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Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling

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The *period* (*per*) and *timeless* (*tim*) genes are intimately involved in the generation and maintenance of *Drosophila* circadian rhythms. Both genes are expressed in a circadian manner, and the two proteins (PER and TIM) participate in feedback regulation which contributes to the mRNA oscillations. Previous studies indicate that the circadian regulation is in part transcriptional. To investigate quantitative features of *per* and *tim* transcription, we analyzed the *in vivo* transcription rate in fly-head nuclei with a nuclear run-on assay. The results show a robust transcriptional regulation, which is similar but not identical for the two genes. In addition, *per* mRNA levels are regulated at a post-transcriptional level. This regulatory mode makes a major contribution to the *per* mRNA oscillations from a previously described *per* transgenic strain as well as to the mRNA oscillations of a recently identified *Drosophila* circadianly regulated gene (*Crg-1*). The data show that circadian mRNA oscillations can take place without evident transcriptional regulation.

Keywords: circadian rhythms/*Drosophila*/*per*/post-transcriptional regulation/*tim*

Introduction

Daily physiological and behavioral oscillations have been extensively studied in a wide variety of organisms, from bacteria to humans. These circadian oscillations are driven by circadian clocks, which are intracellular time-keeping mechanisms that keep running under constant environmental conditions, but can be reset by environmental stimuli such as light (Edmunds, 1988).

Genetic screens for mutants with altered circadian rhythms identified the *period* (*per*) gene in *Drosophila melanogaster* (Konopka and Benzer, 1971). Molecular and genetic evidence indicates that the *per* gene product, PER, is a bona fide clock component (Hardin *et al.*, 1990; Edery *et al.*, 1994a). More recently, the *timeless* gene (*tim*) was identified as a second clock component (Sehgal *et al.*, 1994). It encodes a protein, TIM, that undergoes rapid light-induced degradation, postulated to be the initial molecular response of the clock to the light (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). TIM forms a heterodimeric complex with PER and contributes to clock function (Gekakis *et al.*, 1995; Zeng *et al.*, 1996). Both PER and TIM are subject to circadian regulation, and it is generally believed that regulated expression of the two genes is intimately tied to clock function (Rosbash *et al.*, 1996).

Previous studies have shown that *per* gene expression is regulated at the transcriptional and post-translational levels. The latter includes circadian changes in protein half-life (Dembinska *et al.*, 1997). At the transcriptional level, *per* undergoes robust changes as a function of circadian time. For example, *per* upstream DNA can drive circadian cycling of unrelated reporter genes (Hardin *et al.*, 1992; Brandes *et al.*, 1996; Stanewsky *et al.*, 1997). Moreover, recent results from Hardin and colleagues have defined a 69 nt cycling box within the *per* promoter (Hao *et al.*, 1997). The resultant circadian changes in *per* and *tim* mRNA levels require their functional gene products, as these mRNA oscillations are altered in PER and TIM mutants (Hardin *et al.*, 1990; Sehgal *et al.*, 1995; Marrus *et al.*, 1996). Experiments of this nature define an autoregulatory feedback loop, in which PER and TIM activities affect their own gene expression (Hardin *et al.*, 1990; Zeng *et al.*, 1994). Autoregulatory feedback regulation is an integral part of another clock gene, *frequency* (*frq*), in *Neurospora crassa* (Aronson *et al.*, 1994). Therefore, this mechanism appears to be a common feature of circadian clock function.

Although the circadian fluctuations of *per* mRNA are under transcriptional regulation, measuring reporter mRNA levels does not indicate the quantitative changes in the temporal rate of transcription. To measure this directly, we developed a nuclear run-on assay for fly heads and analyzed the *in vivo* transcription rate of the *per* and *tim* genes. The results obtained, and similar measurements on another clock-relevant gene, suggest that there is important circadian regulation at a post-transcriptional level. We postulate that this additional regulatory mode serves to ensure proper circadian fluctuations of clock gene expression.

Results

Expression of per and tim is transcriptionally controlled

To measure *per* and *tim* transcription as a function of circadian time, we applied the nuclear run-on assay to nuclei made from fly heads frozen at different circadian times. This assay has been used to measure the *in vivo* transcription rate in many cell culture systems (Weber *et al.*, 1977; Love and Minton, 1985; Rougvie and Lis, 1988). The nuclei are incubated in a buffer containing [α-^{32}P]UTP, and previously initiated RNA polymerases will continue to elongate and incorporate radioactive triphosphates into nascent RNA chains. The nascent RNA of a specific gene can be detected by hybridization of the radioactive RNA to a nylon membrane containing cloned DNA. Therefore, the nuclear run-on assay measures the

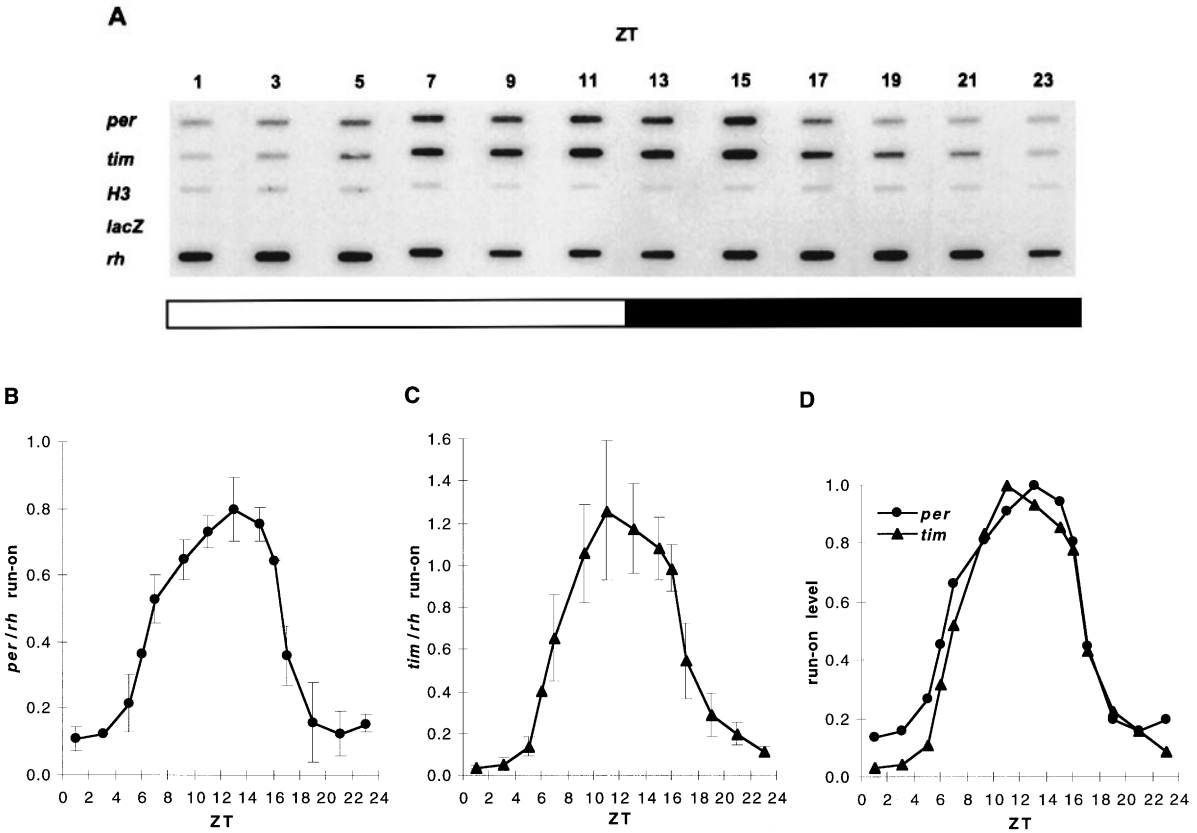

Fig. 1. Rates of *per* and *tim* transcription in wild-type flies. (**A**) A representative blot from a nuclear run-on assay. Each column is an individual hybridization blot from wild-type flies (Canton-S) entrained and collected at the time indicated above the blot (see Materials and methods for details). Each row shows hybridization signals from the genes indicated on the left. Note that *per* and *tim* show oscillating rates of transcription whereas the internal controls, histone 3 (*H3*) and rhodopsin (*rh*) genes, show relatively constant rates. The negative control, the *lacZ* gene, shows no detectable signal. The open and filled bars represent the time when light was on (ZT0–12) and off (ZT12–24), respectively. (**B**) Quantification of rate of *per* transcription in wild-type, showing the average of several experiments. The run-on signals were normalized to the *rh* signals. Error bars indicate the standard deviation (n = 2–6). (**C**) Quantification of transcription rate of *tim* in wild-type. Data were analyzed in the same way as in panel B. (**D**) Peak values of the *per* and *tim* curves in panel B were set to 1. The comparison shows the similar phase of the two curves.

relative amount of nascent transcripts made from the previously initiated RNA polymerases and hence the *in vivo* rate of transcription (O'Brien and Lis, 1993).

As predicted from reporter gene measurements (Hardin *et al.*, 1992; Brandes *et al.*, 1996), *per* and *tim* transcription rates in wild-type flies cycle with robust amplitudes and similar phases (Figure 1A–D). They start to increase at approximately zeitgeber time (ZT) 5 and stay at a high level from ZT9 to ZT16 (zeitgeber time is the time in hours from the start of a normal 12 h light–12 h dark cycle). The timing of the transcriptional upregulation correlates with low levels of PER and TIM, and is consistent with derepression and negative feedback as suggested previously (Zeng *et al.*, 1994). The broad peaks are followed by a sharp drop beginning at ZT16, which reflects strong and rapid gene repression.

As expected, the transcription curves are displaced even further from their respective protein curves than the mRNA curves (Figure 2), indicating an extensive delay in protein accumulation relative to transcription. The delay may be relevant to aspects of negative feedback and emphasizes the importance of examining transcription rather than mRNA curves.

Although the patterns and phases are similar, the *per* and *tim* curves differ in their maximal transcription rates as well as their cycling amplitudes. Both parameters can be compared by using DNA probes of the same size. This approach indicates that the *tim* transcription rate is about two to three times higher than that of *per* at the peak time point (Figure 3, ZT13). Moreover, the cycles of *tim* transcription have an amplitude (maximal/minimal level) of ~30 whereas those of *per* transcription have an amplitude of 7–8 (Figure 3). These differences cannot be due solely to hybridization differences between the transcripts, as these would not simultaneously increase relative *tim* peak values and decrease relative *tim* trough values.

The transcription rates of several *per* and *tim* mutants were also investigated using this assay. We examined transcription in the per^S genotype, which has a ~19 h period. The *per* and *tim* profiles show advanced phases (data not shown) which correlate with both the behavioral and the mRNA data (Hamblen-Coyle *et al.*, 1992; Marrus *et al.*, 1996). In the arrhythmic per^{01} and tim^0 null mutants, both genes are transcribed at intermediate levels compared with the wild-type control strain (Figure 3). This correlates roughly with the mRNA levels observed in these mutants (Hardin *et al.*, 1990; Sehgal *et al.*, 1994) and also correlates with the non-cycling and intermediate RNA levels observed in constant light (Qiu and Hardin, 1996). In contrast, in the arrhythmic frq^9 null mutant of *Neurospora*, there are high constitutive levels of *frq* mRNA, which is more consistent with a simple negative feedback model

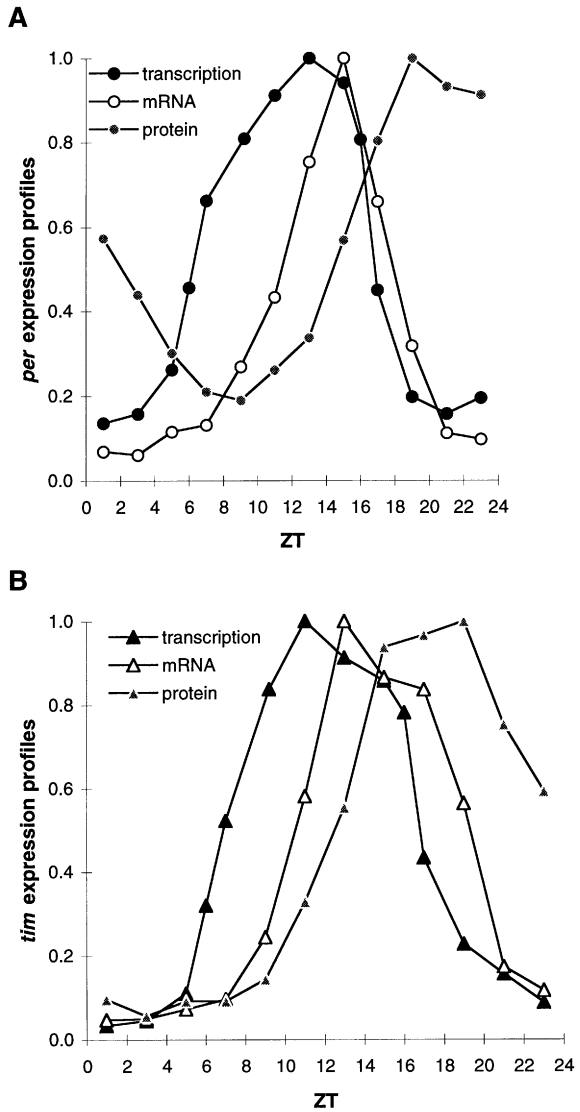

Fig. 2. Transcription, mRNA and protein curves of *per* (**A**) and *tim* (**B**) overlapped to show the delay of the protein profiles behind the transcription and mRNA curves. The transcription curves are from Figure 1 panels B and C, the mRNA curves are from Figure 5B and the protein curves are adapted from Marrus *et al.* (1996). All the curves have the peak values set to 1 for comparison.

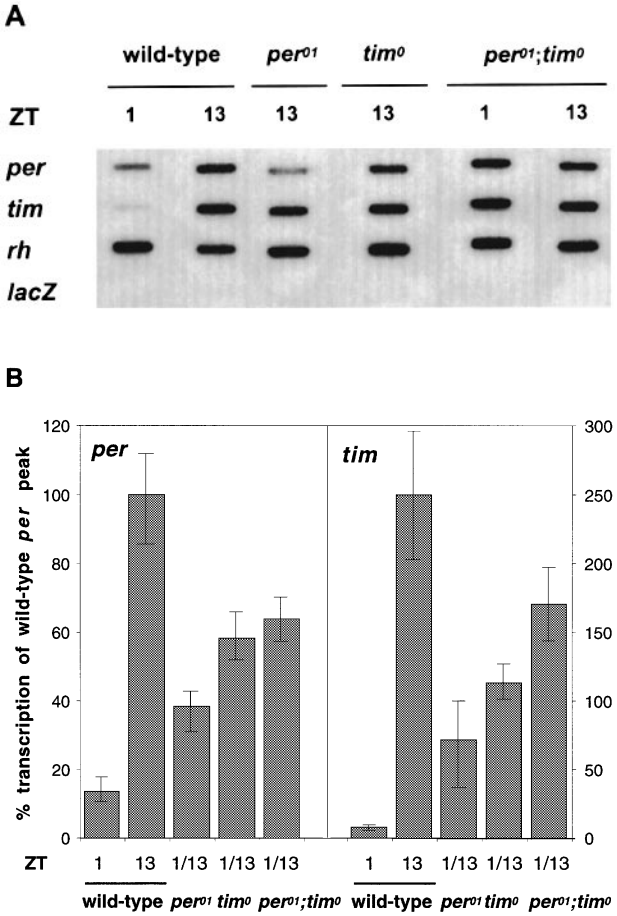

Fig. 3. Transcription rates in per^{01}, tim^{0} and $per^{01};tim^{0}$ mutants. (**A**) A representative blot from a nuclear run-on assay. Each column is an individual hybridization blot from the different genotypes. The flies were entrained and collected at the time indicated (ZT1 or ZT13). Each row shows hybridization signals from the genes indicated on the left. The negative control, *lacZ* gene, shows no detectable signal. (**B**) Quantification showing both *per* and *tim* transcription in the mutants are at an intermediate level as compared with wild-type. For the arrhythmic mutants, data from the two time-points (ZT1 and ZT13) were averaged. Note that the transcription scale of *tim* is 2.5-fold larger than that of *per*. The higher peak level of *tim* transcription was obtained from two independent experiments. Error bars indicate the standard deviation (n = 2–6, 5, 3 and 2 for the wild-type, per^{01}, tim^{0} and $per^{01};tim^{0}$, respectively).

(Aronson *et al.*, 1994). The intermediate transcription values in per^{01} and tim^{0} suggest that simple negative feedback is an insufficent model in the *Drosophila* system and that additional transcription regulators, an activator for example, are also under circadian control (Qiu and Hardin, 1996). Alternatively, PER and TIM may continue to manifest some biological activities in the arrhythmic backgrounds. This possibility is insufficient to explain completely the intermediate levels, because the double per^{01} tim^{0} mutant strain (which lacks both PER and TIM) still has an intermediate level of *per* and *tim* transcription (Figure 3).

Post-transcriptional regulation is also involved in per mRNA expression

Is the circadian regulation of transcription sufficient to explain the resulting mRNA patterns? To address this question, the *per* and *tim* mRNA curves can be compared with their transcription curves. In theory, for an mRNA with a temporally constant half-life, the mRNA cycling curve will have a delayed phase angle and a reduced amplitude compared with the transcription curve. An mRNA with a longer half-life will have an even greater delay and a lower amplitude compared with the transcription curve (Wuarin *et al.*, 1992; see also Figure 4). To obtain an independent measure of the mRNA profiles, both *per* and *tim* mRNA levels were measured by a standard RNase protection assay using the same head samples used for the run-on assays (Figure 5A and B). The results show that the *per* mRNA curve peaks at ZT15, as expected from previous experiments of this kind (e.g. Zeng *et al.*, 1994). The *tim* mRNA curve is very similar but has a slightly earlier phase than that of *per* and peaks at ZT13. Its level remains high and decreases only after ZT17.

A comparison of the *per* mRNA and the transcription

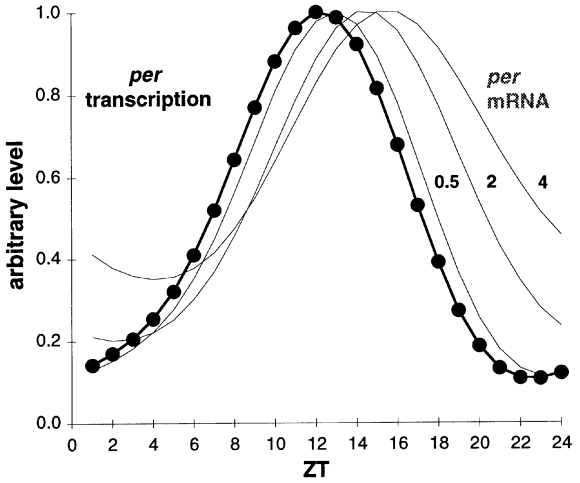

Fig. 4. Comparison of mRNA and transcription profiles by modeling (see Materials and methods). The transcription curve (thick line, T_{per}) were obtained from curve fitting of the experimental *per* transcription profile (Figure 1B). The mRNA curves (thin lines, *R*) are generated from the transcription curve by assuming constant mRNA half-lives of 0.5, 2 and 4 h. The peak values of all the curves were set to 1 for comparison of phase and amplitude. Note that the phase difference, amplitudes and shapes of the mRNA and transcription curves are different from those of the experimental curves (Figure 5). Comparison of *tim* mRNA and transcription profiles by modeling shows similar pattern.

profiles reveals a discrepancy with the simple model in which there is no post-transcriptional control (compare Figure 4 with Figure 5C). During the rising phase of *per* expression, the mRNA curves are much more delayed than during the declining phase. These differences are independent of the sources of the *per* mRNA profiles, which include previously published eye and brain *per* mRNA curves (Zeng *et al.*, 1994) and a head *per* mRNA curve generated by Northern blotting (Rouyer *et al.*, 1997). All three profiles show a similar pattern, i.e. a significant delay during the rising phase but coincident with the transcriptional curve during the declining phase (Figure 5C). Importantly, they also have amplitudes that are comparable to if not larger than the amplitude of the transcription curve. Taken together, these two features suggest a temporal regulation of *per* mRNA half-life (see Discussion): a more stable mRNA during the rising phase of the mRNA curve (especially from ZT9 to ZT15), and a less stable mRNA during the declining phase (after ZT15). Modeling suggests that the half-life change is between 2- and 4-fold (see Materials and methods).

We also considered that the discrepancy between the observed and the theoretical curves might be due to transcriptional control by RNA polymerase stalling, which might be poorly reflected in the run-on assay. However, we were unable to detect polymerase stalling at the key time-points, as DNA probes from the 5′ and 3′ ends of

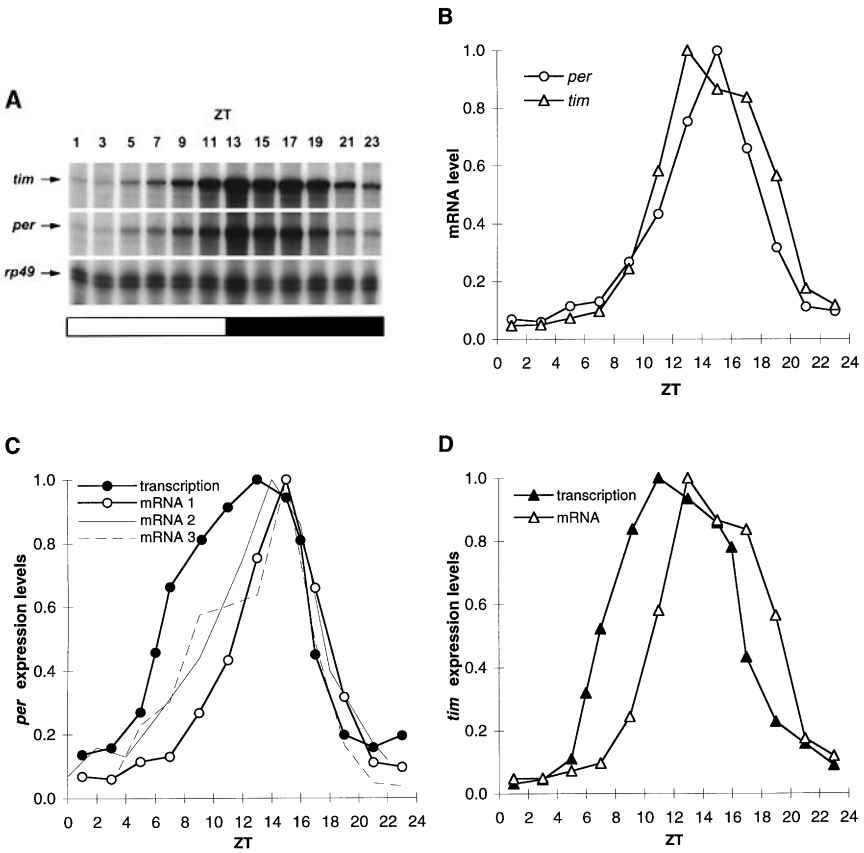

Fig. 5. Comparison of mRNA and transcription profiles. (**A**) RNase protection assay of mRNA from wild-type flies (the Canton-S strain) entrained and collected at the same time as the flies used in the nuclear run-on assay in Figure 1. Open and filled bars represent the time when light was on (ZT0–12) and off (ZT12–24), respectively. (**B**) Quantification of mRNA abundance in *A*. The *per* (○) and *tim* (△) mRNA signals were normalized to the *rp49* signals. Peak values were set to 1. (**C**) Comparison of *per* rate of transcription curve (●) from Figure 1 with *per* mRNA curves from three different sources: from panel B of this figure (○), from the Northern blot in Rouyer *et al.* (1997) (thin line) and from the RNase protection assay of eye mRNA in Zeng *et al.* (1994) (dashed line). Note the phase difference between the mRNA and transcription curves at the rising and declining phases. (**D**) Comparison of *tim* rate of transcription curve from Figure 1 with *tim* mRNA curve from panel B of this figure.

the genes showed similar run-on signals (data not shown; see Materials and methods).

A comparison of the *tim* mRNA and transcription profiles does not show the same differences, at least with the same degree of confidence (compare Figure 4 with Figure 5D). Since the *tim* mRNA curve rises somewhat earlier than the *per* mRNA curve (Figure 5B), the *tim* mRNA curve has a shorter delay behind the *tim* transcription curve during the rising zone. Furthermore, *tim* mRNA levels decrease somewhat later than *per* mRNA levels at ~ZT17, so *tim* mRNA has a greater delay behind *tim* transcription in the declining zone. These phase differences between the *per* and *tim* mRNA curves have been seen in two independent previous experiments (S.Marrus and J.E.Rutila, personal communication). Finally, the *tim* transcription amplitude is so high that it is difficult to be certain that the *tim* mRNA amplitude is comparable; a lower amplitude mRNA curve would be consistent with a more constant half-life. These considerations make it possible that the extent of post-transcriptional regulation of *tim* expression is not comparable to that of *per*, but this is not certain.

mRNA oscillation from the promoterless transgene 7.2 is regulated by mRNA stability

The circadian post-transcriptional regulation of *per* mRNA suggests that this could be important for RNA oscillations, especially in situations where the amplitude is modest. A prominent case is the promoterless *per* gene in the *7.2* transgenic lines. These flies are transformed with a 7.2 kb *per* genomic DNA fragment, which has the entire coding region but is devoid of the 5′ flanking region (Hamblen *et al.*, 1986; Figure 6A). In the line that exhibits the most robust rescue (Hamblen *et al.*, 1986), there is modest (2- to 3-fold) cycling of *per* mRNA transcribed from the *7.2 per* transgene (Frisch *et al.*, 1994). To investigate the quantitative feature of the protein cycling, we performed Western analysis (Figure 6B). Both PER and TIM clearly cycle in abundance, consistent with the PER cycling shown by histochemistry (Frisch *et al.*, 1994). However, PER cycles with a lower amplitude, as compared with wild-type PER peak and trough values assayed on the same gel. PER also has a delayed phase, as indicated by the later trough relative to the expected wild-type trough time of ZT7–9 (compare Figure 6B with Figure 5 of Zeng *et al.*, 1996). These features of PER cycling correlate well with the *7.2* mRNA cycling profile, which also has a lower amplitude and a later phase than that of wild-type (Frisch *et al.*, 1994). It also correlates with the rescued behavioral rhythmicity, which has a long period of 25.5 h [Hamblen *et al.*, 1986; Frisch *et al.*, 1994; the period of locomotor activity was 25.5 ± 0.3 h for per^{01};*7.2:9* flies (n = 8) and 25.8 ± 0.7 for per^{-};*7.2:9* flies (n = 28)]. The likely causes of the cycling *7.2* mRNA levels are: (i) fortuitous insertion under the control of an enhancer that can drive circadian cycling; (ii) an internal transcription element in the *7.2* region that can function independently of the promoter and drive mRNA cycling; (iii) cycling post-transcriptional regulation. The first two possibilities predict that transcriptional cycling will resemble mRNA cycling. If transcription of the *7.2* transgene is temporally constant, then the mRNA cycling is most likely due to post-transcriptional regulation.

To measure transcription from the *7.2* transgene and to distinguish it from endogenous per^{01} expression, we used two *per* gene probes simultaneously: one from the middle of the gene that recognizes both the per^{01} and the *7.2* transcripts and the other from the 5′ end of the gene that recognizes only the per^{01} transcript. Both produced rather low signals and approximately 2- to 3-fold cycling, with peak and trough values at the expected phases (Figure 6C). Subtraction of the two signals gives an approximate *7.2* transcription rate (Figure 6D). This representative curve has a very low amplitude, one-point peak at a time that does not correspond to an expected peak time. This point was not reproducible (data not shown). The low amplitude and bizarre phase of the *7.2* curve can be compared with that of *tim* gene cycling (Figure 6D), which was measured simultaneously and shows clear cycling; both amplitude and phase are appropriate for this gene (see Discussion).

Because this subtraction strategy is not optimal and to confirm the non-cycling transcription of this transgene, we measured its transcription in a *per* DNA null [Df(1)64j4/Df(1)TEM202] background (see Materials and methods, a gift from M.J.Hamblen). Since the behaviors of the two genotypes, per^{01};*7.2* and per^{-};*7.2*, are similar if not identical (see above), we expected the *7.2* transgene expression to be similar as well. The absence of the per^{01} gene allowed us to use a single probe that detects only *7.2*-derived transcripts. Run-on signals from this transgene confirmed the absence of circadian transcription (Figure 7) and indicated that cycling of *7.2* mRNA is probably the result of post-transcriptional regulation.

Post-transcriptional regulation of Crg-1 expression

In the course of these experiments, we sought to test another relevant gene for its transcriptional cycling. There is a newly identified circadianly regulated gene, *Crg-1* (Rouyer *et al.*, 1997), which encodes a putative transcription factor. *Crg-1* mRNA manifests circadian cycling, with a phase rather similar to that of *per* and *tim* mRNA and a peak:trough ratio of 3–4 in wild-type flies (Rouyer *et al.*, 1997; Figure 8A). However, the run-on signals of *Crg-1* show no obvious cycling pattern expected from the cycling mRNA profile but only a 1.5-fold fluctuation (Figure 8B). This varied somewhat in magnitude from experiment to experiment but was never more than 1.5-fold (data not shown). Therefore, some post-transcriptional regulation is required to generate the *Crg-1* mRNA curve.

Discussion

Reporter gene experiments indicate that *per* transcription is under circadian regulation, and our results are in good agreement with these previous studies (Hardin *et al.*, 1992; Brandes *et al.*, 1996; Stanewsky *et al.*, 1997). Both *per* and *tim* show a strong increase in transcription beginning at ZT5 and a rapid decrease beginning at ZT16. A negative feedback view suggests that these times correspond to transcriptional derepression and repression, respectively. Neither derepression at ZT5 nor repression at ZT16 requires a direct role of the PER–TIM system. Indeed, the transcription decrease at ZT16 is clearly earlier than the detected nuclear entry time of PER at ZT18 in lateral neurons (Curtin *et al.*, 1995). This is consistent with a

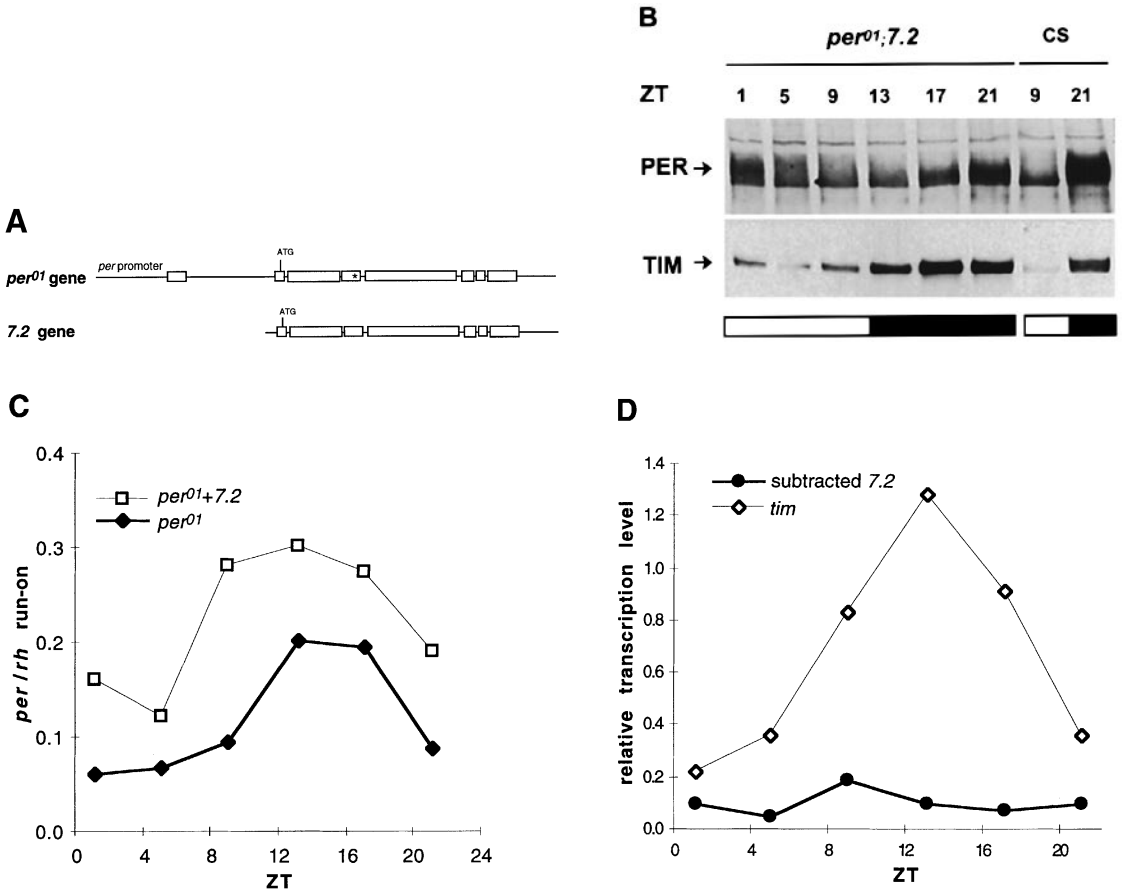

Fig. 6. Molecular cycling in *per*^{01}*;7.2* transgenic flies. (**A**) Schematic representation of the host *per*^{01} gene and the *7.2* transformation construct. The *per*^{01} gene has a premature stop codon (indicated by a star) and produces no observable protein. The 7.2 kb *per* transformation construct contains the start codon (ATG) and so when expressed, can produce functional protein. Open boxes denote exons and lines denote non-coding regions. (**B**) Western blot showing cycling of PER and TIM proteins in *per*^{01}*;7.2* flies as compared with wild-type (CS). Flies were entrained and collected at the time indicated above the blot. Open and filled bars represent times when light was on (0–12 h) and off (12–24 h), respectively. (**C**) Rate of transcription from two individual blots: one gives the sum of the *per*^{01} host gene and the *7.2* transgene (■) (detected by using a DNA probe in the overlapping region of the two genes), and the other gives the *per*^{01} host gene alone (◆) (detected by using a DNA probe in the 5′ region of the *per*^{01} gene). The run-on signals were normalized to the *rh* signals. (**D**) *7.2*-derived rate of transcription obtained by subtraction of the *per*^{01}-derived signal (◆ in panel C) from the sum of *per*^{01}- and *7.2*-derived signals (■ in panel C). The relative transcription level refers to *7.2*/*rh* or *tim*/*rh* values. The *tim* transcription profile is the average of the two individual blots in the same run-on assay. The levels were adjusted to the same probe length used in wild-type (Figure 1A), assuming a linear relationship between probe length and run-on signal.

quite indirect mode of action of PER–TIM on its own transcription, for example through the regulation of a cytoplasmic factor which then enters the nucleus. However, it is possible that a low level of PER–TIM enters the nucleus at ZT16. Alternatively, or in addition, our biochemical measurements may reflect nuclear gating that varies between tissues, so that PER–TIM may enter the nucleus earlier and/or interact with different nuclear transcription factors in other tissues (namely eye photoreceptor cells) that make a prominent contribution to the biochemical assays.

The detailed pattern of PER expression in *per*^{01}*;7.2* flies suggests that PER has a more prominent role than TIM in the negative feedback of transcription. In this strain, PER cycles with a low amplitude, with substantially lower peak levels than in a wild-type strain (Figure 6B; the PER level at ZT21 in *7.2* transgenic flies is less than half that of the wild-type). Interestingly, transcription of both the *per*^{01} and *tim* genes was also reproducibly observed to have a lower amplitude and a higher trough values than in a wild-type strain (e.g. Figure 6C and D). This correlation is consistent with negative feedback and a role for PER in determining the transcriptional trough values. Surprisingly, TIM cycling in the transgenic strain is very similar to that of wild-type (Figure 6B), indicating that post-translational mechanisms contribute strongly to the amplitude of TIM cycling (e.g. Zeng *et al.*, 1996) and suggesting that TIM levels are less tightly coupled to the transcriptional feedback loop. This suggests further that the PER monomer or the PER–TIM heterodimer is the relevant negative feedback effector (cf. Marrus *et al.*, 1996).

Several of our experiments indicate that *per* mRNA expression is regulated at the post-transcriptional level. The difference between the transcription and mRNA curves, in particular, is consistent with recent experiments (Stanewsky *et al.*, 1997). These authors identified an intragenic element contributing to gene expression, but their reporter gene assays could not distinguish transcriptional from post-transcriptional control. The direct measurements of transcription by run-on assay indicate that the regulation is at least in part post-transcriptional.

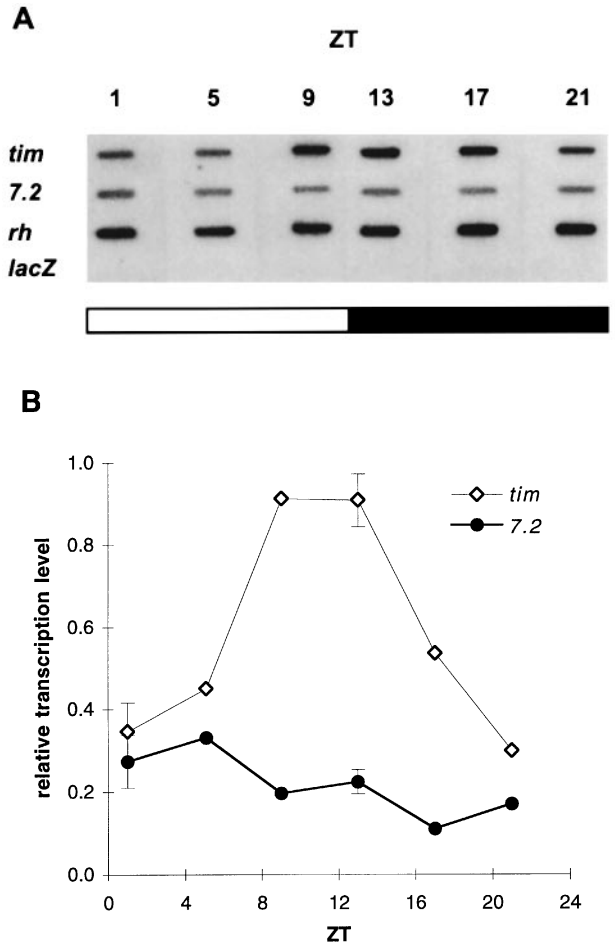

Fig. 7. Rate of transcription of the *7.2* transgene in *per⁻;7.2* transgenic flies. (**A**) A blot from a nuclear run-on assay. Each column is an individual hybridization blot from the *per⁻;7.2* transgenic flies entrained and collected at the time indicated above the blot. Each row shows hybridization signals from the genes indicated on the left. Note the cycling rate of transcription from *tim* and the relatively constant rate of transcription from the *7.2* transgene and the rhodopsin (*rh*) internal control. The negative control, the *lacZ* gene, shows no detectable signal. (**B**) Quantification of rates of *7.2* and *tim* transcription. Relative transcription level refers to *7.2*/*rh* or *tim*/*rh* values. Error bars indicate standard deviation ($n = 2$).

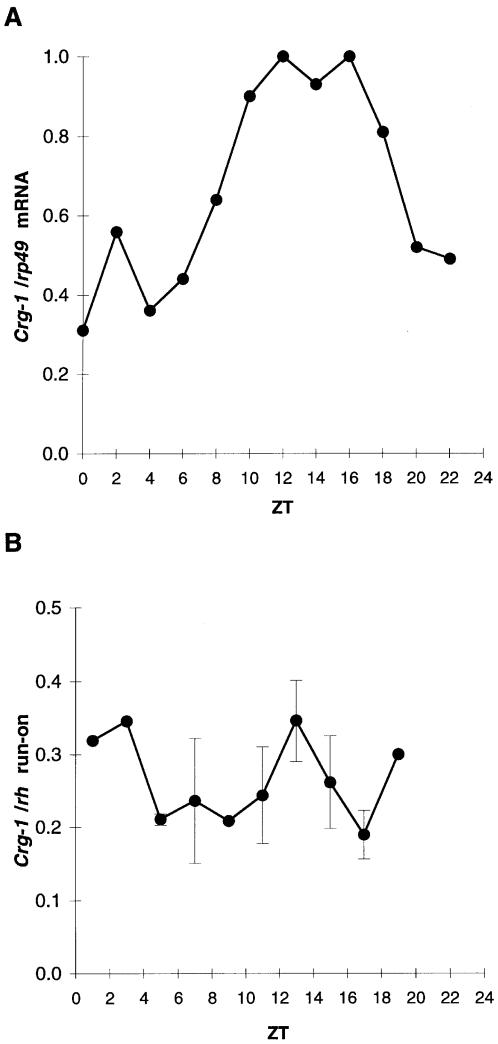

Fig. 8. Molecular analysis of the circadianly regulated gene (*Crg-1*) in wild-type. (**A**) *Crg-1* mRNA cycling in wild-type adapted from Rouyer *et al.* (1997). The mRNA signals were normalized to the *rp49* signal and the peak value was set to 1. (**B**) Quantification of rate of *Crg-1* transcription in wild-type. Run-on signals were normalized to rhodopsin (*rh*) signals. Three representative sets of the data were analyzed. Error bars indicate standard deviation ($n = 2$ or 3).

Although the run-on assays could obscure a transcriptional control mechanism by polymerase stalling, our control experiments indicate that this possibility is unlikely (data not shown). It is also important to exercise caution in comparing and interpreting results obtained by different assays, e.g. comparing the results of run-on and RNase protection assays. However, the curves have similar amplitudes (e.g. Figure 5D), suggesting that the two assays are in a similar signal range. Moreover, the magnitude of per^{01} transcription in the *7.2* transgenic flies (Figure 6C) is similar to that of the *7.2* and *Crg-1* genes and shows an obvious and expected cycling pattern. This indicates that the non-cycling *7.2* transcription and the very low amplitude *Crg-1* cycling are probably not due to the fact that the run-on assay is not in a sufficiently sensitive signal range. Finally, we considered that transcriptional cycling (of *Crg-1*, for example) in some cells might be masked by higher levels of non-cycling transcription in other cells. For this explanation to be viable, the non-cycling cells would have to generate little or no mRNA (due, for example, to very rapid degradation of nascent transcripts or nuclear pre-mRNA) to accommodate the observed mRNA cycling. However, the transcription rates of *7.2* and *Crg-1* (Figures 7B and 8B, respectively) are not unexpectedly high relative to their respective mRNA levels (Frisch *et al.*, 1994; Figure 8A), as compared with the ratio of transcription to mRNA levels for the wild-type *per* gene.

Although we have good evidence for post-transcriptional control, we cannot distinguish nuclear pre-mRNA processing from cytoplasmic mRNA turnover. Either regulatory mode is possible and might contribute to a required feedback delay, to widen the gap between the synthesis of *per* transcripts and the ability of PER to contribute to subsequent inhibition of its own transcription (Figure 2). Alternatively, the regulation might function to provide more mRNA during the accumulating phase of the cycle, especially during ZT9–15, and relatively less RNA during the declining phase, after ZT15. Modeling indicates that the post-transcriptional effect is modest, probably from

2- to 4-fold (see Materials and methods). In the absence of transcriptional cycling, the resultant mRNA cycling curve will have a comparable 2- to 4-fold amplitude, consistent with the observed low amplitude cycling of the *7.2* mRNA (Frisch *et al.*, 1994).

Reports of post-transcriptional regulation and mRNA stability regulation in particular are not unprecedented in *Drosophila* (Riedl and Jacobs-Lorena, 1996). The post-transcriptional regulation that we describe may be related to the well established circadian regulation of translation (Morse *et al.*, 1990; Mittag *et al.*, 1994), as translation and mRNA half-life regulation share common molecular components [e.g. poly(A) shortening and poly(A) binding protein]. Moreover, there are notable examples in development where translation is temporally delayed (masked mRNA) (Ranjan *et al.*, 1993); something similar might contribute to the gap between the transcription and protein accumulation curves.

Since mRNA stability/destability elements have been found mostly in the 3′ untranslated region (3′-UTR; Chen and Shyu, 1995), we checked the sequence conservation in the *per* 3′-UTR. A comparison between *Drosophila melanogaster* and *Drosophila yakuba* (DDBJ/EMBL/GenBank accession number X61127) shows 82% sequence identity in the ~500 bp 3′-UTR. This is high relative to sequence similarities in other non-coding regions of *per* (data not shown). Moreover, the putative 3′ end of *Crg-1* is highly adenylate/uridylate-rich (50% A and 21% U). Adenylate/uridylate-rich elements (AREs) are important for mRNA instability in the 3′-UTRs of some mRNAs that encode protooncogenes, nuclear transcription factors and cytokines in mammalian cells (Chen and Shyu, 1995). Of course, the presence of an ARE does not guarantee a destabilizing function nor that this function would fall under circadian control. Finally, we note that comparable circadian post-transcriptional regulation has been reported in a plant system (Pilgrim *et al.*, 1993).

Although we cannot draw a definitive conclusion about the possibility of post-transcriptional regulation in *tim* mRNA expression, the initial curve comparisons (Figure 5D) do not strongly indicate temporal regulation at this level. This may be related to the substantial quantitative differences in *per* and *tim* transcriptional regulation (see above): the 4-fold higher levels of *tim* transcriptional regulation may not require the additional post-transcriptional regulation. Moreover, TIM is subjected to a robust degradation mechanism that is light-induced (Hunter-Ensor *et al.*, 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996), which may contribute to the potent TIM cycling observed in the *7.2* strain (compare Figure 6B with Figure 5 in Zeng *et al.*, 1996).

As *per* mRNA cycling is abolished in per^{01} and tim^{0} mutants, the post-transcriptional as well as the transcriptional regulation requires a functional clock. Although it is possible that the PER–TIM complex feeds back to effect the changes in mRNA half-life, it is not known if the feedback is direct or indirect. Moreover, TIM and PER exist in different forms, and it is unclear which are effective in post-transcriptional feedback. It is not even certain whether the action of PER–TIM on *per* mRNA half-life is positive or negative. One simple scenario posits that PER–TIM functions in the cytoplasm to increase mRNA stability during the rising phase during the late day and early night. This positive feedback may terminate when the proteins become more highly phosphorylated or when they begin the nuclear translocation process. As the mRNA and transcription curves decrease at about the same time (~ZT15), there may be a switch from cytoplasmic RNA stabilization to nuclear transcriptional repression. Alternatively, the more highly phosphorylated form of PER–TIM may decrease both mRNA levels and transcription at about the same time (~ZT15). Preliminary calculations suggest that this latter possibility is more likely, as mRNA levels in a per^{01} background are high not low relative to transcription levels. A positive contribution to the lowering of RNA levels may even involve some concerted mode of action; for example, inhibition of 3′ cleavage-polyadenylation could occur on nascent RNA, which might allow for a simultaneous contribution to post-transcriptional and transcriptional regulation during negative feedback.

Our observations on *per* and *Crg-1* post-transcriptional regulation raise two more general points. First, it is possible that transcriptional regulation is not necessary for PER cycling or even for circadian rhythms. Our previous observations on the *7.2* transgene suggested that regulatory information within the gene might be sufficient to provide rhythmic function (Frisch *et al.*, 1994). Taken together with experiments indicating additional post-transcriptional regulation at the level of protein half-life (Dembinska *et al.*, 1997), transcriptional regulation of *per* may be unnecessary for free-running circadian rhythms of locomotor activity. Rescue of rhythmic function with a *glass–per* construct is consistent with this suggestion (Vosshall and Young, 1995), although RNA cycling of this transgene was not assayed. Comparable transgenic constructs do not yet exist for *tim*, although our data suggest that this gene might be more dependent on transcriptional regulation than *per* or *crg-1*. Moreover, it is possible that the post-transcriptional regulation of *per* mRNA and protein is dependent on the transcriptional regulation of some other clock genes. Second, these considerations might apply to the *per–tim* regulatory system in other species. In silkmoth adults, *per* mRNA undergoes only modest (2- to 5-fold) cycling, and immunohistochemistry results indicate that PER expression in brain cells is predominantly cytoplasmic (Sauman and Reppert, 1996). As no transcription measurements have been made in this species, our observations would be nicely accommodated were the mRNA cycling largely due to circadian post-transcriptional regulation. They also add the more general cautionary note that RNA cycling does not *ipso facto* indicate transcriptional regulation.

Materials and methods

Fly strains

The strain of wild-type flies used was Canton-S. The 7.2 kb *per* genomic DNA in the per^{01}*;7.2* transgenic flies has been described (Hamblen *et al.*, 1986; Frisch *et al.*, 1994) and is schematically illustrated in Figure 3A. Of the two rhythmic lines (*7.2:2* and *7.2:9*), the 7.2:9 line was used because it has a wider distribution of expression (in photoreceptor, lateral neurons and dorsal neurons). The resultant higher expression level results in more reliable run-on signals. The *7.2:9* line was crossed to overlapping deletions, Df(1)64j4 and Df(1)TEM202 on the X chromosome, which delete *per* (Reddy *et al.*, 1984), resulting in a per^{-}*;7.2:9* stock. Female flies containing both deficiencies were used for analyses.

Nuclear run-on assay

All the flies used were kept at 25°C in a 12 h light–12 h dark cycle for 2–3 days before being collected on dry ice at the appropriate time. Frozen heads were isolated and 50–100 µl of heads was used for each time point. Crude nuclei were isolated using the following protocol developed based on Love and Minton (1985) and Lichtsteiner *et al.* (1987). All steps were performed at 0–4°C unless otherwise specified. The heads were put in a 1 ml Dounce homogenizer (Wheaton) with 800 ml homogenization buffer [10 mM HEPES pH 7.5, 10 mM KCl, 0.8 M sucrose and 1 mM EDTA, with freshly added 0.5 mM phenylmethylsulfonylfluoride (PMSF), 0.5 mM dithiothreitol (DTT) and 100 µg/ml yeast tRNA]. They were homogenized with 5–10 strokes of the 'loose' pestle and then with about 20 strokes of the 'tight' pestle. The homogenate was filtered through a column polymer bed support (Bio-Rad unfilled Bio-Spin Column) to remove the cuticle. An additional 200 µl of homogenization buffer was used to rinse the homogenizer and the mixture was passed through the column bed support. The filtrate was overlaid on a 1 ml cushion (10 mM HEPES pH 7.5, 10 mM KCl, 1.0 M sucrose, 10 % glycerol and 1 mM EDTA) and centrifuged in an HB-4 rotor (Sorvall) at 11 000 r.p.m. for 10 min. The pelleted nuclei were washed once by resuspending in 500 µl of nuclear resuspension buffer (40 mM HEPES pH 8.0, 25% glycerol, 5 mM magnesium acetate and 0.1 mM EDTA, with freshly added 0.25 mM PMSF and 1 mM DTT) and centrifuging at 8000 r.p.m. for 2 min. The nuclear pellet was resuspended in 190 µl of nuclear resuspension buffer. The above nuclear isolation protocol was performed as quickly as possible to avoid losing nucleotide incorporation efficiency in the subsequent run-on reaction.

The run-on reactions and isolation of radiolabeled RNA were performed based on Love and Minton (1985), Srivastava and Schonfeld (1994) and Ausubel *et al.* (1994), with modifications as described. The reaction was initiated by adding a 5× reaction mix containing 5 mM magnesium acetate, 750 mM KCl, 2.5 mM each of ATP, CTP and GTP, 10 mM DTT, 1 µl RNasin (Promega) and 100 µCi [α-^{32}P]UTP (10 µl of 800 Ci/mmol, NEN). Incubation was carried out at 23°C for 30 min. The reaction was terminated by adding 25 µl of RNase-free DNase (RQ1, Promega) at 37°C for 5 min. Twenty microliters of 15× proteinase buffer (7.5% SDS and 150 mM EDTA) and 3 µl of proteinase K (10 mg/ml) were added and the reaction mixture was incubated at 37°C for 30 min. Total RNA was purified by phenol–chloroform extraction with 30 µl sodium acetate pH 5.3. The aqueous layer was passed through a BioSpin 6 column (Bio-Rad) and 1 µl of the flowthrough was used to detect nucleotide incorporation by trichloroacetic acid precipitation (Sambrook *et al.*, 1989). The column flowthrough was ethanol precipitated, washed in 70% ethanol, dried and resuspended in 100 µl of Church buffer [0.5 M $NaHPO_4$ pH 7.2, 7% SDS, 1% bovine serum albumin (BSA) and 1 mM EDTA; Church and Gilbert, 1984]. It was added directly to prehybridized membranes containing DNA probes of genes of interest.

All the DNA probes used are purified PCR products amplified from bgenomic constructs unless otherwise stated. The *per* probe used in Figure 1A is a combination of three DNA pieces that covers the entire transcribing region; a piece from nt 2932 to nt 4887 of the transcription unit was used in Figure 3 to compare transcription rates with that of *tim* and in Figure 6C to detect signals from both per^{01} and *7.2* genes; a piece from nt 1 to nt 2028 was used to detect per^{01} signal in Figure 6C. The *tim* probe in Figure 1A and Figure 7 is a combination of two DNA pieces: nt 163–2338 and nt 2319–3714 of the coding region amplified from cDNA construct; the transcription rate comparison shown in Figure 3 used a piece that is nt 163–1736 of the coding region amplified from genomic DNA; the nt 2319–3714 piece was used in Figure 6D. DNA pieces of the transcribing region nt 54–2589, nt 1–409 and nt 213–867 were used for *Crg-1*, histone 3 (*H3*) and *rhodopsin* (*rh*), respectively. DNA of the coding region nt 1615–2067 was used for *lacZ*.

The DNA pieces were transferred to GeneScreen membranes (NEN) according to the manufacturer's instruction. Half a picomole of DNA was transferred for all probes, except *rh* and *H3* for which 2 pmol was applied. The membrane was prehybridized in 500 µl of Church buffer at 65°C for at least 15 min. The resuspended radiolabeled RNAs from run-on reactions were added directly and hybridized at 65°C overnight (12–20 h). The membrane was washed in washing buffer (40 mM $NaHPO_4$ pH 7.2, 1% SDS and 1 mM EDTA; Church and Gilbert, 1984), twice briefly at room temperature, and then twice at 50°C for 15 min. Signals were monitored using a hand-held Survey meter and additional washes at 60°C for 5 min were performed when appropriate. The run-on signals were quantified using a PhosphorImager and the Molecular Analyst software (Bio-Rad).

RNA polymerase stalling was tested using three membranes; each had DNA probe derived from the 5′, middle or 3′ region of the *per* and *tim* transcribing regions. Each membrane was hybridized to one-third of run-on products prepared from wild-type fly heads collected at ZT7 (when the transcription and mRNA curves do not match) and at ZT15 (when the two curves almost coincide with each other).

Modeling

The *per* and *tim* transcription curves in Figure 2A were fitted using Kaleidagraph (Abelbeck), by applying the standard Fourier series approximation for a function with a 24 h period. The fitted equations are :

$$T_{per} = 0.399 + 0.008\sin(\pi t/12) - 0.366\cos(\pi t/12) + 0.025\sin(\pi t/6) + 0.066\cos(\pi t/6)$$

and

$$T_{tim} = 0.439 - 0.039\sin(\pi t/12) - 0.489\cos(\pi t/12) - 0.022\sin(\pi t/6) + 0.087\cos(\pi t/6),$$

where T_{per} and T_{tim} are transcription rates of *per* and *tim*, respectively, and t is circadian time.

mRNA curves were generated from the transcription curves by assuming that the rate of synthesis of mRNA is proportional to the transcription rate and that the mRNAs were degraded according to the first order decay kinetics (Parker *et al.*, 1991); i.e. $dR/dt = aT - kR$, where dR/dt denotes the rate of the change of the mRNA level, t is circadian time, T is the transcription rate, R is the mRNA level, a is the mRNA synthesis rate constant and k is the mRNA decay rate constant which is equal to $\ln 2/t_{1/2}$, where $t_{1/2}$ is the mRNA half-life. The general equation of the derived mRNA curve is:

$$R = ae^{-kt}\int e^{kt}T(t)dt,$$

where R is the mRNA level and $T(t)$ is the transcription rate function (T_{per} and T_{tim}) shown above.

The change of *per* mRNA half-life as a function of circadian time was estimated using two approaches. The first assumed continuous half-life change along the day and calculated the half-life as a function of time from the known transcription and mRNA functions. The second assumed two different mRNA half-lives at various time frames of the day. mRNA curves were generated to fit the experimental mRNA curves by inputting different half-life values. Using the above differential equation, a range of mRNA half-life values can be estimated assuming different values of the mRNA synthesis rate constant (a).

RNase protection assay

The RNase protection assay was performed as described by Marrus *et al.* (1996). Quantification was carried out with a PhosphorImager and the Molecular Analyst software (Bio-Rad).

Behavioral analysis

Behavioral analysis was performed as described by Rutila *et al.* (1996). Female per^{-}*;7.2* flies were analyzed.

Western blot

Protein extracts from 30 fly heads for each time point was prepared in 30 µl of extraction buffer (Edery *et al.*, 1994b) with the addition of 20 mM β-glycerophosphate and 100 mM Na_3VO_4. Thirty microliters of 2× SDS sample buffer was added and the sample boiled. Thirty microliters of the denatured product was loaded on to a 6% SDS–polyacrylamide gel (29.6:0.4, acrylamide:bisacrylamide ratio). Following electrophoresis, gels were electroblotted on to nitrocellulose for 35 min at 0.15 A using a semidry blotting apparatus according to the manufacturer's instructions (ISS).

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References

Aronson,B.D., Johnson,K.A., Loros,J.J. and Dunlap,J.C. (1994) Negative feedback defines a circadian clock: autoregulation of the clock gene *frequency*. *Science*, **263**, 1578–1584.

Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1994) *Current Protocols in Molecular Biology*. 2nd edn. John Wiley and Sons Inc., New York, NY.

Brandes,C., Plautz,J.D., Stanewsky,R., Jamison,C.F., Straume,M., Wood,K.V., Kay,S.A. and Hall,J.C. (1996) Novel features of *Drosophila period* transcription revealed by real-time luciferase reporting. *Neuron*, **16**, 687–692.

Chen,C.-Y.A. and Shyu,A.-B. (1995) AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem. Sci*., **20**, 465–470.

Church,G.M. and Gilbert,W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA*, **81**, 1991–1995.

Curtin,K., Huang,Z.J. and Rosbash,M. (1995) Temporally regulated nuclear entry of the *Drosophila period* protein contributes to the circadian clock. *Neuron*, **14**, 365–372.

Dembinska,M.E., Stanewsky,R., Hall,J.C. and Rosbash,M. (1997) Circadian cycling of a *period–lacZ* fusion protein in *Drosophila*: evidence for an instability cycling element in PER. *J. Biol. Rhythms*, **12**, 157–172.

Edery,I., Rutila,J.E. and Rosbash,M. (1994a) Phase shifting of the circadian clock by induction of the *Drosophila period* protein. *Science*, **263**, 237–240.

Edery,I., Zwiebel,L.J., Dembinska,M.E. and Rosbash,M. (1994b) Temporal phosphorylation of the *Drosophila period* protein. *Proc. Natl Acad. Sci. USA*, **91**, 2260–2264.

Edmunds,L.N. (1988). *Cellular and Molecular Bases of Biological Clocks—Models and Mechanisms for Circadian Timekeeping*. Springer-Verlag, New York.

Frisch,B., Hardin,P.E., Hamblen-Coyle,M.J., Rosbash,M. and Hall,J.C. (1994) A promoterless DNA fragment from the *period* locus rescues behavioral rhythmicity and mediates cyclical gene expression in a restricted subset of the *Drosophila* nervous system. *Neuron*, **12**, 555–570.

Gekakis,N., Saez,L., Delahaye-Brown,A.-M., Myers,M.P., Sehgal,A., Young,M.W. and Weitz,C.J. (1995) Isolation of *timeless* by PER protein interactions: defective interaction between *timeless* protein and long-period mutant PER^{L}. *Science*, **270**, 811–815.

Hamblen,M., Zehring,W.A., Kyriacou,C.P., Reddy,P., Yu,Q., Wheeler, D.A., Zwiebel,L.J., Konopka,R.J., Rosbash,M. and Hall,J.C. (1986) Germ-line transformation involving DNA from the *period* locus in *Drosophila melanogaster*: Overlapping genomic fragments that restore circadian and ultradian rhythmicity to per^{0} and per^{-} mutants. *J. Neurogenet*., **3**, 249–291.

Hamblen-Coyle,M.J., Wheeler,D.A., Rutila,J.E., Rosbash,M. and Hall,J.C. (1992) Behavior of period-altered circadian rhythm mutants of *Drosophila* in light:dark cycles. *J. Insect Behav.*, **5**, 417–446.

Hao,H., Allen,D.L. and Hardin,P.E. (1997) A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila*. *Mol. Cell. Biol*., **17**, 3687–3693

Hardin,P.E., Hall,J.C. and Rosbash,M. (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature*, **343**, 536–540.

Hardin,P.E., Hall,J.C. and Rosbash,M. (1992) Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. *Proc. Natl Acad. Sci. USA*, **89**, 11711–11715.

Hunter-Ensor,M., Ousley,A. and Sehgal,A. (1996) Regulation of the *Drosophila* protein *timeless* suggests a mechanism for resetting the circadian clock by light. *Cell*, **84**, 677–685.

Konopka,R.J. and Benzer,S. (1971) Clock mutants of *Drosophila melanogaster. Proc. Natl Acad. Sci. USA*, **68**, 2112–2116.

Lee,C., Parikh,V., Itsukaichi,T., Bae,K. and Edery,I. (1996) Resetting the *Drosophila* clock by photic regulation of PER and a PER–TIM complex. *Science*, **271**, 1740–1744.

Lichtsteiner,S., Wuarin,J. and Schibler,U. (1987) The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. *Cell*, **51**, 963–973.

Love,J.D. and Minton,K.W. (1985) Screening of library for differentially expressed genes using *in vitro* transcripts. *Anal. Biochem.*, **150**, 429–441.

Marrus,S.B., Zeng,H. and Rosbash,M. (1996) Effect of constant light and circadian entrainment of per^{S} flies: evidence for light-mediated delay of the negative feedback loop in *Drosophila*. *EMBO J.*, **15**, 6877–6886.

Mittag,M., Lee,D.-H. and Hastings,J.W. (1994) Circadian expression of the luciferin-binding protein correlates with the binding of a protein to the 3′ untranslated region of its mRNA. *Proc. Natl Acad. Sci. USA*, **91**, 5257–5261.

Morse,D.S., Fritz,L. and Hastings,J.W. (1990) What is the clock? Translational regulation of circadian bioluminescence. *Trends Genet.*, **15**, 262–265.

Myers,M.P., Wager-Smith,K., Rothenfluh-Hilfiker,A. and Young,M.W. (1996) Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. *Science*, **271**, 1736–1740.

O'Brien,T. and Lis,J.T. (1993) Rapid changes in *Drosophila* transcription after an instantaneous heat shock. *Mol. Cell. Biol.*, **13**, 3456–3463.

Parker,R., Herrick,D., Peltz,W. and Jacobson,A. (1991) Measurement of mRNA decay rates in *Saccharomyces cerevisiae*. *Methods Enzymol*., **194**, 415–423.

Pilgrim,M.L., Caspar,T., Quail,P.H. and McClung,C.R. (1993) Circadian and light-regulated expression of nitrate reductase in *Arabidopsis*. *Plant Mol. Biol*., **23**, 349–364.

Qiu,J. and Hardin,P.E. (1996) *per* mRNA cycling is locked to lights-off under photoperiodic conditions that support circadian feedback loop function. *Mol. Cell. Biol.*, **16**, 4182–4188.

Ranjan,M., Tafuri,S.R. and Wolffe,A.P. (1993) Masking mRNA from translation in somatic cells. *Genes Dev.*, **7**, 1725–1736.

Reddy,P., Zehring,W.A., Wheeler,D.A., Pirrotta,V., Hadfield,C., Hall,J.C. and Rosbash,M. (1984) Molecular analysis of the *period* locus in *Drosophila melanogaster* and identification of a transcript involved in biological rhythms. *Cell*, **38**, 701–710.

Riedl,A. and Jacobs-Lorena,M. (1996) Determinants of *Drosophila fushi tarazu* mRNA instability. *Mol. Cell. Biol.*, **16**, 3047–3053.

Rosbash,M., Rutila,J.E. and Zeng,H. (1996) Molecular control of circadian rhythms. In Hiroshige,T. and Honma,K.-I. (eds), *Circadian Organization and Oscillatory Coupling*. Hokkaido University Press, Sapporo, pp. 25–38.

Rougvie,A.E. and Lis,J.L. (1988) The RNA polymerase II molecule at the 5′ end of the uninduced *hsp70* gene of *D. melanogaster* is transcriptionally engaged. *Cell*, **54**, 795–804.

Rouyer,F., Rachidi,M., Pikielny,C. and Rosbash,M. (1997) A new clock gene regulated by the circadian clock in the *Drosophila* head. *EMBO J*., **16**, 3944–3954.

Rutila,J.E., Zeng,H., Le,M., Curtin,K.D., Hall,J.C. and Rosbash,M. (1996) The tim^{SL} mutant of the *Drosophila* rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. *Neuron*, **17**, 921–929.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning*: *A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sauman,I. and Reppert,S.M. (1996) Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of *period* protein regulation. *Neuron*, **17**, 889–900.

Sehgal,A., Price,J.L., Man,B. and Young,M.W. (1994) Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science*, **263**, 1603–1606.

Sehgal,A., Rothenfluh-Hilfiker,A., Hunter-Ensor,M., Chen,Y., Myers,M. and Young,M.W. (1995) Circadian oscillations and autoregulation of *timeless* RNA. *Science*, **270**, 808–810.

Srivastava,R.A.K. and Schonfeld,G. (1994) Measurements of rate of transcription in isolated nuclei by nuclear 'run-off' assay. In Harwood,A.J. and Totowa,N.J. (eds), *Methods in Molecular Biology*. Humana Press Inc., NJ, pp. 281–288.

Stanewsky,R., Jamison,C.F., Plautz,J.D., Kay,S.A. and Hall,J.C. (1997) Multiple circadian-regulated elements contribute to cycling *period* gene expression in *Drosophila*. *EMBO J*., **16**, 5006–5018.

Vosshall,L.B. and Young,M.W. (1995) Circadian rhythms in *Drosophila* can be driven by *period* expression in a restricted group of central brain cells. *Neuron*, **15**, 345–360.

Weber,J., Jelinek,W. and Darnell,J.E. (1977) The definition of a large viral transcription unit late in Ad2 infection of HeLa cells: mapping of nascent RNA molecules labeled in isolated nuclei. *Cell*, **10**, 611–616.

Wuarin,J., Falvey,E., Lavery,D., Talbot,D., Schmidt,E., Ossipow,V., Fonjallaz,P. and Schibler,U. (1992) The role of the transcriptional activator protein DBP in circadian liver gene expression. *J. Cell Sci.*, Suppl. 16, 123–127.

Zeng,H., Hardin,P.E. and Rosbash,M. (1994) Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. *EMBO J.*, **13**, 3590–3598.

Zeng,H., Qian,Z., Myers,M.P. and Rosbash,M. (1996) A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature*, **380**, 129–135.

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