expressed in the baculovirus system as an immunogen. Rabbit anti-pigment dispensing factor (PDF) antibodies were raised using synthesized amidated PDF peptide.

Adult flies were isolated from stocks and placed in 5% sucrose, 1% agar bottles for collection. They were then entrained for at least three full cycles in a 12 hr L/D incubator at 25°C. Flies were then collected at either Zeitgeber (ZT) 16.5 (PER Westerns) or ZT 21 (TIM Westerns) on dry ice. Protein extracts were prepared from isolated fly heads by homogenization using a small handheld homogenizer (IKA Labortechnik) on ice in homogenization buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1 × Roche Complete protease inhibitor mixture, prepared according to manufacturer's instructions). Protein samples (80 μg) were loaded onto a 6% SDS-polyacrylamide gel, separated, and transferred overnight to a nitrocellulose blot. The blots were blocked either for 1 hr at 25°C or overnight at 4°C using blocking solution (8% milk, 2% BSA in PBS). Primary antibodies were diluted in the same blocking solution at 1:1000 for UPR 8 and 1:10,000 for UPR 34. Blots were then incubated with secondary antibodies (HRP-conjugated anti-rat) at a dilution of 1:1000, and PER/TIM was visualized with an ECL detection kit (Amersham Biosciences, Arlington Heights, IL). After stripping, blots were restained with commercial monoclonal antibodies to HSP70 (Sigma, St. Louis, MO) for use as a loading control in PER blots.

S2 cell transfections and immunofluorescence. For staining, *Drosophila* S2 cells were seeded onto six-well plates at ~1 × 10⁶ cells per well. After

6–12 hr, 500 ng of *hs-tim* or pAct-*per* DNA, or both, was transfected into the cells via Cellfectin-assisted transformation (Invitrogen, San Diego, CA). Forty-eight hours after transfection, cells were heat shocked at 37°C for 30 min to induce TIM expression. In some experiments the cells were heat shocked with leptomycin B (LMB) (or vehicle) added to the medium (at a final concentration of 1–10 ng/ml, as indicated in Figure legends). Two hours after induction, cells were gently rinsed with 1 × PBS, spun at 1000 × g for 1 min, resuspended in 100–200 μl of 1 × PBS, and spread on 22 × 50 mm coverslips (~100 μl each slip). After 10 min, the PBS was gently removed, and attached cells were fixed in fresh 4% paraformaldehyde in PBS. Cells were rinsed with PBS and then blocked for 1 hr in blocking solution (10% normal donkey serum, 0.1% Triton X-100 in PBS), incubated in primary antibody (1:10,000 in block), and rinsed three times in PBS (10 min each). Cells were then incubated in secondary antibody (FITC-conjugated donkey anti-rat and/or cyanine 3 (CY3*)-conjugated donkey anti-rabbit, each diluted 1:10,000 in block) for 1 hr, rinsed three times (10 min), and mounted in 4'-6'-diamidino-2-phenylindole (DAPI)-containing medium (Vector Laboratories, Burlingame, CA).

Each sample slide was scored blind (50 cells per slide), using DAPI to determine the approximate location of the nucleus. Cells were divided into categories: cells in which most of the TIM/PER signal was nuclear, cells in which most of the signal was cytoplasmic, and cells in which the signal was distributed evenly (uniform). Some slides were scored independently by a second experimenter, and the results were compared for consistency.

In vitro transcription assays. The effects of various TIM deletions on dCLK-CYC-mediated transcription in S2 cells were tested as described in Darlington et al. (1998) with a few modifications. A luciferase reporter plasmid, *perE-hsp-luc* (E-box *luc*), containing four tandem repeats of *per* E-box and a minimal *hsp* promoter (10 ng) was transfected into S2 cells using Cellfectin (Invitrogen). Cotransfection of pAct-*dClock* (1 ng) activated transcription from the E-box reporter construct. The effect of PER and TIM on dCLK-CYC-mediated transcription was tested by cotransfecting pAct-*dper* and *hs-tim* (or one of the nuclear *hs-tim* constructs). Twenty-five nanograms of a β-gal reporter gene under a baculoviral immediate early gene promoter were used as a transfection control, and the total DNA amounts per well were kept constant by adding pAct (empty vector only). S2 cells (0.75 million per well, in 12-well plates) were incubated with the DNA–Cellfectin mix in a serum-free medium (Life Technologies). After 3 hr 10% FBS containing medium was added. In some experiments, cells were heat shocked once every 12 hr for 30 min at 37°C. LMB was added to the medium during the heat shocks, at a final concentration of 5 ng/ml. In these cases the cells were allowed to recover in the presence of LMB for 4 hr, after which the medium was replaced with fresh LMB-free medium. Cells were harvested 48 hr after transfection, washed in PBS, and lysed in 1 × lysis buffer (Promega). Aliquots of the cell extracts were assayed for β-galactosidase and luciferase activities (Tropix and Promega, respectively) in a luminometer (Hewlett Packard Top Count). The average ratios of luciferase activity to baseline β-galactosidase activity were calculated and normalized. Each experimental condition was done in triplicate, and the mean and SEM of three independent experiments were calculated.

Whole-mount immunofluorescence. Populations of developing *per*⁰¹ and *yw* *Drosophila* were entrained to light/dark cycles for 4 d before collection of third-instar larvae. Entrained larvae were collected on ice (in the dark) at times indicated in Results. Whole larval brains were dissected on ice in Schneider's *Drosophila* cell culture medium supplemented with 10% FBS and proteosomal inhibitor MG132 [200 μM Z-Leu-Leu-Leu-H (Peptide Institute, Inc.) in DMSO], with or without Leptomycin B (in 70% methanol; Sigma). Tissue was then incubated with supplemented medium for 1.5–5 hr (as indicated in Results and Figs. 4, 5) in foil-wrapped 12-well cell culture plates, with shaking. Brain tissue was then fixed for 20 min with 4% paraformaldehyde in PBS and washed three times with PBS-Triton X-100 (0.5%) for 20 min each. Brains were blocked for 1 hr using 10% normal donkey serum in PBS. To all tissues, anti-TIM (UPR8 1:2500) or anti-PER (UPR34 at 1:500) and rabbit anti-PDF (1:1000) antibodies were added and incubated overnight at 4°C. The following day the brains were washed three times for 45 min

Table 1. Activity rhythms in flies expressing multiple copies of TIM

	Genotype	No. of copies TIM	n (No. of rhythmic)	Average period ± SEM	% Rhythmic
Locomotor data from Tim 4 lines <i>tim</i> ⁺ / <i>tim</i> ⁺ background	Tim 4-1	++	36 (26)	23.21 ± 0.21	72.2
		++	47 (42)	23.21 ± 0.12	89.4
		++TT	49 (42)	23.35 ± 0.10	85.7
	Tim 4-6	++	30 (14)	23.67 ± 0.21	46.7
		++T	22 (15)	23.71 ± 0.09	68.2
		++TT	26 (22)	23.71 ± 0.13	84.6
	Tim 4-7	++	12 (12)	23.79 ± 0.14	100
		++T	72 (68)	23.60 ± 0.06	94.4
		++TT	90 (81)	23.63 ± .046	90
	Tim 4-7 × Tim4-1	++TT	12 (9)	24.11 ± 0.33*	75
		++TTT	54 (45)	23.58 ± 0.10	83.3
		++TTTT	76 (70)	23.57 ± 0.06	92.1
	Tim 4-7 × Tim4-6	++TT	16 (16)	23.66 ± 0.19	100
		++TTT	72 (65)	23.36 ± 0.06*	90.3
		++TTTT	96 (76)	23.38 ± 0.07*	79.2
Locomotor data from Tim 7 lines <i>tim</i> ⁰¹ / <i>tim</i> ⁰¹ background	Tim 7-5	T	18 (14)	24.00 ± 0.15*	77.8
		TT	39 (29)	24.22 ± 0.07*	74.4
	Tim 7-4	TT ^a	49 (38)	24.30 ± 0.06*	77.6
<i>tim</i> ⁺ / <i>tim</i> ⁺ background	Tim 7-5	++	10 (6)	23.33 ± 0.11	60.0
		++T	16 (14)	23.39 ± 0.09	87.5
		++TT	17 (16)	23.09 ± 0.15	94.1
	Tim 7-4	++	22 (19)	23.00 ± 0.19	86.4
		++TT ^a	43 (40)	23.20 ± 0.06	93.0

Increased TIM has no effect on period length. To measure locomotor activity, flies were placed in locomotor activity monitors under constant conditions at 25°C for 7 d. + refers to one copy of endogenous *tim*. T refers to one copy of Tim 4 or Tim 7 transgenic *tim*. An asterisk indicates that the average period of this genotype is significantly longer or shorter than *yw* control flies (nontransgenic flies from the line in a wild-type background) according to a Student's *t* test (*p* < 0.05 for all). It is clear that these mild differences are not related to transgene dose and are also not consistent from one Tim 4 transgenic line to another.

^a Because Tim 7-4 was located on the X chromosome, one copy of this transgene was counted as two because of dosage compensation in males.

with rocking. Brains were incubated with secondary antibodies at 1:500 (cyanin 3-conjugated anti-rabbit and fluorescein isothiocyanate-conjugated anti-rat; Jackson ImmunoResearch, West Grove, PA) for 1 hr at room temperature. Tissue was then washed for 45 min three times and mounted with VectaShield (Vector Laboratories).

Sample tissue slides were scored blind. For each head, the total number of visible lateral neurons was counted (on the basis of the PDF stain). These were then given a rating on the basis of the clarity of the morphology and whether the cytoplasmic-nuclear boundary could be determined on the basis of PDF staining alone, with "0" being unscorable and "+" being clear. Each + neuron was then scored for TIM or PER visibility and clarity. Only lateral neurons in which most of the TIM or PER signal was clearly visible as more nuclear, more cytoplasmic, or uniformly distributed were counted in the final analysis. TIM and PER localization were reported as percentages of the total number of neurons in these three categories. The initial *per*⁰¹ experimental parameters with respect to final DMSO concentration (2–3%), LMB concentration (20–40 ng/ml), and incubation time with LMB (1.5–5 hr) were varied over seven independent experiments, with similar results each time. The *yw* experiments used 3% DMSO and 40 ng/ml LMB, incubated for 1.5 hr.

Motif search in TIM. The reported TIMELESS sequence (id: TIM_DROME) was scanned for common protein motifs using Motif Scan (<http://hits.isb-sib.ch/cgi-bin/PFSCAN>). A Prosite scan (<http://us.expasy.org/tools/scanprosite/>) was used to scan TIM for occurrences of the leucine-rich consensus sequence for CRM1-exportin-dependent nuclear export Lx_(1–3)Lx_(1–3)LxL(V/I/M) (in which "x" indicates any amino acid) (Yagita et al., 2002).

Results

Increasing Tim dosage does not shorten circadian period

Some clock genes, most notably *per*, are dosage sensitive (Smith and Konopka, 1982). Smith and Konopka (1982) showed, through a series of experiments using deletions and duplications of the *per* locus, that there is an inverse relationship between dosage of *per* and length of the circadian period. Transgenic manipulations of PER levels yield similar results (Baylies et al., 1987; Chen et al., 1998; Hao et al., 1999). To determine whether TIM

levels also affect circadian period length in this manner, we increased copies of *tim* in flies via two different transgenes. One transgene (Tim 4) consisted of *tim* cDNA fused to 5 kb of upstream *tim* promoter region; the other (Tim 7) consisted of genomic *tim* also fused to the same promoter region (Ousley et al., 1998). A total of five lines with independent insertions were examined.

Tim 4 has been shown to rescue locomotor rhythms in *tim*⁰¹ flies (Ousley et al., 1998) and does not interfere with normal rhythms in a wild-type background (Table 1). In addition, extra copies of Tim 4 in a wild-type background did not significantly shorten period. The Tim 4 transcript, however, fails to display robust mRNA cycling (Wang et al., 2001), indicating that Tim 4 lacks sequences required for appropriate regulation of *tim*. Thus, there is the possibility that although Tim 4 is producing high levels of TIM protein throughout the head (Fig. 1A), it may not be regulated appropriately enough in the clock-relevant neurons to produce a significant effect. Therefore we also looked at two lines, Tim 7-4 and Tim 7-5, that contain a genomic *tim* transgene and in which the *tim* transcript cycles like wild-type *tim* (Wang et al., 2001). The Tim 7 transgene effectively rescues rhythms in *tim*⁰¹ flies (Wang et al., 2001). Flies with additional copies of Tim 7 expressed in a wild-type background have periods of ~23 hr, but their nontransgenic *yw* siblings also have a 23 hr period, so the shorter periods cannot be attributed to high TIM expression (Table 1). Western analysis of Tim 4 and Tim 7 lines confirmed that TIM is present at higher than wild-type levels (Fig. 1A,B). These data suggest that the function of TIM in the clock is dosage insensitive, which supports previous findings that decreasing *tim* dosage also does not affect period length (Rothenfluh et al., 2000b).

PER and TIM form a heterodimer in a presumably 1:1 ratio (Zeng et al., 1996). Because PER is thought to require TIM as a partner both for stability and to enter the nucleus to effect feed-

Figure 1. TIM and PER levels in overexpression lines. Protein extracted from adult heads was separated on a 6% polyacrylamide gel. TIM levels are increased over wild-type (*yw*) levels in Tim 4 (*A*) and Tim 7 (*B*) lines collected at ZT 21. Spaces indicate noncontiguous lanes from the same blot. Asterisk indicates a common nonspecific band, used here as a loading control. All Tim 4 and 7 lines were tested, but only one representative line each is shown here for simplicity. In *C*, flies were collected at an earlier time point, ZT 16.5. PER levels are unaltered in Tim 4 (lanes 3, 5, and 6) and Tim 7 (lane 4) overexpression lines. PER is only mildly increased in Per 1-A and decreased in Per 1-B. Surprisingly, Per 1-B generally has a stronger behavioral phenotype than Per 1-A (Table 2), which probably indicates that Per 1-B has stronger negative feedback (see Discussion). HSP 70 (bottom blot) was used as a loading control. Another analysis of PER levels at ZT 21 showed similar results.

back (Vosshall et al., 1994; Price et al., 1995; Saez and Young, 1996), we hypothesized that it is not PER that is the state variable in this system, but the PER–TIM heterodimer. Thus, changes in PER that increase or decrease the length of the circadian period in flies do so by decreasing or increasing PER–TIM levels; however, on the basis of the following data we infer that TIM is present in excess and thus not a limiting factor in the *Drosophila* clock: (1) TIM is present in molar excess of PER for part of the night and is present in PER-immunodepleted extracts (Zeng et al., 1996); (2) the *tim* promoter shows an approximately threefold higher transcription rate than the *per* promoter (So and Rosbash, 1997); and (3) alterations of TIM levels alone do not alter period length (Rothenfluh et al., 2000a) (data presented here). If TIM is in excess and PER is the limiting factor, we would expect alterations in TIM levels to affect period length only if PER levels are also increased. Despite the increased TIM levels in our Tim 4 and Tim 7 transgenic lines, we did not see altered PER levels over wild type (Fig. 1C), which explains the lack of a short-period phenotype. It might be possible, however, by transgenically manipulating PER levels, to increase PER to the point at which TIM becomes limiting. When Smith and Konopka (1982) increased *per* dosage via duplications, they found that they never shortened the period of the clock by >1.5–2 hr even with five copies of endogenous PER. We hypothesized, therefore, that in the Smith and Konopka study (1982) TIM became limiting when PER levels were increased, and thus the period could not be shortened beyond a certain point.

We intended, therefore, to raise both PER and TIM levels together. To this end, we expressed a *per* cDNA under control of the same *tim* promoter region used in the Tim 4 and Tim 7 lines because *tim* appeared to be the stronger promoter (So and Rosbash, 1997). The Per 1 transgenes rescue rhythms in *per*⁰¹ flies, with a period that is 1–2 hr shorter than wild type (Table 2), as would be expected from lines that overproduce PER. Per 1 also shortened period in a wild-type background, the Per 1-B insertion generally more so than Per 1-A. Two copies of the Per 1-B transgene shortened period to 22 hr and also decreased the proportion of rhythmic flies in the line.

We then crossed Tim 4 flies to Per 1 flies with the goal of increasing both PER and TIM together. If TIM becomes limiting at high PER concentrations, then increasing both proteins should shorten the period even further. As is evident from the locomotor data in the second half of Table 2, additional copies of Tim 4 into the Per 1 lines did not shorten period any further than Per 1 alone. Statistical analysis of all groups by two-way ANOVA revealed a significant effect of the Per 1 transgenes on period, but no significant effect of Tim 4. We conclude, therefore, that the clock is relatively insensitive to *tim* dosage, and TIM is generally present in excess for most of the night (until levels decline around dawn). Manipulation of *per* levels can only shorten the period of the clock so far (~2 hr) before either another clock factor becomes limiting or some other critical regulatory mechanism is overcome and the molecular cycle runs into arrhythmia.

Immunohistochemistry of adult heads from Per-1 lines revealed PER expression in cells that normally express PER and TIM (data not shown). Surprisingly, Western blot analysis showed only a small increase in steady-state PER levels in the Per 1A line and a decrease in the Per1B line (Fig. 1C). The lack of a robust increase of PER levels in these lines could be attributable to amplified negative feedback by the overproduced PER, which would ultimately result in decreased protein levels and an accelerated cycle. There may also be differential *per* expression in peripheral oscillators versus LN_vs, such that whole-head extracts show lower than expected PER levels. In these lines, however, PER is driven by the same promoter that drives elevated TIM expression in Tim 4 and TIM 7 lines (Fig. 1A,B). Therefore we favor the hypothesis that excess PER is negatively regulating its own expression but TIM is not (see Discussion).

TIM cannot repress dCLK-CYC-mediated transcription unless PER is present

It is thought that PER must bind to TIM to gain entry into the nucleus and effect transcriptional changes (Vosshall et al., 1994; Saez and Young, 1996). Both proteins contain an NLS as well as a CLD and removing the CLD from either protein frees it from the dimerization requirement for nuclear entry (Saez and Young, 1996); however, PER does not require TIM to exert its effects on transcription. Rothenfluh et al. (2000a) demonstrated that deletion of the CLD allows PER to repress dCLK-CYC-activated transcription in the absence of TIM, and flies expressing this altered PER display a high degree of arrhythmicity (Rothenfluh et al., 2000a). Also, they showed that maximal transcriptional repression in flies occurs during the time that TIM has degraded and only PER remains (Rothenfluh et al., 2000a). Shafer et al. (2002) recently reported that some PER is visible in the nucleus 3–4 hr ahead of TIM in the behavioral clock relevant LN_vs, and they noted that the time of PER nuclear entry coincides with the onset of transcriptional repression.

Thus, PER clearly has a role in circadian transcription repression, but the role of TIM in this process is unknown. Some evidence that TIM might have a role in transcription came from a *Drosophila* mutant defective in PER feedback, *per*^{ΔC2}. dCLK-CYC-activated gene expression remains high in these lines even during the late evening, but a light pulse that degrades TIM causes a further increase in dCLK-CYC-activated transcripts (Schotland et al., 2000). TIM has also been shown to disrupt dCLK and CYC binding to a circadian E-box *in vitro* (Lee et al., 1999). Efforts to confirm a transcriptional role of TIM alone in cell culture or *Drosophila*, however, were hampered by the mechanisms that sequester TIM in the cytoplasm in the absence of PER. To address whether TIM can directly regulate transcriptional activity, with-

Table 2. Activity rhythms of flies expressing PER and TIM

	Genotype	No. of copies PER	n (No. of rhythmic)	Average period ± SEM	% Rhythmic
Locomotor data from Per 1 lines <i>per</i> ⁰¹ / <i>per</i> ⁰¹ background	Per 1-A/+	P	46 (24)	21.69 ± 0.84*	52.2
	Per 1-A/Per 1-A	PP	39 (24)	22.38 ± 0.11*	61.5
	Per 1-B/+	P	16 (14)	22.43 ± 0.10*	87.5
	Per 1-B/Per 1-B	PP	58 (12)	22.54 ± 0.18*	20.7
	+/+ (Per 1-A siblings)	++	28 (26)	23.58 ± 0.09	92.9
	Per 1-A/+	++P	85 (77)	22.39 ± 0.05*	90.6
	+/+ (Per 1-B siblings)	++	32 (29)	23.53 ± 0.07	90.6
	Per 1-B/+	++P	72 (67)	22.68 ± 0.07*	93.1
	Per 1-B/Per 1-B	++PP	32 (10)	22.10 ± 0.16*	31.3
Locomotor data from Per 1 × Tim 4 lines <i>per</i> ⁺ / <i>per</i> ⁺ , <i>tim</i> ⁺ / <i>tim</i> ⁺ Background	Per 1-A × Tim 4-7	P T	97 (82)	22.79 ± 0.07*	84.5
		P TT	122 (84)	22.68 ± 0.06*	68.9
		PPT	19 (12)	22.38 ± 0.24*	63.2
		PPTT	20 (9)	22.28 ± 0.15*	45.0
	Per 1-B × Tim 4-7	P T	52 (49)	22.65 ± 0.06*	94.2
		P TT	78 (66)	22.27 ± 0.07*	84.6
		PPT	64 (8)	21.69 ± 0.25*	12.5
		PPTT	62 (8)	21.94 ± 0.31*	12.9

Increased PER, but not TIM, affects period length. To measure locomotor activity, flies were placed in locomotor activity monitors under constant conditions at 25°C for 7 d. The endogenous *per* gene is on the X chromosome. In males, *per* is dosage compensated, such that the effect of one *per* copy in males is equivalent to two in females. We count it here as two copies. P represents one copy of Per 1; T represents one copy of Tim 4-7. + represents one copy of endogenous *per*. Although it is not indicated in the bottom of the Table with a +, all flies in Per 1 × Tim 4 lines had endogenous *per* and *tim*. An asterisk indicates that the average period of this genotype is significantly different from its nontransgenic siblings ($p < 0.05$; Student's *t* test). Note: The wild-type Per 1-A stock developed a spontaneous independent mutation that significantly reduced the viability of this line, making it difficult to obtain reliable locomotor data from homozygous flies. There was a significant effect of PER on period length in the Per 1 × Tim 4 lines when measured by two-way ANOVA ($p < 0.000$), but not of TIM in the same lines ($p = 0.53$). Thus, additional TIM in the circadian system does not significantly alter period.

out PER, we modified TIM to increase its nuclear localization either by deleting the CLD region (*hs-tim*^{CLD}) or by adding a heterologous NLS (*hs-tim*^{NLS}). We then tested the ability of these modified TIM proteins to affect *in vitro* transcription in the absence of PER.

dCLK/CYC-mediated transcriptional activation in S2 cells was assayed using a *per* E-box reporter system developed by Darlington et al. (1998). We wanted to study the effect of nuclear TIM on dCLK/CYC activity, with and without PER. Using a luciferase reporter (E-box *luc*) to measure transcription levels, we cotransfected into S2 cells dCLK and one of the three inducible versions of *tim* (Fig. 2A): *hs-tim*, *hs-tim*^{CLD}, and *hs-tim*^{NLS}. The activation by dCLK alone (~100- to 200-fold) was normalized to 100%. Both full-length and modified forms of TIM interact with PER to repress transcription at concentrations in which neither protein can repress alone, showing that the two modifications did not interfere with the ability of TIM to enable repression (Fig. 2B, left). A 10-fold increase of PER (which presumably enables PER to overcome cytoplasmic retention mechanisms) allows it to repress transcription without cotransfection of TIM. In contrast, when likewise increased 10-fold, none of the versions of TIM could repress transcription (Fig. 2B, right). Strangely, high concentrations of TIM caused a two- to threefold increase in the luciferase reporter signal. This could not be attributed to a general increase in transcription caused by the heat shock, because it also occurred when TIM was expressed at high levels via an actin promoter (data not shown). Also, the addition of 100 ng of PER at this higher concentration resulted in the expected repression equivalent to high concentrations of PER alone (data not shown). We propose several explanations for this unexpected effect of TIM in Discussion.

There are two possible explanations for lack of repression by TIM alone in our first transcription assay. The first is that TIM requires PER to exert a negative effect on transcription. A second possibility is that these TIMs are not nuclear, despite the modifications. To test this, we expressed the aforementioned modified TIM proteins in S2 cells and visualized the subcellular localization of TIM through immunofluorescence (Fig. 3A). Two hours

after induction of TIM expression by heat shock, cells were scored for relative staining intensities in the nucleus and cytoplasm. TIM^{NLS} protein was visible in the nucleus 25% of the time (Fig. 3B). Of the cells expressing TIM^{CLD}, 5% demonstrated nuclear staining, and there was a significant increase (30%) in the number of uniformly stained cells. In contrast, full-length TIM was almost entirely cytoplasmic. It was shown previously that TIM^{CLD} is more nuclear when assayed 4 hr after induction (Saez and Young, 1996, their Fig. 3); however, similarly modified PER (CLD deleted) is nuclear after 2 hr (Saez and Young, 1996, their Fig. 2). We suggest that there is another mechanism differentially regulating the nuclear entry of TIM that is not necessarily acting through the CLD.

Subcellular localization of TIM is regulated in part by active nuclear export

In mammals, mCRY and mPER were also thought to require heterodimerization for PER nuclear entry (Kume et al., 1999). A recent study by Yagita et al. (2002), however, revealed that mPER2 is regulated by nuclear export and does not require dimerization with mCRY for nuclear entry but only for nuclear accumulation. It seemed quite possible, given our results with the TIM proteins that were still essentially cytoplasmic despite the modification to transport them to the nucleus, that *Drosophila* TIM is also actively exported from the nucleus. Therefore, we made use of the specific nuclear export inhibitor LMB to block nuclear export. LMB binds to the nuclear export protein CRM1, inhibiting its ability to bind to proteins containing the most common nuclear export signal (Nishi et al., 1994; Henderson and Eleftheriou, 2000). The results, shown in Figure 4, A and B, were dramatic; within 2 hr after induction, unmodified TIM localized to the nucleus in a dose-dependent manner, becoming almost entirely nuclear at a concentration of 2 ng/ml of LMB. Even the smallest dose of 1 ng/ml significantly increased nuclear TIM.

Because the previous experiments were performed in embryonic cultured cells instead of clock-relevant lateral neurons, we decided to repeat this test in flies lacking functional endogenous

Figure 2. TIM alone cannot repress dCLK-CYC-mediated transcription of a circadian E-box reporter. An inducible *tim* gene was modified to permit nuclear entry of TIM in the absence of PER, as shown in *A* (see Results). Although the modifications still allowed TIM and PER together to repress transcription (asterisk indicates signal is significantly less than *pAct-dClock* alone; $p < 0.01$; t test), even high doses of nuclear TIM cannot repress transcription without PER. Surprisingly, TIM alone appeared to activate transcription (double asterisk indicates that signal is significantly greater than dCLK activation alone; $p < 0.01$; t test). Differences among *hs-tim*, *tim^{+NLS}*, and *tim^{ΔCLD}* are not significant. *Drosophila* S2 cells were transiently transfected with two reporter genes (E-box *luc* and i.e. β -gal), and a combination of *pAct-dClock*, full-length or mutated *hs-tim*, and *pAct-per*, as indicated by +. After 48 hr, cells were harvested and assayed for luciferase and β -gal activity. Data were normalized to β -gal expression to control for transfection efficiency. Results are presented as percentage of full activation seen in the *dClock* alone condition and expressed as averages ($\pm$ SEM) of three triplicate measures. Two other identical independent experiments showed similar results.

PER. In *per⁰¹* flies, TIM accumulates to high levels in the dark (Myers et al., 1996; Zeng et al., 1996). We therefore entrained *per⁰¹* larvae to an LD schedule and collected them at ZT 20–21, a time during which TIM has been shown to be nuclear in adult wild-type lateral neurons (Shafer et al., 2002). The CNS was dissected and incubated for 1–5 hr in medium containing LMB. The whole-mounts were then fixed and double labeled with antibodies to PDF and TIM. PDF, a peptide normally transported to axon terminals and thus cytoplasmic in lateral neurons (Helfrich-Forster, 1995; Shafer et al., 2002), was used to locate the lateral neurons in the optic lobes of developing larvae (Kaneko et al., 1997) as well as to determine the location of the cytoplasm. TIM localization was scored as described previously. In control *per⁰¹* larvae, most of the TIM (78%) was found to localize to the cytoplasm as reported previously (Myers et al., 1996). In experiments where LMB blocked nuclear export, however, there was a significant increase in TIM localization in the nucleus. Samples of TIM fluorescence (red) are shown with their corresponding PDF signals (green) in Figure 4C. We present the pooled results of seven independent experiments in graphic form in Figure 4D. Thus, in both cultured cells and larval clock cells, TIM is imported into the nucleus in the absence of PER and exported again through CRM1-dependent mechanisms.

Figure 3. TIM localizes predominantly to the cytoplasm in transiently transfected S2 cells despite modifications to increase nuclear entry. Shown in *A* are representative samples of the subcellular distribution patterns of TIM with and without modifications to increase nuclear entry. Quantification of three independent experiments (*B*) revealed that although the modifications do tend to increase TIM distribution in the nucleus, the effect is not as robust as we expected, indicating that there may be alternative mechanisms acting on TIM localization. S2 cells transfected with 500 ng of full-length or modified *hs-tim* (in 6-well plates) were fixed 2 hr after heat shock induction of TIM expression. TIM localization was scored as mostly nuclear, mostly cytoplasmic, or uniformly distributed (left columns, in red), and nuclei were visualized with DAPI (right columns, overlaid against TIM signal). Data are presented as mean percentages of cells in each category ($\pm$ SEM). Asterisk indicates that this group is significantly different from full-length TIM for that subcellular localization ($p < 0.01$; one-way ANOVA).

Nuclear export of TIM is affected by PER

LMB has no significant effect on the localization of PER alone in S2 cells (data not shown); however, the subcellular localization of TIM in S2 cells changed after coexpression with PER (Fig. 5*A,B*). Although TIM alone was completely nuclear when export was blocked, it was significantly more cytoplasmic when PER was present. The effect is PER specific; equivalent amounts of β -galactosidase coexpressed with TIM did not have any effect on TIM localization with or without LMB (data not shown). Note that at this time point (2 hr after induction), PER and TIM were also still mostly cytoplasmic in the work of Saez and Young (1996), indicating that the nuclear expression of the heterodimer is normally delayed even in S2 cells. Because TIM alone (with LMB) is nuclear at 2 hr, we infer that PER sequesters TIM and delays the nuclear entry of the heterodimer (see Discussion).

Our *per⁰¹* larval data led us to hypothesize that PER is necessary for TIM nuclear retention (as opposed to its transport to the nucleus, as originally thought). The cell culture data indicated that PER could also retain TIM in the cytoplasm, suggesting that the effects of PER on TIM could be different under different conditions. To determine the effect of PER on TIM *in vivo*, we repeated our larval CNS assay, this time looking at both TIM and PER localization in *yw* larvae. Because these flies have a working clock, and thus PER and TIM localization varies during the course of the evening, we looked at two different time points, ZT 15.5 and ZT 19.5 ($n = 55$ and 261 LNs scored, respectively). Our

results are summarized in Figure 5, C and D. At both time points, LMB treatment resulted in an increase in nuclear expression of TIM and also of PER, indicating that TIM does influence PER localization to some extent. The effect of blocking nuclear export on nuclear expression of both proteins was greater at ZT 19.5, perhaps because PER is more nuclear at this time point (Shafer et al., 2002). Thus, as in the S2 cells, PER most likely has a profound effect on the subcellular localization of TIM.

TIM does not repress dCLK-CYC-mediated transcription even when its export from the nucleus is blocked

Having shown that the addition of LMB causes a dramatic increase in nuclear TIM, we repeated the transcription assay described in Figure 2, modified to block nuclear export of TIM. S2 cells were transiently transfected with the various reporter constructs, pAct-*dClock* and *hs-tim* or pAct-*per*, or both. TIM expression was again induced via heat shocks, this time in the presence of LMB at a high dose shown to result in nuclear TIM (10 ng/ml; see Materials and Methods for details). Figure 6 shows the result of a representative experiment performed in triplicate, normalized to 100% activation by dCLK. Because we used smaller doses of TIM, we did not see the transcriptional activation that is evident at larger doses. The addition of LMB did not result in any repressive effect of TIM on transcription. Also, LMB had no effect on the ability of PER and TIM together to repress transcription.

Discussion

We used behavioral and genetic assays to analyze the role and regulation of TIM in the circadian clock, on the basis of predictions of the current clock model. Our results show that the clock is insensitive to *tim* dosage. We also present evidence that TIM alone cannot repress dCLK-CYC-mediated transcription of an E-box reporter in S2 cells, despite a report that TIM disrupts the DNA-binding ability of dCLK and CYC *in vitro* (Lee et al., 1999). Finally, we present evidence that TIM does not require dimerization with PER for nuclear entry, although PER is involved in TIM nuclear accumulation and retention.

We found that increased levels of TIM do not shorten period length. This is not surprising, because Zeng et al. (1996) found that TIM is present in molar excess of PER for part of the night. TIM is present in PER-immunodepleted extracts (Zeng et al., 1996), the *tim* promoter is stronger than the *per* promoter (So and Rosbash, 1997), and decreasing *tim* dosage does not affect period length (Rothenfluh et al., 2000b). Thus, there is ample evidence that TIM levels exceed PER levels for most of the night (until dawn, when TIM levels drop), and alterations of TIM levels have little effect on PER function.

Surprisingly, PER levels in whole-head extracts were not

Figure 4. Nucleocytoplasmic shuttling of TIM in cell culture and *per*⁰¹ lateral neurons. Localization of TIM was measured in the presence of a nuclear export blocker (LMB) in transiently transfected S2 cells and larvae. In both cases, blocking nuclear export led to an increased accumulation of TIM trapped in the nucleus, indicating that even in the absence of PER, TIM is transported into the nucleus and actively exported. *A*, Shows representative examples of TIM subcellular localization in S2 cells. Five hundred nanograms of *hs-tim* (full-length only) were transfected, expressed, and scored as described in Figure 3, with one exception: LMB (10 ng/ml) was added to the medium before heat shock. Sample cells (*A*) were fixed 2 hr after induction, but we noted that TIM nuclear expression occurs extremely rapidly, even within 30 min after induction (data not shown). In *B*, TIM localization was quantified as described in Figure 3. LMB dosage was varied, and TIM was localized to the nucleus in a dose-dependent manner. *C*, Representative TIM (red) and PDF (green overlay) signals in *per*⁰¹ larval LNs. TIM is cytoplasmic in *per*⁰¹ flies (above), and LMB addition significantly increased the proportion of TIM found in the nucleus in *per*⁰¹ flies (below) in seven independent experiments, quantified in *D* (asterisk indicates that this group is significantly different from vehicle control; $p < 0.01$; *t* test). Whole brains from *per*⁰¹ third-instar larvae were dissected at ZT 20–21 and incubated for 3 hr in medium containing (bottom) or lacking (top) LMB (40 ng/ml) and supplemented with MG132 (a proteasomal inhibitor) to block TIM degradation. After the incubation period, the tissue was fixed and probed with antibodies to TIM and PDF. For the purpose of this Figure, the contrast of the TIM signal was adjusted slightly to enhance the signal over the background. As with the cell culture quantification, data are presented as percentages of LNs in which most of the TIM signal was nuclear, cytoplasmic, or uniformly distributed. Only LNs with clear cytoplasmic–nuclear boundaries (visualized by the PDF signal) were scored (blind) for TIM localization. LNs in which the TIM signal was too faint to score were discarded. A total of seven independent experiments were performed in which the incubation time (1.5–5 hr), LMB dosage (20–40 ng/ml), and DMSO (1–3%) concentration were varied, with similar results.

markedly increased when overexpressed via the *tim* promoter, despite the dramatic short-period phenotypes (Fig. 1C, Table 2). This is most likely attributable to elevated negative feedback of PER onto the *tim* promoter, which would result in decreased steady-state levels of protein despite increased synthesis. Feedback is presumably required for the shorter period, because we have noticed that overexpression of *per* by heterologous promoters, such as actin and *elav*, actually causes a lengthening of period as well as arrhythmia (Yang and Sehgal, 2001), whereas overexpression of *per* with an E box-containing promoter leads to shorter periods (Hao et al., 1999). This may also explain why the period length cannot be shortened by PER overexpression beyond 21.5–22 hr without loss of protein oscillations and overt behavioral rhythms (Smith and Konopka, 1982; Yang and Sehgal, 2001; this study). Beyond this point the clock may not be able to compensate for the increased PER feedback. Thus, the notion that higher levels of PER shorten period, and vice versa, may be true only when *per* can inhibit its own expression. Otherwise, increased PER lengthens period (because of prolonged protein expression) or causes arrhythmia. On the other hand, TIM levels

Figure 5. PER alters TIM localization *in vitro* and *in vivo*. PER sequesters TIM in the cytoplasm of S2 cells during LMB treatment, indicating that the presence of PER inhibits TIM from entering the nucleus when PER is cytoplasmic. In *yw* larvae, however, there is still a slight trend toward more nuclear expression of TIM and PER when nuclear export is blocked. The trend is more pronounced when PER is already more nuclear. *A* shows representative examples of S2 cells transiently transfected with 500 ng each of *hs-tim* and *pAct-per*. Quantification of TIM and PER localization is presented in *B*, scored as described in Figure 3. S2 cells were treated with LMB during heat shock as described in Figure 4, fixed 2 hr after induction, and stained for either TIM (left, red) or PER (right, red) and DAPI (blue overlay). Data in *B* were pooled from two independent experiments, and in each experiment 100–300 cells per group were scored. *C* presents examples of PER (top) and TIM (bottom) signals in *yw* larvae, collected at ZT 19.5, incubated with LMB or vehicle for 1.5 hr. At this time point, PER is more nuclear. The sample cells show an example of nuclear and uniform PER distribution without LMB and nuclear distribution with LMB. TIM is normally still cytoplasmic, as shown in the (–) LMB example, but there is a definite trend toward nuclear localization when export is blocked by LMB (bottom). PER localization is also affected, as seen in the quantifications of two independent experiments (*D*). Whole brains from *yw* third-instar larvae were dissected at ZT 20–21 and incubated for 3 hr in medium containing (bottom) or lacking (top) LMB (40 ng/ml) and supplemented with MG132 (a proteasomal inhibitor) to block TIM degradation. After the incubation period, the tissue was fixed and probed with antibodies to TIM or PER (visualized with Cy3-conjugated secondary antibodies, red) and compared with the cytoplasmic PDF signal detected with FITC (green). All LNs were scored blind as described in Figure 4.

were increased in the TIM overexpression lines, perhaps because TIM does not directly effect negative feedback (discussed below).

In two different experimental designs, nuclear versions of TIM caused no repression of transcription without coexpression of PER. Our behavioral data also indicate that, unlike PER, higher levels of TIM apparently cannot feed back to repress transcription and alter period length (Lee et al., 1999). This was contrary to our prediction based on the light-induced increase of *per* transcripts in flies expressing a mutant PER (Schotland et al., 2000). The assumption was that the light induced degradation of TIM relieved the repression of *per* transcription. There are alternative explanations, however, such as an undescribed transcriptional role of dCRY, which is also degraded by light (Lin et al., 2001). Mammalian CRY plays an active role in clock feedback (Kume et al., 1999), as do some zebrafish CRYs (Kobayashi et al., 2000; Hirayama et al., 2003). In *Drosophila*, CRY interacts directly with

PER (Rosato et al., 2001) and is a functional component of peripheral clocks (Ivachenko et al., 2001; Krishnan et al., 2001). It is predominantly peripheral head clocks that are reported by the RNase protection assays used by Schotland et al. (2000).

The activating effect of TIM on the luciferase signal was surprising (Fig. 2). This effect is independent of heat shock and abrogated by addition of PER (data not shown). TIM has been shown to promote RNA stability, but this is specific to *per* RNA and requires the presence of functional PER protein (Suri et al., 1999). We identified a glutamine-rich region in TIM (position 397–412) using Motif Scan; these transcriptional activation domains are found in a number of *Drosophila* transcription factors, including *Sp1* (Gill et al., 1994; Roberts, 2000). This raises an intriguing possibility for a PER-independent role of TIM in the upregulation of other clock-controlled genes during the late evening (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ceriani et al., 2002; Lin et al., 2002); however, we believe that this “activation” more likely reflects a positive effect of TIM on *dClk* RNA (Bae et al., 1998; Lee et al., 1998), either by increasing *dClk* RNA stability or perhaps by derepressing the endogenous *dClk* gene by blocking VRILLE repression (Glossop et al., 1999, 2003; Cyran et al., 2003).

The effect of exogenous PER on TIM in the presence of LMB indicates a strong influence of PER on TIM localization. We note that PER and TIM were cytoplasmic at earlier time points (shortly after they are coexpressed) in the Saez and Young (1996) study. Thus, in S2 cells, as in flies, nuclear entry of the heterodimer is delayed. In the LMB experiments, TIM is almost immediately nuclear without PER, indicating that TIM nuclear import is not delayed in the absence of PER. A likely model is that PER sequesters TIM in the

cytoplasm until PER itself is transported actively to the nucleus. Thereafter, it retains TIM in the nucleus. These results are supported by our *in vivo* data, where we found that the effect of LMB on PER and TIM localization in larval lateral neurons varied depending on the time point chosen (and the location of PER). Thus, PER may be the dominant determinant of PER–TIM localization and thus the more likely candidate for mechanisms regulating nuclear entry.

TIM, however, must also play a role in this process the basis of the following lines of evidence: (1) a PER-βGal fusion protein is cytoplasmic in *tim⁰¹* flies, although nuclear in wild type (Vosshall et al., 1994); (2) the presence of TIM promotes nuclear expression of PER at later time points in S2 cells (Saez and Young, 1996); (3) *per^L* mutants, defective in TIM binding, also display a delay in nuclear entry (Curtin et al., 1995; Gekakis et al., 1995); (4) over-

Figure 6. TIM alone does not repress dCLK-CYC-mediated transcription even when its nuclear export is blocked. LMB treatment had no significant effect on either TIM repression or TIM + PER repression. After transient transfection of S2 cells (described previously in Fig. 2), cells were heat shocked for 30 min every 12 hr, in medium supplemented with 5 ng/ml LMB. Four hours after induction, the medium was replaced with LMB-free medium. After 36 hr, cells were collected and analyzed for luciferase and β -gal expression as described in Figure 2. Results are expressed as averages ($\pm$ SEM) of three triplicate measures. Two other identical independent experiments showed similar results. Asterisk indicates that the amount of activation is significantly less than activation by dCLK alone ($p < 0.01$; t test).

expression of *shaggy* (*sgg*), a kinase that phosphorylates TIM, causes a 2 hr advance in PER nuclear entry (Martinek et al., 2001); and (5) LMB, which does not affect PER localization directly, increases PER localization to the nucleus, presumably via the PER interaction with TIM (Fig. 5). Interestingly, PER has been shown to interact with TIM via the TIM NLS, whereas TIM interacts with the PER CLD (Saez and Young, 1996). We suggest that these interactions contribute to the delay in nuclear entry of the heterodimer, which eventually becomes nuclear through active regulation–modification of PER. In light of the recently reported differential timing of PER and TIM nuclear entry (Shafer et al., 2002), a possible explanation is that TIM and PER enter the nucleus as a dimer, but initially TIM is actively exported from the nucleus. A subsequent regulatory event, perhaps phosphorylation, may be required for nuclear retention of TIM by PER.

In mammals, mPER2 stability is regulated by the mCRY proteins (Yagita et al., 2002), reminiscent of the manner in which TIM promotes PER stability in *Drosophila*. It was also shown recently that mCRY is not necessary for mPER2 nuclear entry but is necessary for its nuclear accumulation. Both proteins are actively exported via the same LMB-sensitive CRM1-exportin nuclear export pathways, and nuclear export is blocked when mCRY-mPER2 dimerization blocks their respective nuclear export NES signals (Yagita et al., 2002). As with many proteins, nuclear export was also linked to ubiquitylation and proteasomal degradation of mCRY and mPER2. *Drosophila* TIM and PER are also ubiquitinated and degraded by the proteasome, but the relevance of this for nuclear export is not known (Naidoo et al., 1999; Ko et al., 2002). Scanning TIM for the leucine-rich consensus sequence for CRM1-exportin-dependent nuclear export Lx_(1–3)Lx_(1–3)LxL(V/I/M) (in which x indicates any amino acid) (Yagita et al., 2002; Nigg, 1997 #797) revealed six potential nuclear export signals (residues 184–193, 737–744, 750–758, 1046–1054, 1095–1102, and 1131–1139), none within the CLD region. We suspect that the modification of these sites by *sgg* phosphorylation will be the mechanism by which TIM becomes nuclear in the late evening. A similar scan of PER using this consensus sequence did not turn up any potential LMB-sensitive nuclear ex-

port signals; however, Vielhaber et al. (2001) describe a conserved region in *per* that is an active NES in *mPer1* and *mPer2*.

This study challenges some of the assumptions of the current clock model concerning the effect of *per* dosage on circadian period and the regulation of PER–TIM nuclear entry and dimer function. It also indicates that TIM does not actively repress dCLK-CYC-mediated transcription and in fact may have a novel role in the nucleus. The active nuclear export of TIM provides yet another level of complexity to the function of TIM in the *Drosophila* circadian clock.

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