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Drosophila Photoreceptors Contain an Autonomous Circadian Oscillator That Can Function without *period* mRNA Cycling

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Circadian oscillations in *period* (*per*) mRNA and *per* protein (PER) constitute, in part, a feedback loop that is required for circadian pacemaker function in *Drosophila melanogaster*. Oscillations in PER are required for oscillations in *per* mRNA, but the converse has not been rigorously tested because of a lack of measurable quantities of *per* mRNA and protein in the same cells. This circadian feedback loop operates synchronously in many neuronal and non-neuronal tissues, including a set of lateral brain neurons (LNs) that mediate rhythms in locomotor activity, but whether a hierarchy among these tissues maintains this synchrony is not known. To determine whether *per* mRNA cycling is necessary for PER cycling and whether cyclic *per* gene expression is tissue autonomous, we have generated per^{01} flies carrying a transgene that constitutively expresses *per* mRNA specifically in photoreceptors, a cell type that supports feedback loop function. These transformants were tested for different aspects of feedback loop function including *per* mRNA cycling, PER cycling, and PER nuclear localization. Under both light/dark (LD) cycling and constant dark (DD) conditions, PER abundance cycles in the absence of circadian cycling of *per* mRNA. These results show that *per* mRNA cycling is not required for PER cycling and indicate that *Drosophila* photoreceptors R1–R6 contain a tissue autonomous circadian oscillator.

Key words: Drosophila*; circadian clock; photoreceptors;* period *gene; transgene; gene expression*

Genetic screens for mutations that affect circadian rhythms in *Drosophila melanogaster* have identified two genes that encode components of the circadian timekeeping apparatus, *period* (*per*) and *timeless* (*tim*). Mutations in these genes can shorten (per^{S}, tim^{SL}), lengthen (per^{L}), or abolish (per^{01}, tim^{01}) circadian rhythms in locomotor activity and eclosion (Konopka and Benzer, 1971; Sehgal et al., 1994; Rutila et al., 1996). In parallel with these behavioral rhythms are molecular rhythms in the abundance of *per* and *tim* gene products; *per* and *tim* RNAs peak early in the evening, whereas *per* protein (PER) and *tim* protein (TIM) peak several hours later (Siwicki et al., 1988; Hardin et al., 1990; Zerr et al., 1990; Edery et al., 1994b). Induced alterations in PER or TIM levels can shift the phase of behavioral rhythms (Edery et al., 1994a; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996), showing that behavioral rhythms are dependent on molecular rhythms.

To account for these molecular rhythms, a negative feedback loop model was proposed in which PER and/or TIM suppress *per* and *tim* gene transcription (Hall, 1995; Hardin and Siwicki, 1995; Sehgal, 1995). After the lights are turned off, PER and TIM accumulate in the cytoplasm as heteromeric complexes (Lee et al., 1996; Zeng et al., 1996) and enter the nucleus over the course of a few hours, starting ~6 hr after the lights are turned off (Curtin et al., 1995). In the nucleus, PER and/or TIM feedback to repress *per* and (probably) *tim* gene transcription (Hardin et al., 1990; Zeng et al., 1994; Sehgal et al., 1995). Destruction of PER and TIM proteins, in turn, coincides with the accumulation of *per* and *tim* transcripts during midday and the start of another circadian cycle.

Little is known about how PER–TIM complexes regulate transcription after they enter the nucleus. However, sequences that mediate PER- and TIM-dependent transcriptional cycling have been localized to a 69 bp fragment lying ~500 bp upstream of the *per* transcription start site (Hao et al., 1997). Surprisingly, *per* genomic fragments lacking a promoter (Hamblen et al., 1986; Frisch et al., 1994) or driven by constitutively active promoters (Ewer et al., 1988, 1990; Vosshall and Young, 1995) drive PER cycling and rescue locomotor activity rhythms in per^{01} flies. Although PER cycling was only measured in 12 hr light/dark (LD) cycles in these studies, such cycling presumably persists because behavioral rescue was measured in constant darkness. Behavioral rescue by these transgenes, however, raises the intriguing possibility that *per* RNA cycling is not necessary for PER cycling and locomotor activity rhythms.

The *per* gene is expressed in a variety of neuronal (i.e., photoreceptors, antennae, brain neurons and glia, and thoracic ganglion) and non-neuronal (i.e., gut, Malpighian tubules, testes, and ovary) tissues (Liu et al., 1988; Saez and Young, 1988; Siwicki et al., 1988; Ewer et al., 1992). The circadian feedback loop operates in all of these tissues except the ovary, where *per* is constitutively expressed (Hardin, 1994). In adults, lateral neurons (LNs) are the only cells within the *per* expression pattern known to control autonomously a rhythmic output, locomotor activity (Ewer et al., 1992; Frisch et al., 1994). Oscillator autonomy has also been observed in the pupal prothoracic gland (Emery et al., 1997), but

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whether oscillators operate autonomously in other adult tissues is unknown.

In this report we drive *per* from a crippled *rhodopsin 1* (*Rh1*) promoter to determine whether *per* mRNA cycling is required for PER cycling and whether photoreceptors contain an autonomous circadian oscillator. PER produced by this transgene cycles in abundance under LD and constant dark (DD) conditions. Because the transgene expresses constitutive levels of *per* mRNA, the rhythms in PER show that an oscillator is running through some post-transcriptional mechanism. In addition, this oscillator is operating exclusively in photoreceptors R1–R6, which suggests that these cells contain an autonomous oscillator.

MATERIALS AND METHODS

Plasmid construction. The *Rh1*(−250)–*LacZ*, *Rh1*(−180)–*LacZ*, and *Rh1*(−120)–*LacZ* transformation constructs were made as follows. *Rh1* promoter fragments starting −250, −180, and −120 bp upstream of the *Rh1* transcription start site, respectively, and extending to the *Rh1* translation initiation site were generated by PCR using *Rh1–per* (Zeng et al., 1994) as a template. The *Rh1* upstream region primers included the *Rh1*(−250)+*Xho*I sense primer (5′-GCCTCGAGACTCAAGAATAA TAC-3′), the *Rh1*(−180)+*Xho*I sense primer (5′-GCCTCGAGCCCAT TGCGATGTG-3′), and the *Rh1*(−120)+*Eco*RI sense primer (5′-CCGA ATTCGCGGCCGCGGTACCTGTCGACACTTT-3′). The antisense primer at the *Rh1* translation initiation site was *Rh1*(ATG)+*Bam*HI (5′-GCGGATCCATTGTGTTTTGGTTAC-3′). The *Rh1*(−250)+*Xho*I/*Rh1*(ATG)+*Bam*HI and *Rh1*(−180)+*Xho*I/*Rh1*(ATG)+*Bam*HI amplification products were digested with *Xho*I and *Bam*HI, and the *Rh1*(−120)+*Eco*RI/*Rh1*(ATG)+*Bam*HI amplification product was digested with *Eco*RI and *Bam*HI and cloned into the pCaSpeR-β-gal transformation vector (Thummel et al., 1988).

The *Rh1*(−180)–*per* transformation construct was generated as follows. A 6.2 kb *Sal*I–*Xba*I DNA fragment from 13.2 (HA/C) (Rutila et al., 1992), consisting of genomic sequences from the *Sal*I site in exon 3 to an *Xba*I site ~2 kb downstream of *per* transcribed sequences and including a C-terminal hemagglutinin tag, was cloned into Bluescript KS−. A 630 bp DNA fragment from the *Sal*I site at −120 bp of the *Rh1* promoter to the *Sal*I site in exon 3 of the *per* genomic sequence was removed from *Rh1–per* (Zeng et al., 1994) and inserted at the *Sal*I site upstream of the *Sal*I to *Xba*I *per* fragment in Bluescript KS−. The orientation of the *Sal*I fragment was checked via PCR and restriction enzyme digests, and the plasmid having the *per* coding region driven by the *Rh1*−120 promoter fragment was named *Rh1*(−120)–*perKS*. To make the *Rh1*(−180)–*perKS*, we first removed an *Xho*I site close to the *Xba*I site at the 3′-end of the *per* genomic sequence by digestion with *Xho*I, then filling in with the Klenow fragment of DNA polymerase I. An *Xho*I–*Spe*I fragment starting −180 bp upstream of the *Rh1* transcription initiation site to the *Sal*I site of *per* exon 3 was generated by PCR using the *Rh1*(−180)+*Xho*I sense primer (see above) and the ex3*Sal*I antisense primer (5′–GCAACGCGTTGTCGACCTTCTGGC-3′) and then was digested with *Xho*I and *Spe*I. This fragment was inserted into the *Rh1*(−120)–*perKS* plasmid after *Rh1* sequences between −120 bp of the transcription start site and the *Spe*I site in the *Rh1* leader sequence were removed, thereby forming *Rh1*(−180)–*perKS*. The inserts from *Rh1*(−180)–*perKS* were removed by digestion with *Xho*I and *Xba*I and were inserted into the CaSpeR4 transformation vector (Thummel and Pirotta, 1991) to form the *Rh1*(−180)–*per* transformation plasmid.

Fly stocks and germ line transformation. The wild-type *D. melanogaster* strain Canton-S and transgenic fly strains were raised on a cornmeal, sugar, agar yeast, and Tegosept (a mold inhibitor) medium at 25°C. P-element-mediated transformation was performed as described previously (Hao et al., 1997). Transformant lines with inserts on the second or third chromosomes were balanced with *In(2LR)Cyo* or *In(3LR)TM2*, respectively, and were crossed into a *y,per*^{01}*,w* genetic background.

Behavioral analysis. Locomotor activity of adult male Canton-S; *y,per*^{01}*,w*; *Rh1*(−180)–*per*-1; *y,per*^{01}*,w*; *Rh1*(−180)–*per*-2; and *y,per*^{01}*,w* flies were monitored and analyzed as described by Hamblen et al. (1986). Flies were entrained in 12 hr light/dark cycles at 25°C for 72 hr; then the lights stayed off for 7 d. Locomotor activity was monitored from the first day of entrainment, and data collected during constant darkness were analyzed to determine the period and strength of the rhythm (Hamblen et al., 1986). Flies were designated rhythmic or arrhythmic based on the criteria of Ewer et al. (1992).

RNase protection assays. Flies used for time course analyses were entrained at 25°C in 12 hr light/dark cycles for at least 96 hr before collection. For each time point, RNA was extracted as described from either whole heads (Hardin et al., 1990) or eyes (Hardin et al., 1992b; Zeng et al., 1994), and 10 μg of whole-head RNA or 5 μg of eye RNA was used for RNase protection assays as described (Hardin et al., 1990). To make an RNase protection probe, we cloned a *per* cDNA fragment from the *Spe*I site in exon 2 to the *Sal*I site in exon 3 into Bluescript KS− vector. The RNase protection probe was linearized by *Spe*I digestion, and an RNA probe was transcribed with T3 RNA polymerase. It protects an endogenous *per* fragment of 324 nucleotides (nt) and a *Rh1*(−180)–*per*-derived transcript of 285 nt. Protected *per* bands were quantitated using a Fuji BAS50 phosphorimager. As a control for the amount of RNA in each lane, an antisense ribosomal protein 49 (RP49) probe was used in each RNase protection assay (Hardin et al., 1990).

Immunohistochemistry. For LD experiments, homozygous *per*^{01}; *Rh1*(−180)–*per*-1 flies were entrained in 12 hr LD cycles for at least 4 d and collected at 4 hr intervals. For each time point, flies were immediately embedded into OCT compound (Tissue-Tek) on dry ice, and 10–12 μm horizontal cryostat sections were prepared. A rabbit polyclonal anti-PER antiserum (kindly provided by Ralf Stanewsky) preabsorbed against *per*^{01} embryos was used for immunostaining. Immunostaining detection was performed using a goat anti-rabbit secondary antibody conjugated to horseradish peroxidase as described (Siwicki et al., 1988). For DD experiments, heterozygous *per*^{01};*Rh1*(−180)–*per*-1/+ flies were entrained for 4 d in 12 hr LD cycles; the lights were then left off, and eight samples were collected every 4 hr starting at circadian time 4 (CT4) during the first and second days of constant darkness. Sample preparation and immunostaining for flies collected in DD conditions were performed as described for flies collected in LD conditions.

Western blotting and quantitation. Fly head extracts were prepared from either homozygous transgenic flies for LD experiments or from heterozygous transgenic flies for DD experiments. Flies were entrained and collected in LD and DD conditions as described above for the immunohistochemistry and then were subjected to Western blotting analyses (Edery et al., 1994b) with the following modifications: the first antibody is the same used for immunohistochemical staining and was diluted to 1:20,000, and the secondary antibody is anti-rabbit IgG horseradish peroxidase-conjugated antibody (Amersham, Arlington Heights, IL) diluted 1:5000 in blocking solution. X-ray film exposures of Western blots were scanned using OFOTO software and quantitated using National Institutes of Health Image 1.6 software. The level of PER at each time point was taken as the PER signal minus the background in each lane. In each independent time course, the highest PER signal was set to 1.0, and all other time points were normalized to this value. The normalized PER values from three independent time courses of Canton-S and *per*^{01}; *Rh1*(−180)–*per*-1/+ and from two independent time courses of *per*^{01}; *Rh1*(−180)–*per*-2/+ were added together to yield pooled data for each genotype. The peak PER value from the pooled data was then set to 1.0. The normalized pooled data from each genotype were used to generate a curve based on its fit to a polynomial function using NIH Image 1.6 software.

RESULTS

A minimal *rhodopsin 1* gene promoter drives constitutive expression in photoreceptors R1–R6

To test whether circadian oscillator function requires *per* mRNA cycling and operates autonomously in peripheral tissue (i.e., cells that are not capable of driving locomotor activity rhythms), we had to find an appropriate promoter to drive *per* expression. Such a promoter should meet the following criteria: (1) it directs gene expression in a tissue that normally expresses *per*, (2) it expresses a constant level of mRNA, and (3) it drives expression at a level comparable with that of *per*. One promoter that meets the first two criteria is the *ninaE* (*rhodopsin 1*, *Rh1*) promoter that constitutively expresses mRNA in the six outer photoreceptor cells of each ommatidium (i.e., R1–R6) but not in the inner photoreceptors R7 and R8. However, this promoter expresses high levels of mRNA and was used to show that PER overexpression represses the endogenous *per* RNA cycling (Zeng et al., 1994).

Table 1. Behavioral analysis of per^{01}; *Rhl*(−180)—*per* transgenic flies

Genotype	No. tested	No. rhy.	No. arr.	Period (hr ± SEM)	Power (±SEM)	Width (±SEM)
Canton-S	24	22	2	23.9 ± 0.1	92.5 ± 10.9	6.0 ± 0.3
per^{01}; *Rhl*(−180)—*per*-1	38	0	38			
per^{01}; *Rhl*(−180)—*per*-2	56	0	56			
per^{01}	57	0	57			

Young male flies from wild-type Canton-S, per^{01}; *Rhl*(−180)—*per*-1, per^{01}; *Rhl*(−180)—*per*-2, and per^{01} were entrained in 12:12 hr light/dark cycle for 3 d; then their locomotor activity was monitored in constant darkness at 25°C for 7 d. Periodogram analysis was done as previously described (Hamblen et al., 1986). Power and width are defined in Frisch et al. (1994) and were used to distinguish between rhythmic and arrhythmic flies (Ewer et al., 1992). No. tested, Total number of tested flies; No. rhy., number of rhythmic flies; No. arr., number of arrhythmic flies.

To make this promoter useful for our purposes, we sought to reduce its level of expression while maintaining its tissue specificity. An earlier study had identified an *Rh1* promoter fragment [*Rh1*(−120/+67)] with these properties (Mismer and Rubin, 1987); however, this promoter fragment was not specific to photoreceptors R1–R6 in our hands (H. Hao, Y. Cheng, and P. E. Hardin, unpublished observations). Therefore, we tested the expression level and tissue specificity of two progressively larger portions of the *Rh1* promoter in transgenic flies using a *lacZ* reporter gene (Fig. 1). Flies transformed with the *Rh1*(−250)–*LacZ* and *Rh1*(−180)–*LacZ* constructs, which contain 250 or 180 bp of *Rh1* upstream sequences, respectively, and leader sequences up to the initiating ATG, were sectioned and stained for β-galactosidase (β-gal) activity. Both constructs showed specific staining in the eye, but *Rh1*(−250)–*LacZ* showed considerably higher levels of expression than did *Rh1*(−180)–*LacZ* (Fig. 1). Although each *Rh1*(−180)–*LacZ* line was expressed specifically in the eye, expression in two of the lines was patched (data not shown). Such a pattern may be caused by position effects because the *Rh1* promoter is adjacent to the 5′-end of the P-element vector. Because we wanted to minimize expression levels, we used the *Rh1*(−180) promoter for subsequent experiments.

The *Rh1*(−180) promoter and its leader sequence were fused to *per* genomic sequences at the translation initiation site to drive low level *per* expression specifically in photoreceptors R1–R6 (Fig. 2). Eight independent transgenic lines were obtained and crossed into a per^{01} background to eliminate endogenous PER expression. The spatial expression pattern of PER in these transgenic strains was examined by anti-PER immunohistochemical staining. Six of the eight strains showed undesirable *per* expression patterns; one expresses PER at high levels, two show PER expression in photoreceptors R1–R6 and the central brain, and three express *per* in only a subset of R1–R6 cells (data not shown). The other two transgenic strains have the desired PER expression pattern: photoreceptor R1–R6-specific expression at levels similar to that of PER. To confirm that PER is not expressed at levels too low to detect by immunohistochemical methods in the central brains of these two transgenic lines, we monitored the lines for locomotor activity rhythms. Both lines are arrhythmic, indicating that they do not have a functional circadian pacemaker in their brain (Table 1). These two lines, designated per^{01};*Rh1*(−180)–*per*-1 and per^{01};*Rh1*(−180)–*per*-2, were used for subsequent molecular analyses.

Rh1(−180) promoter activity is constitutive in DD conditions and fluctuates in LD cycles

Previous studies showed that *Rh1* mRNA in wild-type flies is expressed at high levels throughout the circadian cycle (Zeng et al., 1994). To ensure that *per* RNA from this crippled *Rh1*(−180) promoter does not cycle in abundance, we entrained per^{01}; *Rh1*(−180)–*per*-1 flies in 12 hr LD cycles for 4 d and collected flies every 4 hr during the fifth LD cycle. Total head RNA was prepared from each time point, and levels of *Rh1*(−180)–*per* mRNA were determined by RNase protection assays. Whole heads were used in these assays because the *Rh1*(−180)–*per* transgene is specifically expressed in eyes (Fig. 2; Table 1), and the transgene-derived transcript can be monitored independently of the endogenous per^{01} transcript. In contrast to wild-type *Rh1* mRNA levels, *Rh1*(−180)–*per* mRNA levels are ~2.5-fold higher during the day than during the night (Fig. 3). To determine whether these mRNA fluctuations are attributable to the *Rh1*(−180) promoter or clock regulatory sequences within, or downstream of, the *per* coding region, we examined mRNA levels from *Rh1*(−180)–*LacZ* transgenic flies under LD conditions. As seen with *Rh1*(−180)–*per* mRNA, *Rh1*(−180)–*LacZ* mRNA was found to be ~2.5-fold higher during the day than during the night (data not shown), indicating that the *Rh1*(−180) promoter, and not PER coding or downstream sequences, mediates these mRNA fluctuations.

To determine whether the *Rh1*(−180) promoter is sensitive to light or is under clock control, we measured *Rh1*(−180)–*per* mRNA under constant dark conditions. Flies were entrained in 12 hr LD cycles for 4 d, transferred to constant darkness, and collected every 4 hr for one circadian cycle. Head RNA extracted from each time point was analyzed by RNase protection assays, and unlike the previous results in LD conditions, *Rh1*(−180)–*per* mRNA levels do not fluctuate under constant dark conditions (Fig. 3). Likewise, the levels of per^{01} transcripts are constant (Fig. 3), because of the lack of oscillation in noneye head tissue where more per^{01} mRNA is expressed. Identical results were obtained when *Rh1*(−180)–*LacZ* mRNA levels were measured under constant dark conditions (data not shown).

Rh1(−180)–*per* mRNA levels are approximately fivefold higher than endogenous per^{01} transcript levels in these transgenic lines (Fig. 3). This difference is mainly caused by the *Rh1*(−180) promoter because *Rh1*(−180)–*LacZ* mRNA levels are two- to threefold higher than wild-type *per* mRNA levels (data not shown). In addition, per^{01} mRNA levels are two- to threefold lower than that of wild-type *per* mRNA (Hardin et al., 1990), which would further magnify *per* mRNA levels for the *Rh1*(−180)–*per* transgenics. These measurements were made on homozygous transgenic lines; however, the levels of *Rh1*(−180)–*per* mRNA may be lower in heterozygotes because one of the dosage compensation elements (i.e., intron 1) is missing in these constructs (Cooper et al., 1994). Consequently, heterozygotes were used in experiments performed under constant conditions (see below).

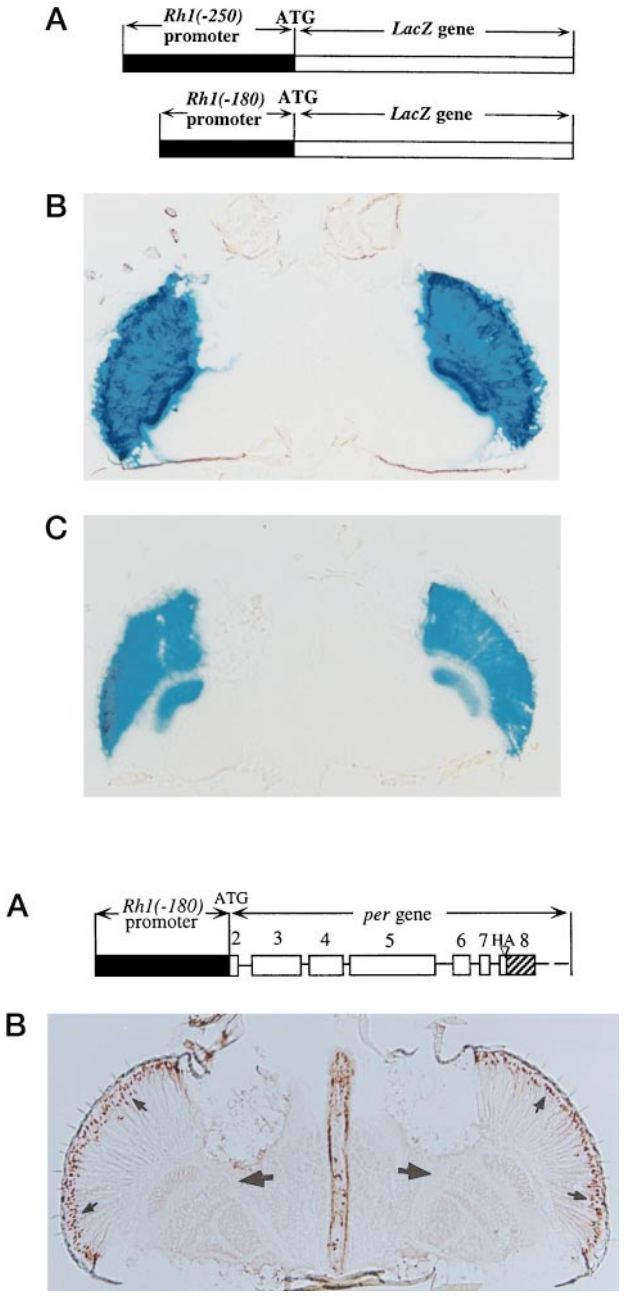

Figure 1. **Top.** A crippled *Rh1* promoter produces low levels of β-gal in eyes. *A*, Schematic representation of the *Rh1*(−250)–*LacZ* and *Rh1*(−180)–*LacZ* DNA constructs. The *Rh1*(−250) and *Rh1*(−180) promoter fragments, which contain 250 and 180 bp of upstream sequences, respectively, and leader sequences up to the translation initiation site, are represented by *black bars*. These promoter fragments were fused to *lacZ* sequences (*white bars*) at the *lacZ* translation initiation site. *B*, Spatial expression and β-gal staining intensity in *Rh1*(−250)–*LacZ* transgenic flies. β-Gal activity was detected via X-gal staining for 12 hr in a horizontal section through a whole head oriented with the anterior end up. β-Gal activity (*blue* stain) is present throughout the eyes of this and four other lines. *C*, Spatial expression and β-gal staining intensity in *Rh1*(−180)–*LacZ* transgenic flies. β-Gal activity was detected in heads as described in *B*. β-Gal activity is present throughout the eyes of this and other lines. Two other lines showed eye-specific staining present in patches within the eye.

Figure 2. **Bottom.** *Rh1*(−180)–*per* expresses specifically in photoreceptors R1–R6. *A*, Schematic representation of the *Rh1*(−180)–*per* DNA construct. The *Rh1*(−180) promoter fragment, which contains sequences from 180 bp upstream of the transcription start site to the translation initiation site, is represented by a *black bar*. The *Rh1*(−180) promoter was fused to *per* genomic sequences at the *per* translation initiation site. *White bars* symbolize *per* protein coding exons 2–8, the *hatched bar* denotes the *per* 3′-untranslated sequence, *thin lines* represent *per* intron sequences, and the *dashed line* denotes genomic sequences downstream from *per*. *HA* refers to the hemagglutinin tag inserted at the C terminus of PER (Rutila et al., 1992). *B*, Spatial expression of PER in *per*^{01};*Rh1*(−180)–*per* transgenic flies. PER immunoreactivity was detected in flies collected at Zeitgeber time 23 (ZT23), sectioned, and incubated with anti-PER antibodies. A horizontal section of a whole head is oriented with the anterior end up. PER immunoreactivity (*dark brown* stain) is only present in the nuclei of photoreceptors R1–R6, which are indicated by *small arrows*. No immunoreactivity is seen in the area of the LNs, indicated by the *large arrows*. Photoreceptor specificity was obtained in a total of five transgenic strains.

PER abundance and nuclear localization cycle during LD conditions

Because TIM is light sensitive and is required for PER accumulation and nuclear localization (Vosshall et al., 1994; Price et al., 1995; Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996), we expected that both PER abundance and nuclear localization would cycle under LD conditions in these transgenic lines. To determine whether PER levels cycle, we entrained *per*^{01}; *Rh1*(−180)–*per*-1 flies to 12 hr LD cycles and collected flies at 4 hr intervals during the final cycle. Protein extracts were prepared from fly heads and analyzed on Western blots with anti-PER antiserum (see Materials and Methods). The results show that PER cycles under these conditions with a lower level during the day than during the night (Fig. 4*A*). The cycling profile under these conditions is different from that of wild-type flies in two respects; it peaks at ZT15 rather than ZT21, and PER is never completely absent during the day. These differences may be caused by either the relatively high levels of *per* RNA from the transgene or a premature (light-driven) rise in mRNA levels from the transgene.

Another parameter that can be used to measure oscillator function is the PER nuclear localization cycle (Curtin et al., 1995). To determine whether PER derived from the *Rh1*(−180)–*per* transgene undergoes nuclear cycling under LD conditions, we measured PER immunohistochemically on sections of flies collected every 4 hr (Fig. 4*B*). Little if any nuclear PER is detected until ZT15, a time point ~2–3 hr earlier than that when PER begins to enter the nucleus in wild-type flies. Nuclear PER peaks between ZT19 and ZT23 and then begins to decline during the light phase until it becomes undetectable at ZT11. These results show that PER nuclear localization cycles under LD conditions. However, in *Rh1*(−180)–*per* flies, PER accumulation peaks at ZT15, but the peak level of nuclear PER occurs at ZT23. This is different than the situation in wild-type flies in which nuclear localization occurs between ZT18 and ZT20 (Curtin et al., 1995), ~4–5 hr before the peak in protein accumulation (Edery et al., 1994a). This uncoupling of PER accumulation and nuclear localization suggests that these two aspects of the circadian cycle are controlled independently because nuclear localization that is solely dependent on PER concentration would result in a premature movement of PER into the nucleus.

Endogenous *per*^{01} mRNA cycling is rescued in the eyes of *per*^{01};*Rh1*(−180)–*per* flies

In the circadian feedback loop, nuclear PER suppresses the transcription of the PER gene, thereby producing fluctuations in mRNA abundance. From this, we would predict that nuclear PER

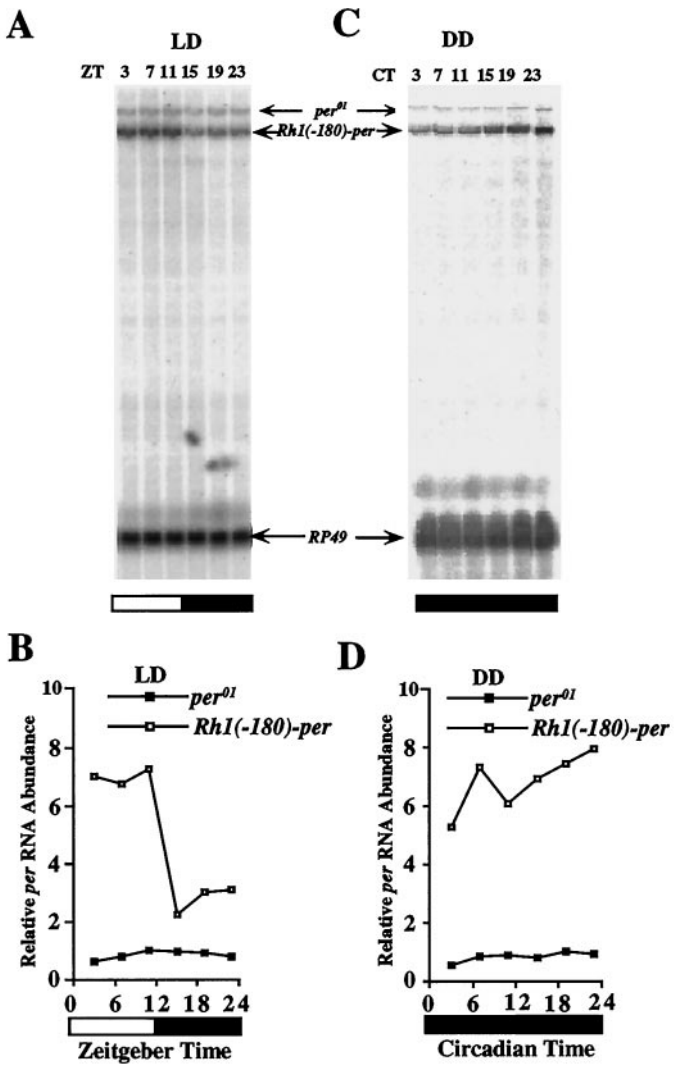

Figure 3. *Rh1*(−180) promoter activity is constitutive in DD conditions and fluctuates in LD cycles. RNase protection assays were performed on total head RNA samples prepared from *per*^{01};*Rh1*(−180)–*per* flies. *A*, Endogenous and transgene-driven *per* RNA levels measured in samples collected during LD cycles at the time points indicated. *Rh1*(−180)–*per* represents the transgene-driven *per*-protected fragment, *per*^{01} represents the endogenous *per*-protected fragment, and *RP49* represents the ribosomal protein 49 mRNA-protected fragment. The *white* and *black boxes* represent times when lights were on or off, respectively. *B*, Quantitation of the data in *A*. Relative RNA abundance refers to the values of endogenous *per*/*RP49* (*filled squares*) or transgene-driven *per*/*RP49* (*open squares*), when the peak value of endogenous *per* mRNA was adjusted to 1.0. The *white* and *black boxes* represent times when lights were on or off, respectively. Similar results were obtained from six independent time courses. *C*, Endogenous and transgene-driven *per* RNA levels measured in samples collected during the first day of DD at the time points indicated. The protected fragments are as designated in *A*. The *hatched* and *black boxes* represent times when lights would have been on or off, respectively, if the light cycle were continued. *D*, Quantitation of the data in *C*. Relative RNA abundance is described in *B*. The *hatched* and *black boxes* represent times when lights would have been on or off, respectively, if the light cycle were continued. Similar results were obtained from three independent time courses.

cycling seen in *per*^{01};*Rh1*(−180)–*per* flies should rescue endogenous *per*^{01} RNA cycling. To test this prediction, we performed RNase protection assays on total RNA isolated from the eyes of homozygous *per*^{01};*Rh1*(−180)–*per*-1 flies collected every 4 hr during LD cycles (see Materials and Methods). The results show that endogenous *per*^{01} mRNA cycling is rescued in the eyes of these transgenic flies and has an amplitude of approximately fourfold (Fig. 5). The peak in *per*^{01} mRNA abundance occurs at ZT11, which is 4–6 hr earlier than the *per* mRNA peak in wild-type flies (Hardin et al., 1990; Brandes et al., 1996). This early *per*^{01} mRNA peak could be accounted for by the premature accumulation of nuclear PER (Fig. 4*B*), which acts to decrease the levels of *per* mRNA. Endogenous *tim* mRNA also exhibited low amplitude cycling, consistent with the notion that *per* and *tim* transcription are coregulated. As expected, the levels of *Rh1*(−180)–*per* mRNA were higher during the light phase because of the sensitivity of the *Rh1*(−180) promoter to light (Figs. 3, 5).

PER abundance cycles under constant dark conditions

To determine whether PER cycling in photoreceptors R1–R6 is a circadian rhythm, we examined both PER levels and nuclear localization in constant darkness. *per*^{01};*Rh1*(−180)–*per*-1/+ flies were entrained in an LD cycle for 4 d and collected during the first 2 d of DD at 4 hr intervals. Head protein extracts were prepared and subjected to Western blotting in parallel with those of wild-type controls. The results show that PER levels in these transgenic flies fluctuate over two circadian cycles. Compared with PER cycling in wild-type flies, in which low levels are present at CT11 and high levels are present at CT23 (Fig. 6*A*), the overall PER levels in *per*^{01};*Rh1*(−180)–*per*-1/+ flies cycle with a different phase and/or period; PER decreases during the first subjective day, reaches its trough level at CT15–19, increases from CT23 to the next CT15, and then decreases (Fig. 6*A*). This phase and/or period difference in PER cycling is also seen in *per*^{01};*Rh1*(−180)–*per*-2/+ flies, although the delay in PER accumulation (vs wild-type PER) is greatly reduced in comparison with that of *per*^{01};*Rh1*(−180)–*per*-1/+ flies (Fig. 6*A*).

Quantification of these data shows that PER from both transgenes cycles over the course of 2 d with a 1.5–2-fold amplitude for *per*^{01};*Rh1*(−180)–*per*-1/+ flies and an approximately twofold amplitude for *per*^{01};*Rh1*(−180)–*per*-2/+ flies (Fig. 6*B*). The cycling amplitudes of these transgenic lines approach that of the 2–2.5-fold cycling seen in wild-type flies collected and tested in parallel with the transgenic lines. This dampened cycling amplitude of wild-type flies in DD conditions is similar to that seen in previous studies (Edery et al., 1994b; Zeng et al., 1996). Because PER abundance could only be measured for a limited number of cycles under DD conditions, it is not possible to determine whether the differences in peaks and troughs in PER levels are caused by period differences, phase differences, or a combination of both. However, it would not be surprising if the higher *per* mRNA levels and the lack of *per* mRNA cycling in the transgenic lines has a negative effect on PER cycling amplitude.

To determine whether nuclear PER cycles under constant dark conditions, we entrained and collected *per*^{01};*Rh1*(−180)–*per*-1/+ flies as described above. Sections were prepared for each time point and stained with anti-PER antibodies. The nuclear PER staining signal is intense as flies enter DD conditions, declines to a minimum value between CT16 and CT20, and then increases during the next circadian day (Fig. 7). Unlike the case in LD cycles, PER staining in *per*^{01};*Rh1*(−180)–*per*-1/+ flies is never completely absent in the nucleus. The fluctuations in PER intensity on sections fit well with overall PER abundance measurements on Western blots (Fig. 6).

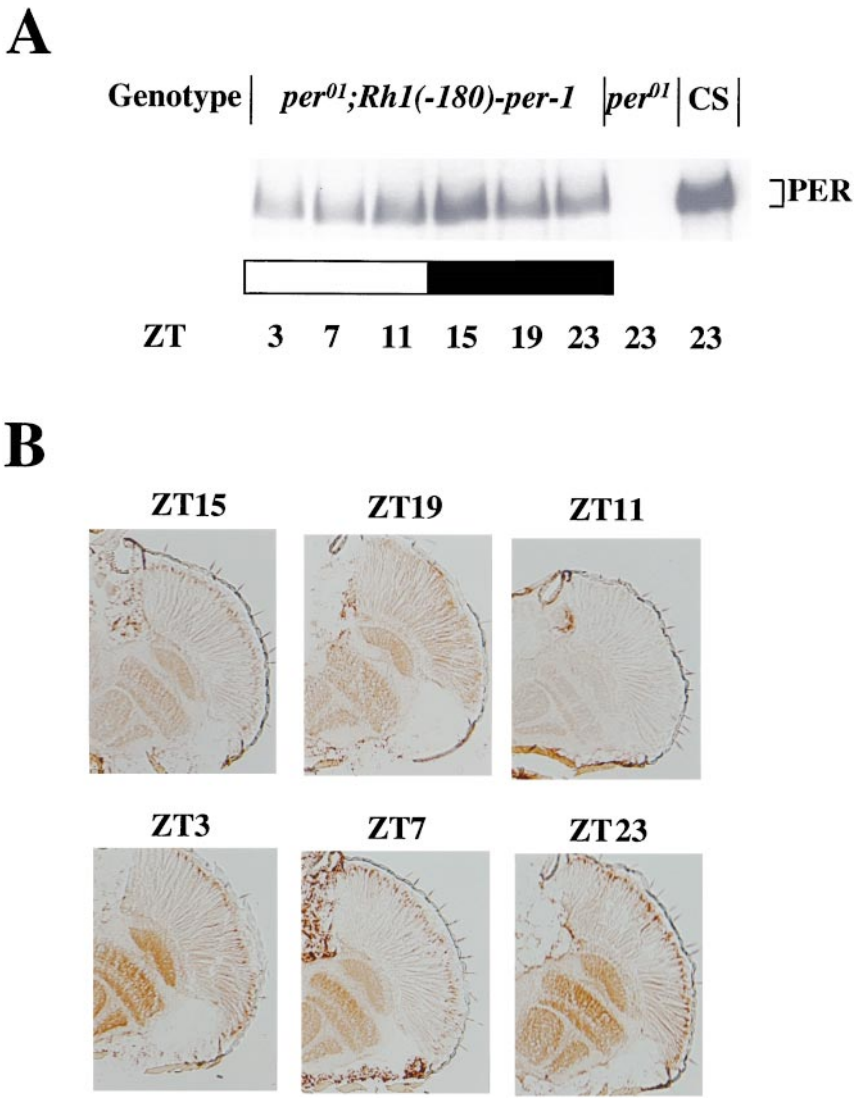

Figure 4. PER abundance and nuclear localization cycles in *Rh1*(−180)–*per* flies under LD conditions. *A*, Head protein extracts were prepared from *per*^{01}; *Rh1*(−180)–*per*-1 transgenic flies collected at the time points indicated, from *per*^{01} flies collected at ZT23, and from wild-type Canton-S (*CS*) flies collected at ZT23. These protein extracts were used to prepare Western blots, which were probed with anti-PER antibodies. The *gel strip* shows the level of PER immunoreactivity for each sample. The *white* and *black boxes* represent times when lights were on or off, respectively. Similar results were obtained from five independent time courses. *B*, *per*^{01};*Rh1*(−180)–*per* flies were entrained for 4 d in 12 hr LD cycles and collected at the time points indicated during the fifth LD cycle. Horizontal sections were prepared and probed with anti-PER antibodies. One hemisphere of a head is shown for each time point and is oriented with the anterior end up. PER immunoreactivity is seen as a *dark brown* stain. Two independent time courses showed similar results.

DISCUSSION

In this study, the *Rh1* promoter was modified so that it would produce low levels of mRNA in photoreceptors R1–R6. This modified promoter, *Rh1*(−180), is >20-fold less active than the native *Rh1* promoter and produces transcripts specifically in photoreceptors R1–R6 at levels approximately fourfold higher than the wild-type *per* mRNA peak (Figs. 1-3, 5). The *Rh1*(−180) promoter is constitutively active in constant darkness and is light inducible, producing 2.5-fold more mRNA in the light phase than in the dark phase (Fig. 3). This light inducibility was not seen in earlier studies with the complete *Rh1* promoter (i.e., having 3 kb of upstream sequences), where high levels of expression may mask differences in mRNA abundance (Zeng et al., 1994). Such diurnal fluctuations in opsin mRNA abundance have been seen in certain species of toads and fish; opsin mRNA levels are 4–10-fold higher during the light phase than in the dark phase and can be induced by light in the dark phase (Korenbrot and Fernald, 1989). However, unlike the case in *Drosophila*, opsin mRNA levels in these species continue to fluctuate under constant darkness, apparently under the control of a circadian oscillator (Korenbrot and Fernald, 1989).

In wild-type flies, PER cycling is mediated, in part, via interactions with the TIM protein. TIM forms heteromeric complexes with PER and is required for both PER accumulation and nuclear localization (Vosshall et al., 1994; Gekakis et al., 1995; Price et al., 1995; Lee et al., 1996; Zeng et al., 1996). Light-induced degradation of TIM, for example, in natural environmental cycling conditions, seems very likely to mediate daily resetting of the *Drosophila* circadian clock such that the oscillator is in synchrony with the environment (Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). In *Rh1*(−180)–*per* flies, *per* mRNA levels are constantly higher than is the wild-type *per* mRNA peak. These high levels of *per* mRNA nevertheless give rise to fluctuations in PER abundance and nuclear localization under LD conditions (Fig. 4). This high amplitude PER cycling is apparently mediated by TIM; when *per*^{01};*Rh1*(−180)–*per* transgenic flies are in LD cycles, light drives TIM cycling in R1–R6 that, in turn, drives both overall PER abundance and nuclear PER to cycle. This light-mediated cycling of TIM and its effect on PER cycling have been seen in previous studies (Myers et al., 1996; Zeng et al., 1996; Dembinska et al., 1997; Stanewsky et al., 1997). However, when extremely high levels of PER are present, as is the case with the *Rh1*–*per* transgenes, TIM cannot maintain PER cycling (Zeng et al., 1996).

The phase of PER cycling in *per*^{01};*Rh1*(−180)–*per* flies is earlier than that of PER in wild-type flies under LD conditions.

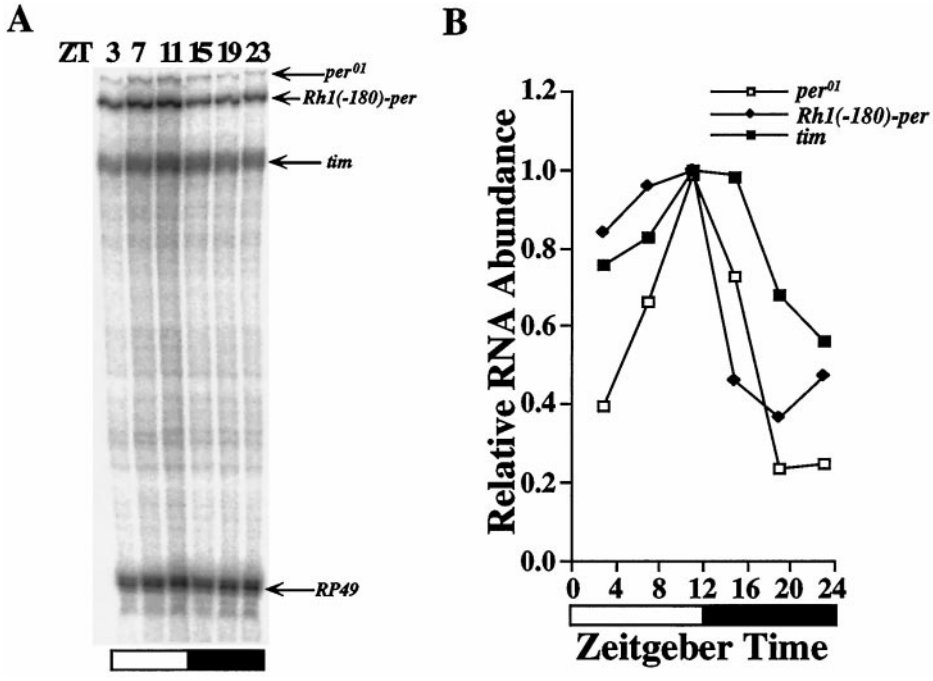

Figure 5. Endogenous per^{01} and *tim* mRNA cycling is rescued in the eyes of *Rh1*(−180)–*per* flies. RNase protection assays were performed on total eye RNA samples (5 μg) prepared from per^{01};*Rh1*(−180)–*per* flies. *A*, Endogenous per^{01}, endogenous *tim*, and transgene-driven *per* RNA levels measured in samples collected during LD cycles at the time points indicated. *Rh1*(−180)–*per* represents the transgene-driven *per*-protected fragment, per^{01} represents the endogenous *per*-protected fragment, *tim* represents the endogenous *tim*-protected fragment, and *RP49* represents the RP49-protected fragment. The *white* and *black boxes* represent times when lights were on or off, respectively. *B*, Quantitation of the data in *A*. Relative RNA abundance refers to the values of endogenous *per*/*RP49* (*open squares*), endogenous *tim*/*RP49* (*filled squares*), or transgene-driven *per*/*RP49* (*filled diamonds*), with each peak adjusted to 1.0. The *white* and *black boxes* represent times when lights were on or off, respectively. Similar results were obtained from three independent time courses.

This early peak in PER abundance (~6–8 hr early) is not followed by an equally early movement into the nucleus, although nuclear localization is ~3 hr early (Fig. 4). This result shows that the PER abundance and nuclear localization cycles can be disconnected, indicating that high levels of PER accumulation alone are not sufficient for nuclear localization. Because PER moves into the nucleus as a complex with TIM (Lee et al., 1996; Zeng et al., 1996), the high levels of PER seen early in the dark phase [in per^{01};*Rh1*(−180)–*per* flies] may not be complexed with TIM and are therefore unable to move into the nucleus.

In LD cycles, endogenous per^{01} mRNA cycling is rescued in photoreceptors R1–R6 of per^{01};*Rh1*(−180)–*per* flies. However, this rescued per^{01} mRNA cycling is different than the cycling seen for wild-type *per* RNA (Hardin et al., 1992b; Zeng et al., 1994). First, the rescued per^{01} mRNA cycling amplitude is low. This may be because PER never drops to zero as it does in wild-type flies (Fig. 4*A*), and constant levels of per^{01} mRNA are seen in 25% of the photoreceptor cells that lack transgene expression. Second, per^{01} mRNA peaks at ZT11, several hours earlier than the wild-type *per* mRNA peak (Fig. 5). This early mRNA peak probably results from higher than normal PER levels, which would lead to premature PER nuclear translocation and a decrease in *per* gene transcription. Nuclear PER in our transgenic flies was detected 3 hr earlier than it was in wild-type flies (ZT15 vs ZT18) (Fig. 4*B*), indicating that the period of time between the *per* mRNA peak and the appearance of nuclear PER in these transgenic flies is similar to that seen in wild-type flies (Hardin et al., 1990; Zerr et al., 1990; Hardin and Siwicki, 1995).

Cycling of PER abundance in per^{01};*Rh1*(−180)–*per* flies persists in constant darkness, showing that *per* RNA cycling is not required for PER cycling (Fig. 6). This result is compelling because expression is restricted to photoreceptors, a cell type in which expression levels are high enough to be quantitatively measured. Previous studies in which mRNA expression levels in certain tissues are inferred, but not directly measured, are consistent with this finding. The *glass* promoter, which is constitutively active in wild-type flies, has been used to drive *per* expression in the LNs and photoreceptors of per^{01} flies. This *glass*–*per* transgene rescues long-period (~28–34 hr) locomotor activity rhythms to varying degrees (25–69% rescue) in each independent line and gives rise to cycling in PER abundance and nuclear localization under LD conditions in photoreceptors (Vosshall and Young, 1995). Even though *per* RNA cycling was not tested in these transgenic lines, constitutive expression from the endogenous *glass* promoter suggested that PER cycling can occur without *per* mRNA cycling (Vosshall and Young, 1995).

Constructs containing a 7.2 kb fragment of *per* genomic DNA lacking the promoter, first exon, and most of the first intron were also used to rescue rhythms in per^{01} flies. One of the two lines that rescue locomotor activity rhythms (7.2:2) expresses PER exclusively in LNs (Hamblen et al., 1986; Frisch et al., 1994). In this line, PER abundance and nuclear localization cycle under LD conditions (Frisch et al., 1994). This cycling is not simply driven by LD cycles, however, because it persists under DD conditions (Fig. 8). As seen in wild-type flies, the PER cycling amplitude in 7.2:2 flies dampens under DD conditions because of PER remaining in the nucleus at times when it would be undetectable in LD cycles (Fig. 8; Zerr et al., 1990). The behavioral rescue seen in the two 7.2 lines was attributed to internal (i.e., within or downstream of the transcribed region) cis-acting transcriptional cycling elements that are active in a permissive genomic environment (Frisch et al., 1994). Our results argue against the existence of internal cycling elements because we see no mRNA cycling in any of the per^{01};*Rh1*(−180)–*per* lines, which only differ from 7.2 lines in that they lack the last 343 bp of the first intron and 46 bp of untranslated second exon sequences. Of these differences, the first intron sequence was incapable of driving cyclic transcription (H. Hao, Y. Cheng, and P. E. Hardin, unpublished observations). Our results, therefore, suggest that rescue of PER cycling and behavioral rhythms in 7.2:2 flies occurs without underlying cycles in *per* mRNA.

If *per* mRNA cycling is not required for PER cycling in general, then what function does transcriptional feedback serve in wild-type flies? We believe that feedback regulation of mRNA cycling

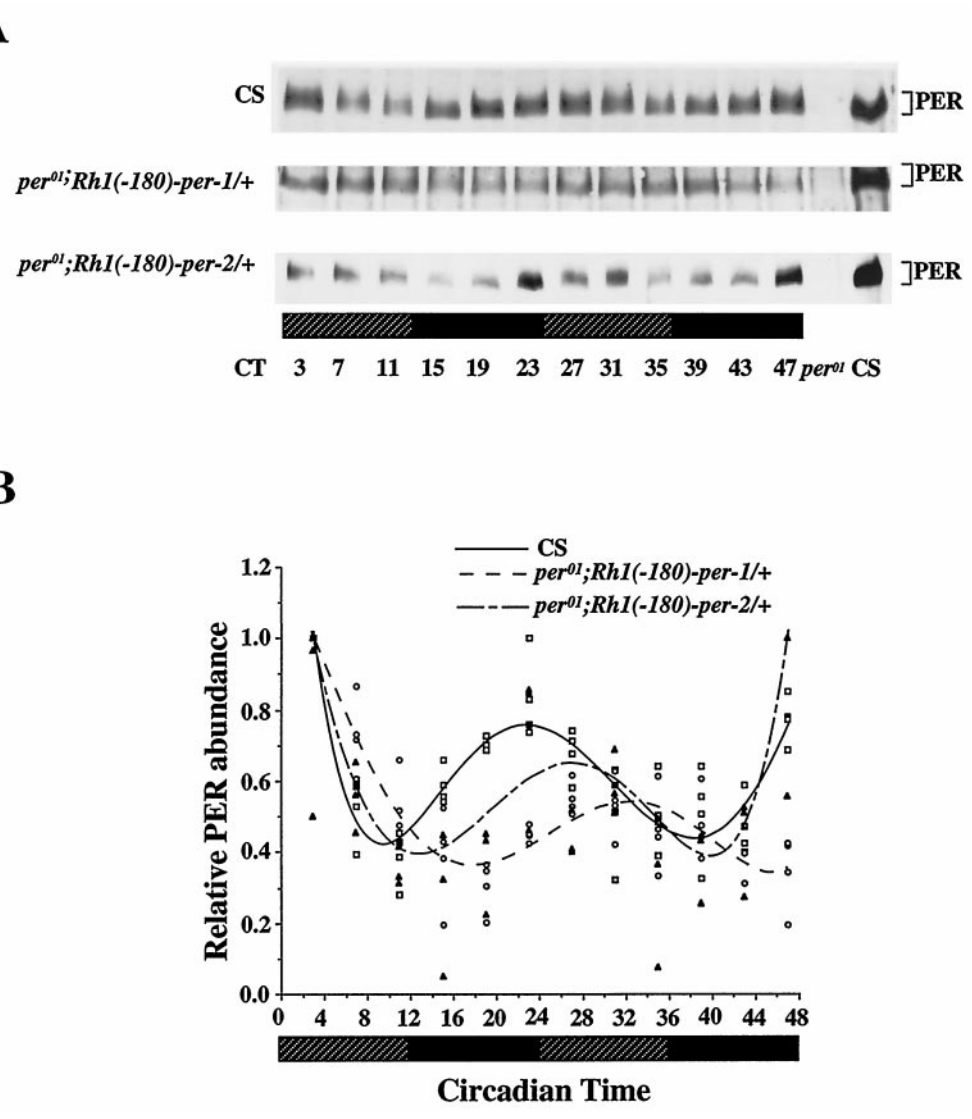

Figure 6. Overall PER abundance cycles under DD conditions. *A*, per^{01}; *Rh1*(−180)–*per*-1/+ and per^{01}; *Rh1*(−180)–*per*-2/+ flies were entrained in LD cycles for 4 d before being transferred to constant darkness and collected at the circadian time points indicated. Head protein extracts were made and subjected to Western blotting as described in Figure 4. The *gel strips* show the level of PER immunoreactivity for each sample. Head extracts from *CS* and per^{01} flies collected at ZT23 were used as positive and negative controls for PER immunoreactivity, respectively (*right two lanes*). The *hatched* and *black boxes* represent times when lights would have been on or off, respectively, if the light cycle were continued. Similar results were obtained from three independent time courses for *CS*, three time courses for per^{01}; *Rh1*(−180)–*per*-1, and two time courses for per^{01};*Rh1*(−180)–*per*-2. *B*, Quantitation of PER cycling under DD conditions is shown. Data from three independent time courses of *CS* (*open squares*; *solid line*), three independent time courses of per^{01}; *Rh1*(−180)–*per*-1/+ (*open circles*; *dashed line*), and two independent time courses of per^{01};*Rh1*(−180)–*per*-2/+ (*filled triangles*; *line* with *short* and *long dashes*) are plotted. The peak time point for each time course was set to 1.0, and all other time points are relative to this value. The curves were generated by fitting the normalized pooled data for each genotype to polynomial functions (see Materials and Methods). The circadian time points and the *hatched* and *black boxes* are described in *A*.

is important for several aspects of circadian clock function. For example, transgenic strains assumed to express constitutive levels of *per* mRNA (i.e., *glass–per*; 7.2:2; *hsp70*-7.2) exhibit period-altered and/or weak locomotor activity rhythms. Likewise, per^{01}; *Rh1*(−180)–*per* flies, which are known to express constant levels of *per* mRNA, exhibit an altered phase and/or period of PER cycling compared with wild type. These examples suggest that *per* mRNA cycling affects PER cycling and behavior; however, inappropriate *per* mRNA or protein expression (i.e., levels that are too high or too low; altered or incomplete spatial expression) could also account for these effects on PER cycling and behavior. Another aspect of clock function that may require *per* and/or *tim* mRNA cycling is phase resetting. Because light acts to decrease TIM abundance, high levels of *tim* mRNA early in the dark phase were proposed to replenish TIM levels, leading to a phase delay, whereas low levels of *tim* mRNA late in the dark phase could not replenish TIM levels, leading to a phase advance (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996). Finally, circadian feedback is believed to control the expression levels of clock output genes. A group of 20 *Drosophila* rhythmically expressed genes (Dregs) have been identified (Van Gelder et al., 1995). Rhythmic expression of several of these transcripts (i.e., Dregs 5, 6, 9, 10, and 15), as measured by mRNA cycling, is dependent on *per* gene function (Van Gelder et al., 1995; Van Gelder and Krasnow, 1996), indicating that circadian feedback loop function is important for rhythmic Dreg expression. Likewise, another clock-regulated gene, *Crg*-1, has been identified that exhibits PER-dependent mRNA cycling (Rouyer et al., 1997).

When *per* is specifically expressed in the photoreceptors R1–R6, PER levels show circadian cycling under both LD and DD conditions. These results demonstrate that *Drosophila* eyes contain an autonomous circadian oscillator (i.e., one that operates in the absence of *per* expression and circadian feedback loop function in other tissues). This finding is consistent with earlier studies on *disconnected* (*disco*) flies, which show that *per* mRNA and protein cycle in heads under both LD and DD conditions even though *disco* flies are behaviorally arrhythmic and lack *per*-expressing lateral neurons (Zerr et al., 1990; Dushay et al., 1992; Hardin et al., 1992a). These results suggest that other circadian oscillators operate in the absence of the pacemaker for locomotor activity.

Like *Drosophila* photoreceptors, hamster suprachiasmatic nucleus (SCN) and retina (Ralph et al., 1990; Tosini and Menaker, 1996), *Xenopus* eyes (Besharse and Iuvone, 1983), *Aplysia* eyes

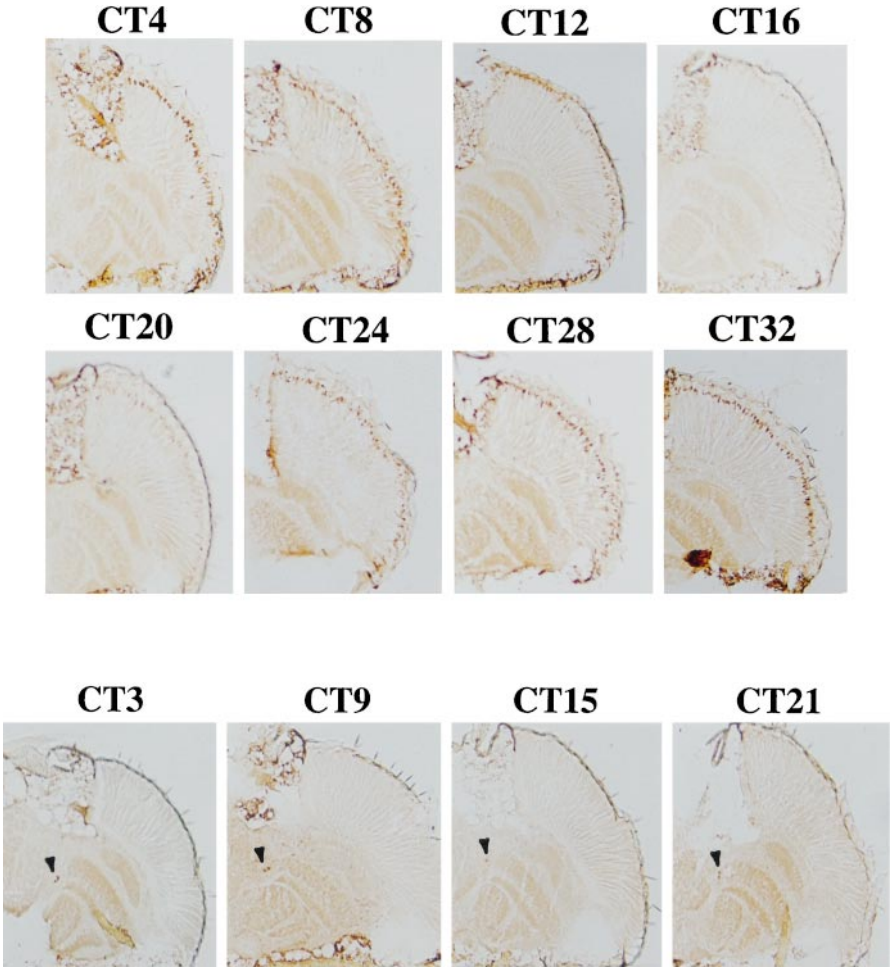

Figure 7. **Top.** Levels of nuclear PER cycle under DD conditions. *per*^{01};*Rh1*(−180)–*per*-1/+ flies were entrained in LD cycles for 4 d before being transferred to constant darkness and collected at the indicated time points. Horizontal sections were prepared and probed with anti-PER antibodies as described in Figure 4*B*. One hemisphere of a head is shown for each time point and is oriented with the anterior end up. PER immunoreactivity is seen as a *dark brown* stain. Two independent time courses showed similar results. Each time point represents similar results from at least four flies.

Figure 8. **Bottom.** Nuclear PER level cycles in lateral neurons of 7.2:2 flies under DD conditions. Homozygous *per*^{01}7.2:2 flies were entrained in LD cycles for 4 d before being transferred to constant darkness and collected at CT3, CT9, CT15, and CT21. Immunohistochemical staining of PER was done as described in Figure 4*B*. One hemisphere of a head is shown for each time point and is oriented with the anterior end up. The *arrowheads* denote anti-PER antibody staining in LNs. Three independent time courses showed high intensity staining at CT3 (n = 5 flies) and CT9 (n = 6 flies), low intensity staining at CT15 (n = 6 flies), and medium intensity staining at CT21 (n = 9 flies).

(Jacklet, 1969), gypsy moth testes (Giebultowicz et al., 1989), and chicken pineal glands (Takahashi et al., 1980) contain tissue autonomous circadian oscillators. This autonomy extends down to individual isolated basal retinal neurons in *Bulla* (Michel et al., 1993), and individual (but not isolated) rat SCN neurons (Welsh et al., 1995) show circadian electrical activity. In contrast, the circadian rhythm of lateral eye sensitivity in horseshoe crab (*Limulus polyphemus*) is a slave oscillator controlled by the brain circadian clock (Barlow et al., 1980; Kaplan and Barlow, 1980; Barlow, 1983; Kass and Barlow, 1992). Thus, depending on the tissue and the organism, circadian oscillators can operate tissue/cell autonomously or can be dependent on oscillators in other tissues. In *Drosophila*, we have shown that the adult eye contains an autonomous circadian oscillator. This, along with clock autonomy in the prothoracic gland (Emery et al., 1997) and lateral neurons (Frisch et al., 1994) and lateral neuron independent oscillators in Malpighian tubules (Giebultowicz and Hege, 1997; Hege et al., 1997), suggests that the *Drosophila* circadian system consists of a collection of autonomous oscillators. Synchrony among these oscillators could be mediated by the light sensitivity of TIM because a hierarchical organization is not apparent.

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