Constructing a Feedback Loop with Circadian Clock Molecules from the
Silkmoth, *Antheraea pernyi*

Dennis C. Chang¹⁻³, Harriet G. McWatters¹,⁴, Julie A. Williams¹,⁵, Anthony L. Gotter¹,³,⁶, Joel D.
Levine¹,⁷, and Steven M. Reppert¹⁻³

1. Laboratory of Developmental Chronobiology
MassGeneral Hospital for Children
Massachusetts General Hospital
Boston, MA  02114

2. Program in Neuroscience
Harvard Medical School
Boston, MA  02115

3. Department of Neurobiology
University of Massachusetts Medical Center
364 Plantation St.
Worcester, MA  01605

Address correspondence to:
Steven M. Reppert
Dept. of Neurobiology
UMass Medical School
364 Plantation St.
Worcester, MA  01605
Phone: (508) 856-6148
Fax: (508) 856-6233
Email: steven.reppert@umassmed.edu

Current addresses:

4. Harriet G. McWatters
Department of Plant Sciences
Oxford University
South Parks Road
Oxford, OX1 3RB, UK

5. Julie A. Williams
Department of Neuroscience
Howard Hughes Medical Institute
University of Pennsylvania
232 Stemmler Hall/6074
Philadelphia, PA 19104

6. Anthony L. Gotter
Division of Human Genetics and Molecular Biology
Children's Hospital of Philadelphia
3516 Civic Center Blvd., ARC 1002
Philadelphia, PA 19104

7. Joel D. Levine
Department of Biology, MS-008
Brandeis University
Waltham, MA 02454
SUMMARY

Circadian clocks are important regulators of behavior and physiology. The circadian clock of *Drosophila* depends on an autoinhibitory feedback loop involving dCLOCK, CYCLE (also called dBMAL, for *Drosophila* Brain and Muscle ARNT-Like protein), dPERIOD, and dTIMELESS. Comparisons between *Drosophila* and mouse indicate that insects and vertebrates inherited their circadian clocks from a common ancestor, but the clock mechanism has since diversified during the course of evolution. Recent studies suggest that the clock mechanism in other insect species may differ strikingly from that of *Drosophila*.

We cloned *Clock*, *Bmal*, and *Timeless* homologs (apClock, apBmal, and apTimeless) from the silkmoth *Antheraea pernyi*, from which a *Period* homolog (apPeriod) has already been cloned. In Schneider 2 (S2) cell culture assays, apCLOCK:apBMAL activate transcription through an E-box enhancer element found in the 5' region of the *apPeriod* gene. Furthermore, apPERIOD can robustly inhibit apCLOCK:apBMAL-mediated transactivation, and apTIMELESS can augment this inhibition. Thus, a complete feedback loop, resembling that found in *Drosophila*, can be constructed from silkmoth CLOCK, BMAL, PERIOD, and TIMELESS. Our results suggest that the circadian autoinhibitory feedback loop discovered in *Drosophila* is likely to be widespread among insects. However, whereas the transactivation domain in *Drosophila* lies in the C-terminus of dCLOCK, in *A. pernyi*, it lies in the C-terminus of apBMAL, which is highly conserved with the C-termini of BMALs in other insects (except *Drosophila*) and in vertebrates. Our analysis sheds light on the molecular function and evolution of clock genes in the animal kingdom.
INTRODUCTION

Circadian rhythms are driven by cell autonomous pacemakers consisting of molecular feedback loops (1). In the fruit fly *Drosophila melanogaster*, circadian feedback loops require the transcription factors dCLOCK (dCLK) and CYCLE (CYC, also called dBMAL) (2). dCLK and CYC heterodimerize to bind E-box enhancer elements, presumably through their basic helix-loop-helix (bHLH) domains (3). In addition, dCLK and CYC each possesses a PER-ARNT-SIM homology (PAS) domain (3, 4, 5), which is thought to facilitate protein-protein interactions (6) and contains two PAS structural motifs (PAS-A and PAS-B) and a region downstream of PAS-B called PAC (7). Further downstream, dCLK possesses a glutamine-rich transactivation domain (3, 4). One of the genes activated by dCLK:CYC, *period* (*dper*), encodes another PAS protein, dPER (2, 3). dPER inhibits dCLK:CYC through a non-PAS, C-terminal domain, called the dCLK:CYC inhibition domain (CCID), thus completing the key circadian negative feedback loop (2, 3, 8). dCLK:CYC also activate transcription of the *timeless* (*dtim*) gene, whose protein product, dTIM, regulates dPER protein stability and nuclear transport and may also contribute to the inhibition of dCLK:CYC (2, 3, 8). dTIM also plays a role in light input to the clock (2).

The circadian feedback loops of mice depend upon mCLK :mBMAL1, homologs of dCLK:CYC (9). Cryptochromes (mCRYs) are the main inhibitors of mCLK:mBMAL1, while mouse PERs seem to regulate mCRY nuclear entry (10). Mammals lack a true ortholog of dTIM (11, 12). The presence of CLOCK, BMAL, and PERIOD (and other molecules) at the heart of circadian clocks in both *Drosophila* and mice suggests that both insects and vertebrates inherited their clocks from a common ancestor (1). However, the differences in fly and mouse clock mechanisms indicate that animal clocks have diversified during the course of evolution.

Indeed, *Drosophila* even differs from other insects in its clock mechanism. The temporally regulated nuclear transport of dPER and dTIM (cytoplasmic during the afternoon,
nuclear at night) in pacemaker neurons (13, 14) is thought to be critical for *Drosophila* clock function (2). In contrast, PER and TIM in the Chinese oak silkworm, *Antheraea pernyi*, (apPER and apTIM) are primarily cytoplasmic at all times of day in brain neurons (15). PER in a damselfly (order Odonata) is also cytoplasmic throughout the day (16). In fact, in the early morning, when dPER is nuclear in *Drosophila* (14), PER is cytoplasmic in representatives of many insect orders: Thysanura, Ephemeroptera, Orthoptera, Plecoptera, Hemiptera, Coleoptera, Hymenoptera, Trichoptera, and even Diptera (16). Thus the pacemaker mechanism in *A. pernyi* (Lepidoptera) may be more representative of insect clocks than the mechanism in *Drosophila*.

To further our understanding of insect clock function and evolution, we cloned *Clock*, *Bmal*, and *Timeless* homologs from *A. pernyi* (*apClk*, *apBmal*, *apTim*). We characterized their functions and interactions with *apPer* using *Drosophila* Schneider 2 (S2) cells, since many aspects of both fly and mammalian circadian biochemistry can be simulated in S2 cells. These include dCLK:CYC-mediated transactivation via E-boxes, dPER inhibition of dCLK:CYC (3, 8), mCLK:mBMAL1 transactivation, and mCRY inhibition of that activation (17, 18). In S2 cells, apCLK:apBMAL can activate transcription through an E-box in the *apPer* promoter, apPER inhibits apCLK:apBMAL-mediated transcription, and apTIM can augment apPER's inhibitory activity. Like dPER, apPER inhibits apCLK:apBMAL through a C-terminal domain that also contains the primary nuclear localization sequence (NLS) of apPER. Thus, despite initial appearances, a *Drosophila*-like negative feedback loop involving CLOCK, BMAL, PERIOD, and TIMELESS may exist in the silkworm *A. pernyi*, and perhaps in other insects with similarly cytoplasmic PER. However, the domain responsible for apCLK:apBMAL-mediated transactivation differs strikingly from that found in *Drosophila*. Our comparative analysis provides new insight into the molecular evolution of circadian pacemakers in animals.
EXPERIMENTAL PROCEDURES

Cloning and Sequence Analysis - Fragments of *apClock*, *apBmal*, and *apTimeless* were cloned by degenerate PCR. cDNA templates for PCR were prepared from RNA purified from *Antheraea pernyi* brains dissected 1-3 days after eclosion. The ends of the coding regions were obtained by rapid amplification of cDNA ends (RACE; Clontech kits). Complete open reading frames were obtained by Pfu Turbo (Stratagene) PCR from cDNA. Clones were sequenced at core facilities at Massachusetts General Hospital and UMass Medical School. Sequence analysis was facilitated by software from the Genetics Computing Group (GCG) and the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

Plasmids - S2 cell expression constructs were generated by subcloning *lacZ*, *apClock*, *apBmal*, *apTimeless*, *apPeriod*, or fragments thereof into the pAc5.1V5/HisA vector (Invitrogen) or a modified pAc5.1 vector with the V5 tag at the N-terminus. Sf9 cell expression constructs were generated by subcloning into a pIB/V5-His vector (Invitrogen) modified so that its multiple cloning site resembled that of pAc5.1V5/HisA. *apPer* promoter constructs were obtained by sequential digests and subcloning of genomic DNA fragments into the promoter-less luciferase vector, pGL3 Basic (Promega). To generate E-box hs-luc constructs, the *hsp70* promoter (from *XhoI* to *NcoI*) from the *dper* 4E-box construct provided by Steve Kay (3) was first subcloned into pGL3 Basic to generate an "hs-luc" construct. Overlapping PCR primers (with restriction sites at the ends) were used to generate fragments containing E-boxes (*apPer Eₕ*, mutant *Eₕ*, *Eₐ*, *Eₐ* and *dper E* and mutant *E*) and 10-11 bp flanking sequences; these were subcloned into hs-luc. A construct with 4 tandem repeats of the *apPer Eₕ* enhancer (with 7-8 bp flanking sequences) upstream of hs-luc ("*apPer 4Eₕ*-hs-luc") was also generated by overlapping primers containing appropriate restriction sites. The pAct-*dClk* construct was provided by Steve Kay (3).
**Site-directed mutagenesis** - NLS mutant apPer M693-4 was generated using complementary primers containing the desired mutation, along with a diagnostic restriction site, to PCR-amplify apPer-V5 template. The PCR reaction was treated with DpnI to remove template DNA, and used to transform E. coli cells (DH5α). apPer M707-9 and apPer M717-9 were generated by designing primers (both sense and antisense) containing the mutation and a restriction site for subcloning. N-terminal and C-terminal fragments, overlapping at the mutation site, were generated by PCR, subcloned, and sequenced. The C-terminal fragment was subcloned downstream to the N-terminal piece to produce the correct, full-length mutant construct.

**Insect cell culture and transfections** - S2 cells were maintained at 25°C in Schneider's Drosophila medium (Gibco) with 9% heat-inactivated fetal bovine serum (FBS; Gibco), and Sf9 cells were maintained at 27°C in Sf-900 II serum-free medium (SFM; Gibco). Transient transfection of plasmids involved mixing DNA with 5-10 µL CellFECTIN reagent (Gibco), and 15 minutes later, applying the mixture to S2 cells in Drosophila SFM (Gibco) supplemented with 2 mM L-glutamine, or to Sf9 cells in Sf-900 II SFM. Approximately four hours later, S2 cells were fed with an equal volume of 9% FBS in Schneider's medium, and Sf9 cells were given fresh SFM. Cells were incubated for approximately 48 hours before use.

**Transcription Assays** - To test the ability of apCLK:apBMAL to activate transcription in S2 cells, we used a modified version of the transcription assay of Darlington and coworkers (3). In brief, 1 ng apClk ± 1 ng apBmal were co-transfected with 10 ng apPer 4E-p-hs-luc and 30 ng βgal pAc5.1V5/HisA (βgal-V5). For each assay, a control transfection including only the reporters, apPer4E-p-hs-luc and βgal-V5, was used to establish baseline reporter activity. In the apPer promoter assays, we substituted apPer genomic DNA constructs for apPer4E-p-hs-luc. To measure the inhibitory activity of apPerlapTim, we co-transfected 2-250 ng of apPer, apTim, or
*apPer* mutant constructs with *apClk, apBmal*, and the reporters. The total amount (ng) of DNA in each transfection was normalized using empty vector (pAc5.1V5). After transfection and a 48 hour incubation at 25°C, cells were harvested, washed in phosphate-buffered saline (PBS, Dulbecco's; Gibco), and lysed in lysis buffer (Promega). Cell extracts were assayed for β-galactosidase and luciferase activities using commercial assay kits (Tropix and Promega, respectively) and an MLX microtiter plate luminometer (Dynex). Average ratios of luciferase activity to β-galactosidase activity were computed, and for the *apPER/apTIM* inhibition assays, the ratios were normalized so that the relative activation by *apCLK:apBMAL* alone equalled 100%. The assays in Sf9 cells were similar, but used ~10-fold higher plasmid doses.

**Immunocytochemistry** - S2 (or Sf9) cells seeded onto glass coverslips in 6-well tissue culture plates were transfected with ca. 300 ng (or 1 µg for Sf9 cells) V5-tagged *apPer, apTim* or *apBmal* constructs. 48 hours after transfection, the cells were washed in PBS and fixed onto the coverslips with 5% paraformaldehyde in PBS. The coverslips were then processed with blocking solution (10% normal goat serum and 0.2% Triton X-100 in PBS), primary antibody (monoclonal mouse anti-V5 IgG (Invitrogen) 1:500 in blocking solution diluted 1:4 in PBS), three PBS washes, secondary antibody (Cy3-conjugated goat anti-mouse IgG (Jackson) 1:300 in diluted blocking solution), two PBS washes, DAPI stain (bisBenzimide 1:500 in PBS), two brief PBS washes, and a rinse in ddH2O, before being mounted onto slides with mounting medium for fluorescence microscopy (Kierkegaard and Perry). Slides were viewed under a fluorescence microscope (Olympus IX70) at the Digital Imaging Core Facility at the UMass Medical Center. For each slide, 30 cells with strong fluorescent signal were classified as having staining in the nucleus, cytoplasm, or both nucleus and cytoplasm. Scoring was done blind to which construct was being analyzed. Each construct was analyzed using three or more separate transfections.
RESULTS

apClock, apBmal, and apTimeless: cloning and sequence analysis

apClock (GenBank: AY330486) encodes a predicted protein of 611 amino acids with clear homology with other CLOCK proteins. For example, the sequence identity between apCLK and dCLK/mCLK bHLH domains was ≥ 67%, PAS-A motifs ≥ 42%, PAS-B ≥ 80% and the PAC ≥ 76% (Table 1). The high N-terminal sequence similarity clearly identifies apCLK as a homolog of CLK, and not of any other bHLH-PAS transcription factor. In contrast, the C-termini (amino acids downstream of the PAS domain) in silkmoth, fly, and mouse CLK proteins are not well conserved (6-17%; Table 1). In particular, while both dCLK and mCLK possess poly-glutamine repeats in their C-termini, apCLK has none (Figure 1A). This is surprising since the glutamine enrichment of mCLK/dCLK C-termini is thought to be indicative of their transactivation function (19, 3, 4). However, the absence of poly-glutamine stretches in apCLK does not rule out the presence of a transactivation domain in its C-terminus. MOP4 (also called NPAS2), a CLK paralog found in mammals, lacks long poly-glutamine repeats, yet is capable of transcriptional activation when partnered with mBMAL1 (20).

A subregion of the C-terminus is relatively well conserved in apCLK, dCLK, and mCLK (Figure 1A). This region corresponds to the peptide sequence encoded by exon 19 of mClk, which is spliced out of the mRNA in Clock mutant mice (19). Since the mutant mCLK protein (mCLKΔ19) is defective for transcriptional activation (21), this region may be an important part of the transactivation domain, even though it does not contain poly-glutamine stretches. Alternatively, the "Δ19" region may be involved in stabilizing the CLK:BMAL heterodimer (22), or function as a binding site for important regulatory proteins, such as PER (see below).
apBmal (GenBank: AY330487) encodes a predicted protein of 589 amino acids with high homology with other BMAL proteins. apBMAL was especially similar to Drosophila CYC or mBMAL1 in the bHLH (≥ 68% identity), PAS-A (≥ 64% identity), and PAS-B (≥ 54% identity) regions (Table 1). Interestingly, whereas CYC terminates at the end of the PAS domain (3,5), the silkmoth and mouse BMAL proteins possess a C-terminal region of a few hundred amino acids downstream of PAS. Moreover, the very C-terminal part of this region (~40 amino acids) is very well conserved (70% identical) between silkmoth and mouse BMALs (Table 1; Figure 1B). This C-terminal end of mBMAL1 was previously implicated in transcriptional activation in both yeast and mammalian cell culture (22). The authors of that study suggested that the C-terminus of mBMAL1 is the primary transactivation domain of mCLK:mBMAL1, and that the C-terminus of mCLK may serve only to augment or stabilize mBMAL1’s activity (22). The conservation of this BMAL C-terminal region (“BCTR”) in apBMAL suggests that apBMAL may also possess a C-terminal transactivation domain.

apTim (GenBank: AF132032) encodes a predicted protein of 1233 amino acids (GenBank: AAF66996) homologous to dTIM. TIMELESS belongs to a protein family that includes a second Drosophila protein, dTIMEOUT, or dTIM2, which is homologous to mouse (mTIM) and Caenorhabditis elegans (ceTIM-1) proteins involved in development (11, 12, 23). However, apTIM is more closely related to dTIM than to dTIMEOUT (11, 12), indicating that it is an ortholog of dTIM, rather than of dTIMEOUT.

Sequence analysis software has suggested that dTIM possesses two domains of Armadillo (Arm)/HEAT motif repeats (24). The role of these structural features in dTIM are unknown, although functional studies in S2 cells have revealed that dTIM contains two dPER-binding sites (25), one overlapping the second Arm/HEAT domain and the other lying within the
second Arm/HEAT domain (24, Figure 1C). In S2 cells, dTIM was also shown to possess a nuclear localization sequence (NLS) and a C-terminal cytoplasmic localization domain (CLD) (25, Figure 1C). apTIM and dTIM are 30% identical overall, and are especially homologous in the Arm/HEAT domains (46 % identity in each, Table 1). apTIM also has a homologous putative NLS in the region of the dTIM NLS, and a region homologous to the CLD in its C-terminus (Figure 1C), but the homology in the C-terminus is poor (Table 1).

**apCLOCK:apBMAL activate transcription via an apPer E-box**

To test whether apCLK:apBMAL can activate transcription from the apPer promoter, we analyzed a genomic DNA fragment containing the first two exons of apPer and approximately 15 kb of upstream sequence (26). Within this clone, there are three putative E-boxes (i.e., CACGTG sequences), approximately 3.5 kb, 9.0 kb, and 13.4 kb upstream of exon 1 (Figure 2A). These we designated E\textsubscript{p}, E\textsubscript{m}, and E\textsubscript{d}, respectively (for proximal, medial, and distal E-boxes). We subcloned genomic fragments of varying lengths upstream from (and including part of) exon 1 into a promoter-less luciferase vector. In S2 cells, we found that apCLK:apBMAL do not activate luciferase transcription from any apPer promoter construct evaluated (data not shown). We performed a similar assay in Sf9 cells, which are derived from the moth Spodoptera frugiperda, but again obtained no activation (data not shown).

There are at least three explanations for this negative result. First, the apPer promoter may not be functional in S2 or Sf9 cells, perhaps because essential activators or co-activators are absent in those cell lines. This possibility is supported by the extreme spatial restriction of apPER expression in silkworm brain to only ~8 cells (15). Second, the apPer genomic fragments may contain a binding site for transcriptional repressors endogenously expressed in S2 and Sf9 cells. The inhibitory action of these repressors could be blocking transcription from the reporter
constructs even in the presence of apCLK:apBMAL. These first two possibilities are supported by the low baseline level of luciferase (in the absence of apClk/apBmal) generated by the apPer promoter compared to that produced by the hsp70 promoter, which was used as a control (data not shown). The third possibility is that the apPer genomic constructs do not contain a viable E-box element to which apCLK:apBMAL can bind to activate transcription.

To test the third possibility, we generated new constructs in which individual E-boxes (the dper E-box or apPer E_p, E_m, or E_d) with 10-11 bp of surrounding sequence were subcloned upstream of the hsp70 promoter in a luciferase reporter construct (Figure 2B). These constructs would not rely on a possibly inactive promoter for transcription initiation, and (because minimal fragments of apPer gDNA are used) they are not likely to contain repressor elements found in larger fragments of the apPer gene 5' region. We tested these constructs in S2 cell transcription assays, and found that apCLK:apBMAL could activate transcription of the dper E-box and the most proximal apPer E-box, E_p, to comparable levels (Figure 2B). apCLK:apBMAL could not robustly activate transcription from the other two apPer E-boxes (E_m and E_d), or from mutated E-boxes reading CTGCAG, instead of CACGTG (Figure 2B). Thus, apCLK:apBMAL specifically activate transcription from the most proximal E-box element in the apPer promoter region. This E-box, the CACGTG and its 4 bp flanking sequences, is extremely similar (12/14 bases identical) to the functional E-box described for the Drosophila clock gene, vrille (27).

To enhance the measurement of apCLK:apBMAL-mediated transcriptional activation, we followed the example of others (3, 27, 28) and generated a reporter construct containing four tandem repeats of the functional apPer E-box, apPer 4E_p-hs-luc, for use in subsequent assays. apCLK:apBMAL activate transcription robustly from the apPer 4E_p-hs-luc construct in both S2 cells (Figure 3) and Sf9 cells (data not shown).
apCLOCK:apBMAL transactivation depends on the C-terminus of apBMAL

To determine the domain(s) responsible for transcriptional activation, we generated deletion mutants of *apClk* and *apBmal* and tested them in the S2 cell transcription assay (Figure 3). We found that the BCTR was essential for activation, since a truncated apBMAL (aa 1-552) possessed no transcriptional activity (Figure 3). The C-terminus of apCLK (everything downstream of PAS), on the other hand, was dispensable for apCLK:apBMAL-mediated transcriptional activation, since a truncated apCLK (aa 1-364) paired with full-length apBMAL was still capable of transcriptional activation (Figure 3). The truncated proteins tagged with the V5 epitope were robustly expressed in S2 cells, as assayed by anti-V5 Western blot (data not shown), and both full-length and C-terminal truncated apBMAL were predominantly nuclear by immunocytochemistry (Table 2). Thus, the effect of the deletion mutations on transcriptional activity could not be ascribed to protein instability or failure of nuclear transport.

apPER inhibits apCLOCK:apBMAL and apTIM augments this inhibition

In the *Drosophila* circadian clock, dPER is the primary inhibitor of dCLK:CYC-mediated transcription, although dTIM has some inhibitory activity as well (8). In mice, although mPERs can modestly inhibit mCLK:BMAL1-mediated transcription, they are not the primary inhibitors (29). To examine the function of silkmoth PER and TIM, we expressed different concentrations of *apPer* and *apTim* in S2 cells along with *apClk* and *apBmal*, and assessed the ability of apPER ± apTIM to inhibit apCLK:apBMAL-mediated transactivation (Figure 4A).

We found that apTIM could not inhibit apCLK:apBMAL on its own (Figure 4A), even though it was robustly nuclear in S2 cells (90% of cells, Table 2). apPER alone could inhibit apCLK:apBMAL-mediated transcription in a dose-dependent manner, although low doses of apTIM could augment the inhibitory activity of apPER. Thus, in this assay, apPER functionally
resembles its *Drosophila* homolog and is likely to be the primary inhibitor of apCLK:apBMAL. It is therefore likely that the silkmoth homologs of *Drosophila* circadian pacemaker elements are capable of producing a complete transcriptional feedback loop.

Interestingly, apPER is not effective at inhibiting the transcriptional activation mediated by the C-terminal truncation mutant apCLK 1-364 paired with apBMAL (Figure 4B). It is therefore possible that the apCLK C-terminus contains a binding site important for apPER-mediated inhibition of apCLK:apBMAL. The "Δ19" region is a candidate for this binding site, since it is relatively well conserved in dCLK (Figure 1A, Table 1), and apPER can also robustly inhibit dCLK:CYC-mediated transcriptional activation (Supplementary Figure S2).

**apPER possesses a C-terminal inhibition domain and a classical bipartite NLS**

apPER inhibition of apCLK:apBMAL-mediated transcription is somewhat surprising in the context of its cytoplasmic localization in brain neurons *in vivo*. It is possible that apPER inhibits apCLK:apBMAL by sequestering one or both of the transcription factors in the cytoplasm. This inhibitory mechanism has been demonstrated for other proteins, such as the aryl hydrocarbon receptor, which is also a bHLH-PAS protein (30). However, since the activity of apPER in S2 cells resembled that of dPER, we investigated another possibility: that apPER contains a C-terminal inhibition domain, homologous to the CCID of dPER (8), and, since the dPER CCID requires nuclear entry (8), a functional nuclear localization sequence (NLS).

By testing deletion mutants of *apPer* in the transcription assay, we found that a C-terminal CCID-like domain of apPER was responsible for its inhibition of apCLK:apBMAL (Figure 5A), similar to what was discovered in *Drosophila* (8). We next examined the subcellular location of apPER and apPER deletion mutants in S2 cells via immunocytochemistry. In contrast to its cytoplasmic localization in brain neurons, apPER was robustly nuclear in S2
cells (96% of cells; Figure 5B, Table 2) and Sf9 cells (97% of cells; Table 2). This indicates that apPER’s inhibition of apCLK:apBMAL is a nuclear activity, similar to dPER (8). The NLS of apPER mapped to a C-terminal region of the protein (aa 684-738; Figure 5B, Table 2). By mutating clusters of basic amino acids, which are typical of classical NLSs (31), we found two clusters in this region (aa 693-4 and aa 707-9) essential for robust nuclear entry (Figure 5B, Table 2). The spacing of these clusters is consistent with that of a classical bipartite NLS (31). Thus, like dPER (8), apPER nuclear entry in S2 cells seems to be mediated by a bipartite NLS. Moreover, the NLSs of apPER and dPER are homologous to each other, and to putative NLSs in other insect PERs (Supplementary Figure S1).

DISCUSSION

Evolution of CLOCK:BMAL Transactivation Domains

In contrast to mouse and Drosophila CLK, apCLK does not possess a C-terminal transactivation domain (Figure 3). Furthermore, in the Anopheles gambiae genome (32) the closest homolog of dClk (agClk = "ebiP4355", GenBank: EAA11642) resembles apCLK in its lack of poly-glutamine repeats. Thus, even among the Diptera, a glutamine-rich CLK protein is not universal. It seems likely that in the common ancestor of flies and moths, the CLK protein resembled that of apCLK and agCLK more than dCLK. Indeed, the putative transactivation domains, including the positions of the poly-glutamine stretches, are not well conserved between vertebrates and flies (Table 1; Figure 1A). This suggests that the glutamine-rich transactivation domains found in Drosophila and mouse CLOCKs are not conserved from an ancestral CLOCK protein. We propose that CLOCK originally lacked poly-glutamine stretches, and acquired them independently in the Drosophila and vertebrate lineages.
In contrast to the C-termini of CLOCK proteins, the C-termini of BMALs are highly conserved in both sequence and (at least for apBMAL and mBMAL1) transactivation function. Thus, we conclude that the BCTR is evolutionarily ancient and likely to be widespread in the animal kingdom. A search of the GenBank database supports this assertion. The only BMAL homolog lacking the BCTR is Drosophila CYCLE (Figure 6A). The Bmal homolog recently cloned from the domestic silkmoth, Bombyx mori (33), contains an extremely well conserved BCTR (87% identical to apBMAL). The Anopheles gambiae genome (32) also contains a Bmal homolog ("ebiP7299", GenBank: EAA11829) containing a bHLH domain, PAS, and the BCTR. Among vertebrates, the BCTR is universally found in close BMAL homologs, such as BMAL1 (also called MOP3 or ARNT3) and BMAL2 (also called MOP9 or CLIF) in mammals (34, 35), and BMAL1 through BMAL3 in the zebrafish, Danio rerio (36, 37). It is not found in ARNT proteins, although ARNT is more closely related to BMAL than most other bHLH-PAS proteins.

Thus, in the currently most parsimonious model for CLOCK:BMAL evolution (Figure 6A), the common ancestor of insects and vertebrates possessed a BMAL homolog possessing a C-terminal transactivation domain and a CLOCK homolog lacking a glutamine-rich domain. CLOCK acquired poly-glutamine repeats very early in the vertebrate lineage, since similar repeats are found in zebrafish and mammals. CLOCK independently acquired poly-glutamine repeats in the Drosophila lineage sometime after brachyceran flies diverged from mosquitoes ca. 250 million years ago (38). Meanwhile, BMAL retained its ancient transactivation domain in vertebrates and in most insects, but lost this domain in the Drosophila lineage, presumably because the newly acquired dCLK transactivation domain made the BCTR redundant. More species must be examined to verify this model of CLOCK-BMAL evolution.

Evolution of TIMELESS
In contrast to the widespread conservation of *Clock* and *Bmal*, the central clock gene *timeless* has not yet been found outside of insects. It is absent from *C. elegans*, humans, puffer fish (*Fugu rubripes*), and sea squirt (*Ciona intestinalis*), four animals whose genomes have been almost completely sequenced (39-43). *timeout*-like genes, on the other hand, are found universally in animals, suggesting that *timeless* evolved from a gene duplication of *timeout*, perhaps in the arthropod lineage (12). *timeless* orthologs have been cloned from a number of dipterans, including *Drosophila* species (44, 45), the drosophilid fruit fly *Chymomyza costata* (GenBank: BAB91179), and the non-drosophilid fly *Sarcophaga crassipalpis* (46). A *timeless* homolog is also present in the genome (32) of the mosquito *Anopheles gambiae* (GenBank: EAA12266). *apTim* is the first *timeless* ortholog identified in a lepidopteran insect, or indeed in any non-dipteran. The cloning of *apTim* establishes that the origin of *timeless* occurred before the evolutionary divergence of Diptera and Lepidoptera ca. 330 million years ago (38).

The sequence homology in the Arm/HEAT domains between dTIM and apTIM suggests that they might share similar functions. Indeed, both apTIM and dTIM augment PER-mediated inhibition of CLK:BMAL-driven transcription in S2 cells (3, 8, Figure 4). dTIM may augment dPER's activity by facilitating dPER nuclear entry (25, 47), but this cannot be how apTIM enhances apPER's inhibitory activity, since apPER is already nuclear in S2 cells (Figure 5B).

apTIM differs from dTIM in at least two ways. First, in S2 cells, apTIM shows little ability to inhibit and at high doses may even enhance apCLK:apBMAL-activated transcription (Figure 4A), whereas dTIM clearly inhibits dCLK:CYC to a moderate level (3, 48, 8). Second, apTIM is nuclear in S2 cells and Sf9 cells (Table 2), while dTIM is cytoplasmic in the same cell lines (25, Table 2). The "CLD" in apTIM is thus unable to function as a CLD in S2 or Sf9 cells. Although sequences similar to the CLD are present in most TIM proteins, such a region is absent
in *Drosophila hydei* TIM (45). Furthermore, the homology in this "CLD" region is very weak outside of the Drosophilidae, with only 14% identity to dTIM in *Anopheles gambiae* TIM. Thus, the generality of the TIM "CLD" for cytoplasmic localization is unclear.

**PERIOD evolution and inhibitory mechanism**

Functional similarity of apPER to dPER was predicted based on the ability of an *apPer* transgene to rescue behavioral rhythms in *dper* null mutant flies (49). Although apPER is only two-thirds the size of dPER, its function is well conserved in our S2 cell assays. Both PERs possess a homologous C-terminal domain that inhibits CLK:BMAL-mediated transcription, and a conserved bipartite NLS that is functional in S2 cells (8, Figure 5). The PAS domain of apPER also resembles that of dPER (50); however, it remains unknown whether the apPER PAS domain can bind apTIM as the dPER PAS domain binds dTIM. Interestingly, the PAC of apPER, which is homologous to the CLD of dPER, does not override the NLS to produce predominantly cytoplasmic immunostaining in S2 cells (Figure 5; Table 2) or Sf9 cells (Table 2).

In S2 cells, apPER robustly inhibits not only apCLK:apBMAL (Figure 4A), but also dCLK:CYC (Supplementary Figure S2), even though the mechanism of transcriptional activation (apBMAL’s BCTR vs. dCLK’s glutamine-rich C-terminus) is different. The versatility of apPER’s inhibitory activity suggests that its CCID does not negate a specific transactivation domain. Instead, the C-terminal inhibitory domain of apPER may be a general transcriptional repressor domain, or it may act on the DNA-binding and/or dimerization of CLK:BMAL, which is mediated by the bHLH and PAS domains and is therefore likely to be independent of the transactivation domain(s). *In vitro* gel-shift assays have shown that dPER binding to dCLK:CYC can inhibit DNA-binding without disrupting dCLK:CYC heterodimer formation (48), suggesting that dPER can interfere with the bHLH domains of dCLK:CYC. However, the
inhibitory activity of apPER is dependent on the C-terminus of apCLK (Figure 4B). Thus apPER cannot act only through the bHLH domains of apCLK:apBMAL.

Towards a General Insect Circadian Clock Model

Our data in S2 cells indicate that a transcriptional feedback loop resembling that found in the circadian clock of *Drosophila* can be constructed from *A. pernyi* CLOCK, BMAL, PERIOD, and TIMELESS proteins, and the *apPeriod* gene (Figure 6B). Such a feedback loop could explain the previously detected in vivo rhythms in apPer RNA and protein levels (15, 26).

However, it remains unclear why apPER is always primarily cytoplasmic in *A. pernyi* brain neurons, when it possesses an evolutionarily ancient, classical bipartite NLS (Figure 5B). We propose that apPER may indeed enter the nucleus, if only transiently or at low levels. The conservation of the NLS in insect PER proteins (Supplementary Figure S1) suggests that although PER has been detected only in the cytoplasm in most insects, PER nuclear entry may be widely true. The regulation of PER subcellular localization in *Drosophila* differs dramatically from that of *A. pernyi* and most other insects (15, 16). The tobacco hawkmoth, *Manduca sexta*, is also atypical in that PER nuclear immunostaining (but not PER rhythmicity) is readily detectable in brain neurons (51). However, regardless of how PER subcellular transport is controlled, a *Drosophila*-like feedback loop may form the core of the circadian clockwork in insects.

Acknowledgements

We thank Sriram Sathyanarayanan for help generating some of the constructs. We thank Aditi V. Chavda, Kurtis N. Gray, and Kasia Macko for expert technical assistance. D.C.C. was supported by a Howard Hughes Medical Institute Predoctoral Fellowship. This work was supported by NIH grant R01 NS39303.
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**Figure Legends**

**Figure 1.A.** Schematic comparison of apCLOCK, dCLOCK, and mCLOCK protein features.

The gray bar represents primary amino acid sequence of each protein (to scale). White box with
"bH" = basic helix-loop-helix (bHLH) domain; remaining white boxes = parts of the PAS domain labeled "A" (PAS-A), "B" (PAS-B), and "C" (PAC); gray box containing "Δ" = the region homologous to exon 19 in mCLK, which is eliminated in Clock mutant mice (generating the mCLKΔ19 mutant protein) (19); "Q" = a poly-glutamine region, where ≥ 60% of the amino acid residues are glutamines; Jrk = the site of the dClkJrk nonsense mutation (4). In the sequence of the "Δ19" region of apCLK, dCLK, and mCLK, amino acids identical in all three species are marked black, those identical in two species are dark gray, and those similar in two or more species are light gray. B. Schematic comparison of apBMAL, CYCLE (dBMAL), and mBMAL1 protein features. The gray bar represents primary amino acid sequence of each protein (to scale). White box with "bH" = basic helix-loop-helix (bHLH) domain; remaining white boxes = parts of the PAS domain labeled "A" (PAS-A), "B" (PAS-B), and "C" (PAC); gray box with "T" = region homologous to the transactivation domain of mBMAL1 (22); called the BMAL C-terminal region (BCTR) in the text. In the sequence of the BCTR of apBMAL and mBMAL1, identical amino acids are marked black and similar amino acids are light gray. C. Schematic comparison of apTIM and dTIM protein features. The gray bar represents primary amino acid sequence (to scale). Black boxes labeled "Arm" = Arm/HEAT domains; gray circle = nuclear localization sequence (NLS); white box labeled "CLD" = region homologous to the cytoplasmic localization domain (CLD) in dTIM. dPER binding sites of dTIM (25) are underlined.

**Figure 2.A.** apPer genomic clone containing the 5' regulatory region. The gray bar represents an ~18 kb genomic DNA clone containing exons 1 and 2 of apPer and three putative E-box elements (CACGTG sequences) upstream of exon 1. Proximal, medial, and distal E-boxes are labeled E_p, E_m, and E_d. Black boxes = apPer exons, labeled 1 and 2; light gray boxes labeled "E"
= CACGTG, putative E-boxes. B. apCLK:apBMAL activate transcription via the most proximal E-box 5' of the apPer gene. Presence (+) or absence (-) of co-transfected apClk-V5 and apBmal (2 ng each plasmid) is indicated to the immediate left of the graph. Luciferase reporter constructs used (described above) are shown further to the left. White box labeled "luc" = luciferase cDNA; white box labeled "hs" = hsp70 minimal promoter; gray boxes = E-boxes, labeled "E" for the dper E-box, E_p for the proximal apPer E-box, E_m for the medial apPer E-box, and E_d for the distal apPer E-box, and with a bold "X" for a mutated E-box. The sequence of each E-box and the flanking regions included in the luciferase construct is shown to the left of the schematics. The luciferase activity relative to the β-galactosidase activity was computed and normalized such that the mean value for the hs-luc construct in the absence of apCLK:apBMAL was 1. Each value is mean ± SEM of three replicates.

**Figure 3.** An essential transactivation domain is found at the C-terminus of apBMAL. Presence (+) or absence (-) of apClk-V5 or apClk 1-364 V5 (1 ng plasmid) is indicated to the immediate left of the graph. Co-transfection (protein illustration) or omittance (-) of 1 ng of apBmal or apBmal deletion mutants is indicated at the far left. The relative luciferase activity (from a luciferase reporter driven by 4 apPer E-boxes, as shown to the far left) was computed relative to the β-galactosidase activity (driven by an actin promoter that lacks E-boxes) and normalized such that the mean value for the apPer 4E_p-hs-luc construct in the absence of apCLK/apBMAL was 1. Each value is the mean ± SEM of three replicates. The numbers associated with each mutant represent the amino acids of full-length apBMAL (589 aa) or apCLK (611 aa) contained in each mutant protein. White box with "b" = bHLH domain, other white boxes = parts of the PAS domain, labeled "A" (PAS-A), "B" (PAS-B), and "C" (PAC); gray box labeled "Δ" = "Δ19"
homology region; gray box labeled "T" = putative transactivation domain (BMAL C-terminal region, BCTR). The results shown are representative of three independent experiments.

**Figure 4.A.** apPER inhibits apCLOCK:apBMAL and low doses of apTIM augment that inhibition. Presence (+) or absence (-) of apClk-V5 and apBmal (1 ng of each plasmid) is indicated to the immediate left of the graph. Presence (# ng plasmid) or absence (-) of apPer-V5 or apTim-V5 is indicated to the far left. The luciferase activity (from the apPer 4E\textsubscript{P}-hs-luc construct) relative to the \( \beta \)-galactosidase activity was computed and normalized such that the mean value in the presence of apCLK:apBMAL alone was 100\%. Each value is mean ± SEM of three replicates. **B.** The C-terminus of apCLOCK is required for apPER-mediated inhibition of apCLOCK:apBMAL. Presence (+) or absence (-) of apClk-V5, apClk 1-364 V5, or apBmal (1 ng plasmid) is indicated in the three columns to the immediate left of the graph. Presence ("20", for 20 ng plasmid) or absence (-) of apPer-V5 or apTim-V5 is indicated in the two leftmost columns. The luciferase activity (from the apPer 4E\textsubscript{P}-hs-luc construct) relative to the \( \beta \)-galactosidase activity was computed and normalized such that the mean value in the presence of apCLK:apBMAL alone was 100\%. Each value is mean ± SEM of three replicates.

**Figure 5.A.** A C-terminal domain in apPER is responsible for its inhibitory action on apCLOCK:apBMAL. Presence (+) or absence (-) of apClk-V5 and apBmal (1 ng of each plasmid) is indicated to the immediate left of the graph. Co-transfection of 100 ng of apPer full-length or deletion mutant constructs is indicated by the protein illustrations and labels at the far left. The luciferase activity relative to the \( \beta \)-galactosidase activity was computed and normalized such that the mean value in the presence of apCLK:apBMAL alone was 100\%. Each value is
mean ± SEM of three replicates. The numbers associated with each mutant represent the amino acids of full-length apPER (849 aa) contained in each mutant protein. White boxes = parts of the PAS domain, labeled "A" (PAS-A), "B" (PAS-B), and "C" (PAC); black box = apCLK:apBMAL inhibition domain. The results shown are representative of three independent experiments. B. apPER nuclear transport is dependent on a bipartite nuclear localization sequence (NLS) C-terminal to PAS. V5-tagged apPer constructs (ca. 300 ng) were transfected into S2 cells and the cellular location of their protein products was assayed by immunocytochemistry using a monoclonal anti-V5 primary antibody, and a Cy3-conjugated secondary antibody. The cells were also DAPI stained to visualize the nuclei. For each cell examined, the V5-tagged protein was classified as having one of three staining patterns: nuclear (N), cytoplasmic (C), or both nuclear and cytoplasmic (B). For each construct, the proportion of cells in each category (N, C, or B) relative to the total number of cells examined was calculated as a percentage; the immunostaining pattern (N, C, or B) of >50% of cells examined is shown in the right-hand column, and the percentage of cells with that staining pattern is shown in parentheses (complete data are given in Table 2). The vertical dashed lines indicate the NLS-containing region (aa 684-738) determined using deletion mutants. The sequence of this region is shown below the constructs. The basic amino acids (lysine, K; arginine, R; histidine, H) are underlined, mutations to alanine are indicated above the sequence, and functional components of the bipartite NLSs, as shown by mutagenesis, are indicated below the sequence. White boxes = parts of the PAS domain, labeled "A" (PAS-A), "B" (PAS-B), and "C" (PAC); black box = apCLK:apBMAL inhibitory domain; "X" = mutagenized putative NLS.
Figure 6.A. Model of CLOCK and BMAL evolution. The tree represents the known phylogenetic relationships among the species indicated at the far right. Next to each species name are symbolic representations of the CLOCK and BMAL homologs present in that animal. CLOCK and BMAL symbols at other points on the tree represent the homologs believed to be present in the ancestral animal at that place in the phylogenetic scheme. Black boxes = transactivators; circle labeled "+" = transactivation domain; white boxes = binding partner lacking a transactivation domain.  

B. Model of circadian clock function in A. pernyi. There are three E-boxes (black bars) in the 5' regulatory region of the apPer gene (horizontal black line). apCLK (white box labeled "CLK") and apBMAL (black box labeled "BMAL" with transactivation domain shown as circle labeled "+") bind and activate transcription specifically from the most proximal E-box. The apPer mRNA (wavy line) translocates to the cytoplasm (above the curved line), where it is translated into apPER protein (gray boxes labeled "PER" with inhibitory domain shown as circle labeled "-"). Some apPER enters the nucleus (below the curved line), where it can inhibit apCLK:apBMAL, thus shutting off its own transcription. Our inability to detect apPER in the nucleus of A. pernyi brain neurons may be due to high rates of nucleus-specific degradation and/or nuclear export (white arrows) and/or cytoplasmic sequestration of apPER.

Supplementary Figure S1. Sequence comparison of insect PER NLSs. Insect species are designated by the prefixes: "ap" = Antheraea pernyi (apPER in GenBank accession AAA64675), "dm" = Drosophila melanogaster (dmPER in GenBank accession P07663), "md" = Musca domestica (house fly; mdPER in GenBank accession AAD39163), "am" = Apis mellifera (honeybee; amPER in GenBank accession AAF70859), "pa" = Periplaneta americana
(American cockroach; PaPER in GenBank accession AAA64677), "bg" = *Blattella germanica* (German cockroach; bgPER in GenBank accession AAN02439). Black regions represent amino acids identical in all six insect species listed, dark gray regions represent amino acids identical in at least three species, and light gray regions represent similar but not identical amino acids. The two parts of the bipartite NLS are indicated at the bottom.

**Supplementary Figure S2.** A C-terminal domain in apPER is responsible for its inhibitory action on dCLK:CYC. Presence (+) or absence (-) of 1 ng pAct-\textit{dClk} plasmid (3) is indicated to the immediate left of the graph. \textit{cyc} is endogenously expressed in S2 cells. Co-transfection of 100 ng of \textit{apPer} full-length or deletion mutant constructs is indicated by the protein illustrations and labels at the far left. The luciferase activity, generated by the \textit{dper} 4E-hs-luc construct provided by Steve Kay (3), relative to the \(\beta\)-galactosidase activity was computed and normalized such that the mean value in the presence of \textit{dClk} alone was 100%. Each value is mean ± SEM of three replicates. The numbers associated with each mutant represent the amino acids of full-length apPER (849 aa) contained in each mutant protein. White boxes = parts of the PAS domain, labeled "A" (PAS-A), "B" (PAS-B), and "C" (PAC); black box = apCLK:apBMAL inhibition domain. The results shown are representative of three independent experiments.

### Tables

**Table 1: Domain-by-domain comparison of amino acid identities among CLOCK/BMAL/TIMELESS homologs.** Peptide sequences for apCLK, dCLK, and mCLK were aligned using the Pileup program in the Genetics Computing Group (GCG) software package. Peptide sequences for apBMAL, CYC, and mBMAL1, and sequences for apTIM and
dTIM were aligned using the same software. bHLH, PAS-A and PAS-B regions were
demarcated according to the SMART protein domain analysis available on the NCBI website
(http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The PAS linker is the region between
PAS-A and PAS-B. The C-terminus of CLK or BMAL was defined as the entire peptide
sequence downstream of the PAS domain. The "Δ19" region was defined according to the entire
exon 19 of mCLOCK (19). The BCTR was defined as the final 40 amino acids of apBMAL
(which corresponds to the final 39 amino acids of mBMAL1). The Arm/HEAT domains were
defined according to the analysis of Vodovar et al. (24), and the "Arm linker" is the region
between the two Arm/HEAT domains. The C-terminus of TIM was defined as the peptide
sequence downstream of Arm/HEAT 2. The CLD was defined according to the deletion mutant
mapping of dTIM by Saez and Young (25). Sequence gaps were counted as amino acids in the
calculation of % identity. GenBank sequences used in this comparison were: apCLK:
AY330486, dCLK: AAD10630, mCLK: O08785, apBMAL: AY330487, CYC: AAC39124,
mBMAL1: BAA81898, apTIM: AAF66996, dTIM: P49021.

<table>
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<th>apCLK v. mCLK</th>
<th>dCLK v. mCLK</th>
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<td>18 %</td>
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<td>bHLH</td>
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<td>67 %</td>
<td>59 %</td>
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<td>84 %</td>
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<tr>
<td>C-terminus</td>
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<td>&quot;Δ19&quot; region</td>
<td>57 %</td>
<td>31 %</td>
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For the comparisons of apBMAL and dCYC, mBMAL1:

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<th>apBMAL v. mBMAL1</th>
<th>dCYC v. mBMAL1</th>
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<tr>
<td>Whole protein</td>
<td>34 %</td>
<td>38 %</td>
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Table 2: Subcellular localization of V5-tagged apPER constructs in S2 and Sf9 cells.

S2 cells seeded onto glass coverslips were transiently transfected with ca. 300 ng plasmid for the expression of the indicated C-terminal V5-tagged apPER or apTIM constructs or N-terminal V5-tagged apBMAL. Sf9 cells (last three rows) were transfected with ca. 1 µg plasmid. Numerical intervals in the leftmost column indicate which amino acids in the 849 aa apPER or the 589 aa apBMAL are present in the expressed protein. Two days after transfection, the cells were processed for immunocytochemistry using a monoclonal anti-V5 primary antibody, and a Cy3-conjugated secondary antibody. The cells were also DAPI stained to visualize the nuclei. Slides were viewed under a fluorescence microscope (Olympus IX70) and 30 cells per slide were categorized as having primarily nuclear staining (N), cytoplasmic staining (C) or staining in both nucleus and cytoplasm (B). Cells were scored blind to which construct was transfected into
them. The scores were converted into percentages (rounded to the nearest integer) and the mean and standard error of three or more replicates are shown on the table.

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Figure 6
Supplementary Figure S1

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<td>bgPER</td>
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NLS
Supplementary Figure S2
Constructing a feedback loop with circadian clock molecules from the silkmoth, *Antheraea pernyi*

Dennis C. Chang, Harriet G. McWatters, Julie A. Williams, Anthony L. Gotter, Joel D. Levine and Steven M. Reppert

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