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The 69 bp Circadian Regulatory Sequence (CRS) Mediates *per*-Like Developmental, Spatial, and Circadian Expression and Behavioral Rescue in *Drosophila*

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The *period* (*per*) gene is an essential component of the circadian timekeeping mechanism in *Drosophila*. This gene is expressed in a circadian manner, giving rise to a protein that feeds-back to regulate its own transcription. A 69 bp clock regulatory sequence (CRS) has been identified previously upstream of the *period* gene. The CRS confers wild-type mRNA cycling when used to drive a *lacZ* reporter gene in transgenic flies. To determine whether the CRS also mediates proper developmental and spatial expression and behavioral rescue, we used the CRS to drive either *lacZ* or *per* in transgenic flies. The results show that the CRS is able to activate expression in pacemaker neuron precursors in larvae and essentially all tissues that normally express *per* in pupae and adults. The CRS is sufficient to rescue circadian feedback loop function and behavioral rhythms in per^{01} flies. However, the period of locomotor activity rhythms shortens if a stronger basal promoter is used. This study shows that regulatory elements sufficient for clock-dependent and tissue-specific *per* expression in larvae, pupae, and adults are present in the CRS and that the period of adult locomotor activity rhythms is dependent, in part, on the overall level of *per* transcripts.

Key words: Drosophila*; circadian clock; transcriptional regulation; behavior;* period *gene; developmental expression*

In *Drosophila melanogaster*, an autoregulatory feedback loop in gene expression is a central feature of the circadian timekeeping mechanism. In this feedback loop, the *period* (*per*) and *timeless* (*tim*) genes are rhythmically expressed such that circadian fluctuations in *per* and *tim* mRNA levels are controlled by fluctuating levels of PER and TIM proteins (Rosato et al., 1997; Hardin and Sehgal, 1998). As PER and TIM accumulate, they bind to each other and move into the nucleus (Vosshall et al., 1994; Curtin et al., 1995; Gekakis et al., 1995; Saez and Young, 1996; Zeng et al., 1996), where they act to repress the transcription of their own genes (Hardin et al., 1992; Zeng et al., 1994; Sehgal et al., 1995; So and Rosbash, 1997; Darlington et al., 1998).

To understand how PER and TIM regulate cyclic transcription, we have identified the sequences that control circadian transcription of the *per* gene. A 69 bp circadian regulatory sequence (CRS), situated ~500 bp upstream of the *per* transcriptional initiation site, mediates mRNA cycling with an amplitude and phase similar to that of the wild-type *per* transcript (Hao et al., 1997). Within the CRS a consensus "E-box" transcription factor binding site is required for high-level *per* transcription (Hao et al., 1997).

E-box–dependent transcriptional activation is mediated by two members of the basic helix-loop-helix-PAS (bHLH-PAS) family of transcription factors, *Drosophila* CLOCK (dCLK) and BMAL1 (Darlington et al., 1998), also known as CYCLE (CYC) (Rutila et al., 1998). Mutations that impair the activity of either dCLK or CYC result in very low levels of *per* mRNA and behavioral arrhythmicity, showing that these proteins are essential for circadian clock function in *Drosophila* (Allada et al., 1998; Rutila et al., 1998). Consistent with the role of PER and TIM as transcriptional repressors, dCLK- or CYC-dependent activation is inhibited by the presence of PER and TIM in *Drosophila* tissue culture (Darlington et al., 1998). A similar regulatory circuit may also occur in mammals because orthologs of dCLK and CYC, called CLOCK (King et al., 1997) and BMAL1 (Ikeda and Nomura, 1997; Gekakis et al., 1998), respectively, activate transcription via E-boxes upstream of the mouse PER1 (mPER1) gene (Gekakis et al., 1998).

Although the *per* CRS is sufficient for circadian transcription in adult *Drosophila*, it is unclear whether the CRS also controls tissue- and developmental stage–specific expression. The spatial expression pattern of *per* has been well characterized and includes neuronal and non-neuronal tissues in the head and body (Hall, 1995). Among these tissues, a set of neurons in the lateral brain (LNs) appears to be the pacemaker cells for locomotor activity rhythms (Frisch et al., 1994). *per* is active in late embryos through adults (Young et al., 1985), and expression in LN precursors

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during development may be important for mediating the "time memory" of adults that were entrained as larvae (Sehgal et al., 1992; Kaneko et al., 1997).

In this study we have tested whether the *per* CRS mediates normal spatial and developmental expression and rescues behavioral rhythms in per^{01} mutants. These studies show that the CRS confers accurate (i.e., *per*-like) spatial expression in larvae, pupae, and adults. CRS-dependent *per* expression rescues behavioral rhythms, resulting in shorter or longer periods depending on whether strong or weak promoters are used, respectively. Thus, the *per* CRS is a target for transcription factors that regulate circadian, spatial, and developmental expression.

MATERIALS AND METHODS

Construction of transformation plasmids. The CRS/P/*lacZ* transgene was constructed as follows. The CRS was amplified from the −563 to −494/hs/*lacZ* construct template with a sense (5′-GAGAATTCGAGAAACC-GTAGG-3′) and an antisense (5′-GTGGATCCGATTTTGCTGGCC-3′) primer pair. This PCR fragment was inserted into the CPLZ vector (Wharton and Crews, 1993) at the *Eco*RI and *Bam*HI sites.

The hs/*cper* transformation vector was constructed as follows. A 5.9 kb *per* cDNA fragment spanning from the *Sal*I site of exon 3 to the *Eco*RI site at the 3′ downstream sequence was cut out from the *hspcper* (Edery et al., 1994a) and cloned into pBluescript KS^- to form Rec2. The remainder of the *per* cDNA and the heat-shock basal promoter were generated by PCR using the *hspcper* as a template, a sense primer (5′-GGCTCGAGGAGCGCCGGAGTATAAATAG-3′), and an antisense primer (5′-GGCTCGTCGACGCCGAG-3′). This PCR product was cloned into the *Xho*I and *Sal*I restriction sites of Rec2 to form the hs/*cper* fusion Rec5. The hs/*cper* fusion gene was then cloned into the *Kpn*I- and *Xba*I-cut polylinker sequences of a modified (i.e., *Xho*I sites deleted) CaSpeR-4 transformation vector (Thummel and Pirotta, 1991).

The CRS DNA fragment was generated using the −563 to −494/hs/*lacZ* construct (Hao et al., 1997) (also called CRS/hs/*lacZ*) as a template for PCR with sense (5′-GAGGTACCTACGGTTTCTCGG-3′) and antisense (5′-CACTCGAGGCGGATTTGCTGGCC-3′) primers. The PCR product was inserted into the hs/*cper* transformation vector at the *Kpn*I and *Xho*I sites to form the CRS/hs/*cper* construct.

Construction of the CRS/P/*cper* transgene was as follows. A 550 bp *Xho*I/*Sal*I fragment containing the *hsp70* basal promoter fused to the 5′ portion of the *per* cDNA was subcloned into pBluescript KS^-, forming X/S-550. The *hsp70* promoter region was removed by digestion with *Xho*I and *Nco*I and replaced with a 100 bp PCR fragment containing the P-element transposase basal promoter generated using CaSpeR-4 as a template for sense (5′-GTCTCGAGAAGCTTACCGAAG-3′) and antisense (5′-GACCATGGTAAGGGTTAATG-3′) primers, ultimately forming X/S-P550. The P-element transposase basal promoter containing the *Xho*I/*Sal*I fragment from X/S-P550 was then removed and used to replace the *hsp70* basal promoter-containing fragment from Rec4, which contains the 3′ portion of the *per* cDNA plus 2.1 kb of downstream *per* sequences, forming Rec4-P. The CRS was removed from the CRS/hs/*cper* construct by digestion with *Kpn*I and *Xho*I and was inserted into Rec4-P, forming CRS-Rec4-P. A *Kpn*I/*Eco*RI fragment containing the CRS, the P-element basal promoter, and the *per* cDNA and 3′-flanking sequences was ligated into CaSpeR-4, forming CRS/P/*cper*.

The nucleotide sequence of all constructs was confirmed using octamer sequencing (Hardin et al., 1996).

Fly stocks and germ-line transformation. D. melanogaster strains were raised on a cornmeal, sugar, agar, yeast, and Tegosept (a mold inhibitor) medium at 25°C. The wild-type *D. melanogaster* strain was Canton-S. P-element–mediated germ-line transformation was performed as described previously (Hao et al., 1997). At least four independent transformant lines with inserts on the second or third chromosomes were generated for each construct and balanced with In(2LR)CyO and In(3LR)TM2, respectively. CRS/P/*lacZ* transformants were crossed into a *y* per^{01} *w* genetic background for β-galactosidase staining as adults and into a *w* genetic background for β-galactosidase staining as larvae and pupae. The BG6 transgene (Dembinska et al., 1997) was used as a positive control for larval staining (Kaneko et al., 1997). To test whether larval staining was dependent on *dClk* or *Cyc*, we crossed BG6 transformants into a homozygous $dClk^{Jrk}$ (Allada et al., 1998) or *Cyc* (Rutila et al., 1998) genetic background. CRS/P/*cper* transformants were crossed into a *y* per^{01} *w* genetic background to test for molecular and behavioral rhythms.

β-Galactosidase staining. Each of five independent CRS/P/*lacZ* transgenic lines was dissected at Zeitgeber time 1 (ZT1) as L1, L2, or L3 larvae and as early, mid, or late pupae and then assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) histochemistry as described (Smith and Shepherd, 1996). CRS/P/*lacZ* adults were sectioned and stained using X-gal histochemistry as described (Liu et al., 1988). At least eight individuals were assayed from each independent CRS/P/*lacZ* line at each developmental stage. Twenty BG6 larvae were assayed at the L3 stage, and each gave the reported staining pattern (Kaneko et al., 1997). Twenty BG6; $dClk^{Jrk}$ and six BG6;*Cyc* larvae were assayed at the L3 stage, and none showed CNS staining.

Locomotor activity analysis. Locomotor activity of adult male Canton-S, *y* per^{01} *w*, *y* per^{01} *w*;CRS/P/*cper*, *y* per^{01} *w*;CRS/hs/*cper*, and *y* per^{01} *w*;hs/*cper* transgenic flies were monitored and analyzed as described (Hamblen et al., 1986). Briefly, flies were entrained in 12:12 hr light/dark (LD) cycles at 25°C for 3 d and then were transferred into constant darkness (DD). Locomotor behavior was monitored continuously starting from the entrainment, and data collected during DD were analyzed using periodogram analysis (Hamblen et al., 1986). Flies with powers >15 and a width greater than two in periodogram analysis were designated rhythmic.

RNase protection assays. Flies used for RNase protection analyses were entrained in 12:12 hr LD cycles at 25°C for 3 d and then transferred into DD and collected during the first day. For each time point, RNA was extracted from the heads and used for RNase protection assays as described (Hardin et al., 1990). The probe used in these studies was Rec5 (used to detect endogenous per^{01} transcript and transgenic hs/*cper* transcript). The Rec5 probe contains a 329 nucleotide (nt) antisense RNA from +208 bp of the heat-shock leader sequences to the *Sal*I site in *per* exon 3. The probe protects a 329 nt fragment from the transgenic *per* transcript (hs/*cper*) and a 283 nt fragment from the endogenous per^{01} transcript. As a control for the amount of RNA in each lane, an antisense ribosomal protein probe (RP49) was included in each RNase protection assay (Hardin et al., 1990).

Immunohistochemistry. Flies were entrained in 12:12 hr LD cycles at 25°C for at least 3 d and then transferred into DD and collected during the first day. Sectioning and staining were performed as described (Siwicki et al., 1988). Polyclonal anti-PER antibody raised in rabbits (a gift from J. Hall and R. Stanewsky) was used with a biotinylated goat anti-rabbit IgG secondary antibody to detect PER via immunostaining at a 1:4000 and a 1:2000 dilution, respectively.

Western blotting. Western-blotting analyses were performed as described (Edery et al., 1994b) with the following modifications. The polyclonal anti-PER antibody was diluted to 1:20,000, and the horseradish peroxidase–linked anti-rabbit IgG was diluted to 1:5000 in blocking solution; the blots were incubated with the primary antibody at 4°C overnight and with the secondary antibody at room temperature for 1 hr.

RESULTS

The CRS mediates *per*-like spatial expression in adults

Our previous reporter gene studies showed that the CRS is capable of driving rhythmic transcription (Hao et al., 1997). The transgenes used in these studies produced cytoplasmic β-galactosidase that was difficult to resolve as individual cells. To improve cellular resolution, we inserted the CRS into the CPLZ transformation vector (Wharton and Crews, 1993), which produces a β-galactosidase product that is localized to the nucleus because it is fused to the N terminal of the P-element transposase that contains a nuclear localization signal (O'Kane and Gehring, 1987). This vector also uses the P-element transposase basal promoter to drive *lacZ* (Fig. 1).

In adults, X-gal staining of each CRS/P/*lacZ* transformant line reveals nuclear β-galactosidase activity in photoreceptors, glial cells of the optic lobe, most LNs and dorsal neurons (DNs), the ventriculus, cardia, fat bodies, and Malpighian tubules (Fig. 2). We have observed that a few cells, including glia in the lamina, dorsal LNs, and the first group of dorsal neurons (DN1s), show little or no staining in CRS/P/*lacZ* transformants compared with

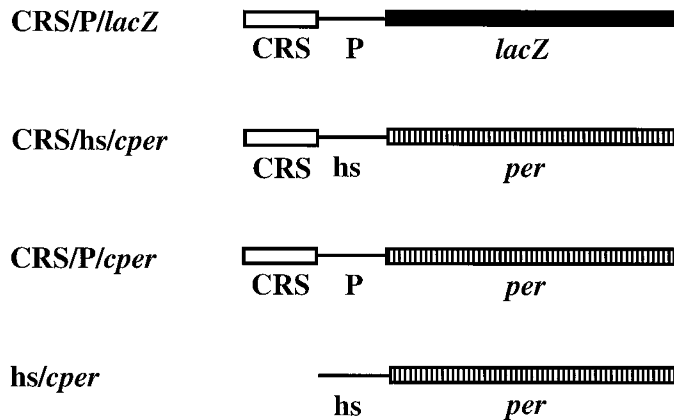

Figure 1. Schematic drawings of transgenic constructs. *CRS*, *per* circadian regulatory sequence; *hs*, *Drosophila* heat-shock protein 70 gene basal promoter plus leader sequences; *lacZ*, fusion of the N terminal of the P-element transposase (including the nuclear localization signal) and the *Escherichia coli lacZ*-coding sequences; *P*, *Drosophila* P-element transposase gene basal promoter plus leader sequences; and *per, per* cDNA plus 2.1 kb of 3′-flanking sequences.

that shown by *lacZ* driven by ~4 kb of *per* upstream sequence (Liu et al., 1988, 1991; Stanewsky et al., 1997) and of PER in wild-type flies (Zerr et al., 1990). Staining throughout the entire cell is seen in the abdomen and represents endogenous β-galactosidase activity (Liu et al., 1988). Thus, the CRS contains not only regulatory sequences capable of driving rhythmic transcription but also regulatory sequences that mediate essentially normal *per* expression in adults.

The CRS drives expression in LN precursors

The *Drosophila* circadian timekeeping system operates from the first larval instar (L1) onward (Sehgal et al., 1992). During the L1 stage, *per* begins to be expressed in one cluster of lateral neurons and in two clusters of dorsal neurons (DN1 and DN2) (Kaneko et al., 1997). Although *per* is rhythmically expressed in all three clusters of larval neurons, the observation that *per* expression is only maintained in the LNs into adulthood suggests that this cluster of neurons conveys circadian phase to adults (Kaneko et al., 1997). Because *per* expression in larval neurons is correlated with the onset of circadian timekeeping, we wanted to determine whether the CRS could drive *per*-like expression in larvae.

Larvae containing the CRS/P/*lacZ* or BG transgenes were stained at the mid-L1, -L2, and -L3 stages. The BG transgene, which serves as a positive control, is a *per–lacZ* fusion gene that is expressed in larval LNs, DN1s, and DN2s (Kaneko et al., 1997). The CRS/P/*lacZ*-staining patterns were similar for each larval instar (data not shown); thus we will only show the results obtained from L3 larvae. In BG and two representative CRS/P/*lacZ* lines, β-galactosidase staining was readily detected in the nuclei of four to five larval LNs (Fig. 3). In contrast to BG, CRS/P/*lacZ* larvae showed no staining in DN1s, and staining in DN2s was detected in only 10% of the brain hemispheres (data not shown). Because the CRS/P/*lacZ* transgene was expressed in larval and adult LNs (Figs. 2, 3), we suspected that this transgene would also be expressed in pupal LNs. When brains from early (12–24 hr), mid (48 hr), and late (72–96 hr) CRS/P/*lacZ* pupae were stained, LN staining was observed (data not shown). These data demonstrate that the CRS mediates expression in LNs from the first larval instar to adults.

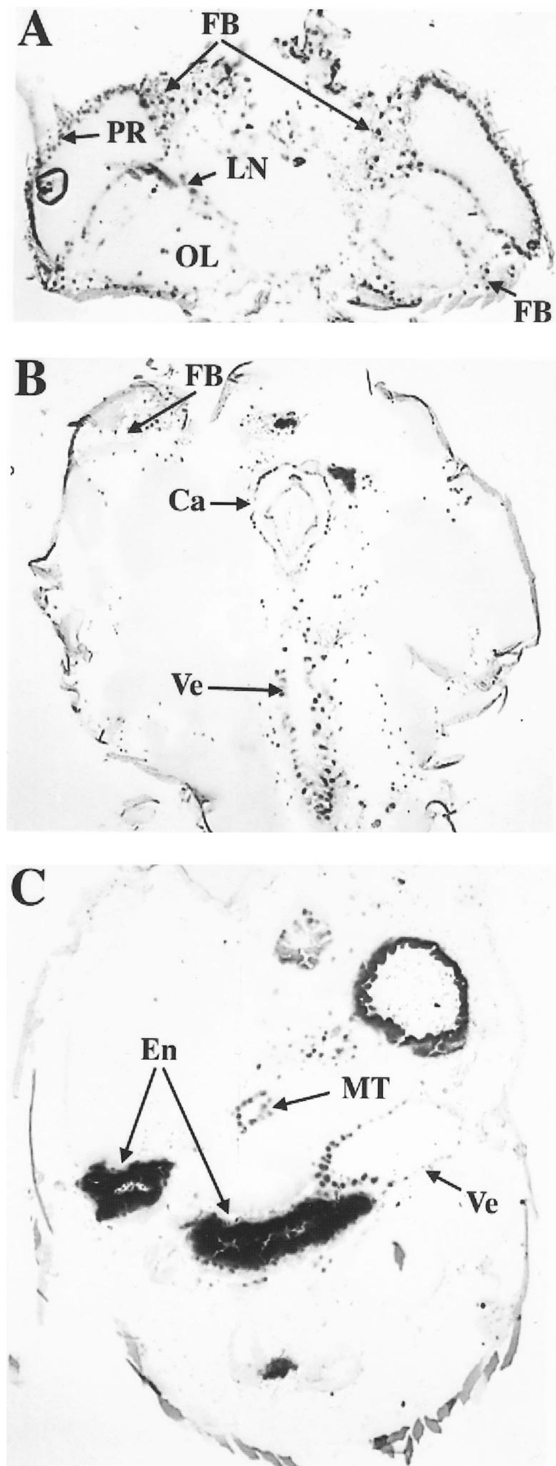

Figure 2. Spatial expression of the CRS/P/*lacZ* transgene in adults. *A*, X-gal–stained head of a male transgenic CRS/P/*lacZ* fly. *B*, X-gal–stained thorax of a male transgenic CRS/P/*lacZ* fly. *C*, X-gal–stained abdomen of a male transgenic CRS/P/*lacZ* fly. *Ca*, Cardia; *En*, endogenous ventricular staining; *FB*, fat bodies; *LN*, lateral neurons; *MT*, Malpighian tubules; *OL*, optic lobe glia; *PR*, photoreceptor cells; and *Ve*, ventriculus.

The CRS regulates correct *per* mRNA and protein expression in adults

Because the CRS is capable of mediating *per*-like circadian, spatial, and developmental expression, we hypothesized that *per*

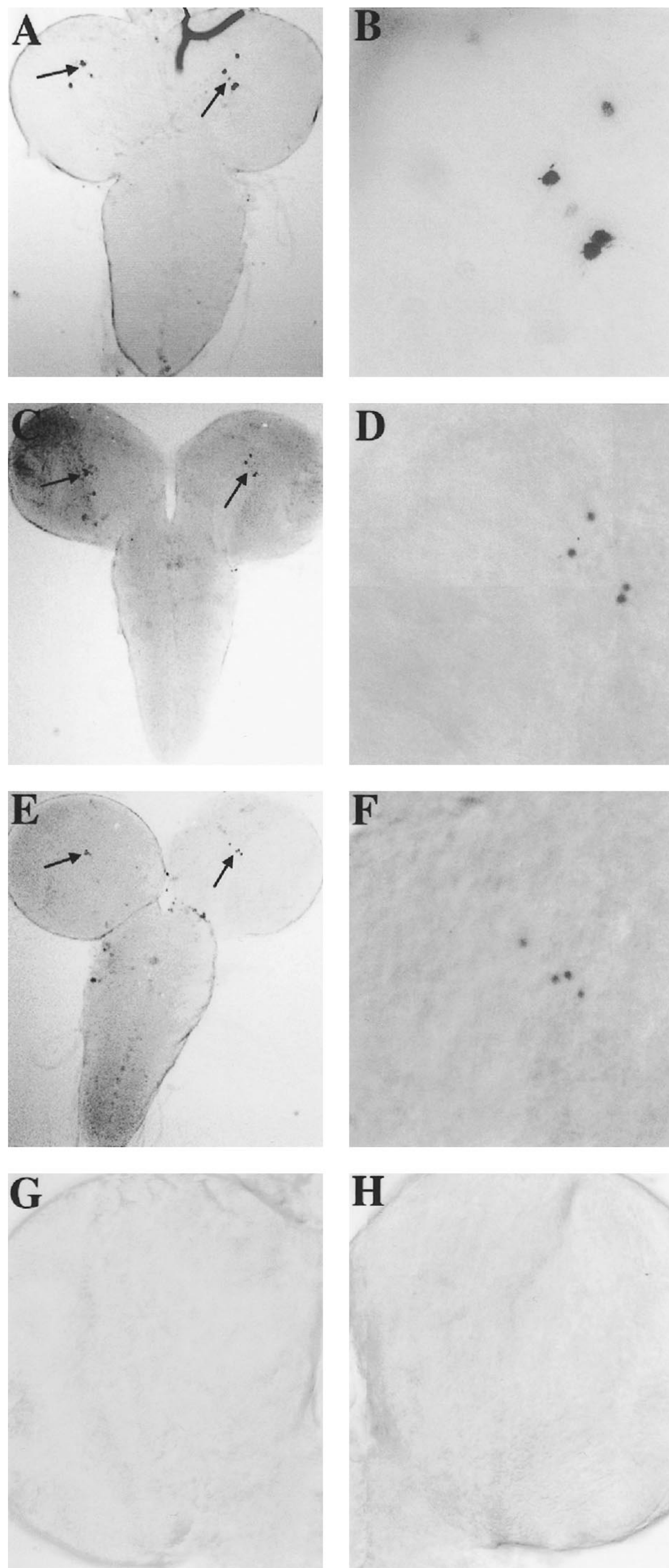

Figure 3. Spatial expression of the CRS/P/*lacZ* transgene in L3 larvae. *A–F*, Anterior on *top*. *A*, CNS of a BG6 third instar larva dissected at ZT1. In this focal plane, staining is restricted to a cluster of four or five LNs (*arrows*) in each hemisphere. *B*, Higher magnification of LN staining from the right hemisphere in *A*. *C*, CNS of a CRS/P/*lacZ*-3 third instar larva dissected at ZT1. Staining is restricted to a cluster of four or five LNs (*arrows*) in each hemisphere. *D*, Higher magnification of LN staining from the right hemisphere in *C*. *E*, CNS of a CRS/P/*lacZ*-8 third instar larva dissected at ZT1. Staining is restricted to a cluster of four or five LNs (*arrows*) in each hemisphere. *F*, Higher magnification of LN staining from the right hemisphere in *E*. *G*, Left optic lobe of a representative BG6;*dClk*^{Jrk} third instar larva dissected at ZT1. *H*, Right optic lobe of a representative BG6;*Cyc* third instar larva dissected at ZT1.

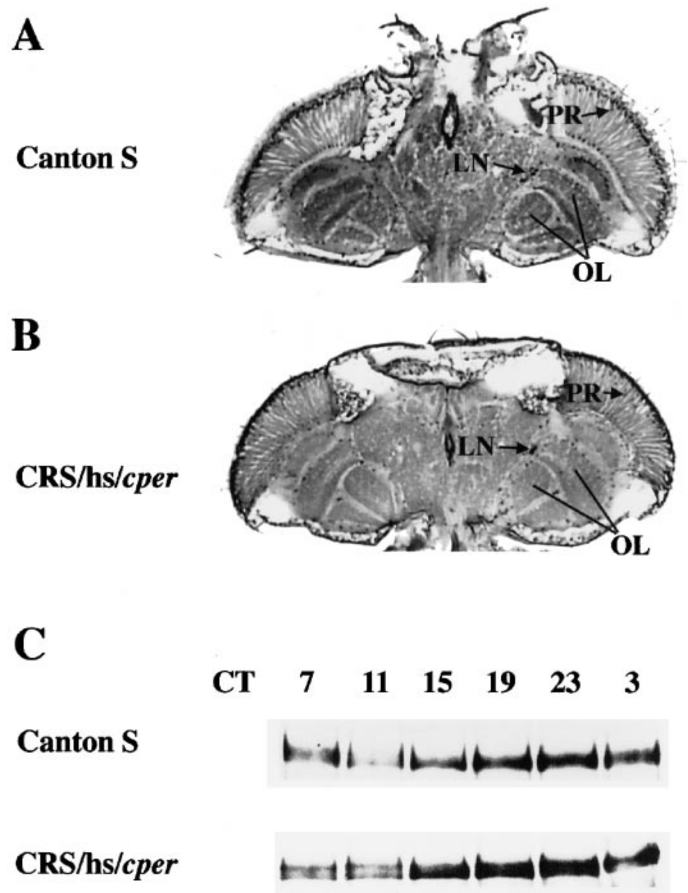

Figure 4. Spatial and circadian expression of PER in *per*^{01};CRS/hs/*cper* transformants. *A*, Spatial distribution of PER staining within the head of a wild-type (Canton-S) fly collected at ZT22. *B*, Spatial distribution of PER staining within the head of a *per*^{01};CRS/hs/*cper* transformant collected at ZT22. *C*, Western blot of PER abundance during constant darkness. Wild-type (Canton-S) and *per*^{01};CRS/hs/*cper* flies were collected every 4 hr during the first day in DD after 3 d of entrainment. The circadian time (*CT*) of each time point is noted *above* the *lane*. For abbreviations, see Figures 1 and 2.

expression driven by this regulatory element would rescue molecular and behavioral rhythms in *per*^{01} flies. To test this hypothesis, we inserted the CRS upstream of a *Drosophila* heat-shock protein 70 (*hsp70*) basal promoter/*per* cDNA (hs/*cper*) fusion gene that contains *per* coding sequences plus 2.1 kb of noncoding genomic sequences (Fig. 1). P-element–mediated germ-line transformation was used to generate four transgenic lines, which were then crossed into a *per*^{01} background.

The PER spatial expression pattern in transformant flies containing CRS/hs/*cper* resembles that of wild-type flies; CRS/hs/*cper* flies collected at circadian time 22 (CT22) and sectioned and stained with anti-PER antibody show PER in photoreceptors, brain glia, and LNs (Fig. 4*A*,*B*). The abundance of head-specific PER generated from the transgene also shows daily fluctuations during the first day of DD by Western blot analysis (Fig. 4*C*). The

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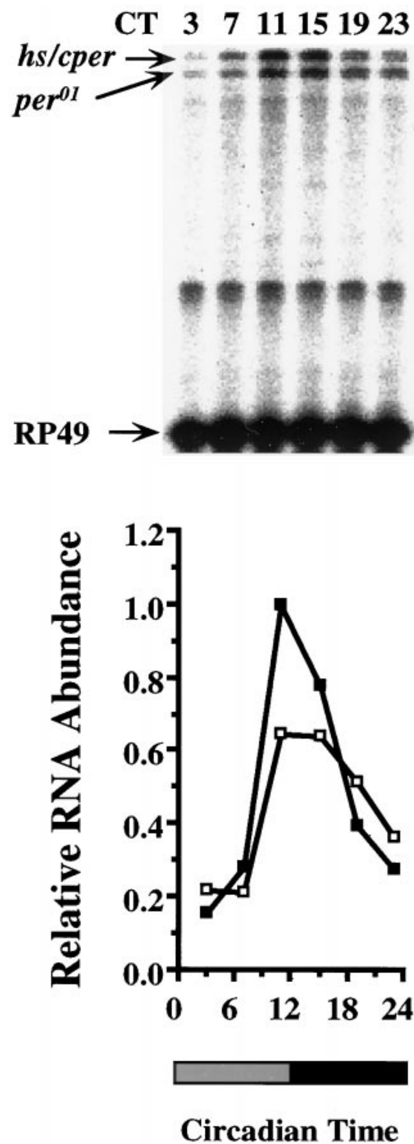

Figure 5. CRS/hs/*cper* drives mRNA cycling in DD. *Top*, RNase protection gels of per^{01};CRS/hs/*cper* transformants taken at CT 3, 7, 11, 15, 19, and 23. *Bottom*, The quantitation of the protection gel. Data are normalized to the peak in *hs/cper* mRNA abundance, which is set to 1.0. The *hs/cper* RNA is shown with *filled squares*, and the endogenous per^{01} RNA is shown with *open squares*. The *shaded* and *filled boxes below* the *x*-axis represent subjective day or night under DD conditions, respectively. This experiment was repeated five times with similar results. Ribosomal protein 49 (*RP49*), Antisense ribosomal protein 49 RNA probe. For other abbreviations, see Figures 1 and 4.

levels of PER in these transformants peak between CT19 and CT23 and fall to their lowest levels between CT7 and CT11. This cycling appears to phase lead PER cycling in wild-type flies, which peaks at CT23 and is least abundant at CT11, consistent with previous observations (Edery et al., 1994b; Zeng et al., 1996).

CRS/hs/*cper*-derived transcripts from heads cycle with an approximately fivefold amplitude during the first day of DD (Fig. 5). Consistent with previous observations, circadian cycling of endogenous per^{01} transcripts is also rescued (Hardin et al., 1990). The peak level for both the endogenous and transformant-derived transcripts is between CT11 and CT15, consistent with that of wild-type transcripts (Hardin et al., 1990). The overall level of CRS/hs/*cper*-derived transcripts is more than twofold higher than that of rescued per^{01} transcripts at their peak levels, which may be attributable to the relatively strong *hsp70* basal promoter (Hao et al., 1997). Thus, the histochemical staining, Western blot, and RNase protection results show that the CRS is capable of regulating the transcriptional aspects of *per* expression needed for feedback loop function in the appropriate cells of the head.

CRS-driven PER expression rescues locomotor activity rhythms

The ability of the CRS/hs/*cper* construct to rescue molecular feedback loop function suggests that it would also rescue behavioral rhythms. Indeed, the CRS/hs/*cper* transgene rescues rhythmic locomotor activity in 50–93% (average, ~77.8%) of the per^{01} flies tested with periods of ~22.5 hr (Table 1). The penetrance of CRS/hs/*cper* flies is strong compared with that of previous transformant flies containing *per* genomic sequences (penetrance ranging from 25 to 100%), and they have shorter periods than other *per* transformant types (periods range from ~23 to ~37 hr) (Bargiello et al., 1984; Hamblen et al., 1986; Baylies et al., 1987, 1992; Citri et al., 1987; Liu et al., 1991). Rhythms in CRS/hs/*cper* flies have an average power of 40 ± 3.5 (Table 1), similar to the 48.5 ± 1.8 value for per^{01} flies transformed with a 13.2 kb *per* genomic DNA fragment containing ~4 kb of upstream sequence, the entire *per* transcribed sequence, and ~2 kb of downstream sequence (Cheng et al., 1998).

Because the CRS/hs/*cper* flies have ~1.5 hr shorter periods than wild-type flies, we postulated that this difference was attributable to the relatively strong *hsp70* basal promoter. Because the P-element basal promoter produces approximately fivefold lower levels of transcript than the *hsp70* basal promoter (Hao et al., 1997), we expected that if the CRS were driving this promoter, the period would be closer to that of wild type. Such a result would agree with previous observations that lower *per* mRNA titers correlate with longer periods (Baylies et al., 1987). Indeed, the CRS/P/*cper* construct rescued locomotor activity rhythms in per^{01} flies (Table 1). Overall, 86% of the CRS/P/*cper* flies were rhythmic with a period of 24.5 ± 0.2 hr and an average power of 54.3 ± 4.0. The penetrance and power values are in line with that of other transformants that mediate *per* behavioral rescue (Bargiello et al., 1984; Hamblen et al., 1986; Baylies et al., 1987, 1992; Citri et al., 1987; Liu et al., 1991), and the period is in line with our expectation given the weakness of the P-element promoter.

To insure that behavioral rescue is CRS dependent, we tested the ability of an hs/*cper* gene lacking any *per* upstream regulatory sequences to rescue locomotor activity rhythms in per^{01} flies. All transgenic lines were arrhythmic (data not shown), showing that behavioral rescue is CRS dependent and that neither the *per* coding region nor the 2.1 kb of downstream sequences contain regulatory elements capable of driving expression in the LNs. These results show that the *per* CRS is sufficient for strong, high penetrance rescue of behavioral rhythms with ~24 hr circadian periods and that the period shortens with a stronger basal promoter.

DISCUSSION

In *Drosophila*, a rapidly expanding list of genes is required for circadian feedback loop function including *per* (Hardin et al., 1990), *tim* (Sehgal et al., 1994), *dClock* (Allada et al., 1998; Darlington et al., 1998), *Cycle* (Darlington et al., 1998; Rutila et

Table 1. Activity rhythms of per^{01}; CRS/hs/*cper* and per^{01}; CRS/P/*cper* flies

Genotype	Line	Number tested	Percent rhythmic (%)	Average period (hr ± SEM)	Average power (±SEM)
CRS/hs/*cper*	4	20	90	23.1 ± 0.1	53.3 ± 7.5
	5	16	81.8	22.7 ± 0.2	40.2 ± 7.6
	10	16	93.8	22.3 ± 0.3	29.8 ± 3.8
	19	20	50	22.5 ± 0.2	30.9 ± 4.9
	pooled	72	77.8	22.7 ± 0.1	40.0 ± 3.5
CRS/P/*cper*	1a	10	60	23.9 ± 0.3	38.9 ± 3.4
	2a	10	100	23.3 ± 0.3	54.5 ± 6.9
	3	10	80	26.0 ± 0.2	65.3 ± 14.2
	1	10	90	25.4 ± 0.1	66.6 ± 9.1
	2	10	100	24.2 ± 0.1	43.4 ± 5.1
	pooled	50	86	24.5 ± 0.2	54.3 ± 4.0
Canton-S		18	94.7	24.2 ± 0.1	78.2 ± 4.2

Young male Canton-S, per^{01}; CRS/hs/*cper* and per^{01}; CRS/P/*cper* flies were entrained in 12 hr light/dark cycles for 3/d, and 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 (Cheng et al., 1998).

al., 1998), and *double-time* (Kloss et al., 1998; Price et al., 1998). These genes act at the transcriptional or post-transcriptional levels to regulate circadian feedback loop function (Hardin et al., 1990, 1992; Vosshall et al., 1994; Price et al., 1995, 1998; So and Rosbash, 1997; Allada et al., 1998; Cheng et al., 1998; Darlington et al., 1998; Kloss et al., 1998; Rutila et al., 1998). Two of these genes, *dClk* and *Cyc*, encode proteins that activate *per* and *tim* transcription via E-boxes located in their respective upstream sequences (Darlington et al., 1998; Gekakis et al., 1998). One of the E-boxes targeted by dCLK and CYC is located within the 69 bp CRS from *per*, which is required for rhythmic transcription (Hao et al., 1997).

In this study, we show that the *per* CRS also mediates *per*-like spatial and developmental expression and that CRS-dependent *per* expression rescues feedback loop function and behavioral rhythms. The fact that the CRS contains all of the regulatory information required for *per*-like developmental, spatial, and circadian expression suggests that dCLK and CYC might mediate all aspects of *per* expression. If true, we might expect *dClk* and/or *Cyc* to be expressed in the same cells as *per*, thereby restricting *per* activation to the proper cell types. In favor of this possibility is the observation that there appear to be no obvious pleiotropic effects from mutations in *dClk* or *Cyc* (Allada et al., 1998; Rutila et al., 1998), thereby tentatively limiting the function of dCLK and CYC to clock gene activation. If the dCLK and CYC proteins are responsible for *per* and *tim* spatial expression, then the minimal E-box within the CRS may well be the only regulatory sequence that is needed to drive correct spatial expression. Although the CRS is capable of mediating many, if not all, aspects of *per* expression, transgenes lacking the CRS can also rescue behavioral rhythms and drive expression in LNs (Ewer et al., 1990; Liu et al., 1991; Frisch et al., 1994), indicating that important *per* regulatory elements are not exclusive to the CRS.

There is evidence that PER- and/or TIM-dependent transcriptional repression also occurs via dCLK and CYC binding at the E-box (Darlington et al., 1998). This repression could occur directly via an interaction between PER and CYC and/or dCLK that disrupts activation (perhaps via PAS domains) or indirectly by PER and/or TIM activating a transcriptional repressor or another factor that acts to disrupt dCLK and/or CYC activation. If repression were caused by the direct disruption of dCLK and/or CYC activation by PER and/or TIM, then the minimal E-box needed for activation would also be sufficient for repression and therefore circadian cycling. There is precedent for such a small DNA binding target being sufficient for correct spatial and developmental activation; the SINGLEMINDED and TANGO bHLH-PAS proteins activate expression along the CNS midline in stage 10 *Drosophila* embryos using four repeats of the 18 bp midline enhancer (Wharton et al., 1994; Sonnenfeld et al., 1997; Darlington et al., 1998).

Several clusters of neurons express *per* in larvae, including putative precursors to the adult LNs (Kaneko et al., 1997). By the use of antibodies to PER and TIM, this larval expression was shown to be rhythmic, but with different phases depending on the neuronal cluster (Kaneko et al., 1997). The CRS/P/*lacZ* transgene is expressed normally in larval, pupal, and adult LNs (Figs. 2, 3) (data not shown). Because the CRS is a target for dCLK and CYC, perhaps these transcription factors also drive *per* expression in larvae. To determine whether this is the case, we tested homozygous BG6;$dClk^{Jrk}$ and BG6;*Cyc* larvae for β-galactosidase expression at the L3 stage and found no staining in the CNS (Fig. 3*G,H*). This result demonstrates that dCLK and CYC are required for *per* expression in the larval CNS. Because PER and TIM levels cycle in larval and pupal LNs (Kaneko et al., 1997) and *per* expression in larval LNs is dependent on *dClk* and *Cyc*, it is likely that the circadian feedback loop is operating in larval and pupal LNs as it does in adult LNs. If so, the feedback loop could convey circadian phase from larvae to adults, thereby accounting for larval time memory.

The period of locomotor activity rhythms is sensitive to the number of *per* gene copies in that half the dosage (one copy of this X-linked gene in females) results in 0.5–1 hr longer periods and two to five times the dosage (two to five *per* copies in males) results in 1–1.5 hr shorter periods (Smith and Konopka, 1982). Dosage-dependent alterations are also seen when the dosage of the *dClk* and *Cyc* genes is lowered, resulting in longer period rhythms (Allada et al., 1998; Rutila et al., 1998). Altering the dosage of *per* or its transcriptional activators presumably alters *per* mRNA titer because lower levels of *per* mRNA have been shown to correlate with longer period locomotor activity rhythms (Baylies et al., 1987). CRS-driven *per* expression results in rhythms that are close to 24 hr, indicating that this regulatory sequence

contains all the information necessary for feedback loop function in LNs. The period of this rhythm in CRS-driven *per* flies, however, is sensitive to the strength of the basal promoter, with longer periods from the weaker P-element transposase basal promoter and shorter periods from the stronger *hsp70* basal promoter. The *hsp70* basal promoter produces approximately fivefold more RNA than the P-element transposase basal promoter (Hao et al., 1997), which results in an ~2 hr difference in behavioral period. This magnitude of difference is consistent with the dosage studies mentioned above and shows that factors other than the number of *per* gene copies or the levels of *per* gene activators affect the period of behavioral rhythms.

In this study we show that a single copy of the CRS is sufficient to drive *per* expression in its normal spatial pattern and to mediate robust behavioral rescue. The *per* coding and downstream-flanking sequences do not mediate behavioral rescue (Table 1), showing that the CRS is a necessary transcriptional element. Among the clock regulatory sequences identified (Anderson and Kay, 1995; Bell-Pederson et al., 1996; Liu et al., 1996; Hao et al., 1997), this is the first case in which a minimal circadian regulatory sequence has been shown to support both clock gene expression and phenotypic rescue. By dissecting the CRS further, we will determine whether the E-box is the key element involved in both circadian and spatial expression or whether other sequences are required for these functions. The discovery of an E-box upstream of *tim* that functions to activate expression suggests that other rhythmically transcribed feedback loop components and perhaps clock output genes will be regulated by the same mechanism in *Drosophila* (Darlington et al., 1998). This work may also provide insight into mammalian clock function because CLOCK and BMAL1 activate mPER1 expression via E-boxes upstream of its putative transcription start site (Gekakis et al., 1998).

REFERENCES

Allada R, White NE, So WV, Hall JC, Rosbash M (1998) A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell 93:791–804.

Anderson SL, Kay SA (1995) Functional dissection of circadian clock and phytochrome regulated transcription of the *Arabidopsis CAB2* gene. Proc Natl Acad Sci USA 92:1500–1504.

Bargiello TA, Jackson FR, Young MW (1984) Restoration of circadian behavioural rhythms by gene transfer in *Drosophila*. Nature 312:752–754.

Baylies MK, Bargiello TA, Jackson FR, Young MW (1987) Changes in abundance and structure of the *per* gene product can alter periodicity of the *Drosophila* clock. Nature 328:390–392.

Baylies MK, Vosshall LB, Sehgal A, Young MW (1992) New short period mutations of the *Drosophila* clock gene *per*. Neuron 9:575–581.

Bell-Pederson D, Dunlap JC, Loros JJ (1996) Distinct cis-acting elements mediate clock, light, and developmental regulation of the *Neurospora crassa eas* (ccg-2) gene. Mol Cell Biol 16:513–521.

Cheng Y, Gvakharia B, Hardin PE (1998) Two alternatively spliced transcripts from the *Drosophila period* gene rescue behavioral rhythms with different periods. Mol Cell Biol 18:6505–6514.

Citri Y, Colot HV, Jacquier AC, Yu Q, Hall JC, Baltimore D, Rosbash M (1987) A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene. Nature 326:42–47.

Curtin KD, Huang ZJ, Rosbash M (1995) Temporally regulated entry of the *Drosophila period* protein contributes to the circadian clock. Neuron 14:365–372.

Darlington TK, Wager-Smith K, Ceriani MF, Staknis D, Gekakis N, Steeves TDL, Weitz CJ, Takahashi JS, Kay SA (1998) Closing the circadian feedback loop: CLOCK induced transcription of its own inhibitors, *period* and *timeless*. Science 280:1599–1603.

Dembinska ME, Stanewsky R, Hall JC, Rosbash M (1997) Circadian cycling of a *period-lacZ* fusion protein in *Drosophila*: evidence for cyclical degradation. J Biol Rhythms 12:157–172.

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

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

Ewer J, Hamblen-Coyle M, Rosbash M, Hall JC (1990) Requirement for *period* gene expression in the adult and not during development for locomotor activity rhythms of imaginal *Drosophila melanogaster*. J Neurogenet 7:31–73.

Frisch B, Hardin PE, Hamblen-Coyle MJ, Rosbash MR, Hall JC (1994) A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the *Drosophila* nervous system. Neuron 12:555–570.

Gekakis N, Saez L, Delahaye-Brown A-M, Myers MP, Sehgal A, Young MW, Weitz CJ (1995) Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PER^{L}. Science 270:815–819.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. Science 280:1564–1569.

Hall JC (1995) Tripping along the trail to the molecular mechanisms of biological clocks. Trends Neurosci 18:230–240.

Hamblen M, Zehring WA, Kyriacou CP, Reddy P, Yu Q, Wheeler DA, Zwiebel LJ, Konopka RJ, Rosbash M, Hall JC (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.

Hao H, Allen DL, Hardin PE (1997) A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila*. Mol Cell Biol 17:3687–3693.

Hardin PE, Sehgal A (1998) Molecular components of a model circadian clock: lessons from *Drosophila*. In: Handbook of behavioral state control: molecular and physiological mechanisms (Lydic R, Baghdoyan H, eds), pp 61–74. Boca Raton, FL: CRC.

Hardin PE, Hall JC, Rosbash M (1990) Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature 342:536–540.

Hardin PE, Hall JC, Rosbash M (1992) Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. Proc Natl Acad Sci USA 89:11711–11715.

Hardin SH, Jones LB, Homayouni R, McCollum JC (1996) Octamer primed cycle sequencing: design of an optimized primer library. Genome Res 6:545–550.

Ikeda M, Nomura M (1997) cDNA cloning and tissue-specific expression of a novel basic- helix-loop-helix/PAS protein (BMAL1) and identification of alternatively spliced variants with alternative translation initiation site usage. Biochem Biophys Res Commun 233:258–264.

Kaneko M, Helfrich-Forster C, Hall JC (1997) Spatial and temporal expression of the *period* and *timeless* genes in the developing nervous system of *Drosophila*: newly identified pacemaker candidates and novel features of clock gene product cycling. J Neurosci 17:6745–6760.

King DP, Zhao Y, Sangoram AM, Wilsbacher LD, Tanaka M, Antoch MP, Steeves TDL, Vitaterna MH, Kornhauser JM, Lowrey PL, Turek FW, Takahashi JS (1997) Positional cloning of the mouse circadian *Clock* gene. Cell 89:641–653.

Kloss B, Price JL, Saez L, Balu J, Rothenfluh A, Wesley C, Young MW (1998) The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iϵ. Cell 94:97–107.

Liu X, Lorenz LJ, Yu Q, Hall JC, Rosbash M (1988) Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. Genes Dev 2:228–238.

Liu X, Yu Q, Huang Z, Zwiebel LJ, Hall JC, Rosbash M (1991) The strength and periodicity of *D. melanogaster* circadian rhythms are differentially affected by alterations in *period* gene expression. Neuron 6:753–766.

Liu Z, Taub CC, McClung CR (1996) Identification of an *Arabidopsis thaliana* ribulose-1,5-bisphosphate caboxylase/oxygenase activase (RCA) minimal promoter regulated by light and the circadian clock. Plant Physiol 112:43–51.

O'Kane C, Gehring WJ (1987) Detection *in situ* of genomic regulatory elements in *Drosophila*. Proc Natl Acad Sci USA 84:9123–9127.

Price JL, Dembinska ME, Young MW, Rosbash M (1995) Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*. EMBO J 14:4044–4049.

Price JL, Blau J, Rothenfluh A, Abodeely M, Kloss B, Young MW (1998) *double-time* is a new *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell 94:83–95.

Rosato E, Piccin A, Kyriacou CP (1997) Circadian rhythms: from behavior to molecules. Bioessays 19:1075–1082.

Rutila JE, Suri V, Le M, So WV, Rosbash M, Hall JC (1998) CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell 93:805–814.

Saez L, Young MW (1996) Regulation of nuclear entry of the *Drosophila* clock proteins *period* and *timeless*. Neuron 17:911–920.

Sehgal A, Price J, Young MW (1992) Ontogeny of a biological clock in *Drosophila melanogaster*. Proc Natl Acad Sci USA 89:1423–1427.

Sehgal A, Price JL, Man B, Young MW (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 MP, Young MW (1995) Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. Science 270:808–810.

Siwicki KK, Eastman C, Petersen G, Rosbash M, Hall JC (1988) Antibodies to the *period* gene product of *Drosophila* reveal diverse distribution and rhythmic changes in the visual system. Neuron 1:141–150.

Smith RF, Konopka RJ (1982) Effects of dosage alterations at the *per* locus on the period of the circadian clock of *Drosophila*. Mol Gen Genet 189:30–36.

Smith SA, Shepherd D (1996) Central afferent projections of proprioceptive sensory neurons in *Drosophila* revealed with the enhancer-trap technique. J Comp Neurol 364:311–323.

So VW, Rosbash M (1997) Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. EMBO J 16:7146–7155.

Sonnenfeld M, Ward M, Nystrom G, Mosher J, Stahl S, Crews S (1997) The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian Arnt and controls CNS midline and tracheal development. Development 124:4571–4582.

Stanewsky R, Frisch B, Brandes C, Hamblen-Coyle MJ, Rosbash M, Hall JC (1997) Temporal and spatial expression patterns of transgenes containing increasing amounts of the *Drosophila* clock gene *period* and a *lacZ* reporter: mapping elements of the PER protein involved in circadian cycling. J Neurosci 17:676–696.

Thummel CS, Pirotta V (1991) New pCaSpeR P-element vectors. Drosoph Inf Serv 71:150.

Vosshall LB, Price JL, Sehgal A, Saez L, Young MW (1994) Block in nuclear localization of *period* protein by a second clock mutation, *timeless*. Science 263:1606–1609.

Wharton KA, Crews ST (1993) CNS midline enhancers of the *Drosophila slit* and *Toll* genes. Mech Dev 40:141–154.

Wharton KA, Franks RG, Kasai Y, Crews ST (1994) Control of CNS midline transcription by asymmetric E-box-like elements: similarity to xenobiotic responsive regulation. Development 120:3563–3569.

Young MW, Jackson FR, Shin H-S, Bargiello TA (1985) A biological clock in *Drosophila*. Cold Spring Harb Symp Quant Biol 50:865–875.

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

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

Zerr DM, Hall JC, Rosbash M, Siwicki KK (1990) Circadian fluctuations of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. J Neurosci 10:2749–2762.