

# A Circadian Enhancer Mediates PER-Dependent mRNA Cycling in *Drosophila melanogaster*

HAIPING HAO,<sup>1</sup> DAVID L. ALLEN,<sup>2</sup> AND PAUL E. HARDIN<sup>2\*</sup>

*Department of Biology, Texas A & M University, College Station, Texas 77843-3258,<sup>1</sup> and  
Department of Biology, University of Houston, Texas 77204-5513<sup>2</sup>*

Received 21 January 1997/Returned for modification 27 February 1997/Accepted 1 April 1997

**Genes expressed under circadian-clock control are found in organisms ranging from prokaryotes to humans. In *Drosophila melanogaster*, the *period* (*per*) gene, which is required for clock function, is transcribed in a circadian manner. We have identified a circadian transcriptional enhancer within a 69-bp DNA fragment upstream of the *per* gene. This enhancer drives high-amplitude mRNA cycling under light-dark-cycling or constant-dark conditions, and this activity is *per* protein (PER) dependent. An E-box sequence within this 69-bp fragment is necessary for high-level expression, but not for rhythmic expression, indicating that PER mediates circadian transcription through other sequences in this fragment.**

A variety of biochemical, physiological, and behavioral processes are expressed as circadian rhythms in prokaryotic and eukaryotic organisms. These rhythms are controlled by an endogenous circadian clock and persist under constant environmental conditions. The circadian timing system is thought to be composed of three components: the pacemaker, which keeps time; an entrainment pathway, which synchronizes the pacemaker to environmental (light-dark) cycles; and output pathways, which activate rhythmic processes in a pacemaker-dependent fashion (11).

Pharmacological studies have shown that both transcription and translation are essential for circadian-clock function in many organisms (44). In *Aplysia*, circadian rhythms are reset by transcription inhibitors applied only at certain times of the circadian cycle, suggesting that one or more cycling mRNAs are involved in clock function (26, 42). Consistent with these pharmacological experiments, rhythmically expressed mRNAs have been discovered in a variety of organisms (51). Many rhythmically expressed transcripts derive from genes involved in controlling outputs of the pacemaker, such as the *Neurospora* CCGs and *Drosophila* DREGs (31, 54, 55); moreover, genes that are integrally involved in circadian pacemaker function, such as *frequency* (*frq*) in *Neurospora crassa* and *period* (*per*) and *timeless* (*tim*) in *Drosophila melanogaster*, also give rise to rhythmic transcripts (3, 21, 31, 48).

Circadian fluctuations in *per* mRNA are regulated through a transcriptional feedback loop that requires *per* protein (PER) and *tim* protein (TIM). As lights go off, the *per* gene is actively transcribed, and *per* mRNA accumulates to high levels during the first 2 to 6 h after lights-off (41). At this time, PER begins to accumulate in the cytoplasm (9), where it complexes with TIM and is stabilized (15, 28, 40, 59). PER is then translocated to the nucleus 5 to 8 h after lights-off (9, 56), where it inhibits its own transcription (58), probably indirectly, as it contains no known DNA binding motifs. This transcriptional inhibition is relieved when PER breaks down early in the light phase (10, 21, 22, 58, 60).

Studies of this feedback loop have focused on the posttranscriptional regulation (accumulation, modification, and nu-

clear translocation) of PER and TIM; however, relatively little is known about how PER (or a PER-TIM complex) feeds back to control transcription. To understand how the transcriptional side of this feedback loop is controlled, we have focused on identifying regulatory elements that drive circadian transcription.

## MATERIALS AND METHODS

**Construction of transformation plasmids.** Two different classes of *per-lacZ* fusion genes were constructed which contained *lacZ* coding sequences driven by *per* sequences appended to either the *Drosophila* heat shock protein 70 gene basal promoter or the *Drosophila* P-element transposase gene basal promoter. The *per* portion of fusion genes including the *hsp70* basal promoter was generated by PCR with a CG construct template (29). Primers that create an *EcoRI* site at the 5' end and a *BamHI* site at the 3' end were used to generate the following fragments: -1313 to -34, -603 to -449, -603 to -550, -563 to -494, -505 to -449, and -449 to -603 (numbering relative to the transcription start site [7]). These fragments were cloned into an *EcoRI-BamHI*-cut polylinker sequence of the transformation vector CaSpeR-HS43- $\beta$ -gal (53). The *per* portion of the fusion genes containing the P-element transposase basal promoter was generated by PCR of the CG template with primers that create an *EcoRI* site at the 5' end and a *BamHI* site at the 3' end for the following fragments: -1313 to -587, -603 to -34, -467 to -34, -341 to -34, -175 to -34, and -603 to -156. These fragments were cloned into an *EcoRI-BamHI*-cut polylinker sequence of the transformation plasmid CPLZ (57). The integrity of *per* sequences in all constructs was confirmed by DNA sequencing (23).

**Germ line transformation.** P-element-mediated transformation was carried out as described previously (49) with some modifications. Dechorionated embryos with the genotype *y, w; ry, Ki, P[ry<sup>+</sup>,  $\Delta$ 2-3] (99B)/+* were injected with plasmids at 300  $\mu$ g/ml in 5 mM KCl-0.1 mM PO<sub>4</sub> (pH 7.8). Transformant lines with inserts on the second and third chromosomes were balanced with *In(2LR)CyO* and *In(3LR)TM2*, respectively. Transformant lines with inserts on the X chromosome were balanced with *In(1)FM7*. At least three independent lines were generated for each transformation construct.

**RNase protection assays.** Flies used for time course analyses were entrained at 25°C in 12-h-light-12-h-dark (LD) cycles for at least 72 h prior to collection. For constant darkness (DD) experiments, flies were transferred to DD after they had been entrained in LD for 3 days. For each time point, heads were isolated, and RNA was extracted as described previously (21). The probes used in these studies were *per 5* (used to detect endogenous *per* mRNA) and  $\beta$ -gal (used to detect mRNA from the transgenes). The *per 5* probe contains RNA from +6383 to +7196 bp and protects a 182-nucleotide fragment of exon 5 when linearized with *NcoI*. The  $\beta$ -gal probe covers a 207-nucleotide portion of *lacZ* as described previously (61). As a control for the amount of RNA in each lane, an antisense ribosomal protein probe (RP49) was included in each RNase protection assay (21).

**Mutagenesis.** To generate E-box (CACGTG) deletions, *per* sequences from -603 to -449 bp were amplified by PCR and cloned into the *EcoRI* and *BamHI* sites of pBluescript and were then linearized with *PmlI*. The linearized plasmid DNA was treated with exonuclease BAL 31 at 30°C for 30 s. The digested plasmid was then treated with T4 DNA polymerase to make blunt ends, ligated, and used to transform *Escherichia coli*. Two clones that were analyzed had 11-bp deletions, which remove at least half of the E box (see Fig. 3A). The mutagenized

\* Corresponding author. Mailing address: Department of Biology, University of Houston, Houston, TX 77204. Phone: (713) 743-2652. Fax: (713) 743-2636. E-mail: phardin@uh.edu.

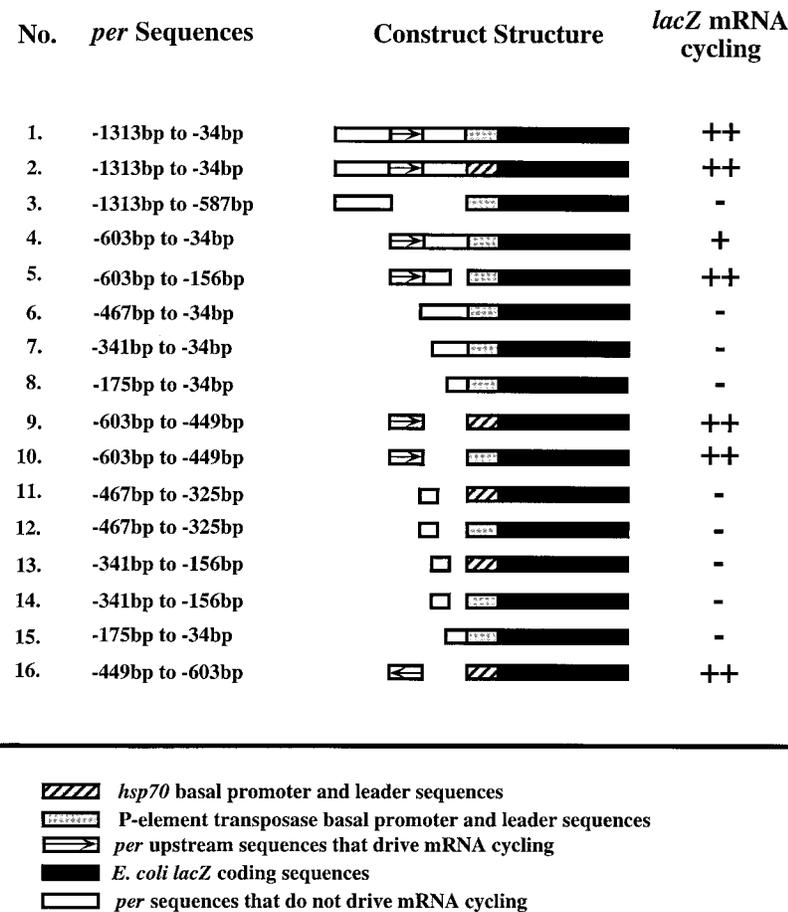


FIG. 1. Mapping of *per* upstream sequences that drive mRNA cycling. Sixteen plasmid constructs used for germ line transformation are shown. *per* sequences in each construct are labeled relative to the *per* transcription start site (+1). Bars represent CaSpeR-HS43- $\beta$ -gal- and CPLZ-based transformation constructs that contain *per* sequences, heterologous basal promoters, and *lacZ* protein-coding sequences as denoted in the figure. RNase protection assays were used to measure *lacZ* mRNA abundance. ++, high-amplitude (5- to 10-fold) cycling; +, low-amplitude (2- to 4-fold) cycling; -, low-level constitutive expression.

*per* sequences were then cloned into the transformation vector CaSpeR-HS43- $\beta$ -gal (53).

## RESULTS

***per* upstream sequences can drive mRNA cycling from heterologous promoters.** Previously, a *per* upstream fragment from -1313 bp to the transcription start site (+1) was shown to drive chloramphenicol acetyltransferase mRNA oscillations at an amplitude and phase similar to those of endogenous *per* mRNA in wild-type flies, though at a lower overall level (22). Such mRNA oscillations could be due to either the *per* basal promoter (i.e., the TATA sequence, transcription start site, and leader sequence) or separate upstream regulatory elements. To determine whether cyclic transcription is mediated by the promoter or by separate upstream regulatory elements, *per* upstream sequences lacking the natural basal promoter (-1313 to -34) were fused to either a *Drosophila hsp70* basal promoter-*lacZ* reporter gene (14) or a *Drosophila* P-element transposase gene basal promoter-*lacZ* reporter gene (37).

Wild-type flies transformed with these fusion genes exhibited *lacZ* RNA cycling which was similar in phase (within the limits of resolution for a 4-h time course) and amplitude to that of the endogenous *per* transcript (Fig. 1, constructs 1 and 2). The overall levels of *lacZ* transcript varied depending on the

promoter used; the *Drosophila hsp70* basal promoter produced ~5-fold more transcript than the *Drosophila* P-element transposase basal promoter (data not shown). These experiments show that a *per* upstream fragment sufficient for mRNA cycling is separable from the basal *per* promoter and that cyclic transcription can be mediated by heterologous promoters. In addition, these results imply that sequences capable of driving mRNA cycling can be identified by testing various 5' and 3' deletions of the *per* upstream region from -1313 to -34.

**A 154-bp clock control region acts in concert with other upstream sequences to mediate high-amplitude mRNA cycling.** To further define *per* regulatory elements that mediate cyclic mRNA expression, we made a series of constructs in which *lacZ* was driven by different portions of the -1313 to -34 *per* upstream fragment fused to either the P-element transposase or *hsp70* basal promoters (Fig. 1). Two or more transformant lines for each construct were tested for *lacZ* RNA cycling. Cycling of mRNA from these transgenes could be classified into three categories: wild-type (five- to tenfold amplitude), low-amplitude (two- to fourfold amplitude), and low-level constitutive expression. The peak values of the constructs that drive mRNA cycling using the *hsp70* basal promoter or the P-element transposase basal promoter varied no more than twofold.

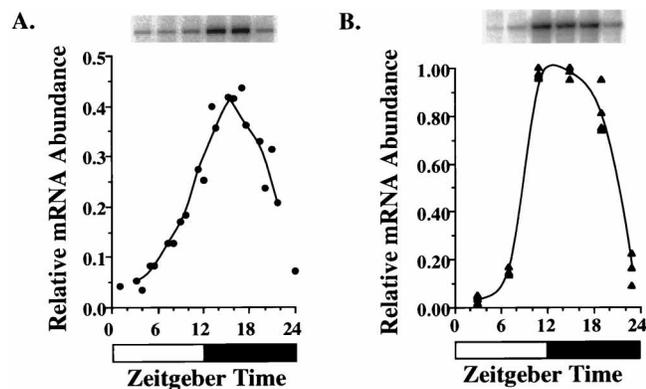


FIG. 2. An enhancer within *per* upstream sequences mediates mRNA cycling. (A) *per* sequences from  $-603$  to  $-449$  were used to drive a P-element transposase basal promoter-*lacZ* fusion gene in wild-type flies under LD conditions. The gel strip represents a typical RNase protection assay experiment from these transformants with samples taken at ZT 1, 5, 9, 13, 17, and 21. Quantitation of data from two experiments (shown as filled circles) was normalized to the peak in *per*<sup>+</sup> mRNA abundance measured in the same experiment, which was set at 1.0. The curve is derived from an average of these data. The white and black bars represent times when lights were on and off, respectively. (B) Inversely oriented *per* sequences from  $-603$  to  $-449$  were used to drive an *hsp70* basal promoter-*lacZ* fusion gene in wild-type flies under LD conditions. The gel strip represents a typical RNase protection assay experiment from these transformants with samples taken at ZT 1, 5, 9, 13, 17, and 21. Quantitation of data from four experiments (shown as filled triangles) was normalized to the peak in *lacZ* mRNA abundance, which was set at 1.0. The curve is derived from an average of the four experiments. The white and black bars are as described for panel A.

All transformants containing a *per* upstream fragment from  $-603$  to  $-449$  show *lacZ* mRNA cycling, while all transformants lacking this fragment exhibit low-level constitutive *lacZ* expression (Fig. 1). The amplitude of cycling depends on the sequences flanking this 154-bp fragment. Removing the first 710 bp of the  $-1313$  to  $-34$  fragment (Fig. 1, construct 4) reduces the cycling amplitude two- to threefold, suggesting that a positive element has been eliminated. In contrast, removal of the sequences between  $-34$  and  $-156$  increases the cycling amplitude (Fig. 1, compare constructs 4 and 5), indicating that a negative element resides within this fragment. Thus, this 154-bp *per* upstream fragment represents a clock

control region that mediates cyclic transcription of variable amplitudes, depending on flanking sequences.

**The *per* clock control region contains a PER-dependent enhancer that drives mRNA cycling.** Transformants carrying the  $-603$  to  $-449$  *per* upstream regulatory fragment express *lacZ* RNA that oscillates with an  $\sim 8$ -fold amplitude and a peak abundance at Zeitgeber time (i.e., time during environmental LD cycles, or ZT) 16 (Fig. 2A), similar to the amplitude and peak phase of the wild-type *per* transcript (21). Moreover, this fragment can activate heterologous basal promoters and can function at different distances from the transcription start site (Fig. 1), suggesting that it may act as a transcriptional enhancer. To further test this possibility, the  $-603$  to  $-449$  *per* upstream fragment was inverted and used to drive *lacZ* from the *hsp70* basal promoter (Fig. 1). The levels of *lacZ* mRNA in wild-type flies carrying this transgene cycled with an amplitude similar to and a phase slightly earlier than those of wild-type *per* mRNA (Fig. 1 and 2B). The orientation-independent function of this *per* clock control region, along with its ability to activate heterologous promoters and act at different distances, indicate that it acts as a transcriptional enhancer.

To determine if this enhancer functions in a PER-dependent manner, the  $-603$  to  $-449$ /*hs*/*lacZ* transgene was moved into a *per*<sup>01</sup> genetic background and tested for *lacZ* mRNA cycling. Under these conditions, *lacZ* cycling was abolished (Fig. 3A), indicating that the rhythmic transcription mediated by this fragment requires PER. Since PER cycling is not driven simply by the LD cycle (10, 60), these sequences should also support *lacZ* RNA cycling during constant DD. When transformants in which *per*  $-603$  to  $-449$  sequences were used to drive *lacZ* mRNA from the P-element promoter ( $-603$  to  $-449$ /P/*lacZ* transformants) were tested in DD, *lacZ* mRNA cycled (Fig. 3B) with a phase and amplitude similar to those previously observed for *per* mRNA in wild-type flies.

**An E-box-containing subfragment of the clock control region drives robust mRNA cycling.** Since a 154-bp *per* upstream fragment could mediate high-amplitude *lacZ* mRNA cycling, the sequence of this clock control region was determined and common transcription factor binding sites were searched for (16). One of the sites identified in this search was a consensus E box (Fig. 4A), which is a target site for basic helix-loop-helix (bHLH) transcription factors (35). This E box is notable be-

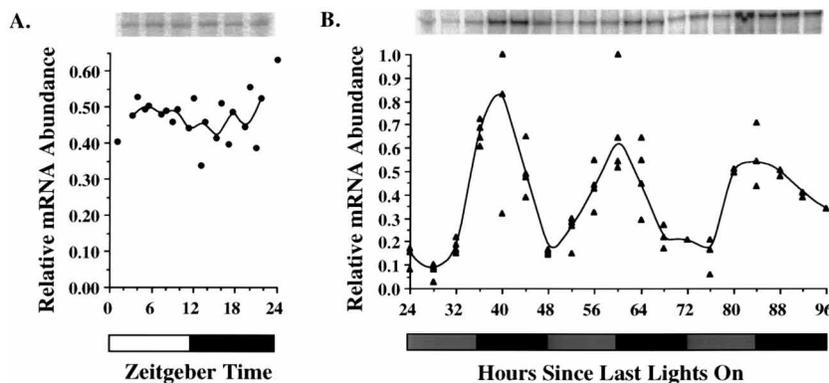


FIG. 3. Enhancer-mediated mRNA cycling is PER dependent. (A) *per* sequences from  $-603$  to  $-449$  were used to drive an *hsp70* basal promoter-*lacZ* fusion gene in *per*<sup>01</sup> flies under LD conditions. The gel strip, quantitation, and normalization of two independent time courses were as described for Fig. 2A. The white and black bars represent times when lights were on and off, respectively. (B) *per* sequences from  $-603$  to  $-449$  were used to drive an *hsp70* basal promoter-*lacZ* fusion gene in wild-type flies under DD conditions (see Materials and Methods). The gel strip shows *lacZ* RNase protection assay results at time points every 4 h for three days, starting at 24 h after the start of the last light phase. Quantitation of data from three experiments (shown as filled triangles) was normalized to the peak in *lacZ* mRNA abundance, which was set at 1.0. The curve is derived from an average of the three experiments. The shaded and black bars represent times when lights would have been on and off, respectively, if the LD cycle had been continued.



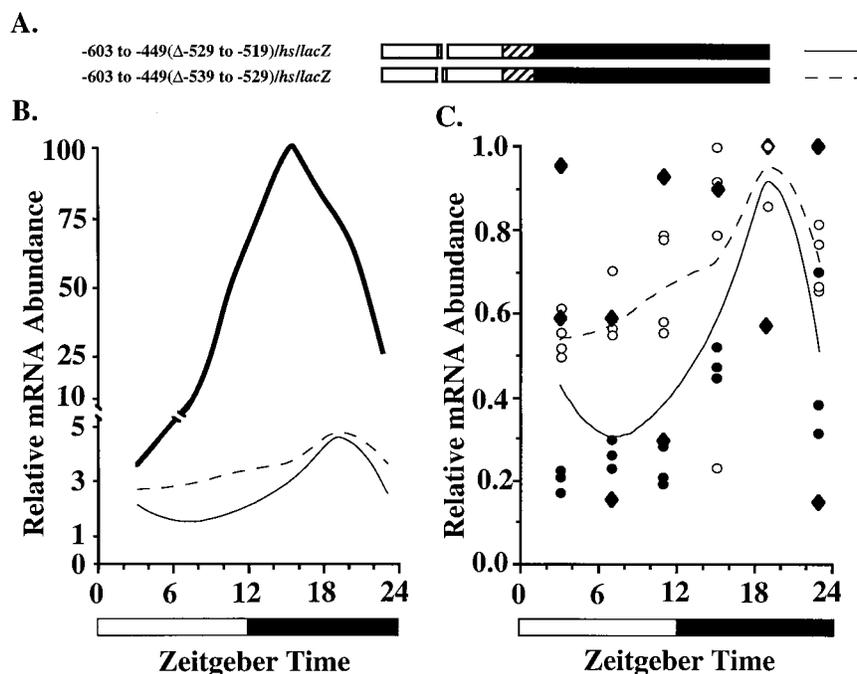


FIG. 5. An E box within the clock control region is required for high-level expression. (A) Constructs used to determine the effects of E-box deletion mutations on mRNA cycling. Symbols denoting the origin of sequences in the constructs and the numbering of *per* sequences are as designated in Fig. 4B. The deletion of sequences between  $-529$  and  $-519$  is designated  $\Delta(-529$  to  $-519)$ , and the deletion of sequences between  $-539$  and  $-529$  is designated  $\Delta(-539$  to  $-529)$ . Line symbols correspond to the *lacZ* mRNA curves for these constructs in panels B and C. (B) Relative *lacZ* mRNA abundances in flies transformed with the mutant constructs from panel A and the wild-type construct (no. 10) from Fig. 1. The line symbols represent *lacZ* mRNA levels from the transgenes shown in panel A. *lacZ* mRNA levels for the wild-type construct (shown as a solid, bold line) are replotted from Fig. 2A. Quantitation was based on at least four experiments for each construct and was normalized to the highest level (100) of *lacZ* mRNA. The ordinate is broken, indicating a change of scale. The white and black bars represent times when lights were on and off, respectively. (C) Expanded scale of the relative *lacZ* mRNA abundances from the E-box mutants. Data from four time courses of  $-603$  to  $-449$   $\Delta(-539$  to  $-529)$ /*hs/lacZ* transformants (open circles), two time courses of an arrhythmic  $-603$  to  $-449$   $\Delta(-529$  to  $-519)$ /*hs/lacZ* transformant line (filled diamonds), and time courses of three rhythmic  $-603$  to  $-449$   $\Delta(-529$  to  $-519)$ /*hs/lacZ* transformant lines (filled circles) are plotted. The average curves calculated for panel B for each construct are shown. The white and black bars are as described for panel B.

*per*, *tim*, and *freq*) as well as components of the pathways that activate rhythmic processes (e.g., output genes). As a first step towards unraveling the molecular mechanisms that govern circadian gene expression, we have identified a transcriptional enhancer that mediates PER-dependent circadian transcription. This enhancer lies within a 69-bp sequence upstream of the *per* gene and is the first circadian transcriptional regulatory sequence in an animal system to be characterized.

In plants and fungi, the expression of several genes is under circadian-clock control (18, 25, 27, 33, 38, 39, 43). Clock regulatory sequences have been localized to a 36-bp DNA fragment upstream of the chlorophyll *a/b* binding protein 2 (CAB2) gene from *Arabidopsis* (1, 2, 6) and a 45-bp activating clock element upstream of the *eas* (*cgg-2*) gene from *Neurospora* (5). The regulation of these genes is similar in that both are morning specific (i.e., transcripts peak soon after actual or subjective dawn) and light inducible (2, 4, 27, 32, 33). Nevertheless, sequence comparisons between the CAB2 and *eas* clock regulatory sequences have revealed no common elements (5). Likewise, no conserved sequence elements were found upon comparison of the *per* clock control region with those from CAB2 or *eas* (data not shown).

In vertebrates, different aspects of circadian transcription are under circadian clock control. Light-induced expression of the *c-fos* gene in the suprachiasmatic nucleus is phase dependent, occurring only during the subjective night (reviewed in reference 46). This clock-dependent inducibility is thought to be mediated by  $Ca^{2+}$ -cyclic AMP (cAMP) response elements in the *c-fos* promoter, which are bound by phosphorylated

cAMP response element binding (CREB) proteins (17). Since light-induced phosphorylation of CREB occurs only during the subjective night, the clock apparently exerts its effect on *c-fos* expression through the phosphorylated form of CREB. Phosphorylated CREB is also involved in regulating cAMP response element modulator gene expression in the pineal gland of rats. During darkness, a novel cAMP response element modular isoform, called induced early cAMP regulator (ICER), can be induced by phosphorylated CREB after adrenergic stimulation of the pineal from the suprachiasmatic nucleus (34, 50). ICER, a transcriptional repressor, then inhibits cAMP-induced expression of several cAMP response element-modulated genes, including its own gene (34). In addition to this clock-dependent gating of inducibility, ICER is rhythmically expressed; the abundance of ICER mRNA peaks during the subjective night (12, 50). Unlike the case of gated ICER inducibility, sequences that mediate circadian expression of ICER have not been characterized.

The *per* gene is rhythmically transcribed, giving rise to mRNA oscillations in which 5- to 10-fold more mRNA is present around ZT 15 than around ZT 1 (21). This cycling was faithfully reproduced by a *per* upstream fragment from  $-1313$  to  $+1$  (22), suggesting that most or all elements of the cycling regulatory apparatus are contained within this fragment. We have now shown that within the first  $\sim 1,300$ -bp of *per* upstream sequence, a 69-bp fragment from  $-563$  to  $-494$  can drive high amplitude mRNA cycling (Fig. 4). Sequences outside this 69-bp portion of the  $\sim 1,300$ -bp *per* upstream fragment can either positively or negatively affect the amplitude of cycling

two- to threefold, but do not themselves appear to be capable of mediating mRNA cycling. Although we cannot exclude the possibility that other sequences outside this ~1,300-bp *per* upstream fragment (i.e., further upstream, within the *per*-transcribed region, or downstream) drive mRNA cycling, the cycling amplitude, phase (within the limits of resolution for a 4-h time course), and overall level of wild-type *per* transcript can be accounted for quite well by the activity of this 69-bp fragment. Since *per* constructs that lack upstream sequences have been found to mediate some degree of cycling (13), we searched for internal and downstream sequences similar to the 69-bp fragment. We found no extended regions of similarity, though we did find three CACGTG E boxes within intron 1, one within exon 4, and one within exon 5 (19a).

The cycling of endogenous *per* mRNA is not simply dependent on environmental cycles of light and dark but is regulated via feedback from PER and TIM (22, 48). Likewise, the 69-bp *per* upstream sequence (within the context of the 154-bp clock control region) requires PER to mediate mRNA cycling; *lacZ* mRNA cycling is abolished when transgenes containing this 69-bp sequence are crossed into *per<sup>01</sup>* flies, whereas *lacZ* mRNA cycling persists during DD when transgenes containing this 69-bp sequence are maintained in wild-type flies (Fig. 3). The clock-regulated elements within this 69-bp sequence are also active in a variety of contexts with respect to the transcription start site (i.e., at various distances, with differing basal promoters and in an inverse orientation) (Fig. 1 to 3). These qualities indicate that clock-regulated elements within this 69-bp sequence act as PER-dependent enhancers that drive rhythmic *per* transcription. From these studies, we have identified a clock-regulated enhancer that accurately reproduces essentially all aspects of *per* mRNA cycling in a PER-dependent fashion.

PER has been proposed to mediate mRNA cycling through transcriptional repression (58). Since PER is nuclear throughout its entire spatial expression pattern (except in the ovaries) in *Drosophila* (30), its role as a repressor was thought to be rather direct (58). Although direct interaction between PER and DNA is unlikely due to the lack of known DNA binding domains in PER (52), the presence of a PAS protein dimerization domain raises the possibility that PER could affect transcription by dimerization with a DNA binding protein (19). This possibility is strengthened by the fact that virtually all other PAS domain-containing proteins are bHLH transcription factors (52). Since bHLH transcription factors specifically bind E-box sequences, the presence of an E box in the 69-bp fragment that mediates PER-dependent mRNA cycling suggested a possible mechanism through which PER could regulate mRNA cycling; PER could disrupt bHLH-PAS activators or activate bHLH repressors that bind the E box. Since PER can stimulate disruption or activation only when it is localized to the nucleus, its rhythm in nuclear localization and breakdown would result in rhythmic transcription. Deletions that disrupt this upstream *per* E box have been tested, and although *lacZ* mRNA levels drop dramatically (due to either a general reduction in each cell or a more restricted spatial pattern), residual *lacZ* mRNA continues to cycle in abundance (Fig. 5). This result argues against (but does not eliminate) the possibility of bHLH protein involvement in cyclic regulation (through its role in enhancing *per* transcription may affect the phase of the rhythm) and shifts the focus to factors that bind other sequences within this 69-bp fragment.

The role of PER in regulating cyclic transcription is a mystery. Although its translocation to the nucleus in *Drosophila* is required for mRNA cycling (20, 47, 56), recent results from the silk moth *Antheraea pernyi* show that PER nuclear localization

is not essential for *per* mRNA cycling (45). In addition, whether silk moth PER remains cytoplasmic (as in brain neurons) or becomes localized to the nucleus (as in the photoreceptors), *per* mRNA cycling is associated with an antisense *per* transcript cycling in the opposite phase (45). Whether this antisense transcript is involved in mediating silk moth *per* mRNA cycling is not known, but if PER is part of the clock in silk moth adults, it could also be part of an (indirect) autoregulatory feedback loop.

A number of mRNAs that undergo circadian oscillations in abundance have recently been identified in *Drosophila*. One of these mRNAs is encoded by the *tim* gene, which along with *per*, is a component of the circadian feedback loop required for clock function in *Drosophila* (15, 36, 48). The phase and amplitude of *tim* mRNA cycling are virtually identical to those of *per* mRNA, and *tim* mRNA cycling is dependent on both TIM and PER function (48). A screen of *Drosophila* head cDNAs identified several oscillating mRNAs (54). Some of these transcripts oscillated out of phase with *per*, though the contribution of *per* to the cycling of these transcripts varied and environmental factors such as food availability, caging conditions, and light-dark cycling played a large role in driving these rhythms (54). Most transcripts from this screen oscillated in phase with *per* mRNA (54), and one transcript that was characterized in detail cycles in a PER-dependent manner (55). It may be that the sequences responsible for cyclic *per* gene expression are also involved in driving the cyclic expression of these other genes. Identification of clock control sequences should allow us to identify the factors that confer cyclic expression, which would enable us to determine how common this regulatory mechanism is in *Drosophila* and perhaps in other organisms.

#### ACKNOWLEDGMENTS

We thank S. Hardin, G. Cahill, A. Eskin, C. Weitz, B. Morrish, and Y. Cheng for discussion and comments on the manuscript. This work was supported by NIH grant R29-NS31214.

#### REFERENCES

- Anderson, S. L., and S. A. Kay. 1995. Functional dissection of circadian clock- and phytochrome-regulated transcription of the *Arabidopsis* CAB2 gene. *Proc. Natl. Acad. Sci. USA* **92**:1500–1504.
- Anderson, S. L., G. R. Teakle, S. J. Martino-Catt, and S. A. Kay. 1994. Circadian clock- and phytochrome-regulated transcription is conferred by a 78-bp cis-acting domain of the *Arabidopsis* CAB2 promoter. *Plant J.* **6**:457–470.
- Aronson, B. D., K. A. Johnson, J. J. Loros, and J. C. Dunlap. 1994. Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. *Science* **263**:1578–1584.
- Arpaia, G., J. J. Loros, J. C. Dunlap, G. Morelli, and G. Macino. 1993. The interplay of light and the circadian clock: independent dual regulation of clock-controlled gene *cgg-2 (eas)*. *Plant Physiol.* **102**:1299–1305.
- Bell-Pedersen, D., J. C. Dunlap, and J. J. Loros. 1996. Distinct cis-acting elements mediate clock, light, and developmental regulation of the *Neurospora crassa eas (cgg-2)* gene. *Mol. Cell. Biol.* **16**:513–521.
- Carre, I. A., and S. A. Kay. 1995. Multiple DNA-protein complexes at a circadian-regulated promoter element. *Plant Cell* **7**:2039–2051.
- Citri, Y., H. V. Colot, A. C. Jacquier, Q. Yu, J. C. Hall, D. Baltimore, and M. Rosbash. 1987. A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene. *Nature* **326**:42–47.
- Crews, S. T., J. B. Thomas, and C. S. Goodman. 1988. The *Drosophila single-minded* gene encodes a nuclear protein with sequence similarity to the *per* gene product. *Cell* **52**:143–151.
- Curtin, K. D., Z. J. Huang, and M. Rosbash. 1995. Temporally regulated entry of the *Drosophila period* protein contributes to the circadian clock. *Neuron* **14**:365–372.
- Ederly, I., L. J. Zwiebel, M. E. Dembinska, and M. Rosbash. 1994. Temporal phosphorylation of the *Drosophila period* protein. *Proc. Natl. Acad. Sci. USA* **91**:2260–2264.
- Eskin, A. 1979. Identification and physiology of circadian pacemakers. *Fed. Proc.* **38**:2570–2572.
- Foulkes, N. S., G. Duval, and P. Sassone-Corsi. 1996. Adaptive inducibility of CREM as transcriptional memory of circadian rhythms. *Nature* **381**:83–85.

13. Frisch, B., P. E. Hardin, M. J. Hamblen-Coyle, M. R. Rosbash, and J. C. Hall. 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.
14. Garabedian, M. J., B. M. Shepherd, and P. C. Wensink. 1986. A tissue-specific transcription enhancer from the *Drosophila* yolk protein 1 gene. *Cell* **45**:859–867.
15. Gekakis, N., L. Saez, A.-M. Delahaye-Brown, M. P. Myers, A. Sehgal, M. W. Young, and C. J. Weitz. 1995. Isolation of *timeless* by PER protein interaction: defective interaction between *timeless* protein and long-period mutant PER<sup>L</sup>. *Science* **270**:815–819.
16. Ghosh, D. 1992. TFD: the transcription factors database. *Nucleic Acids Res.* **20**:2091–2093.
17. Ginty, D. D., J. M. Kornhauser, M. A. Thompson, H. Bading, K. E. Mayo, J. S. Takahashi, and M. E. Greenberg. 1993. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science* **260**:238–241.
18. Giuliano, G., N. E. Hoffman, K. Ko, P. A. Scolnik, and A. R. Cashmore. 1988. A light-entrained circadian clock controls transcription of several plant genes. *EMBO J.* **7**:3635–3642.
19. Hall, J. C. 1995. Tripping along the trail to the molecular mechanisms of biological clocks. *Trends Neurosci.* **18**:230–240.
- 19a. Hao, H. Unpublished data.
20. Hardin, P. E. 1994. Analysis of *period* mRNA cycling in *Drosophila* head and body tissues suggests that body oscillators are subservient to head oscillators. *Mol. Cell. Biol.* **14**:7211–7218.
21. Hardin, P. E., J. C. Hall, and M. Rosbash. 1990. Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. *Nature* **342**:536–540.
22. Hardin, P. E., J. C. Hall, and M. Rosbash. 1992. Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. *Proc. Natl. Acad. Sci. USA* **89**:11711–11715.
23. Hardin, S. H., L. B. Jones, R. Homayouni, and J. C. McCollum. 1996. Octamer primed cycle sequencing: design of an optimized primer library. *Genome Res.* **6**:545–550.
24. Huang, Z. J., I. Edery, and M. Rosbash. 1993. PAS is a dimerization domain common to *Drosophila* Period and several transcription factors. *Nature* **364**:259–262.
25. Kay, S. A., and A. J. Millar. 1993. Circadian-regulated cab gene transcription in higher plants, p. 73–89. In M. W. Young (ed.), *Molecular genetics of biological rhythms*. Marcel Dekker, New York, N.Y.
26. Koumenis, C., Q. Tran, and A. Eskin. 1996. The use of a reversible transcription inhibitor, DRB, to investigate the involvement of specific proteins in the ocular circadian system of *Aplysia*. *J. Biol. Rhythms* **11**:45–56.
27. Lauter, F.-R., V. E. Russo, and C. Yanofsky. 1992. Developmental and light regulation of *eas*, the structural gene for the rodlet protein of *Neurospora*. *Genes Dev.* **6**:2373–2381.
28. Lee, C., V. Parikh, T. Itsukaichi, K. Bae, and I. Edery. 1996. Resetting the *Drosophila* clock by photic regulation of PER and PER-TIM complex. *Science* **271**:1740–1744.
29. Liu, X., Q. Yu, Z. Huang, L. J. Zwiebel, J. C. Hall, and M. Rosbash. 1991. The strength and periodicity of *D. melanogaster* circadian rhythms are differentially affected by alterations in *period* gene expression. *Neuron* **6**:753–766.
30. Liu, X., L. J. Zwiebel, D. Hinton, S. Benzer, J. C. Hall, and M. Rosbash. 1992. The *period* gene encodes a predominantly nuclear protein in adult *Drosophila*. *J. Neurosci.* **12**:2735–2744.
31. Loros, J. 1995. The molecular basis of the *Neurospora* clock. *Semin. Neurosci.* **7**:3–13.
32. Loros, J., and J. C. Dunlap. 1991. *Neurospora crassa* clock-controlled genes are regulated at the level of transcription. *Mol. Cell. Biol.* **11**:558–563.
33. Loros, J. J., S. A. Denome, and J. C. Dunlap. 1989. Molecular cloning of genes under the control of the circadian clock in *Neurospora*. *Science* **243**:385–388.
34. Molina, C. A., N. S. Foulkes, E. Lalli, and P. Sassone-Corsi. 1993. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell* **75**:875–886.
35. Murre, C., P. S. McCaw, and D. Baltimore. 1989. A new DNA binding and dimerization motif in immunoglobulin, enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. *Cell* **56**:777–783.
36. Myers, M. P., K. Wager-Smith, C. S. Wesley, M. W. Young, and A. Sehgal. 1995. Positional cloning and sequence analysis of the *Drosophila* clock gene, *timeless*. *Science* **270**:805–808.
37. O'Kane, C. J., and W. J. Gehring. 1987. Detection *in situ* of genomic regulatory elements in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **84**:9123–9127.
38. Piechula, B., and J. Grissem. 1987. Diurnal mRNA fluctuations of nuclear and plasmid genes in developing tomato fruits. *EMBO J.* **6**:3593–3599.
39. Pilgrim, M. L., T. Caspar, P. H. Quail, and C. R. McClung. 1993. Circadian- and light-regulated expression of nitrate reductase in *Arabidopsis*. *Plant Mol. Biol.* **23**:349–364.
40. Price, J. L., M. E. Dembinska, M. W. Young, and M. Rosbash. 1995. Suppression of PERIOD protein abundance and circadian cycling by the *Drosophila* clock mutation *timeless*. *EMBO J.* **14**:4044–4049.
41. Qiu, J., and P. E. Hardin. 1996. *per* mRNA cycling is locked to lights-off under photoperiodic conditions that support circadian feedback loop function. *Mol. Cell. Biol.* **16**:4182–4188.
42. Raju, U., C. Koumenis, M. Nunez-Reguero, and A. Eskin. 1991. Alteration of the phase period of a circadian oscillator by a reversible transcription inhibitor. *Science* **253**:673–675.
43. Redinbaugh, M., M. Sabre, and J. Scandalios. 1990. Expression of the maize Cat3 catalase gene is under the influence of a circadian rhythm. *Proc. Natl. Acad. Sci. USA* **87**:6853–6857.
44. Rosbash, M., and J. C. Hall. 1989. The molecular biology of circadian rhythms. *Neuron* **3**:387–398.
45. Saumann, I., and S. M. Reppert. 1996. Circadian clock neurons in the silkworm *Antheraea pernyi*: novel mechanisms of *period* protein regulation. *Neuron* **17**:889–900.
46. Schwartz, W. J., N. Aronin, J. Takeuchi, M. R. Bennett, and R. R. Peters. 1995. Towards a molecular biology of the suprachiasmatic nucleus: photic and temporal regulation of *c-fos* gene expression. *Semin. Neurosci.* **7**:53–60.
47. Sehgal, A., J. L. Price, B. Man, and M. W. Young. 1994. Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. *Science* **263**:1603–1606.
48. Sehgal, A., A. Rothenfluh-Hilfiker, M. Hunter-Ensor, Y. Chen, M. P. Myers, and M. W. Young. 1995. Rhythmic expression of *timeless*: a basis for promoting circadian cycles in *period* gene autoregulation. *Science* **270**:808–810.
49. Spradling, A. C. 1986. P element-mediated transformation, p. 175–196. In D. B. Roberts (ed.), *Drosophila: a practical approach*. IRL Press, Oxford, United Kingdom.
50. Stehle, J. H., N. S. Foulkes, C. A. Molina, V. Simmoneaux, P. Pevet, and P. Sassone-Corsi. 1993. Adrenergic signals direct rhythmic expression of transcriptional repressor CREM in the pineal gland. *Nature* **365**:314–320.
51. Takahashi, J. 1993. Circadian-clock regulation of gene expression. *Curr. Opin. Genet. Dev.* **3**:301–309.
52. Takahashi, J. S. 1992. Circadian clock genes are ticking. *Science* **258**:238–240.
53. Thummel, C. S., and V. Pirotta. 1991. New pCaSpeR P-element vectors. *Drosoph. Inf. Serv.* **71**:150.
54. Van Gelder, R., H. Bae, M. Palazzolo, and M. Krasnow. 1995. Extent and character of circadian gene expression in *Drosophila melanogaster*: identification of twenty oscillating mRNAs in the fly head. *Curr. Biol.* **5**:1424–1436.
55. Van Gelder, R. N., and M. A. Krasnow. 1996. A novel circadianly expressed *Drosophila melanogaster* gene dependent on the *period* gene for its rhythmic expression. *EMBO J.* **15**:1625–1631.
56. Voshall, L. B., J. L. Price, A. Sehgal, L. Saez, and M. W. Young. 1994. Block in nuclear localization of *period* protein by a second clock mutation, *timeless*. *Science* **263**:1606–1609.
57. Wharton, K. A., and S. T. Crews. 1993. CNS midline enhancers of the *Drosophila slit* and *Toll* genes. *Mech. Dev.* **40**:141–154.
58. Zeng, H., P. E. Hardin, and M. Rosbash. 1994. Constitutive overexpression of the *Drosophila period* protein inhibits period mRNA cycling. *EMBO J.* **13**:3590–3598.
59. Zeng, H., Z. Qian, M. P. Myers, and M. Rosbash. 1996. A light-entrainment mechanism for the *Drosophila* circadian clock. *Nature* **380**:129–135.
60. Zerr, D. M., J. C. Hall, M. Rosbash, and K. K. Siwicki. 1990. Circadian fluctuations of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. *J. Neurosci.* **10**:2749–2762.
61. Zwiebel, L. J., P. E. Hardin, X. Liu, J. C. Hall, and M. Rosbash. 1991. A post-transcriptional mechanism contributes to circadian cycling of a *per*-b-galactose fusion protein. *Proc. Natl. Acad. Sci. USA* **88**:3882–3886.