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Molecular Mechanism of Cell-autonomous Circadian Gene Expression of *Period2*, a Crucial Regulator of the Mammalian Circadian Clock[D]

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Although circadian transcription of *Period2* (*Per2*) is fundamental for the generation of circadian rhythm, the molecular mechanism remains unclear. Here we report that cell-autonomous circadian transcription of *Per2* is driven by two transcriptional elements, one for rhythm generation and the other for phase control. The former contains the E-box-like sequence (CACGTT) that is sufficient and indispensable to drive oscillation, and indeed circadian transcription factors site-specifically bind to it. Furthermore, the nature of this atypical E-box is different from that of the classical circadian E-box. The current feedback loop model is based mainly on *Period1*. Our results provide not only compelling evidence in support of this model but also an explanation for a general basic mechanism to produce various patterns in the phase and amplitude of cell-autonomous circadian gene expression.

INTRODUCTION

In nearly all organisms, behavioral and physiological processes display ~24 h rhythms that are controlled by circadian pacemakers (Pittendrigh, 1993). The circadian organization of physiology and behavior in mammals is governed by the suprachiasmatic nuclei (SCN), a defined pair of cell clusters in the anteroventral hypothalamus (Ralph *et al.*, 1990). Circadian clocks can count time only approximately and must be adjusted every day by the photoperiod in order to be in harmony with the outside world (Menaker, 2003). Circadian oscillators also exist in most peripheral cells and even in cultured cells (Balsalobre *et al.*, 1998; Yamazaki *et al.*, 2000). It is thought that the phase of these peripheral timekeepers is reset by signals regulated by the SCN pacemaker (Akashi and Nishida, 2000; Schibler and Sassone-Corsi, 2002).

The molecular makeup of circadian clocks has been the subject of intense genetic and biochemical investigation in various organisms, including cyanobacteria, *Neurospora*, higher plants, *Drosophila*, and mammals (Dunlap, 1999; Kondo and Ishiura, 2000; Allada *et al.*, 2001; Williams and Sehgal, 2001; Young and Kay, 2001; Reppert and Weaver, 2002). Over the last several years, orthologues of most *Drosophila* circadian clock genes have been cloned from mammals (Albrecht and Eichele, 2003; Lowrey and Takahashi, 2004). Although *mPer2* was literally cloned as a secondary mammalian *period* gene (Albrecht *et al.*, 1997; Takumi *et al.*, 1998), gene-knockout analysis revealed that an *mPer2* mutant displays a loss of circadian rhythmicity, revealing a prominent role for mPER2 in the mammalian clock (Zheng *et al.*, 1999). Additionally familial advanced sleep phase syndrome has been attributed to a missense mutation in *hPer2* (Toh *et al.*, 2001). These studies demonstrate that a robust circadian fluctuation in *Per2* transcription is an essential event for the generation of circadian rhythm.

Circadian oscillators appear to have been highly conserved throughout evolution and to involve transcription–translation negative feedback loops for the regulation of clock genes (Dunlap, 1999; Young and Kay, 2001). In mammals, in vitro studies have shown that the expression of *Per1* (*Period1*) is driven by the CLOCK/BMAL1 transcription complex through an E-box enhancer and that PER proteins, together with CRY (Cryptochrome) proteins, serve to regulate the CLOCK/BMAL1 transcription complex negatively (Gekakis *et al.*, 1998; Kume *et al.*, 1999; Hida *et al.*, 2000). This model has been thought to be applicable to other *Per* and *Cry* genes. However, several problems remain to be solved in the current model. First, there is a wide variation in the phase and amplitude of circadian accumulation of *Per1*, *Per2*, *Per3*, *Cry1*, and *Cry2* mRNA levels in tissues and cultured cells (Yamamoto *et al.*, 2004). The current feedback loop model in mammals cannot explain the mechanism to generate this variation. It is importantly to note that the phase and amplitude of circadian transcription cannot be verified by using an overexpression-based transient reporter assay. Second, although *Per1*, which has been analyzed in detail, serves as a foundation for the mammalian model, it has been reported recently that *Per1* is not absolutely required for the generation and maintenance of circadian rhythms (Cermakian *et al.*, 2001; Zheng *et al.*, 2001). This issue remains controversial, therefore, because Bae *et al.* (2001) found that the loss of *Per1* can result in arrhythmic mice. Thus, it is unclear whether the current model really reflects the core clock mechanism. Gene knockout studies of clock genes support the current model; for example, genetic deficiency of *Bmal1* results in the down-regulation of *Per* expression (Vitaterna *et al.*, 1999; Bunger *et al.*, 2000; Bae *et al.*, 2001). However, these studies cannot

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prove whether the regulation is direct or indirect. Overexpression-based transient reporter assay has shown that transcription of the *Per2* gene, a key component for rhythm generation, is also up-regulated by coexpression of circadian transcription factors (BMAL1, CLOCK, and NPAS2), as shown for the *Per1* gene (Travnickova-Bendova *et al.*, 2002; Kaasik and Lee, 2004). Yet, although *Per1* contains 5 classical circadian E-boxes (Hida *et al.*, 2000; Yamamoto *et al.*, 2004), no evolutionarily conserved E-box (the classical circadian E-box) has been identified in the upstream sequence of *Per2*. Therefore it remains unknown whether these circadian transcription factors directly activate the *Per2* gene in a site-specific manner.

Herein we report the detailed analysis of the mechanism of a robust cell-autonomous circadian fluctuation in *Per2* transcription, not by transient reporter assays but by monitoring transcriptional fluctuation of the luciferase reporter gene over several days. An ~20-base pair region located near the transcription start site (TSS) was indispensable to drive cell-autonomous rhythmic transcription of *Per2*, whereas another region, located upstream from it, was shown to be responsible for phase control of cell-autonomous circadian transcription. An E-box-like sequence exists in the core region (the former region), and indeed circadian transcription factors activated *Per2* transcription through site-specific binding to this element. The fact that this identified small region, indispensable for cell-autonomous rhythmic transcription of *Per2*, contains a functional E-box-like sequence is of great significance. Our results thus validate the current model by demonstrating that this model is actually applicable to cell-autonomous circadian fluctuation of *Per2*, a core component for the generation of circadian rhythm. We also show that this atypical E-box has properties different from those of the classical circadian E-box, which result in a pattern of circadian transcription different from that of *Per1*. Our results provide not only compelling evidence in support of the current feedback loop model in mammals but also an explanation for a general mechanism that generates wide variation in the expression patterns of circadian genes.

MATERIALS AND METHODS

Plasmid Construction

A bacterial artificial chromosome (BAC) clone containing the complete genomic sequence of the mouse *Per2* (*mPer2*) gene was purchased from the BACPAC Resource Center (BPRC) at Children's Hospital Oakland Research Institute. The *mPer2* promoter region was isolated and cloned in the pGL3-Basic vector (Promega, Madison, WI). The *mPer2* region spans from −2811 to +110 (+1 is the putative TSS).

Cell Culture, Transfection, and Reporter Assay

NIH3T3 cells were cultured and transfected as described previously (Akashi *et al.*, 2002). For real-time PCR analyses, cells were immediately frozen in liquid nitrogen and stored at −80°C until processed for RNA. Cell lysates were used in the Dual Luciferase assay System (Promega) as described previously (Akashi and Takumi, 2005).

Real-Time Monitoring of Luciferase Activity in Living Cells

NIH3T3 cells were cultured, transfected with *mPer2*-luc and incubated for 24 h. Then the medium was exchanged for serum-rich medium (DMEM, supplemented with 50% serum). Two hours later this medium was replaced with normal culture medium. In the presence of 0.1 mM luciferin, light emission was measured and integrated for 1 min at intervals of 15 min with a photomultiplier tube (Hamamatsu Photonics, Hamamatsu, Japan) as described previously (Akashi and Takumi, 2005).

Data Analysis

Phase and period measurements were calculated as in previous studies (Abe *et al.*, 2002; Yamazaki *et al.*, 2002; Yoo *et al.*, 2004). Data sets were detrended by subtracting the 24-h running average from the raw data. The maximum differences between the smoothed curves for each cycle (the peak and the trough) were used to calculate the amplitude of each cycle.

Animals

Mice were housed under a strict 12:12 h light/dark condition. Tissues were immediately frozen in liquid nitrogen and stored at −80°C until processed for RNA. All protocols of experiments using animals in this study were approved by the OBI (Osaka Bioscience Institute) Animal Research Committee.

Pulldown Experiment

Mouse liver extracts were prepared at 4-h intervals by homogenizing the tissue in ice-cold incubation buffer (Akashi *et al.*, 2002), and then the extracts were incubated with an 80-base pair double-stranded biotinylated oligonucleotide that had been immobilized on streptavidin-Sepharose beads (Amersham, Piscataway, NJ). After having been washed with the incubation buffer, the resulting bound protein was subjected to immunoblot analysis. The designed sequences (response elements underlined) were the following: *wild-type* (*mPer2*, −105 to +15): 5′CTCAGGTTCCGCCCCGCCAGTATGCAAATGAGGTGGCACTCCGACCAATGGCGCGCGCAGGGGCGGGCTCAGCGCGCGCGGTCACGTTTTCCACTATGTGACAGCGGAGGGCGACGCGGC3′; *mutant* (*mPer2*, −105 to +15): 5′CTCAGGTTCCGCCCCGCCAGTATGCAAATGAGGTGGCACTCCGACCAATGGCGCGCGCAGGGGCGGGCTCAGCGCGCGCGGTCTTTCCACTATGTGACAGCGGAGGGCGACGCGGC3′; *wild-type* (*mPer1*, 3E-box): 5′GAAAGCTTTAGCCACGTGACAGTGAGGGGCACCCCTTAACGACACGTGGGCCCTCAATTGAGCACCCAAGTCCACGTGCAGGGATGTGTGGGGGCAGGGCCTGGCATTATGCAACCCGCCTCCCAGCCTC3′; and *mutant* (*mPer1*, 3E-box): 5′GAAAGCTTTAGCCGACAGTGAGGGGCACCCCTTAACGACGGGCCCTCAATTGAGCACCCAAGTCCGCAGGGATGTGTGGGGGCAGGGCCTGGCATTAACCCGCCTCCCAGCCTC3′.

Real-Time Quantitative RT-PCR

Quantification of relative RNA levels by the SYBER Green real-time PCR technology was done as described previously (Yamamoto *et al.*, 2004). Briefly, DNase-treated total RNA (2.5 μg) was reverse-transcribed by using an oligo(dT) primer and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA equivalent to 20 ng total RNA was PCR-amplified in an ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Forward primers and reverse primers were as follows: *Gapdh* forward: 5′-CATCCACTGGTGCTGCCAAGGCTGT-3′; *Gapdh* reverse: 5′-ACAACCTGGTCCTCAGTGTAGCCCA-3′; *mPer1* forward: 5′-CAGGCTAACCAGGAATATTACCAGC-3′; *mPer1* reverse: 5′-CACAGCCACAGAGAAGGTGTCCTGG-3′; *mPer2* forward: 5′-GGCTTCACCATGCCTGTTGT-3′; and *mPer2* reverse: 5′-GGAGTTATTTCGGAGGCAAGTGT-3′.

The relative levels of each RNA were normalized to the corresponding *Gapdh* RNA levels. Relative RNA levels were then expressed as percentage of the maximal value obtained for each experiment.

RESULTS AND DISCUSSION

Cell-autonomous Circadian Transcription of Per2 Is Regulated by Two or More Transcriptional Regulatory Elements

The core clock is a cell-autonomous system. Therefore, we should examine whether transcriptional regulatory elements function under a cell-autonomous condition. Our approach enables us to exclude internal environmental cues such as blood-borne factors and body temperature. To identify the transcriptional regulatory elements for cell-autonomous circadian transcription of *Per2*, we monitored transcriptional fluctuation of *Per2* in real-time by using a deletion series of *Per2* promoter-reporter constructs (Figure 1A). Note that exogenously transfected transgenes have no effect on endogenous cell-autonomous circadian oscillation and, therefore, that the deletion or mutation of constructs does not affect the endogenous pacemaker. NIH3T3 cells were transfected with the *Per2*-luc construct and then stimulated with a high concentration of serum. After the serum shock, in the presence of luciferin, light emission was measured and integrated for 1 min at intervals of 15 min. By using this in vitro luminescence reporter system, we can monitor cell-autonomous oscillators. Clearly, *Per2*-controlled fluctuations of luminescence resulted in a series of readily appreciable peaks and troughs, as exemplified in Figure 1. Almost the same phases

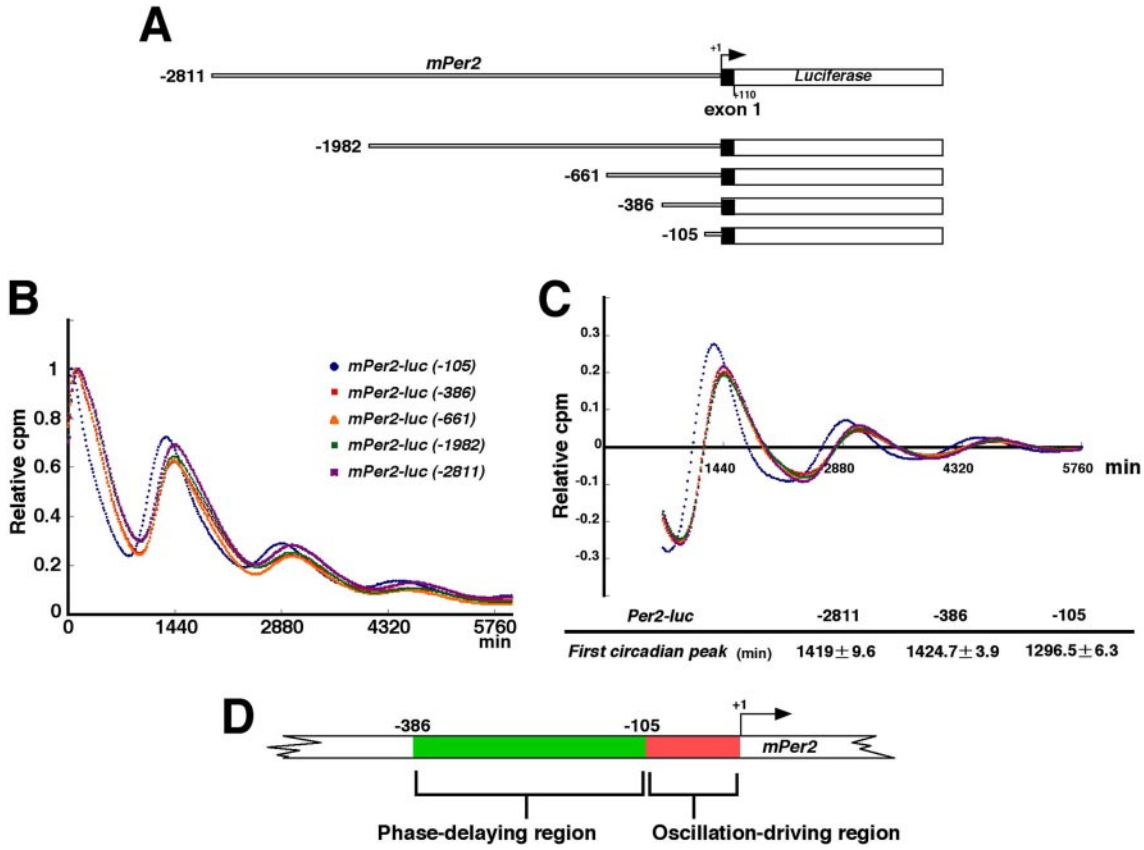

Figure 1. A phase-delaying element and a rhythm-generating element are required for robust cell-autonomous circadian gene expression of *Per2*. (A) Schematic representation of deletion mutants of the *mPer2* promoter. +1 corresponds to the transcription start site. (B) Transcriptional oscillation of *mPer2* was monitored by using the cell culture-based luminescent reporter assay. NIH3T3 cells were transfected with the *mPer2*-luc construct and then stimulated with a high concentration of serum. After the serum shock, in the presence of luciferin, light emission was measured and integrated for 1 min at intervals of 15 min (vertical scale: relative cpm; horizontal scale: 1440 min = 1 d). Peak values of the curves were set to 1. A representative result of three independent experiments is shown. (C) The signals obtained in B were detrended. The time of the first peak was calculated as a phase marker (mean ± SEM; n = 4). (D) Schematic representation of the results obtained from the deletion analysis.

and amplitudes were observed in the cells transfected with the constructs from −2811 to −386, whereas a phase advance (~2 h earlier) was detected only when using the *Per2* (−105) construct (Figure 1B). To better calculate phase differences, data sets were detrended, and the time of the first peak was used as a phase marker (Figure 1C). Again the *Per2* (−105) construct showed a 2-h phase advance compared with the other constructs, as expected in Figure 1B. Thus, these data suggest that a phase delaying element is located between −386 and −106 base pairs upstream from the TSS(s) and that a rhythm-generating element exists between −105 and +1 base pairs upstream from the TSS(s) (Figure 1D). These results demonstrate that two or more transcriptional regulatory elements, i.e., a phase-delaying element and a rhythm-generating element located upstream from the TSS(s), are required for cell-autonomous circadian gene expression of *Per2*. Consistent with this cell-autonomous phenomenon, Yoo *et al.* (2005) has very recently reported that a 210-base pair fragment upstream from the TSS(s) drives *Per2* circadian oscillation in vivo.

An ~20-Base Pair Region Located near the TSS(s) Was Indispensable to Drive Cell-autonomous Rhythmic Transcription of Per2

To identify transcription factors required to drive cell-autonomous rhythmic transcription of *Per2*, we exactly narrowed the rhythm-generating region by monitoring in real-time *Per2*-controlled fluctuations of luminescence in cells transfected with another set of constructs, in which a fragment from *Per2* (−105) was inserted into the upstream of the SV40 promoter (Figure 2, A and C). When 40-base pair fragments overlapping by 20 base pairs were used (Figure 2A), the *Per2* (−45 to −6)-SV40-luc construct and the *Per2* (−25 to +15)-SV40-luc construct clearly showed *Per2*-controlled fluctuations of luminescence as compared with the other constructs (Figure 2B). The detrended data suggests that the SV40 promoter activity exhibits not a drastic but significant circadian fluctuation (Supplementary Figure 1A), indicating that this promoter contains some circadian enhancer elements. The detrended bioluminescence data sets make clear that only the *Per2* (−45 to −6)-SV40-luc and *Per2* (−25 to +15)-SV40-luc construct exhibit a higher amplitude of oscillation than the SV40 promoter. Next, to compare the magnitude of fluctuation in these two constructs, the amplitude in each cycle was calculated by subtracting the value of the trough from that of the peak (Supplementary Figure 1B). The higher amplitude of oscillation was maintained over the cycles in *Per2* (−45 to −6)-SV40-luc. The *Per2* (−45 to −6)-SV40-luc/*Per2* (−25 to +15)-SV40-luc amplitude ratio was obtained by dividing the *Per2* (−45 to −6)-SV40-luc wave amplitude by the *Per2* (−25 to +15)-SV40-luc wave amplitude in each

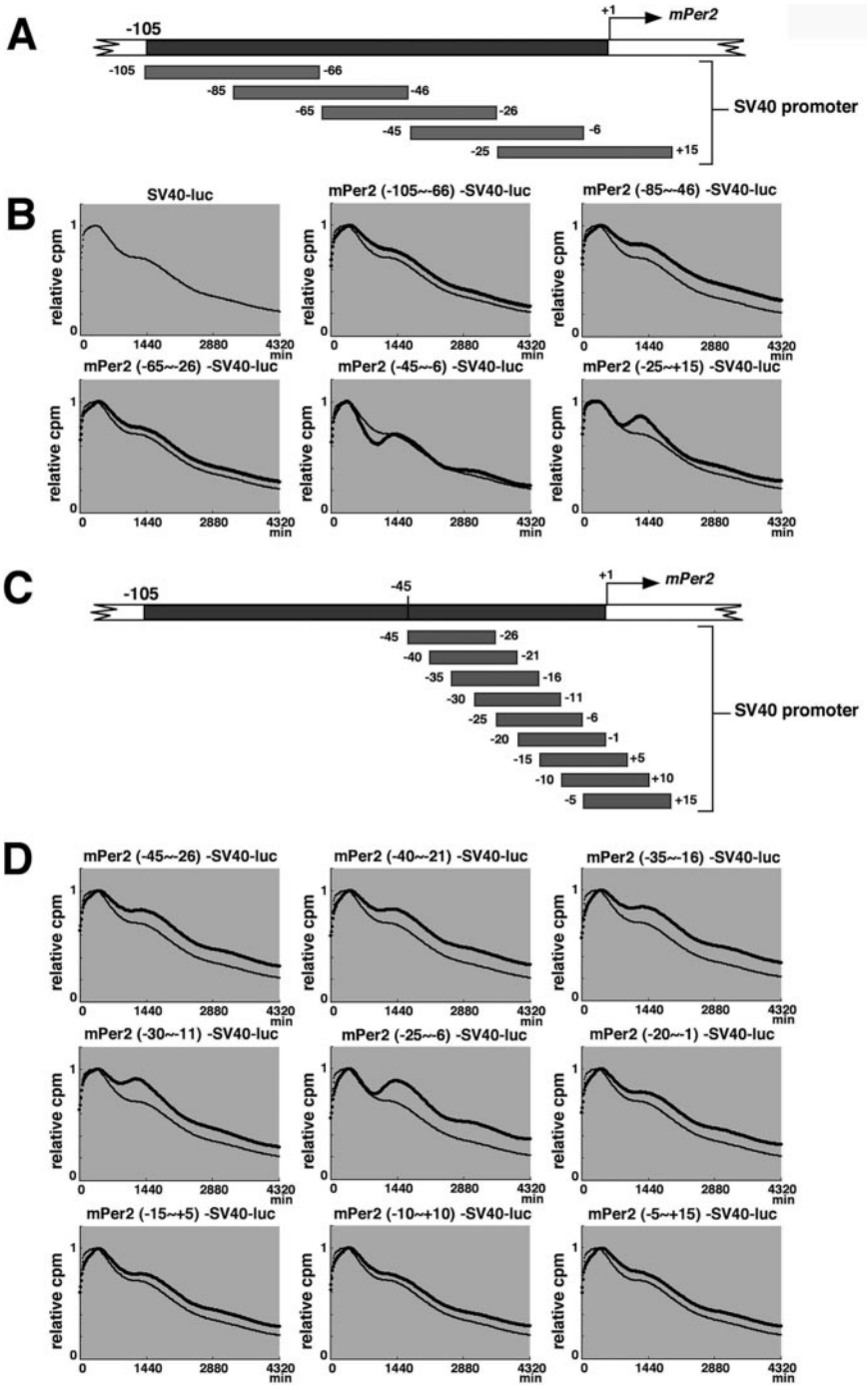

Figure 2. Identification of the region responsible for driving cell-autonomous circadian oscillation of *Per2* transcription. (A) Schematic representation of a set of constructs in which a 40-base pair fragment from *Per2* (−105 to +15) was inserted into the upstream of the SV40 promoter attached to the luciferase gene. These fragments overlap by 20 base pairs. (B) Transcriptional oscillation of *mPer2*-SV40-luc was monitored. NIH3T3 cells were transfected and then stimulated with a high concentration of serum. After the serum shock, light emission was measured and integrated for 1 min at intervals of 15 min (vertical scale: relative cpm; horizontal scale: 1440 min = 1 d). Peak values of the curves were set to 1. A representative result of three independent experiments is shown. For accurate comparison, thin lines show the curve for "SV40-luc." (C) Schematic representation of another set of constructs, in which a 20-base pair fragment from *Per2* (−45 to +15) was inserted into the upstream of the SV40 promoter attached to the luciferase gene. These fragments overlap by 15 base pairs. (D) Transcriptional oscillation of *mPer2*-SV40-luc was monitored. Peak values of the curves were set to 1 (horizontal scale: 1440 min = 1 d). A representative result of three independent experiments is shown. For accurate comparison, thin lines show the curve for "SV40-luc."

cycle (Supplementary Figure 1C), demonstrating that the amplitude of *Per2* (−45 to −6)-*SV40-luc* gradually grew to be twofold higher than that of *Per2* (−25 to +15)-SV40-luc, as cycle number increases. Taken together, the region ranging from −45 to +15 was shown to possess the ability to drive circadian oscillation of transcription.

Next, to further define this region, we used 20-base pair fragments overlapping by 15 base pairs (Figure 2C). The results show that the *Per2* (−25 to −6) fragment possessed the most potent ability to oscillate SV40 promoter activity (Figure 2D). The detrended data highlighted the *Per2* (−25 to −6) and *Per2* (−30 to −11) fragments' ability to drive oscillation (Supplementary Figure 1D). The *Per2* (−25 to −6)-SV40-luc maintained a higher amplitude in each cycle than that of *Per2* (−30 to −11)-SV40-luc (Supplementary Figure 1E). Additionally, the *Per2* (−25 to −6)-SV40-luc/*Per2* (−30 to −11)-SV40-luc amplitude ratio in each cycle demonstrated that the amplitude of *Per2* (−25 to −6)-SV40-luc was also considered, being twofold higher over cycles than that of *Per2* (−30 to −11)-SV40-luc (Supplementary Figure 1F). The merge of *Per2* (−25 to −6)-SV40-luc (Supplementary Figure 1D) and *Per2* (−45 to −6)-SV40-luc (Supplementary Figure 1A) illustrated almost the same pattern of oscillation, confirming that the *Per2* (−25 to −6) region is necessary and sufficient to drive *Per2* (−45 to −6)-SV40-luc oscillation (Supplementary Figure 1G). This region (−25 to

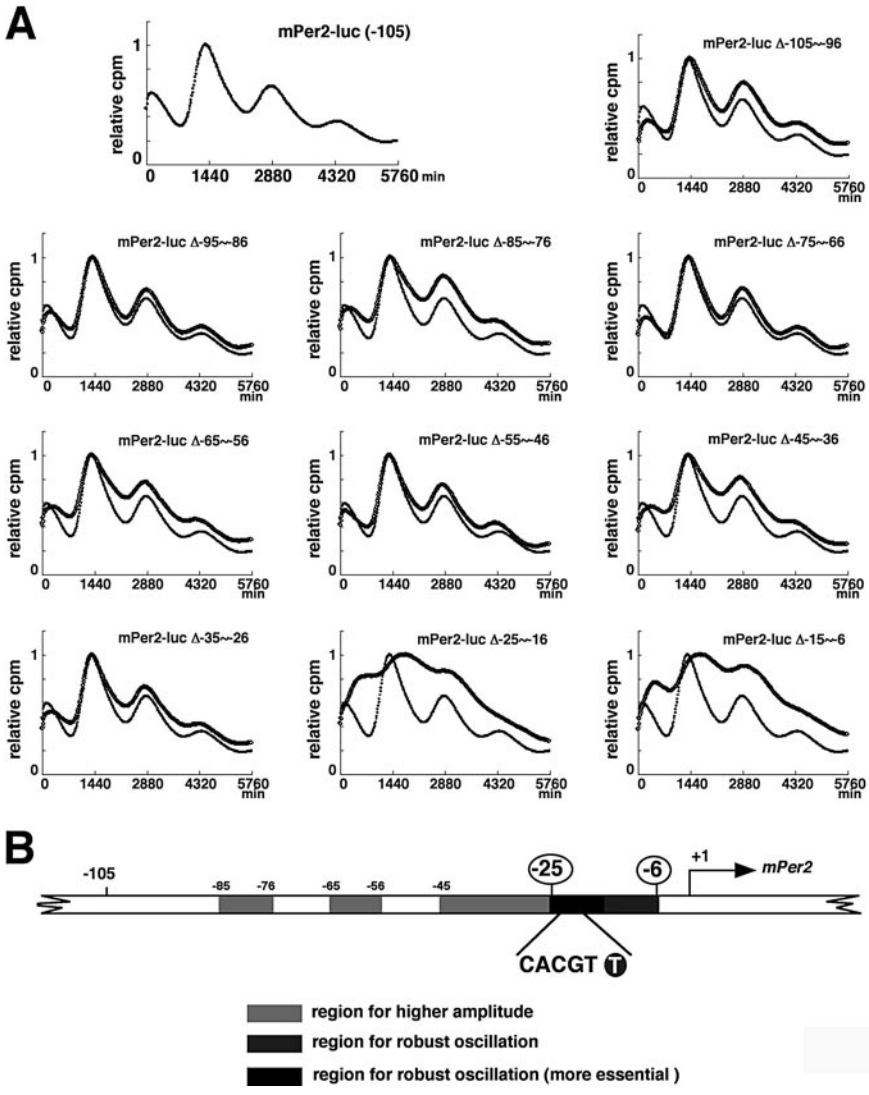

Figure 3. Mapping of the rhythm-generating element by using deletion mutants of *Per2*. (A) Transcriptional oscillation of a series of *mPer2*-luc 10-base pair deletion constructs was monitored in real time. NIH3T3 cells were transfected and then stimulated with a high concentration of serum. Peak values of the curves were set to 1 (vertical scale: relative cpm; horizontal scale: 1440 min = 1 d). A representative result of three independent experiments is shown. For accurate comparison, thin lines show the curve for "*mPer2*-luc (−105)." (B) Schematic representation of the results obtained from the 10-base pair deletion analysis, taken together with those in Figure 2. Sequence inspection reveals an E-box-like sequence (CACGTT) in the region essential for circadian transcription of *Per2*.

−6), therefore, contains the core element for rhythmic transcription of *Per2*.

We also constructed mutants lacking 10 base pairs in the −105 to +6 region, and monitored transcriptional fluctuation of *Per2* in real-time (Figure 3A). The amplitude of circadian transcription of *Per2* was strongly diminished in *mPer2*-luc Δ (−25 to −16) and *mPer2*-luc Δ (−15 to −6). Furthermore, both of these two constructs exhibited a phase delay, illustrating that both 10-base pair regions contribute to maintain the original phase. Other regions were required for amplification, whereas these two regions were essential for driving rhythmic transcription of *Per2*. After removal of baseline changes (Supplementary Figure 2A), the period was obtained from regression analysis of a circadian marker (trough) in Supplementary Figure 2B. As compared with wild-type *Per2*-luc, *mPer2*-luc Δ (−95 to −86), *mPer2*-luc Δ (−85 to −76), *mPer2*-luc Δ (−75 to −66), *mPer2*-luc Δ (−65 to −56), *mPer2*-luc Δ (−55 to −46), and *mPer2*-luc Δ (−45 to −36) showed a 20–40-min shorter period. Notably, the −75 to −46 region seemed to largely contribute to the determination of period length. Basically, exogenously transfected transgenes have no effect on endogenous cell-autonomous circadian oscillation. Therefore, these slight changes in period lengths may be attributable to desynchronization of reporter expression from the endogenous oscillator and elicitation of transcriptional noise, because these mutations resulted in loss of robust transcriptional oscillation. The amplitude of *mPer2*-luc Δ (−85 to −76), *mPer2*-luc Δ (65 to −56), *mPer2*-luc Δ (−45 to −36), or *mPer2*-luc Δ (−35 to −26) was significantly smaller over cycles than that of wild-type *Per2*-luc (Supplementary Figure 2C), and deletion of −15 to −6 or −25 to −16 caused a remarkable inhibition of circadian fluctuation, as shown in Figure 3A. The others showed only a slight decrease of the initial amplitude. To compare the damping pattern in the constructs, the amplitude in the initial cycle was set to 100 (Supplementary Figure 2D). Although *mPer2*-luc Δ (−25 to −16) showed a slightly irregular damping pattern, the others had almost the same damping pattern as that of wild-type *Per2*-luc. Taken together, the −85 to −76, −65 to −56, −45 to −36, and −35 to −26 regions work to enhance the amplitude of transcriptional oscillation, whereas the −15 to −6 and −25 to −16 regions are most essential to drive circadian oscillation. There are no regions that obviously affect the period length and damping rate.

To rule out the possibility that these mutations disrupt the core promoter activity, we examined the basal transcriptional activity of these constructs by conducting reporter

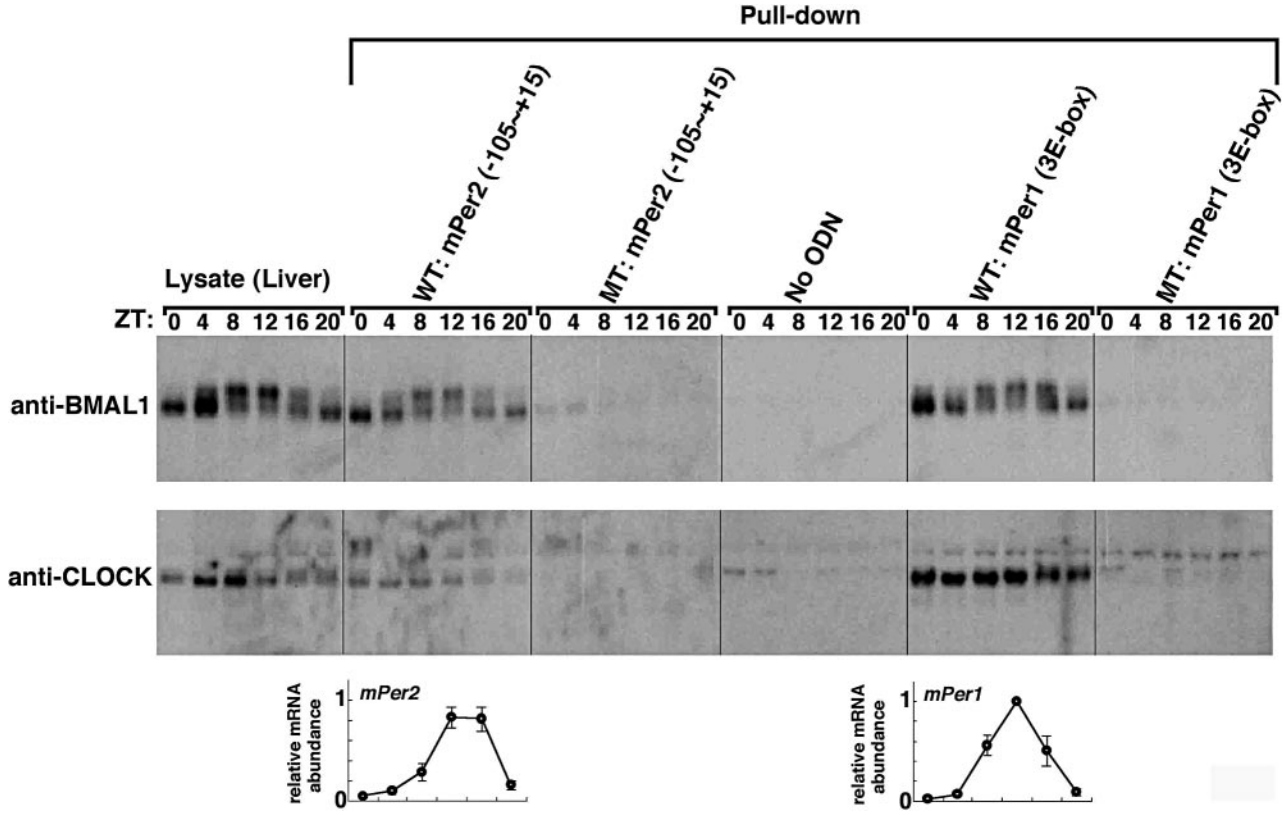

Figure 4. Temporal patterns of the site-specific binding of endogenous circadian transcription factors to the *Per2* E-box-like sequence. Mouse liver extracts were harvested at 4-h intervals and then subjected to immunoblot analysis with anti-BMAL1 antibody (top panels) or anti-CLOCK antibody (middle panels). Mouse liver extracts were incubated with a double-stranded biotinylated oligonucleotide including the consensus-predicted Per2 E-box-like sequence (CACGTT) or three different *Per1* E-boxes (wild-type, WT; mutant, MT), which was immobilized on streptavidin-Sepharose beads. The negative control samples were treated with the beads without an oligonucleotide (No ODN). The resulting precipitates were subjected to immunoblot analysis with anti-BMAL1 antibody (top panels) or anti-CLOCK antibody (middle panels). Temporal expression patterns of the *mPer2* and *mPer1* genes in mouse liver were assayed by real-time quantitative RT-PCR (bottom panels). Each value represents the average of three independent RT-PCR experiments. The relative levels were normalized to the corresponding *Gapdh* RNA levels. Peak values of the *mPer2* and *mPer1* curves were set to 1.

experiments. These mutations did not interfere with the core promoter activity, because these constructs did not show severe attenuation of the transcriptional activity found for the full-length construct (Supplementary Figure 3). These results are consistent with those in Figure 2, B and D. Taken together, our data indicate that the −25 to −6 region upstream from the TSS is indispensable for robust cell-autonomous circadian gene expression of *Per2*. Sequence inspection revealed an E-box-like sequence (CACGTT, instead of the known clock E-box sequence CACGTG) in this region (Figure 3B).

Endogenous Circadian Transcription Factors Bind Site-specifically to the Per2 E-Box-like Sequence

To examine whether circadian transcription factors indeed bind to the *Per2* E-box-like sequence, we investigated the binding of endogenous BMAL1 and CLOCK to *Per2* (−105 to +15) double-stranded DNA fragments immobilized on streptavidin beads (Figure 4). After having been entrained to LD (12-h light/12-h dark) cycles, BALB/c mice were transferred to DD (constant dark). Liver lysates were prepared at 4-h intervals and immunoblotted with anti-BMAL1 or anti-CLOCK antibody. The shifted bands correspond to phosphorylated BMAL1, as reported recently (Lee *et al.*, 2001; Kondratov *et al.*, 2003; Tamaru *et al.*, 2003). The phosphorylation levels peaked at ZT (Zeitgeber Time) 8 to ZT12. By pulldown experiments using the *Per2* promoter fragment immobilized to beads, protein precipitation of BMAL1 and phosphorylated BMAL1 was observed. The peak of phosphorylated BMAL1 bound to the *Per2* promoter correlated with that of *Per2* mRNA expression in the liver (bottom panel). This correlation indicates that phosphorylated BMAL1 activated transcription of the *Per2* gene, consistent with the report that formation of the CLOCK/BMAL1 complex is followed by their codependent phosphorylation (Kondratov *et al.*, 2003). When we used the *Per2* promoter fragment with a mutated E-box-like sequence, precipitated BMAL1 protein and phosphorylated BMAL1 were almost completely undetectable. Thus, BMAL1 and phosphorylated BMAL1 were confirmed to specifically recognize and bind to the *Per2* E-box-like sequence. We also confirmed that the BMAL1-CLOCK-mediated transcription of the *Per2* gene was dependent on this E-box-like sequence by performing traditional luciferase assays (unpublished data). As a positive control, when a DNA fragment containing three different *Per1* E-boxes was used for pulldown assays, a similar

pattern of BMAL1 binding was detected. Mutation of these *Per1* E-boxes completely inhibited BMAL1 binding. As observed in the *Per2* pulldown assays, the peak of phosphorylated BMAL1 bound to the *Per1* E-boxes correlated with that of *Per1* mRNA expression (bottom panel). Our results indicate that the *Per2* E-box-like sequence, as well as the classical circadian E-box, binds to endogenous circadian transcription factors and that transcriptional activation of *Per2* and *Per1* correlates with phosphorylation of BMAL1.

The Per2 E-Box-like Sequence Has Functional Characteristics Different from Those of the Classical Circadian E-Box

Both *Per2* and *Per1* mRNA expression showed circadian oscillations with high amplitude in peripheral tissues (Figure 5A), whereas the amplitude of *Per1* mRNA rhythms was significantly lower than that of *Per2* mRNA rhythms in serum-stimulated NIH3T3 cells (Figure 5B). This observation was reproducible when Rat-1 fibroblasts were used (unpublished data; Balsalobre *et al.,* 1998). A very recent report demonstrated that in vitro cultured fibroblasts harbor self-sustained and cell-autonomous circadian clocks similar to those operative in SCN neurons (Nagoshi *et al.,* 2004). Therefore, the high amplitude of *Per1* mRNA oscillation in peripheral tissues largely depends on the extracellular environment such as blood-borne factors and body temperature, which changes cyclically around the clock, rather than on the cell autonomous core clock. In support of this idea, in *mPer1*::*Luc* transgenic animals, peripheral organs fail to express persistent circadian rhythms in reporter gene activity (Yamazaki *et al.,* 2000). In contrast, in *mPer2-Luciferase* knockin mice, peripheral tissues in explant cultures show robust and self-sustained circadian rhythms (Yoo *et al.,* 2004). On the other hand, gene knockout studies have indicated that *mPer1*-deficient mice display a persistent circadian rhythm (Bae *et al.,* 2001; Cermakian *et al.,* 2001; Zheng *et al.,* 2001), whereas mice deficient for *mPer2* have no circadian rhythms in locomotor activity (Zheng *et al.,* 1999; Bae *et al.,* 2001; Zheng *et al.,* 2001). Consistent with these behavioral phenotypes, disruption of *mPer2* results in reduced levels of clock gene expression in the SCN; and in contrast, mice homozygous for the targeted *mPer1* allele have unaltered SCN gene expression rhythms (Bae *et al.,* 2001). Taken together, these reports also indicate that the *Per1* gene might not be tightly incorporated into the cell-autonomous core clock mechanism. However, this issue remains controversial, because the loss of *Per1* can result in arrhythmic mice, in prolonged constant dark conditions (Bae *et al.,* 2001). Also, as for the above in vivo experiments, the difference between transgenics and knockins should be recognized; the latter are in their native context and have significantly more potential *cis*-acting sequences surrounding them. If there are important elements that are distant from the TSS, they might be lost in the *Per1* transgenics.

Per1 is highly sensitive to various extracellular stimuli in vitro (Akashi and Nishida, 2000; Balsalobre *et al.,* 2000); and also in vivo, circadian changes in the extracellular environment can readily induce expression of the *Per1* gene. Thus, the dramatic circadian changes in *Per1* mRNA accumulation in peripheral tissues (Figure 5A) may be attributed to this mechanism. Increased expression of the *Per1* gene in response to changes in the extracellular environment may be functional in the entrainment of peripheral oscillators. In fact, in peripheral tissues of *mPer1*-deficient mice, the phase of clock gene expression is not only delayed, but the peak of expression is broadened (Cermakian *et al.,* 2001)

A Mechanism by Which the Per2 E-Box-like Sequence Generates a Higher Amplitude of Circadian Gene Expression than Does the Classical Circadian E-Box

As shown in Figure 3A, even when the region including the E-box-like sequence was deleted, the promoter activity still fluctuated in a circadian manner. These data suggest that the neighboring −15 to −6 region also contains another element that regulates circadian transcription of *Per2* by cooperating with the E-box-like sequence. This −15 to −6 region contains a consensus E4BP4 binding site (8/10 base pairs match), suggesting that DBP and E4BP4 may cooperate with BMAL1-CLOCK/PER-CRY to drive robust circadian gene expression of *Per2*. In Figure 5C, we substituted the *Per2* E-box-like sequence (CACGTT) with the classical circadian E-box (CACGTG) by site-directed mutagenesis and monitored in real-time its transcriptional fluctuation. This substitution did not markedly affect either basal promoter activity or BMAL1-CLOCK-induced transactivation (unpublished data). Interestingly, this 1-base pair substitution resulted in small amplitude of circadian gene expression of *Per2* (Figure 5C, left panel), as observed in *Per1*-luc (Figure 5C, right panel). The detrended bioluminescence data sets made it clear that compared with *Per2*-luc, *Per2*-luc (E-box) showed a small amplitude, and additionally that its period time-dependently became longer (Figure 5D). Interestingly, *Per2*-luc (E-box) had a very similar pattern of amplitude, period, and damping rate to those of *Per1*-luc (Figure 5E). Thus, our data suggest that the *Per2* E-box-like sequence generates high amplitude of circadian gene expression through cooperation between these two distinct elements (the −25 to −16 region and the −15 to −6 region) and that the 1-base pair difference (CACGTT) is indispensable for this cooperation.

To examine whether E4BP4 controls transcriptional oscillation of *Per2*, we studied the binding of E4BP4 to *Per2* (−45 to +15). To confirm that the E4BP4-LUCIFERASE fusion protein binds to the *Per2* fragment, we pulled down the fusion protein by using biotinylated double-strand *Per2* (−45 to +15) containing the putative E4BP4 consensus (Figure 5F). In the presence of this *Per2* fragment, the luciferase activity in the precipitate was enhanced, demonstrating the E4BP4 binding to this fragment. Next, in order to examine the role of E4BP4 protein in cell-autonomous transcriptional oscillation of *Per2*, we monitored the *Per2* (−105)-luc activity in real time. As shown in Figure 5G, coexpression of E4BP4 resulted in a gradual reduction of the basal transcriptional activity (left) and a shortened period length of circadian transcription (right, detrended data). The expression of E4BP4 shortened the period in a dose-dependent manner (Figure 5H). These results indicate that E4BP4 may be a transcriptional repressor in *Per2* transcription and control the period length of *Per2* oscillation. As shown in Figures 3B and 5C, *Per2* Δ (−15 to −6)-luc and *Per2* (E-box)-luc did not show deep troughs in circadian transcription, as wild-type *Per2*-luc did, and therefore, we speculate that E4BP4 may be required for the trough formation in circadian transcription of *Per2*.

CONCLUSIONS

The current feedback loop model has been based mainly on *Per1*. A publication that appeared after this article was submitted demonstrated that a 210-base pair region including the E-box-like sequence is sufficient for *Per2* oscillation in vivo (Yoo *et al.,* 2005). Consistent with this report, our data indicate that the −25 to −6 region upstream from the TSS, including the same E-box-like sequence, is indispensable for

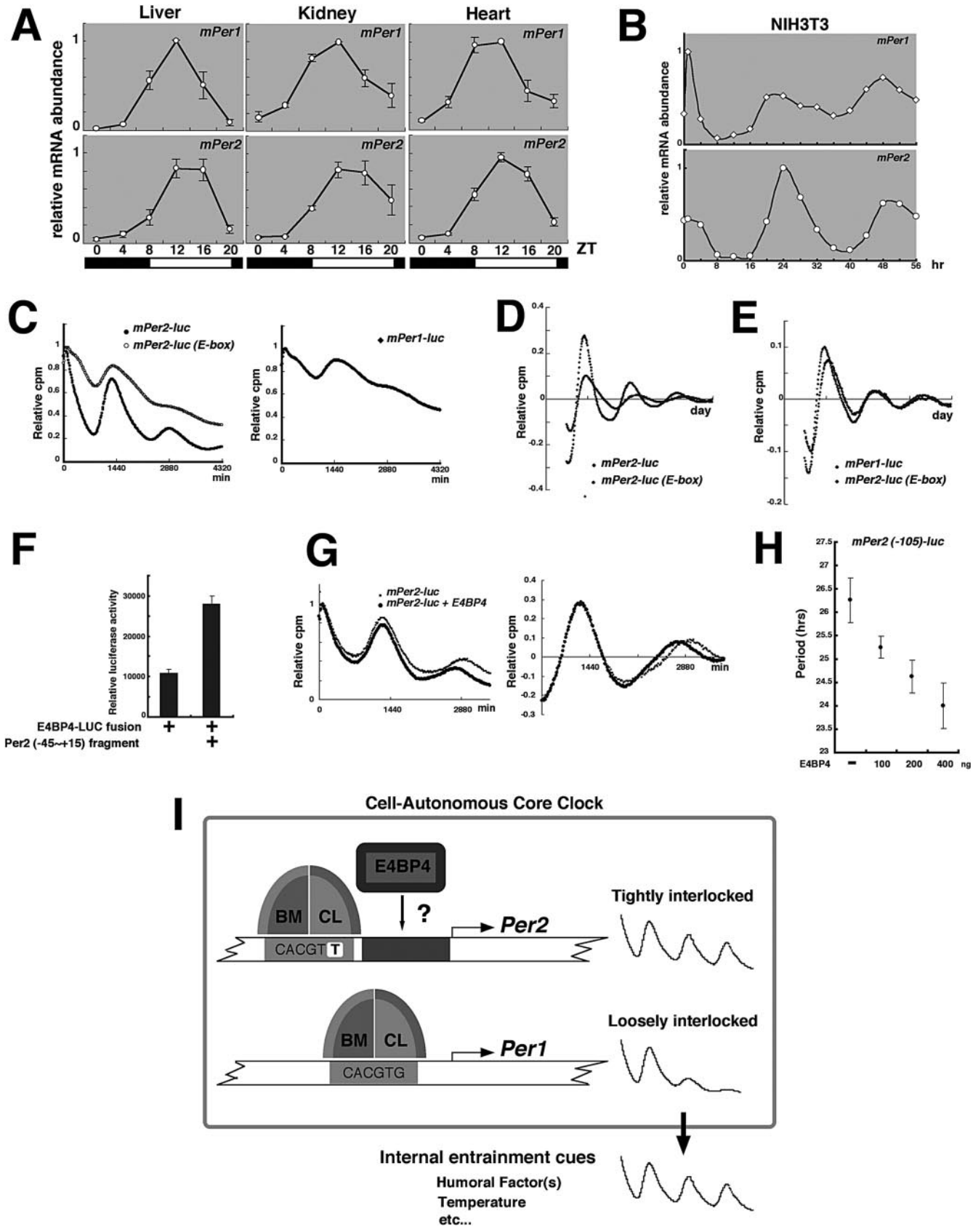

Figure 5. A potential mechanism by which the cell-autonomous core clock generates more overt circadian oscillations in *Per2* transcription than in *Per1* transcription. (A and B) Temporal expression patterns of the *mPer1* and *mPer2* genes, in mouse peripheral tissues (A) and in serum-stimulated NIH3T3 cells (B) were assayed by real-time quantitative RT-PCR. Mice were kept in a 12-h light:12-h dark cycle (LD, lights

cell-autonomous circadian gene expression of *Per2*. Not only the E-box described by Yoo *et al.* (2005) and Ueda *et al.* (2005) but also the 10-base pair nucleotides next to the E-box are necessary for this oscillatory regulation. Furthermore, our biochemical data show that endogenous BMAL1 and CLOCK site-specifically bind to the E-box in a phosphorylation- and time-dependent manner (Figure 4), which is novel observation. Importantly, our experiments were mainly performed on the cell-based real-time assay, because the core clock is a cell-autonomous system. To investigate if transcriptional oscillation is driven in the core clock system, we need to examine whether transcriptional regulatory elements function under a cell-autonomous condition. Our approach enables us to exclude internal environmental cues such as blood-borne factors and body temperature. Thus, these two reports verified that the current feedback loop model reflects a core clock mechanism.

The current molecular model for the mammalian circadian clock has been established mainly on the basis of data obtained by conventional reporter assays on the *Per1* promoter, implying that several issues remain to be clarified. First, it is difficult to interpret whether the experimental system based on ectopic overexpression reflects physiological phenomena. The E-box consensus sequence, which consists of only six-base pair nucleotides, could appear at some frequency in the promoter region of many genes, leading to the possibility that overexpressed BMAL1 and CLOCK nonspecifically bind to E-box and E-box-like sequences and activate transcription (Munoz *et al.*, 2002). Second, these transient assays are not suitable to monitor temporal changes, and therefore the obtained data do not reflect mechanisms of circadian rhythm generation. Mutant mice also could not clarify the role of BMAL1 and CLOCK in *Per1* oscillation, because of the possibility of indirect effects. Thus, to date there is no compelling evidence that BMAL1 and CLOCK regulate circadian transcription of *Per1* and that E-boxes are indispensable for circadian fluctuation of *Per1* transcription. Third, the BMAL1:CLOCK regulation might be specific for *Per1* transcription, because detailed analyses have not been performed on the involvement of BMAL1 and CLOCK in circadian transcription of other clock genes. So far at least we could not exclude the possibility that other as yet unknown components have pivotal roles in circadian transcription of other clock genes. In this report, by the real-time monitoring of bioluminescence in cultured cells, we demonstrated that the BMAL1 and CLOCK binding region is included in only the 20-base pair region essential for circadian transcription of *Per2*, an indispensable gene for the mammalian circadian clock. Our monitoring system was performed under the condition that the endogenous cell-autonomous circadian pacemaker was indeed operating, without the use of overexpression.

Our results are summarized in Figure 5I. Next to the *Per2* E-box-like sequence, there is another region that generates more robust cell-autonomous oscillation in transcription of *Per2*. A combination of the E-box-like sequence and this cooperating element increases the amplitude of cell-autonomous rhythmic transcription of *Per2*, and consequently the amplitude of *Per2* mRNA rhythms is significantly higher than that of *Per1* mRNA rhythms. The 1-base pair difference (CACGTT) from the classical circadian E-box is indispensable for this combination. This discovery suggests that the atypical E-box (CACGTT) might be more functional for circadian gene expression rather than the classical E-box (CACGTG) that has been believed to be as the BMAL1:CLOCK binding consensus. However, we still need to note that the reduction in clock-gene amplitude may not necessarily indicate that the pacemaker amplitude is reduced. Future studies will define the precise relationship between the amplitude of clock gene oscillation and that of pacemaker function. The cell-autonomous core clock generates overt circadian oscillations in *Per2* transcription, whereas the high amplitude of *Per1* oscillation in vivo largely depends on the extracellular environment, which changes cyclically around the clock, rather than on the core clock. This increased expression of the *Per1* gene in response to circadian changes of the extracellular environment may be functional in the entrainment of peripheral oscillators.

Among genes that are expressed in a circadian manner, there are clock genes that are central to the timing mechanism and output genes that directly or indirectly mediate physiology under circadian control. The phases and amplitudes in the circadian expression of these genes are different from each other (Panda *et al.*, 2002; Storch *et al.*, 2002). Nevertheless, it is thought that a few enhancer elements, such as E-box, RORE, and DBPE, generate a broad range of phases and amplitudes in circadian gene expression (Yamamoto *et al.*, 2004). Our results provide an explanation for why a small number of elements generate various patterns of circadian gene expression. We showed that two or more transcriptional regulatory elements, such as a phase-delaying element and a rhythm-generating element, are required for robust circadian gene expression of *Per2*, illustrating that even when the same rhythm-generating element regulates several different genes, combinations with other

Figure 5 (cont.) on 8 a.m.; lights off 8 p.m.) for 2 wk to establish entrainment. Three animals were killed at the times given on the abscissas of the diagrams. Each value represents the average of three independent RT-PCR experiments. The relative levels of each RNA were normalized to the corresponding *Gapdh* RNA levels. Peak values of the *mPer1* and *mPer2* curves were set to 1. (C) Transcriptional oscillation of *mPer2*-luc, *mPer2*-luc (E-box), and *mPer1*-luc was monitored in real time. NIH3T3 cells were transfected and then stimulated with a high concentration of serum. Peak values of the curves were set to 1 (vertical scale: relative cpm; horizontal scale: 1440 min = 1 d). A representative result of three independent experiments is shown. (D and E) The signals obtained in C were detrended. (F) COS7 cells were transfected with the E4BP4-luciferase fusion expression vector. Cell extracts were incubated with the double-stranded biotinylated *Per2* (−45 to +15) oligonucleotide, including the consensus-predicted E4BP4 response elements, which was immobilized on streptavidin-Sepharose beads. The negative control samples were treated with streptavidin-Sepharose beads without an oligonucleotide. The resulting precipitates were subjected to luciferase assays. Data represent the mean ± SEM of triplicate samples. (G) Transcriptional oscillation of *Per2* (−105)-luc was monitored, in the presence or absence of E4BP4 (left). The signals obtained were detrended (right). (H) With increasing dose of the E4BP4 expression plasmid, transcriptional oscillation of *Per2* (−105)-luc was monitored. The periods were obtained from analysis of a circadian marker. Data represent the mean ± SEM of triplicate samples. (I) Schematic model representing BMAL1:CLOCK-mediated control of cell-autonomous *Per1* and *Per2* oscillation. A combination of the E-box-like sequence and a cooperating element increases the amplitude of rhythmic transcription of *Per2*, and consequently the amplitude of *Per2* mRNA rhythms is significantly higher than that of *Per1* mRNA rhythms. The 1-base pairs difference (CACGTT) from the classical circadian E-box may be indispensable for this combination. The cell-autonomous core clock generates overt circadian oscillations in *Per2* transcription, whereas the high amplitude of *Per1* oscillation in vivo largely depends on the extracellular environment, which changes cyclically around the clock, rather than on the core clock. Thus, the *Per1* gene might not be tightly incorporated into the cell-autonomous core clock mechanism.

enhancer elements can advance or delay the phase of these circadian gene expressions. The copy number and various combinations of elements would create unlimited patterns in phases. On the other hand, we showed that the *Per2* E-box-like element might generate high amplitude of circadian gene expression by cooperating with a neighboring element. This result demonstrates that synergistic cooperation of several elements may generate more dynamic oscillation in circadian gene expression. In fact, we found that 2 ROR response elements synergistically function in circadian transcription of *Bmal1* (Akashi and Takumi, 2005), illustrating that cooperation of elements can enhance the amplitude. The molecular mechanism by which circadian expression of clock and clock-related genes shows a variety of phases and amplitudes will be more clearly revealed by further detailed analyses of the regulatory mechanism for circadian transcription of each known clock gene.

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REFERENCES

Abe, M., Herzog, E. D., Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., and Block, G. D. (2002). Circadian rhythms in isolated brain regions. J. Neurosci. *22*, 350–356.

Akashi, M., and Nishida, E. (2000). Involvement of the MAP kinase cascade in resetting of the mammalian circadian clock. Genes Dev. *14*, 645–649.

Akashi, M., and Takumi, T. (2005). The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. Nat. Struct. Mol. Biol. *12*, 441–448.

Akashi, M., Tsuchiya, Y., Yoshino, T., and Nishida, E. (2002). Control of intracellular dynamics of mammalian period proteins by casein kinase I epsilon (CKIepsilon) and CKIdelta in cultured cells. Mol. Cell. Biol. *22*, 1693–1703.

Albrecht, U., and Eichele, G. (2003). The mammalian circadian clock. Curr. Opin. Genet. Dev. *13*, 271–277.

Albrecht, U., Sun, Z. S., Eichele, G., and Lee, C. C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell *91*, 1055–1064.

Allada, R., Emery, P., Takahashi, J. S., and Rosbash, M. (2001). Stopping time: the genetics of fly and mouse circadian clocks. Annu. Rev. Neurosci. *24*, 1091–1119.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., and Weaver, D. R. (2001). Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron *30*, 525–536.

Balsalobre, A., Damiola, F., and Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell *93*, 929–937.

Balsalobre, A., Marcacci, L., and Schibler, U. (2000). Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. Curr. Biol. *10*, 1291–1294.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. Cell *103*, 1009–1017.

Cermakian, N., Monaco, L., Pando, M. P., Dierich, A., and Sassone-Corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the Period1 gene. EMBO J. *20*, 3967–3974.

Dunlap, J. C. (1999). Molecular bases for circadian clocks. Cell *96*, 271–290.

Gekakis, N., Staknis, D., Nguyen, H. B., Davis, F. C., Wilsbacher, L. D., King, D. P., Takahashi, J. S., and Weitz, C. J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

Hida, A., Koike, N., Hirose, M., Hattori, M., Sakaki, Y., and Tei, H. (2000). The human and mouse Period1 genes: five well-conserved E-boxes additively contribute to the enhancement of mPer1 transcription. Genomics *65*, 224–233.

Kaasik, K., and Lee, C. C. (2004). Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature *430*, 467–471.

Kondo, T., and Ishiura, M. (2000). The circadian clock of cyanobacteria. Bioessays *22*, 10–15.

Kondratov, R. V., Chernov, M. V., Kondratova, A. A., Gorbacheva, V. Y., Gudkov, A. V., and Antoch, M. P. (2003). BMAL1-dependent circadian oscillation of nuclear CLOCK: posttranslational events induced by dimerization of transcriptional activators of the mammalian clock system. Genes Dev. *17*, 1921–1932.

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H., and Reppert, S. M. (1999). mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell *98*, 193–205.

Lee, C., Etchegaray, J. P., Cagampang, F. R., Loudon, A. S., and Reppert, S. M. (2001). Posttranslational mechanisms regulate the mammalian circadian clock. Cell *107*, 855–867.

Lowrey, P. L., and Takahashi, J. S. (2004). Mammalian circadian biology: elucidating genome-wide levels of temporal organization. Annu. Rev. Genomics Hum. Genet. *5*, 407–441.

Menaker, M. (2003). Circadian rhythms. Circadian photoreception. Science *299*, 213–214.

Munoz, E., Brewer, M., and Baler, R. (2002). Circadian transcription. Thinking outside the E-Box. J. Biol. Chem. *277*, 36009–36017.

Nagoshi, E., Saini, C., Bauer, C., Laroche, T., Naef, F., and Schibler, U. (2004). Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. Cell *119*, 693–705.

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., and Hogenesch, J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. Cell *109*, 307–320.

Pittendrigh, C. S. (1993). Temporal organization: reflections of a Darwinian clock-watcher. Annu. Rev. Physiol. *55*, 16–54.

Ralph, M. R., Foster, R. G., Davis, F. C., and Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. Science *247*, 975–978.

Reppert, S. M., and Weaver, D. R. (2002). Coordination of circadian timing in mammals. Nature *418*, 935–941.

Schibler, U., and Sassone-Corsi, P. (2002). A web of circadian pacemakers. Cell *111*, 919–922.

Storch, K. F., Lipan, O., Leykin, I., Viswanathan, N., Davis, F. C., Wong, W. H., and Weitz, C. J. (2002). Extensive and divergent circadian gene expression in liver and heart. Nature *417*, 78–83.

Takumi, T., Matsubara, C., Shigeyoshi, Y., Taguchi, K., Yagita, K., Maebayashi, Y., Sakakida, Y., Okumura, K., Takashima, N., and Okamura, H. (1998). A new mammalian period gene predominantly expressed in the suprachiasmatic nucleus. Genes Cells *3*, 167–176.

Tamaru, T., Isojima, Y., van der Horst, G. T., Takei, K., Nagai, K., and Takamatsu, K. (2003). Nucleocytoplasmic shuttling and phosphorylation of BMAL1 are regulated by circadian clock in cultured fibroblasts. Genes Cells *8*, 973–983.

Toh, K. L., Jones, C. R., He, Y., Eide, E. J., Hinz, W. A., Virshup, D. M., Ptacek, L. J., and Fu, Y. H. (2001). An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. Science *291*, 1040–1043.

Travnickova-Bendova, Z., Cermakian, N., Reppert, S. M., and Sassone-Corsi, P. (2002). Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. Proc. Natl. Acad. Sci. USA *99*, 7728–7733.

Ueda, H. R., Hayashi, S., Chen, W., Sano, M., Machida, M., Shigeyoshi, Y., Iino, M., and Hashimoto, S. (2005). System-level identification of transcriptional circuits underlying mammalian circadian clocks. Nat Genet *37*, 187–192.

Vitaterna, M. H., Selby, C. P., Todo, T., Niwa, H., Thompson, C., Fruechte, E. M., Hitomi, K., Thresher, R. J., Ishikawa, T., Miyazaki, J., Takahashi, J. S., and Sancar, A. (1999). Differential regulation of mammalian period genes and circadian rhythmicity by cryptochromes 1 and 2. Proc. Natl. Acad. Sci. USA *96*, 12114–12119.

Williams, J. A., and Sehgal, A. (2001). Molecular components of the circadian system in *Drosophila*. Annu. Rev. Physiol. *63*, 729–755.

Yamamoto, T., Nakahata, Y., Soma, H., Akashi, M., Mamine, T., and Takumi, T. (2004). Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. BMC Mol. Biol. *5*, 18

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G. D., Sakaki, Y., Menaker, M., and Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. Science *288*, 682–685.

Yamazaki, S., Straume, M., Tei, H., Sakaki, Y., Menaker, M., and Block, G. D. (2002). Effects of aging on central and peripheral mammalian clocks. Proc. Natl. Acad. Sci. USA *99*, 10801–10806.

Yoo, S. H., Ko, C. H., Lowrey, P. L., Buhr, E. D., Song, E. J., Chang, S., Yoo, O. J., Yamazaki, S., Lee, C., and Takahashi, J. S. (2005). A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proc. Natl. Acad. Sci. USA *102*, 2608–2613.

Yoo, S. H. *et al.* (2004). PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc. Natl. Acad. Sci. USA *101*, 5339–5346.

Young, M. W., and Kay, S. A. (2001). Time zones: a comparative genetics of circadian clocks. Nat. Rev. Genet. *2*, 702–715.

Zheng, B. *et al.* (2001). Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell *105*, 683–694.

Zheng, B., Larkin, D. W., Albrecht, U., Sun, Z. S., Sage, M., Eichele, G., Lee, C. C., and Bradley, A. (1999). The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature *400*, 169–173.