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TIMELESS-dependent positive and negative autoregulation in the *Drosophila* circadian clock

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The *timeless* protein (TIM) is a central component of the circadian pacemaker machinery of the fruitfly *Drosophila melanogaster*. Both TIM and its partner protein, the *period* protein PER, show robust circadian oscillations in mRNA and protein levels. Yet the role of TIM in the rhythm generation mechanism is largely unknown. To analyze TIM function, we constructed transgenic flies that carry a heat shock-inducible copy of the *timeless* gene (*tim*) in an arrhythmic *tim* loss-of-function genetic background. When heat shocked, TIM levels in these flies rapidly increased and initiated a molecular cycle of PER accumulation and processing with dynamics very similar to the PER cycle observed in wild-type flies. Analysis of *period* (*per*) mRNA levels and transcription uncovered a novel role for TIM in clock regulation: TIM increases *per* mRNA levels through a post-transcriptional mechanism. Our results suggest positive as well as negative autoregulation in the *Drosophila* circadian clock.

Keywords: autoregulation/circadian rhythms/*Drosophila*/TIMELESS

Introduction

Most organisms have evolved systems to modulate their behavior and physiology in anticipation of varying environmental conditions. Circadian rhythms are perhaps the most widespread of such mechanisms and allow organisms to adapt to diurnal changes in illumination and temperature. These rhythms are entrainable, persist in constant conditions, can be phase shifted and are temperature compensated (Edmunds, 1988). The genetics and molecular biology that underlie circadian rhythms are well characterized in *Neurospora crassa* (Dunlap, 1996). There has also been recent progress in plants (Millar and Kay, 1997; Schaffer *et al.*, 1998; Wang and Tobin, 1998), cyanobacteria (Ishiura *et al.*, 1998), mice (Reppert, 1998) and even in cultured rat fibroblasts (Balsalobre *et al.*, 1998).

The circadian system of *Drosophila melanogaster* controls a variety of behaviors, including locomotor activity and pupal eclosion (Saunders, 1982; Young, 1998). Mutations at *period* (*per*) and *timeless* (*tim*) loci were found to alter the periodicity of locomotor activity and eclosion rhythms (Konopka and Benzer, 1971; Sehgal *et al.*, 1994), and the subsequent cloning of these genes allowed a molecular approach to the *Drosophila* rhythm system (Rosbash *et al.*, 1996; Young *et al.*, 1996). Both *per* and *tim* undergo rhythmic transcription (So and Rosbash, 1997) and show circadian oscillation in mRNA amounts, which is a cornerstone of the original clock model (Hardin *et al.*, 1990; Sehgal *et al.*, 1994). However, there is evidence for multiple levels of regulation. The two genes manifest temporally controlled protein accumulation and phosphorylation (Edery *et al.*, 1994; Zeng *et al.*, 1996). PER and TIM form a heterodimeric complex which is believed to be important for their clock functions (Gekakis *et al.*, 1995; Rutila *et al.*, 1996; Zeng *et al.*, 1996). These may include a specific role in nuclear import as well as a more general role in transcriptional repression of their own genes (Vosshall *et al.*, 1994; Saez and Young, 1996; Darlington *et al.*, 1998). The earliest observable effect of light, the major environmental time cue, is a disappearance of TIM protein, and there is evidence that post-transcriptional TIM regulation is relevant to light entrainment and phase shifting (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996; Suri *et al.*, 1998; Yang *et al.*, 1998).

Subsequent genetic screens and biochemical approaches have also led to the recent identification of additional *Drosophila* clock components. These include the *doubletime* protein (DBT), a putative PER kinase that may contribute to post-transcriptional regulation of PER levels (Kloss *et al.*, 1998; Price *et al.*, 1998). They also include two basic-helix–loop–helix (bHLH) transcription factors, the *clock* protein (CLK; Allada *et al.*, 1998; Bae *et al.*, 1998; Darlington *et al.*, 1998) and the *cycle* protein (CYC; Rutila *et al.*, 1998b), which have been shown to drive transcription from *per* and *tim* promoter elements. CLK and CYC are believed to be important for the autoregulatory negative feedback loop that turns down transcription at the *per* and *tim* regulatory elements. This widely accepted model of the *Drosophila* clock is based on the altered mRNA rhythms in *per* mutants (Hardin *et al.*, 1990; Marrus *et al.*, 1996) as well as non-cycling mRNA expression in the per^{01} null mutant (Hardin *et al.*, 1990) and the tim^{01} null mutant (Sehgal *et al.*, 1994). Support for this mechanism also comes from an experiment in which constitutively expressed PER was found to inhibit endogenous *per* mRNA rhythms (Zeng *et al.*, 1994). Additionally, PER and TIM co-expression in cultured *Drosophila* cells inhibits transcriptional activation at the *per* regulatory element, which is a putative target site of the transcription factors CLK and CYC (Darlington *et al.*, 1998). This last observation, along with others (Allada *et al.*, 1998; Rutila *et al.*, 1998b), has led to the current formulation of the original autoregulatory feedback loop model: CLK and CYC activate PER and TIM transcription, and PER and TIM inhibit their own transcription (Dunlap,

1998; Schibler, 1998). In addition, PER and TIM have been suggested recently to be positive regulators of CLK, as levels of *clk* mRNA and CLK protein are very low in per^{01} and tim^{01} mutant backgrounds (Bae *et al.*, 1998; Lee *et al.*, 1998). Interestingly, CLK has been shown to be present in complexes containing PER and TIM, although the precise activity or function of these complexes is unclear (Lee *et al.*, 1998).

A detailed and accurate explanation of the *Drosophila* clock, however, remains elusive. It must include the basis for the precisely controlled phase and amplitude of *per* and *tim* expression. Although the *per* and *tim cis*-acting regulatory elements are required for high level expression, their specific role in rhythmic expression remains uncertain. Deletion of this element from the *per* promoter, for example, reduced the expression level but significant cycling properties remained (Hao *et al.*, 1997). Also, promoter-less *per* transgenes such as the *per* 7.2 transgene rescue behavior and continue to show RNA oscillations, albeit with a reduced amplitude (Frisch *et al.*, 1994). A partial explanation of these observations lies in recent studies that defined a post-transcriptional contribution to *per* RNA oscillations (So and Rosbash, 1997). This is consistent with another recent investigation (Stanewsky *et al.*, 1997) which identified a second element in the *per* coding region contributing to the amplitude and phase of *per* RNA oscillations. Neither the mechanisms nor the factors involved in this type of *per* mRNA regulation are known.

Although several other studies have addressed the issue of clock regulation by constructing different *per* transgenic strains (Ewer *et al.*, 1988; Frisch *et al.*, 1994; Zeng *et al.*, 1994; Cheng and Hardin, 1998), transgenic rescue with *tim* has been reported only recently (Ousley *et al.*, 1998; Rutila *et al.*, 1998a). Moreover, there are as yet no published studies in which different *tim*-expressing constructs have been used to study clock regulation. To address the role of TIM in the *Drosophila* circadian cycle, we transformed arrhythmic tim^{01} flies with *tim* cDNA under the control of a heat shock-inducible promoter. As tim^{01} is a nonsense mutation, the mutant strain does not express any detectable TIM protein. The transgenic strain allowed us to investigate the biochemical and behavioral consequences of rapidly introducing TIM into these flies. TIM induction is sufficient to start off a complete and wild-type-like cycle of *per* mRNA. In addition, PER protein accumulation, processing and degradation also appear normal. A quasi-normal behavioral cycle even ensues. Our results strongly suggest a major role for TIM in positive, post-transcriptional regulation of *per* gene expression. Additionally, by assaying the effect of TIM overexpression in the cyc^{0} null mutant, we show that PER protein rhythms can be generated in the absence of CYC-mediated transcription.

Results

TIM overexpression rescues the wild-type-like accumulation and phosphorylation pattern of PER protein

We were interested in exploring the behavioral and molecular effects of TIM overexpression on the *Drosophila* clock. To this end, we generated transgenic flies carrying a heat shock-inducible copy of *tim* cDNA in a background tim^{01} mutant strain (Figure 1A). We tested the behavior of these flies in both light:dark (LD) and constant darkness (DD) at 18, 25 and 29°C. All the transgenic lines tested were behaviorally arrhythmic at either 18, 25 or 29°C (Table I). We assayed TIM protein levels in the transgenic flies under LD conditions and found low, non-cycling TIM levels in these animals at 25 and 29°C (data not shown). High-level cyclical expression of TIM therefore appears to be a pre-requisite for periodicity of locomotor activity. In contrast, a 30 min heat shock at 37°C caused TIM levels in these animals to increase ~50-fold after 4 h (Figure 1B and C; 0 time is immediately after the 30 min heat shock).

To investigate the molecular consequences of this dramatic TIM induction, we assayed changes in PER levels as well as the PER phosphorylation status following the 30 min heat shock. In the background tim^{01} mutant strain, PER levels and phosphorylation remain temporally constant, whereas PER manifests robust oscillations in abundance and phosphorylation in wild-type flies (Price *et al.*, 1995). Following TIM induction in tim^{01}*;hs-tim* flies, PER levels also showed a rapid and dramatic rise from pre-heat shock levels. Furthermore, the accumulation and phosphorylation profiles were reminiscent of PER cycling in wild-type flies (Figure 2A and B; Marrus *et al.*, 1996). PER levels peaked at ~10 h after the heat shock, and the *hs-tim* PER accumulation curve superimposed well on the wild-type accumulation curve (Figure 2C). A comparison of the accumulation profile of PER in wild-type flies and heat-shocked tim^{01}*;hs-tim* flies suggested that the time of the heat shock corresponded to approximately Zeitgeber time (ZT) 7–8 of the wild-type curve. As this is almost precisely the nadir of the PER cycle, it suggests that the molecular program in the tim^{01} mutant strain is not only static but also stuck at about ZT 7–8 values. TIM induction then releases the cycle from that point into a fairly normal accumulation pattern. However, we cannot rule out an apparent lack of oscillations due to asynchrony between individual cells and tissues. In this case, TIM induction may reset all phases to approximately ZT 8 values, so that individual *per*-expressing tissues now show synchronous mRNA and protein rhythms.

Peak values of PER during the *hs-tim* cycle are ~4- to 5-fold higher than pre-heat shock levels. As the latter are ~30–40% of wild-type peak levels [PER levels in tim^{01}*; hs-tim* flies are marginally higher than the 20–30% reported for the tim^{01} strain (Price *et al.*, 1995)], the *hs-tim* strain generates ~1.5- to 2-fold more PER than the wild-type peak levels. We were especially impressed by the apparent recapitulation of the striking phosphorylation program that is a characteristic feature of PER cycling in wild-type flies (Edery *et al.*, 1994); clear decreases in PER mobility were visible ~8–10 h after the heat shock. PER phosphorylation probably regulates stability, as mutations in the PER kinase *DBT* cause large amounts of hypophosphorylated forms to accumulate in *Drosophila* larvae (Kloss *et al.*, 1998). On Western blots, newly translated, less phosphorylated forms appear as rapidly migrating species in the late day–early night (ZT 8–14). During the late night and early day, these forms are converted to slowly migrating more phosphorylated forms which are subsequently degraded (Edery *et al.*, 1994; Marrus *et al.*,

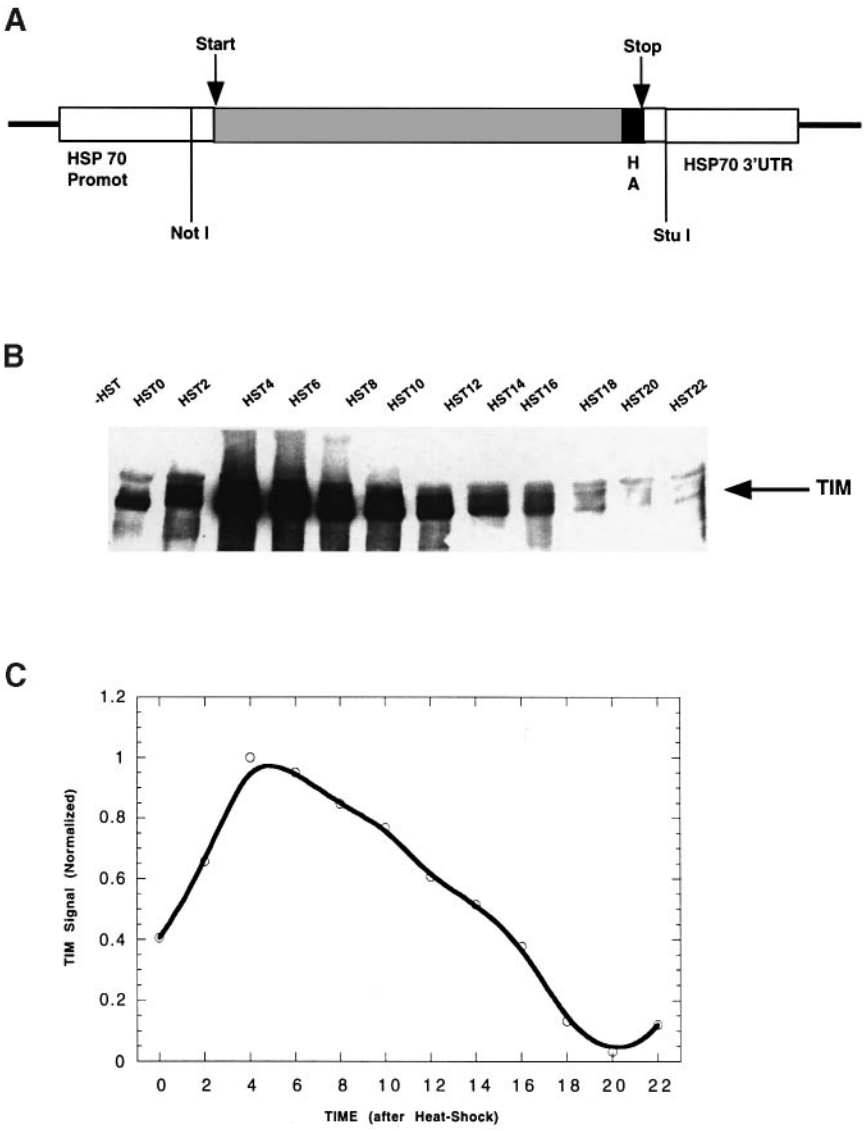


Fig. 1. Construction of transgenic flies carrying *tim* under the control of the heat shock promoter. (**A**) Injection construct, where the coding sequences are denoted by the shaded box. Vector sequences containing important heat shock elements are shown by the open box. (**B**) A 30 min heat shock (37°C) causes rapid induction of TIM. Flies were heat shocked at 37°C for 30 min and then collected on dry ice. Head extracts were run on a 6% SDS–PAGE gel and blotted with anti-TIM antibody. The first lane (–HST) is the non-heat-shocked control. HST2, HST4, etc. are heat-shocked samples recovered in the dark for 2, 4 h, etc. (**C**) Quantitation of the Western blot shown in (B).

Table I. Locomotor activity analysis of flies carrying *hs-tim* transgene

Genotype	Light cycle	Temperature (°C)	No. of flies tested	Percent rhythmic	Mean period (± SEM)
yw;tim^0	LD	25	32	90	24.15 (± 0.28)
yw;tim^0;F/TM2	LD	25	32	100	24.29 (± 0.12)
yw	DD	18	5	80	23.9 (± 0.8)
yw;tim^0	DD	18	4	0	–
yw;tim^0;F/TM2	DD	18	5	20	26.5
yw	DD	25	16	94	23.5 (± 0.72)
yw;tim^0	DD	25	32	3	36.7
yw;tim^0;F/TM2	DD	25	32	3	32.2
yw	DD	29	11	91	23.17 (± 0.46)
yw;tim^0	DD	29	4	0	–
yw;tim^0;F/TM2	DD	29	11	0	–

Line F represents the hs-tim transgenic flies. Several lines were tested with similar results. In the above analysis, flies with penetrance <10 were considered arrhythmic.

1996). PER degradation in heat-shocked *hs-tim* flies showed a similar temporal profile, as fast migrating species were converted to slow migrating species, which were then degraded. No comparable changes in PER levels were observed when the same heat shock protocol was applied to the control *yw;tim*^{01} background (data not shown; see also Sidote *et al.*, 1998). These results clearly indicate a role for TIM in the regulation of PER protein levels and phosphorylation.

Further analysis using glycerol gradient centrifugation showed that PER appeared exclusively as a PER–TIM heterodimer at initial time points (2–9 h), whereas at later times much of PER traveled in the top fractions and was apparently free from TIM (Figure 3A and B). TIM always sedimented as a high molecular weight species, as previously reported (Zeng *et al.*, 1996; data not shown). Co-immunoprecipitation experiments also supported a similar pattern of temporal dynamics of the PER–TIM complex (data not shown). Thus, all temporal features of PER, TIM and the PER–TIM complex resembled the wild-type pattern (Zeng *et al.*, 1996).

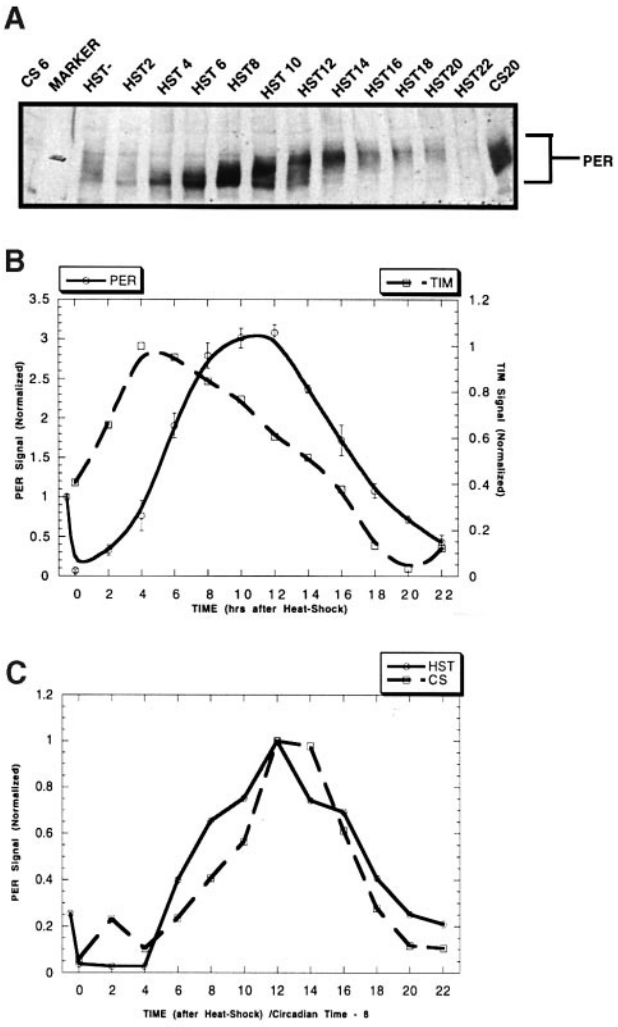


Fig. 2. TIM induction causes an increase in PER levels. (**A**) PER protein levels increase dramatically after TIM induction in *hs-tim* flies. Flies were heat shocked as described in the legend to Figure 1B and blotted with anti-PER antibody. (**B**) Quantitation of the Western blot shown in (A). The blot was stained with Ponceau-S to ensure equal loading of lanes. All values are plotted relative to the pre-heat shock values. The experiment was repeated three times. Error bars represent standard error of the mean. (**C**) A comparison of the phase of TIM-induced PER cycling in *tim*^{01}*;hs-tim* flies and PER cycling in wild-type Canton-S flies. The *x*-axis is time after heat shock for *tim*^{01}*; hs-tim* flies and ZT 8 for Canton-S flies.

Increase in PER levels is partly due to an increase in per RNA

As previously described, PER levels are temporally constant in *tim*^{01} flies, at ~20–30% of the peak levels in wild-type flies (Price *et al.*, 1995). TIM was therefore suggested to be important for PER stability, presumably by sequestering PER in a heterodimeric complex and increasing its half-life. To investigate if post-translational mechanisms are solely responsible for the *hs-tim*-mediated increase in PER levels, we assayed *per* RNA levels following the heat shock. RNA levels initially declined by ~50–60% and then returned to their initial values ~2–4 h later, in both *hs-tim* flies and in the control *yw;tim*^{01} flies (Figure 4A and B, and data not shown). However, only in *hs-tim* flies did *per* RNA levels continue to increase over the next 4–6 h to ~2- to 3-fold over the starting value. Considering that *per* RNA in *tim*^{01} flies is ~50–70% of the peak wild-type levels (Sehgal *et al.*, 1995), this represents a 1.5- to 2-fold increase in RNA over

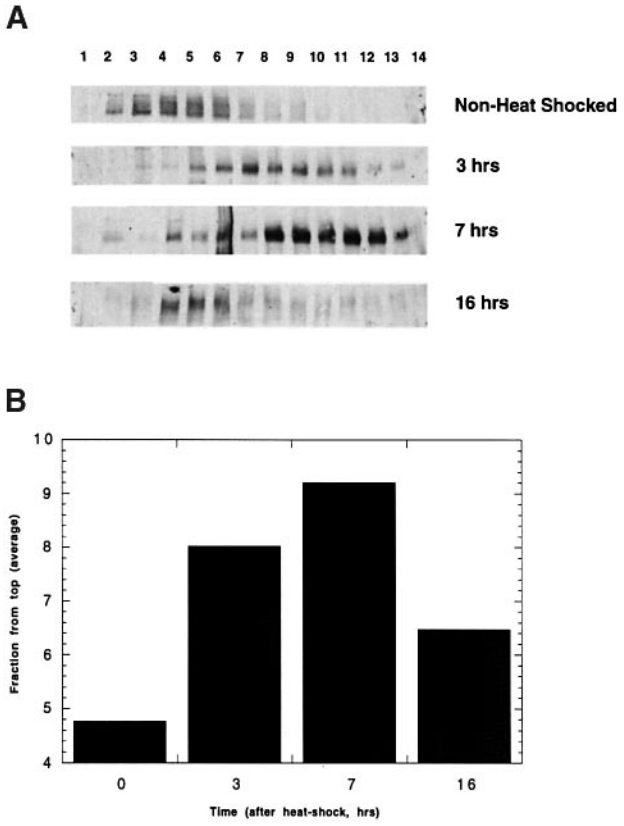
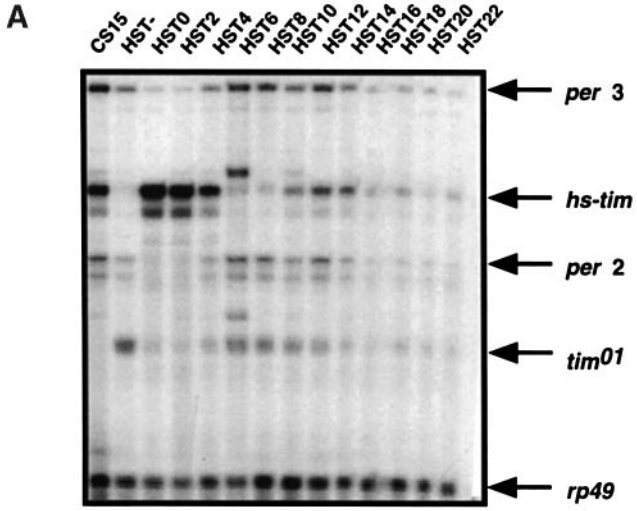
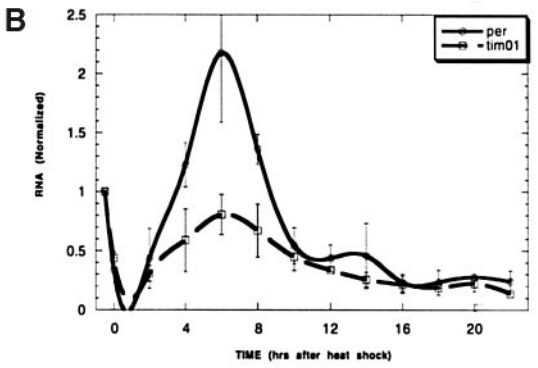
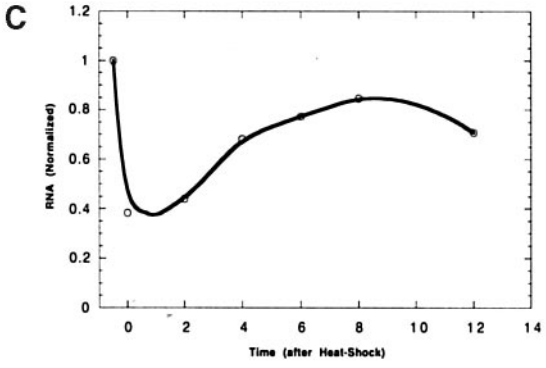


Fig. 3. Glycerol gradient analysis of PER indicates that most of the newly synthesized PER sediments as a high molecular weight species. (**A**) Flies were heat shocked for 30 min in a water bath and recovered for the indicated times. Fly head extract equivalent to 40 heads was run on a 2 ml 5–30% glycerol gradient. Fractions of 100 µl fractions were collected sequentially from the top, TCA precipitated, run on a Western blot and probed for PER. (**B**) The average fraction number from the top was calculated using the formula: $F = \Sigma(C_i*F_i)/\Sigma C_i$, where C_i is the amount of PER protein in each 100 µl fraction, F_i is the fraction number and the summation is over all fractions. PER protein amount was calculated by quantitation of the PER signal in the Western blot shown in (A).

wild-type peak levels. The results clearly indicate a TIM-dependent positive effect on *per* mRNA levels. We then assayed the levels of endogenous *tim* (i.e. tim^{01}) mRNA. Initially, tim^{01} mRNA levels declined and rapidly recovered, presumably due to general heat shock effects. Importantly, however, they did not overshoot their initial levels, indicating that the mechanism is *per* mRNA specific. About 8 h post-heat shock, *per* mRNA then experienced a strong decline in levels. The endogenous tim^{01} mRNA also showed a comparable decrease. This indicates that both transcripts are downregulated through a common mechanism, in contrast to the earlier upregulation phenomenon, specific to the *per* transcript.







We further determined whether the up regulation required PER itself by analyzing a per^{01};*hs-tim* strain. per^{01} flies carry a nonsense mutation at the *per* locus and do not express any detectable protein. As shown in Figure 4C, TIM overexpression in this strain had no detectable effect on *per* mRNA levels, indicating that the mechanism required functional PER and suggesting that the PER–TIM dimer is the active species driving *per* mRNA upregulation. It is possible, however, that the effect is more indirect, e.g. the per^{01} strain may be deficient in PER-regulated proteins that are needed for *per* mRNA upregulation.

Transcriptional mechanisms are insufficient to explain the increase in per RNA

To assess the contribution of transcriptional regulation to the *per* RNA level changes following TIM induction, we carried out a nuclear run-on analysis of polymerase density at the *per* locus (So and Rosbash, 1997). As shown in Figure 5A and B, we assayed transcription before the 30 min of *hs-tim* induction and after a subsequent 0, 3, 6, 9 or 12 h. In these assays, we used *rh*1 rhodopsin and *lacZ* genes as controls. The heat shock caused a transient decrease in both *per* and *rh*1 transcription rates. A decline in transcription and subsequent recovery after a heat shock has been reported for several non-heat shock genes (Vazquez *et al.*, 1993). Indeed, after 3 h, *per* transcription relative to rhodopsin transcription was very similar to that in non-heat shocked flies, i.e. it had returned to the pre-heat shock rate. In other experiments, we assayed *per* transcription rates 30 min, and 2 and 5 h after the heat shock and also failed to observe any appreciable rise compared with pre-heat shock values (data not shown). Hence, transcriptional mechanisms cannot account for the TIM-driven increase in *per* mRNA levels. It must depend on post-transcriptional mechanisms, which may include *per* mRNA stabilization. A similar conclusion was reached in a recent study comparing *per* and *tim* transcriptional oscillations with their respective mRNA oscillations in wild-type flies (So and Rosbash, 1997).

The subsequent *per* RNA decline, however, was clearly due to transcriptional repression: complete transcriptional shut-off was observed ~6–9 h after TIM induction (Figure 5A and B). While we did not assay transcription at the endogenous tim^{01} locus, the very similar kinetics and magnitude of the tim^{01} mRNA decline suggest a

Fig. 4. RNase protection analysis of RNA levels in *hs-tim* flies following TIM induction. (**A**) RNase protection analysis of *per*, *hs-tim*, tim^{01} and ribosomal protein 49 (rp49) transcripts. Flies were heat shocked as described in the legend to Figure 1B. Total RNA was then isolated and hybridized with a mixture of *per*, *tim* and *rp49* probes. Excess probe was digested with RNase A and the samples were run on a 5% denaturing gel. The *per* probe is derived from genomic DNA and protects two bands, *per*2 in exon 2 and *per*3 in exon 3. The *tim* probe protects a 167 nucleotide band in tim^{+} message and a 97 nucleotide band in the tim^{01} mRNA. (**B**) Quantitation of the *per* and tim^{01} signals. The signal intensity was normalized to *rp49*. All values are plotted relative to the pre-heat shock values. The experiment was repeated three times. Error bars represent standard error of the mean. (**C**) *per* mRNA does not increase in per^{01} flies carrying a *hs-tim* transgene. per^{01};*hs-tim* flies were heat shocked and *per* RNA levels were assayed every 2 h. The experiment was repeated twice with similar results.

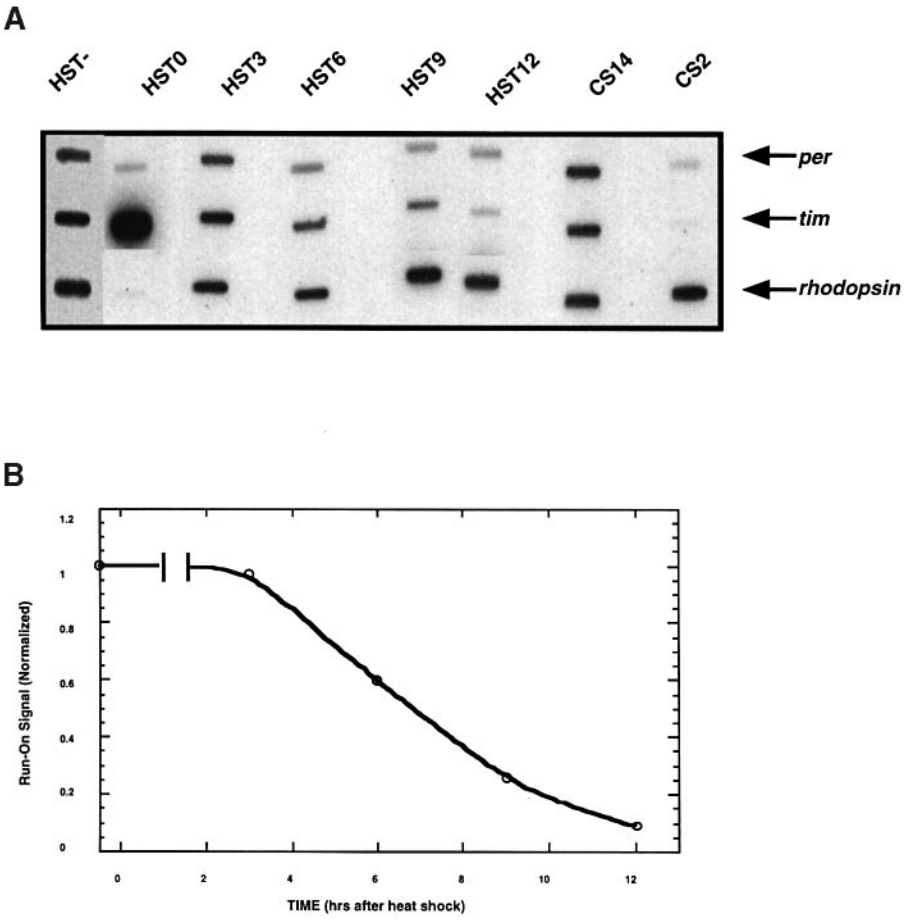


Fig. 5. Transcriptional regulation cannot explain the rise in *per* RNA. (**A**) Flies were heat shocked for 30 min at 37°C and recovered for the times indicated. Nuclear run-on analysis was then performed as described in So and Rosbash (1997). The blot was exposed to X-ray film for ~24 h. (**B**) Quantitation of the *per* run-on signal normalized to the rhodopsin signal is shown. The experiment was repeated twice with similar results. All the points shown are from a single experiment.

common transcriptional mechanism for the repression of both *per* and *tim* transcripts.

TIM-induced PER increase does not require the transcription factor CYC

CYC is a *Drosophila* bHLH-PAS transcription factor which, together with its dimerization partner CLK, drives transcription from the *per* and *tim* promoters (Allada *et al.*, 1998; Darlington *et al.*, 1998; Rutila *et al.*, 1998b). In the cyc^0 null mutant, *per* and *tim* transcripts levels are very low and apparently do not cycle. We crossed the *hs-tim* transgene into a *cycle* mutant background. In this case, a homozygous viable line (line G) carrying the *hs-tim* insert on the second chromosome was used. This line expressed somewhat higher amount of TIM at 25°C when compared with line F (which was used in all other experiments). We assayed PER protein and *per* mRNA levels in cyc^0 flies carrying the *hs-tim* transgene. Consistent with the post-transcriptional hypothesis, a heat shock caused a 4-fold rise in PER levels (Figure 6A). The time course of the increase is very similar to what occurs in the original tim^{01}*;hs-tim* experiment (Figure 2). We also assayed changes in *per* RNA levels following a heat shock in flies maintained at either 18 or 25°C. In both cases, an increase in *per* mRNA was observed 8–10 h after heat shock (Figure 6B). In the case of flies maintained at 18°C, a robust 2-fold increase in *per* mRNA was observed ~10 h after heat shock. When flies maintained at 25°C were heat shocked, *per* mRNA levels rose to ~40% of the maximal values observed in wild-type flies at ZT 15, an increase of 1.5-fold from the 25% of wild-type ZT 15 levels present in the pre-heat-shocked flies. In both cases, the values plotted are normalized to the starting RNA value. Since line G expresses higher amounts of TIM at 25°C when compared with 18°C, *per* mRNA amounts are higher to start with and a less impressive increase is observed. It is also possible that the smaller *per* RNA increase observed in cyc^0 flies carrying *hs-tim* may be due to lower levels of important *cyc*-transcribed factors in the mutant strain. These may even include PER, which is also present at much lower levels in this strain. Importantly, no strong repression occurred subsequent to the *per* RNA rise in either the 18 or 25°C case, as both protein and RNA levels slowly returned to pre-heat shock values. The data suggest that robust transcriptional repression requires the CLK–CYC system but that the TIM-mediated post-transcriptional increase in PER and *per* mRNA can occur without the transcriptional circuit.

TIM overexpression rescues restricted features of the Drosophila circadian activity cycle

Lastly, we asked whether HS-TIM induction could rescue some aspects of the activity–rest cycle. We preferred

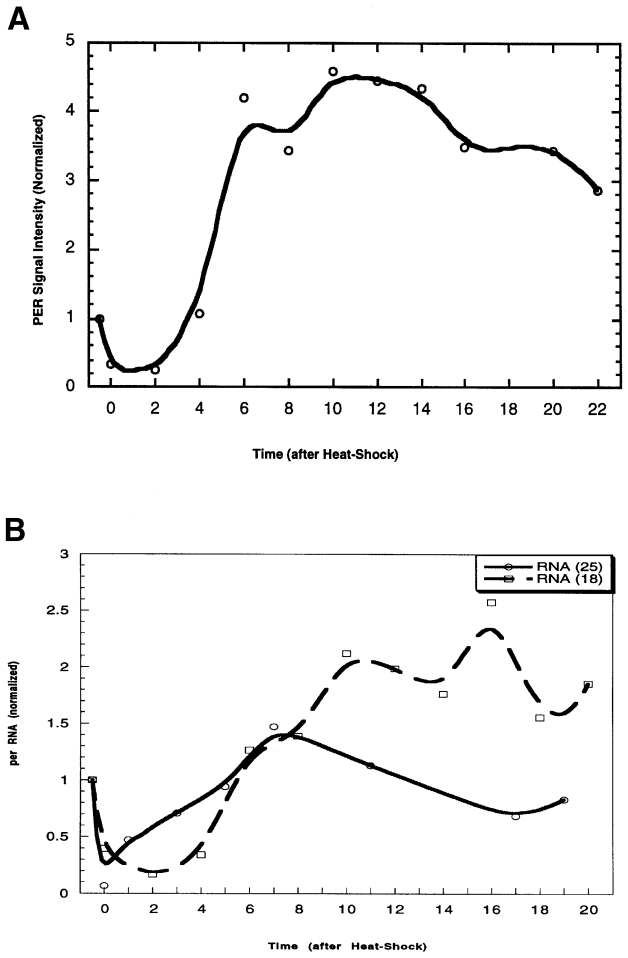


Fig. 6. TIM induction causes an increase in *per* mRNA and PER protein in cyc^0 mutant flies. (**A**) PER levels increase several-fold in cyc^0 mutant flies following TIM induction. Flies were maintained at 25°C. Heat shock and recovery were as described in the legend to Figure 1B. Three independent experiments were performed. Quantitation of one of the Western blots is shown. (**B**) *per* mRNA also increases following TIM induction in cyc^0 mutant flies. The RNA (18) experiment was done on flies maintained at 18°C, heat shocked for 30 min at 37°C and then returned to 18°C for recovery. The RNA (25) experiment was done identically except that the flies were maintained and recovered at 25°C. The PER signal was quantitated and plotted as described in the legend to Figure 4.

average activity analysis to period analysis, since the former affords a clear view of locomotor activity changes irrespective of periodicity (Hamblen-Coyle *et al.*, 1992). tim^{01}*;hs-tim* flies were entrained for one LD cycle, placed in DD and then heat pulsed for five consecutive days at CT10:30 for 30 min at 37°C. Data from several flies were then pooled to generate the average activity plot (average No. of activity events/30 min bin). Remarkably, TIM induction resulted in the generation of a clear wave of activity (Figure 7). The result was dependent upon the presence of the *hs-tim* transgene and the heat shock; control tim^{01} flies were not affected appreciably by the heat shocks, and the tim^{01}*;hs-tim* strain showed the random activity pattern characteristic of an arrhythmic strain without the heat shocks. In addition, the activity values of the controls showed much more variability, suggesting that induction of TIM leads to a more uniform behavioral profile (note scale change in Figure 7B). Taken together, the results indicate that the heat shock TIM cycle is comparable in several aspects with a wild-type cycle, including the induction of the downstream locomotor activity output machinery.

In summary, our observations suggest that the circadian cycle in tim^{01} flies is frozen at ZT 7–8 (relative to a wild-type cycle). TIM induction initiates accumulation of PER–TIM dimer during the first 2–4 h after heat shock. The subsequent *per* mRNA level increase requires PER, suggesting that the PER–TIM dimer positively feeds back to increase *per* mRNA levels during the subsequent 2–4 h. The increased mRNA levels then contribute to additional PER accumulation. Together with post-translational protein stabilization and processing, this results in sufficient PER and TIM levels and activity to cause the subsequent decrease in *per* and *tim* transcription. This series of events nicely recapitulates the wild-type cycle between about ZT 8 and 24.

Discussion

The recent identification and cloning of *Clk* and *cyc* fills a major gap in our understanding of circadian transcription (Allada *et al.*, 1998; Bae *et al.*, 1998; Darlington *et al.*, 1998; Lee *et al.*, 1998; Rutila *et al.*, 1998b). These clock genes have defined clock components with biochemical functions that link directly to transcriptional regulation, perhaps directly to the robust transcriptional oscillations characteristic of the normal PER and TIM cycle. However, the *Drosophila* circadian clock has apparently evolved multiple regulatory mechanisms that govern these RNA and protein oscillations (Dembinska *et al.*, 1997; So and Rosbash, 1997; Stanewsky *et al.*, 1997; Kloss *et al.*, 1998; Price *et al.*, 1998). The extent to which transcriptional regulation is necessary or sufficient for rhythmicity is therefore not entirely clear.

Recently, Hardin and colleagues have reported strong PER protein cycling in photoreceptor cells from a PER-expressing transgene with a constitutive promoter (Cheng and Hardin, 1998). In this strain, there was only limited *per* mRNA cycling, indicating that translational and post-translational processes can drive PER protein rhythms adequately. Other experiments, with promoter-less *per* transgenes for example, also indicate that multiple regulatory modes govern *per* mRNA and protein rhythms (Frisch *et al.*, 1994). Although these results suggest that circadian transcriptional regulation of *per* is dispensable, normal regulation of *tim* may be critical for the observed post-transcriptional regulation of *per* mRNA and protein. In the *Clock* and *cycle* mutants, *per* and *tim* transcription are low and non-cycling, and there are no detectable molecular or activity rhythms. The *hs-tim* rescue of some aspects of PER cycling in the cyc^0 mutant is consistent with the notion that a sharp increase in TIM levels is important for continuing molecular oscillations during the mid–late morning. This may normally require a strong increase in *tim* transcription, indicating that TIM as the lead molecule (see below) may be more dependent on transcriptional induction or de-repression than PER. It will be interesting to see if constitutive TIM expression can function as well as constitutive PER expression (Ewer *et al.*, 1988; Vosshall and Young, 1995; Cheng and Hardin, 1998) in rescuing features of the circadian program. Temporal regulation of transcription may be even more

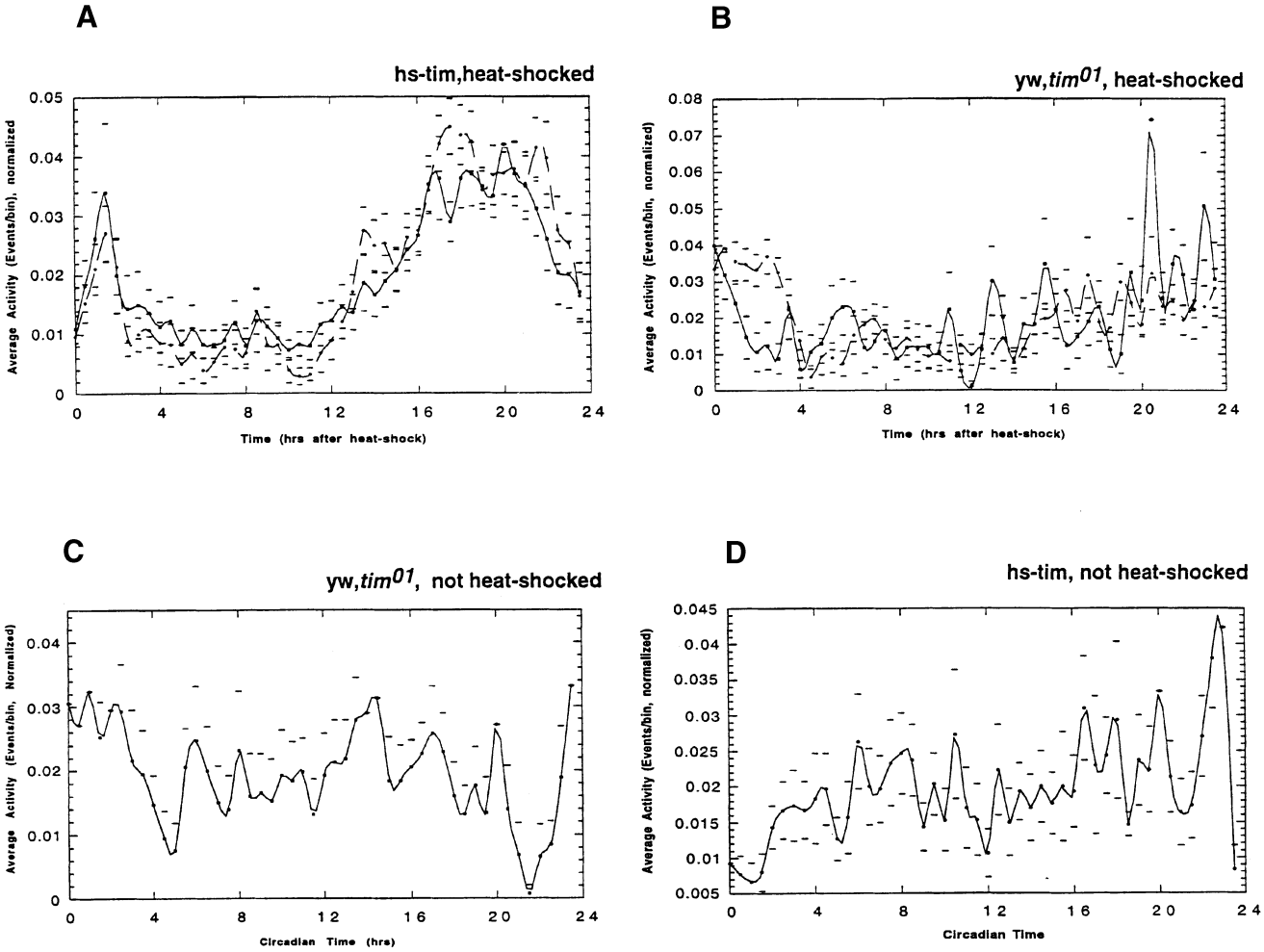


Fig. 7. TIM induction rescues features of the *Drosophila* activity cycle. (**A**) Activity profiles for heat-shocked *yw;tim*^{01}*;hs-tim* flies. The animals were entrained for two LD cycles and then transferred to DD and heat shocked at 37°C for five consecutive days from CT10:30 to CT11:00. The *y*-axis represents normalized activity in arbitrary units. The *x*-axis represents time after heat shock. Data from 16 flies averaged over 4 days (solid line) and 32 flies averaged over 4 days (dashed line) are shown. Standard deviations are noted by the horizontal dashes. (**B**) Activity profiles for heat-shocked *yw;tim*^{01} flies. The experiment was done exactly as described above. Two different experiments are plotted. Data from 16 flies averaged over 4 days (solid line) and 32 flies averaged over 4 days (dashed lines) are shown. (**C**) Activity profiles of *yw;tim*^{01}*;hs-tim* flies entrained for two LD cycles and then transferred to DD. Data from 32 flies averaged over 4 days in DD are plotted. (**D**) Activity profiles of *yw;tim*^{01} flies entrained for two LD cycles and then transferred to DD. Data from 32 flies averaged over 4 days in DD are plotted.

important for other clock genes, and it should be interesting to test whether constitutive TIM and PER constructs can rescue molecular or behavioral cycling in *cyc*^{0} flies.

However, the most straightforward conclusion of our TIM induction experiments is that normal transcriptional regulation at the *tim* promoter is not strictly required for many aspects of the circadian program. The function of *tim* transcriptional regulation may be just to aid the appearance and disappearance of the protein, which is apparently phenocopied well by the 30 min heat shock. The experiments also suggest that TIM overexpression is not problematic, at least under this pulse protocol. Even aspects of the behavioral program are induced by TIM induction: there is a broad activity peak subsequent to the 30 min heat shock in the *tim*^{01}*;hs-tim* strain (Figure 7). However, the timing of this activity wave does not correspond perfectly to what normally occurs in DD (data not shown), which suggests that aspects of the normal circadian sequence of events are aberrant. This may reflect peculiarities of heat shock TIM synthesis; an excess quickly and then less than wild-type amounts somewhat later. Alternatively, it may be due to some downstream consequence of this unusual model of TIM synthesis. In this context, it is worth noting that it is not known how the molecular oscillations link to the behavioral program in DD, neither the temporal relationships nor the biochemical links between the two cycles.

PER–TIM dimer as a positive effector in the Drosophila circadian clock

To gain insight into the molecular events initiated by TIM induction, we first assayed PER protein levels in *tim*^{01}*; hs-tim* flies following TIM induction. PER showed a striking pattern of accumulation, phosphorylation and disappearance. The kinetics were indistinguishable from a wild-type cycle (Marrus *et al.*, 1996) although PER levels were somewhat higher. TIM has been suggested to be required for stabilization of PER (Price *et al.*, 1995). This is because PER levels in *tim*^{01} are low (~20–30% of the peak levels in wild-type), whereas *per* mRNA levels in this strain are ~50–70% of their peak levels in wild-type flies. All of these relationships are consistent with

the notion that TIM helps drive the PER increase in wild-type flies (Zeng *et al.*, 1996). PER and TIM oscillate with an ~5- to 10-fold amplitude in wild-type flies, and TIM phase-leads PER by ~2 h. TIM levels are low in the day, with a trough at about ZT 3–5. They increase dramatically between ZT 11 and 15 and peak around ZT 17–19. The PER trough and peak are later, at about ZT 7–9 and ZT 19–21, respectively. Moreover, TIM levels are greater than those of PER during the early phase of the accumulation cycle (Zeng *et al.*, 1996).

We then measured changes in steady-state *per* RNA levels following the heat shock. *per* mRNA levels initially declined post-heat shock but then rapidly increased to ~2- to 3-fold the levels in non-heat-shocked flies. *per* mRNA levels peaked ~6 h after the heat shock, whereas protein levels peaked ~10 h after the heat shock, suggesting that the increase in PER protein levels was due in part to the mRNA rise. This phase difference is very similar to that observed in wild-type flies (~3–4 h; Zeng *et al.*, 1996). We also assayed endogenous tim^{01} mRNA levels in the *hs-tim* strain, and they did not show any increase over initial levels. This served as a good control in our assays and suggested that the post-transcriptional mechanism is specific for *per* mRNA, consistent with previous observations (So *et al.*, 1997).

To assess the contribution of transcription to the mRNA rise, we directly measured relative transcription rates using a nuclear run-on assay. Although the heat shock caused an initial decline, the rates recovered to their pre-heat shock levels but did not show any further rise. We conclude that TIM acts positively to upregulate *per* mRNA levels through a post-transcriptional mechanism. This hypothesis is also supported by the PER and *per* mRNA increases in the arrhythmic cyc^{0} strain.

We observed no overshoot of per^{01} mRNA levels in a per^{01} background, suggesting that the mechanism requires PER. Although per^{01} nonsense mRNA may have unusual characteristics, it has been shown to cycle robustly in a wild-type PER background (Frisch *et al.*, 1994). The relevant defect in the per^{01}*;hs-tim* strain is therefore probably the absence of PER. We suggest, therefore, that the positive effector is the PER–TIM dimer. Consistent with this notion are the glycerol gradient centrifugation and co-immunoprecipitation experiments, which indicated that most of the PER synthesized in the first 6–8 h of a cycle is sequestered within a PER–TIM dimer (Figure 3). However, there are other genes that are poorly expressed in a per^{01} strain (Rouyer *et al.*, 1997), indicating that the *per* requirement does not demand a direct role of PER or the PER–TIM dimer in the feedback mechanism. In any case, the target is probably cytoplasmic *per* mRNA, which increases in half-life. However, *per* mRNA is probably not an exclusive target, as other mRNAs may also be up-regulated by TIM at the post-transcriptional level (So and Rosbash, 1997). These other genes may even participate in subsequent clock-relevant regulatory events (Figure 8).

Several components of the circadian machinery are well conserved across large evolutionary distances. However, there are also variations in the mechanisms that underlie molecular oscillations in different organisms (Dunlap, 1998). In insects other than *Drosophila*, clock molecules are best characterized in *Antheraea pernyi* (Sauman and Reppert, 1996a,b) In this organism, PER and TIM immunoreactivity in the brain is limited to the cytoplasm of eight neurons, with no discernible nuclear staining, in contrast to the *Drosophila* paradigm where PER nuclear entry in the pacemaker neurons appears to be gated temporally (Vosshall *et al.*, 1994; Curtin *et al.*, 1995; Saez and Young, 1996). These differences can be reconciled by suggesting that the modest amplitude of *A.pernyi per* mRNA cycling is due to post-transcriptional regulation by cytoplasmic TIM or by the PER–TIM dimer. Indeed, this may be the dominant mode of regulation in *A.pernyi* pacemaker neurons, with little or no transcriptional regulation. However, *A.pernyi* photoreceptor cells appear to be more similar to the *Drosophila* paradigm, with intense nuclear PER staining (Sauman and Reppert, 1996a), so different modes of regulation may operate, even within different tissues of a single animal (Hardin, 1994).

Transcriptional repression and its relationship to post-transcriptional regulation

We used RNase protection analysis and nuclear run-on assays to analyze the subsequent decline of the *per* mRNA curve. A pronounced, rapid decrease in steady-state *per* as well as tim^{01} mRNAs occurred ~10–12 h after the heat shock, and run-on analysis indicated that this was largely or exclusively a transcriptional event. This correlates well with PER–TIM accumulation and the beginning of the phosphorylation program, indicating that PER and/or TIM might be reversing the accumulation of their own mRNAs through an effect on transcription. The relationship is consistent with the recent suggestion that PER and TIM inhibit CLK- and CYC-mediated transcription of the *per* and *tim* genes (Darlington *et al.*, 1998). There is probably no difference between these features of the transcriptional program initiated by *hs-tim* induction and those of a wild-type cycle. PER and TIM may carry out transcriptional inhibition directly, or they may influence transcription only more indirectly (Figure 8).

Although these experiments cannot distinguish between these two possibilities, they offer a novel perspective. The inducible TIM strain initiates the circadian program as a linear series. In principle, this facilitates the establishment of cause and effect relationships or facilitates addressing certain possibilities. TIM induction mimics events that normally begin at about ZT 8, and it recapitulates events that occur during the ZT 8–16 phase of the wild-type cycle. These include PER–TIM dimer accumulation and the increase in *per* mRNA levels that takes place during the first 6–8 h post-heat shock. PER levels increase in parallel with PER–TIM dimer levels, and PER then becomes phosphorylated and free from TIM, 8–16 h after the initial heat shock. This resembles the wild-type pattern during the last two-thirds of the night (ZT 16–24). It is therefore possible that phosphorylated forms of the proteins, free of each other, negatively regulate transcription and lead to a sharp decrease in *per* as well as *tim* mRNA levels, as previously suggested (Rosbash *et al.*, 1996). Although complexes with other proteins may be important (Lee *et al.*, 1998), the observations suggest that the PER–TIM dimer is not directly involved in transcriptional repression.

However, a more conservative view suggests that even PER and/or TIM monomers may not act on transcription directly, because of the many hours between their

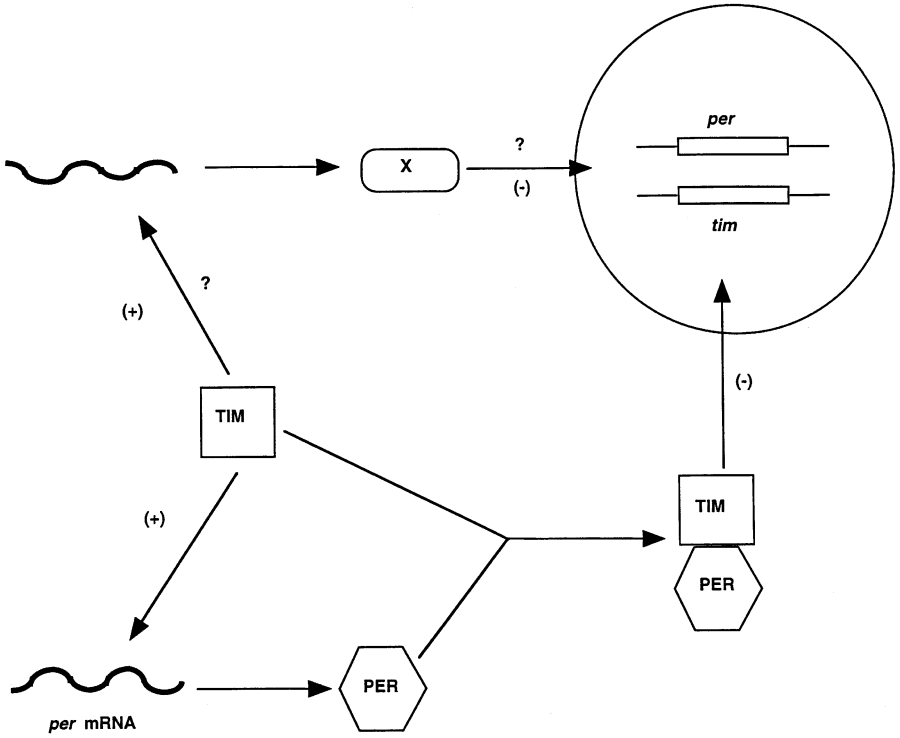


Fig. 8. A model for the generation of high amplitude oscillations in *per* and *tim* mRNA through autoregulatory transcriptional and post-transcriptional regulation. Transcription factors CLK and CYC generate high levels of *per* and *tim* RNA. TIM and PER then post-transcriptionally increase *per* mRNA to peak levels. This form of post-transcriptional regulation may also be applicable to other transcripts, including those that may be involved in subsequent transcriptional repression. It is also possible that the PER–TIM dimer or monomeric PER and TIM themselves repress transcription. The arrows do not necessarily imply direct regulation.

synthesis and the initiation of transcriptional repression. Importantly, there is still no evidence for a direct role for either protein in transcriptional regulation, and an earlier role for PER and/or TIM is more likely to take place without requiring intervening steps. Therefore, the transcriptional effect may be indirect and may depend on a more direct role in the earlier post-transcriptional upregulation. This could include other mRNAs in addition to *per* (So and Rosbash, 1997), some of which might encode direct transcriptional repressors (Figure 8). Identification of additional up-regulated mRNAs might provide more support for this hypothesis.

Materials and methods

Stocks and generation of hs-tim transgenic flies

The DNA construct for injections was made by cloning a *Not*I–*Xho*I fragment of TIM (Rutila *et al.*, 1998a), containing full-length *tim* cDNA, with a hemagglutinin (HA) peptide tag (YPYDVPDYA) at the C-terminus, into pCasper-hs. *w;tim*^{01} flies were then transformed using P-element-mediated transformation (Spradling *et al.*, 1995). Several lines were obtained. The flies were later crossed into a *yw;tim*^{01} background. For all the biochemical and behavioral analyses reported here, line F, which carries the transgene on the third chromosome, or line G, which carries the transgene on the second chromosome, were used. Similar results were obtained with the other tested lines (data not shown). All lines were behaviorally arrhythmic in a *tim*^{01} background at 18, 25 or 29°C (Table I; data not shown).

Behavioral analysis

Locomotor activity assays were performed on adult males as described in Hamblen-Coyle *et al.* (1992). For activity profile analysis, flies were entrained for two 12 h:12 h L:D cycles. Where needed, the flies were heat shocked at CT10:30–CT11:00 during the dark cycle by transferring the entire activity monitor into a 37°C hybridization oven for 30 min at the appropriate time. Average activity plots were generated as described in Rutila *et al.* (1998a).

Glycerol gradient centrifugation and Western blotting analysis

For all the biochemical assays, 50–80 flies in glass vials were heat shocked in a water bath at 37°C for 30 min. The flies were then allowed to recover in the dark at 25°C, and samples were collected every 2 h. The non-heat shocked sample was also kept in darkness during this period. For glycerol gradient analysis, 40 flies (for each time point) were homogenized in extraction buffer [20 mM HEPES pH 7.5, 100 mM KCl, 5% glycerol, 20 mM β-glycerophosphate, 100 μM Na_3VO_4, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A]. The samples were microcentrifuged at 13 000 *g* for 10 min. The supernatant was loaded on a 2 ml 5–30% glycerol gradient and centrifuged at 55 000 r.p.m. for 4 h at 4°C in a TLS55 rotor using a Beckman TL100 centrifuge. Fractions of 100 μl were then withdrawn sequentially from the top. Proteins in each fraction were precipitated with 5% trichloroacetic acid (TCA), washed with acetone, dried, resuspended in SDS–PAGE sample buffer, run on 6% SDS–PAGE and analyzed by Western blotting as described below.

To measure protein levels by Western blotting, protein extracts were made and analyzed as in Zeng *et al.* (1996). Protein extracts (10 heads per sample) were run on a 6% SDS–PAGE gel, electroblotted onto nitrocellulose membrane and probed for PER using rabbit anti-PER antibody (Dembinska *et al.*, 1997) at 1:10 000 dilution. Horseradish peroxidase (HRP)-conjugated anti-rabbit serum (Amersham) was used as the secondary antibody at 1:1000 dilution. TIM was probed with rat anti-TIM antibody (Suri *et al.*, 1998) at 1:10 000 dilution. HRP-conjugated anti-rat serum (Amersham) was used as the secondary antibody at 1:1000 dilution. Where needed, blots were quantitated using a phosphoimager (Bio-Rad, CA) or an ARCUS scanner (AGFA, NV), in both cases using Molecular Analyst software (Bio-Rad, CA).

RNase protection assays and nuclear run-on analysis

RNase protection assays were performed using an RPAII kit (Ambion, TX) following the procedures previously described (Zeng *et al.*, 1994)

with minor modifications. Total RNA was isolated from 50 fly heads for each sample, treated with RQ1 RNase-free DNase (Promega) and hybridized overnight (~12–14 h) with *per2/3*, tim^{01} and *rp49* probes. *per2/3* and *rp49* probes are identical to those used by Zeng *et al*. (1994). The tim^{01} probe was generated by placing a T7 promoter in front of a 167 nucleotide *tim* cDNA fragment, nucleotides 2283–2450 (DDBJ/EMBL/GenBank accession No. U37018). This probe gives two bands with RNA from *yw*;tim^{01};*hs-tim* flies corresponding to *tim* RNA (167 nucleotides) and tim^{01} RNA (97 nucleotides) respectively. *per2/3* and tim^{01} probes were used at 10^6 c.p.m./reaction and *rp49* was used at 2×10^5 c.p.m./reaction. Excess RNA was digested with 10 U of RNase ONE (Promega) for 1 h at 37°C. Quantitation was done using a phosphoimager (Bio-Rad, CA).

Nuclear run-on analysis of transcription was carried out as previously described (So and Rosbash, 1997) using the same probes for *per*, *tim*, rhodopsin, histone 4B and *lacZ*. Bands were quantitated using a phosophoimager (Bio-Rad, CA).

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