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

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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 nuclear 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 *Eco*RI site at the 5′ end and a *Bam*HI 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 *Eco*RI-*Bam*HI-cut polylinker sequence of the transformation vector CaSpeR-HS43-β-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 *Eco*RI site at the 5′ end and a *Bam*HI 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 *Eco*RI-*Bam*HI-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*$^{+}$, *Δ2-3]* (99B)/+ were injected with plasmids at 300 μg/ml in 5 mM KCl–0.1 mM PO_4 (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 β-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 *Nco*I. The β-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 *Eco*RI and *Bam*HI sites of pBluescript and were then linearized with *Pml*I. 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

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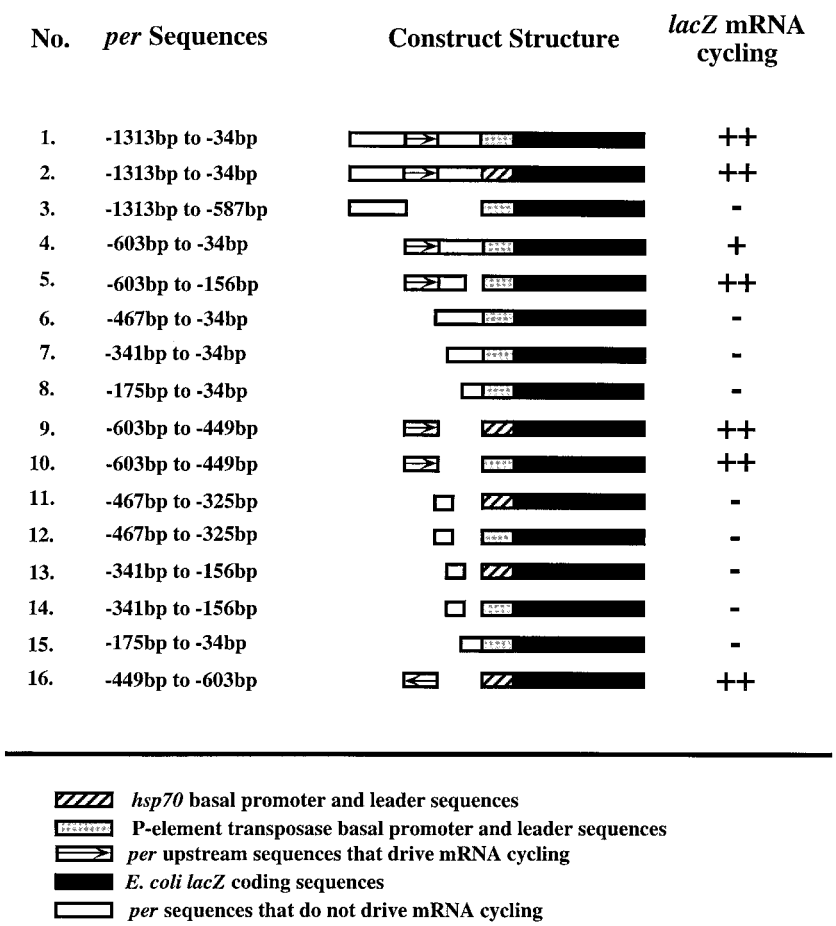

FIG. 1. Mapping of *per* upstream sequences that drive mRNA cycling. Sixteen plasmid constructs used for germ line transformation are shown. *per* sequences in each construct are labeled relative to the *per* transcription start site (+1). Bars represent CaSpeR-HS43-β-gal- and CPLZ-based transformation 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-β-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.

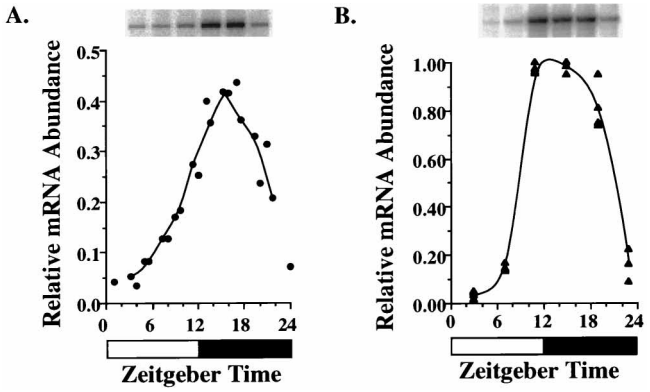

FIG. 2. An enhancer within *per* upstream sequences mediates mRNA cycling. (A) *per* sequences from −603 to −449 were used to drive a P-element transposase basal promoter-*lacZ* fusion gene in wild-type flies under LD conditions. The gel strip represents a typical RNase protection assay experiment from 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^{+} 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 ~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^{01} 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-

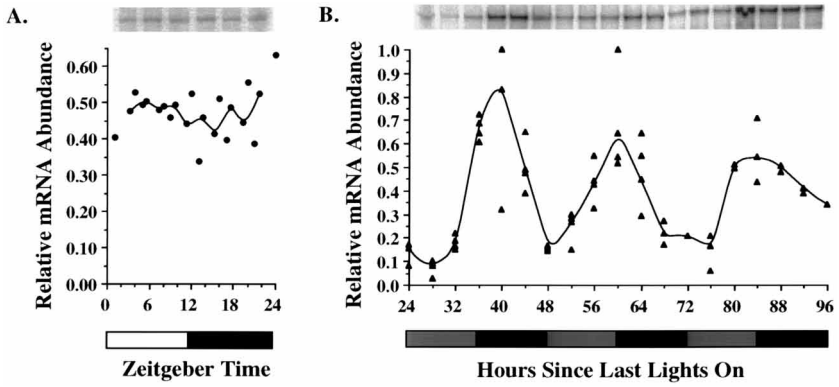

FIG. 3. Enhancer-mediated mRNA cycling is PER dependent. (A) *per* sequences from −603 to −449 were used to drive an *hsp70* basal promoter-*lacZ* fusion gene in per^{01} flies under LD conditions. The gel strip, quantitation, and normalization of two independent time courses were as described for Fig. 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.

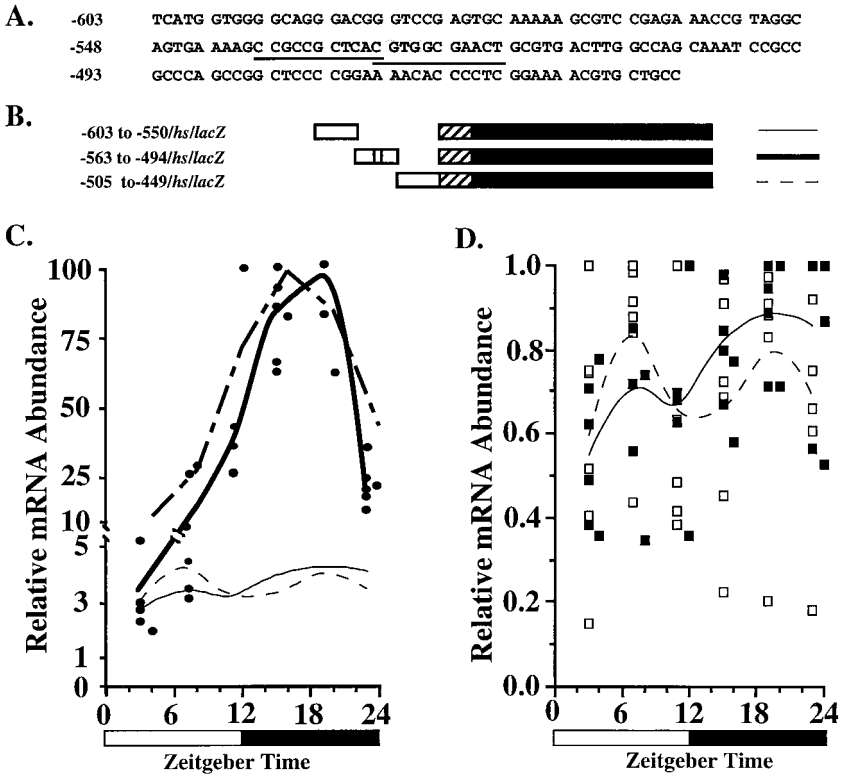

FIG. 4. An E-box-containing subfragment of the clock control region supports mRNA cycling. (A) *per* upstream sequences from −603 to −449. The shaded bases constitute the E box. The underlined sequences represent E-box deletions. (B) Constructs used to further define the *per* clock control region. □, *per* upstream sequences; ▨, the E box in *per* upstream sequences; ▨, *Drosophila* heat shock protein 70 gene basal promoter sequences; ■, *E. coli lacZ* gene coding sequences. The numbers delimit the end point of *per* upstream sequences in each construct and are relative to the *per* gene transcription start site (+1). Line symbols correspond to the *lacZ* mRNA curves for these constructs in panels C and D. (C) Relative *lacZ* mRNA abundances in flies transformed with the constructs in panel B. Filled circles represent data points for the −563 to −494/*hs*/*lacZ* construct. The line symbols represent *lacZ* mRNA levels from the transgenes shown in panel B. An idealized *per*$^+$ mRNA curve is depicted as a bold, dashed line whose peak value was adjusted to 100. Quantitation was based on at least three experiments for each construct and was normalized to the highest level (designated 100) of *lacZ* mRNA from the −563 to −494/*hs*/*lacZ* construct. The break in the ordinate indicates a change of scale. The white and black bars represent times when lights were on and off, respectively. (D) Expanded scale of the relative *lacZ* mRNA abundances from non-E-box-containing clock control region fragments. Data from six independent time courses of −603 to −550/*hs*/*lacZ* transformants (filled squares) and −505 to −449/*hs*/*lacZ* transformants (open squares) are plotted. The average curves calculated for panel C are shown. White and black bars are as described for panel C.

cause a subgroup of bHLH transcription factors share a region with PER called the PAS domain (8), which is capable of mediating homotypic and heterotypic protein-protein complexes (15, 24).

To determine whether an E-box-containing portion of the 154-bp clock control region is important for *per* gene expression, three subfragments (one containing the E box and two lacking the E box) were placed upstream of the *hsp70* basal promoter driving *lacZ*, transformed into wild-type flies, and analyzed by RNase protection (Fig. 4B). The 69-bp E-box-containing subfragment of the −603 to −449 *per* upstream sequence (−563 to −494/*hs*/*lacZ*) is capable of driving *lacZ* cycling with a comparable phase and an amplitude equivalent to or higher (due to a higher peak level) than those of the wild-type transcript, while *per* upstream fragments lacking the E box show an ~20-fold drop in expression (Fig. 4C). The residual *lacZ* mRNAs from *per* upstream fragments lacking the E box are widely scattered in abundance and vary less than twofold on average (Fig. 4D). The inability of the non-E-box-containing fragments to drive mRNA cycling indicates that all the information needed for mRNA cycling lies within the central 69-bp E-box-containing fragment of the clock control region.

The E box within the clock control region is required for high-level transcription. To determine whether the E box within the *per* clock control region is required for high-level and/or cyclic transcription, two deletions which remove a portion of the E box [−603 to −449 Δ(−529 to −519) and −603 to −449 Δ(−539 to −529)] were generated (Fig. 4A and 5A), transformed into wild-type flies, and analyzed for their abilities to drive *lacZ* mRNA cycling. Each of these E-box deletion lines expresses *lacZ* mRNA at levels close to the trough level of wild-type *per* mRNA (Fig. 5B), making mRNA cycling difficult to measure. When the signal from these deletion lines is plotted relative to the peak time point for each time course, the residual expression from both sets of mutant lines cycles with an overall low amplitude (Fig. 5C). For the two −603 to −449 Δ(−529 to −519) lines, cycling amplitude was consistently ~2-fold, whereas in the four [−603 to −449 Δ(−539 to −529)] lines, cycling amplitudes ranged from 0 (two time courses from one line) to ~4- to 6-fold (one time course from three independent lines). These data suggest that PER mediates mRNA cycling through sequences outside the E box.

DISCUSSION

Rhythmic gene expression is a key mechanism by which the circadian clock regulates physiological and behavioral processes in animal, plant, and microbial systems. Such regulation encompasses key elements of the time-keeping apparatus (e.g.,

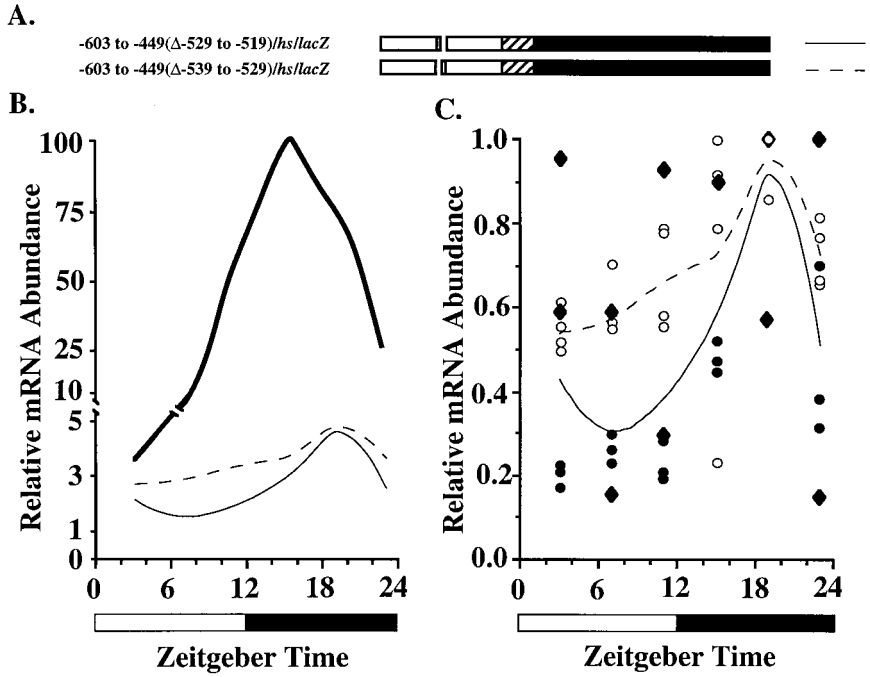

FIG. 5. An E box within the clock control region is required for high-level expression. (A) Constructs used to determine the effects of E-box deletion mutations on mRNA cycling. Symbols denoting the origin of sequences in the constructs and the numbering of *per* sequences are as designated in Fig. 4B. The deletion of sequences between −529 and −519 is designated Δ(−529 to −519), and the deletion of sequences between −539 and −529 is designated Δ(−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 Δ(−539 to −529)/*hs*/*lacZ* transformants (open circles), two time courses of an arrhythmic −603 to −449 Δ(−529 to −519)/*hs*/*lacZ* transformant line (filled diamonds), and time courses of three rhythmic −603 to −449 Δ(−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 *frq*) 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* (*ccg-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 ~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 ~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^{01} 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.

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