Molecular mechanism of cell-autonomous circadian gene expression of *Period2*, a crucial regulator of the mammalian circadian clock

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A running title: Molecular mechanism of *Per2* oscillation

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Abstract

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 approximately 24 hr 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, orthologs 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 \textit{Per1}, which has been analyzed in detail, serves as a foundation for the mammalian model, it has been reported recently that \textit{Per1} is not absolutely required for the generation and maintenance of circadian rhythms (Cermakian \textit{et al.}, 2001; Zheng \textit{et al.}, 2001). This issue remains controversial, therefore, since Bae \textit{et al.} found that the loss of \textit{Per1} can result in arrhythmic mice (Bae \textit{et al.}, 2001). 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 \textit{Bmal1} results in the down-regulation of \textit{Per} expression (Vitaterna \textit{et al.}, 1999; Bunger \textit{et al.}, 2000; Bae \textit{et al.}, 2001). However, these studies cannot prove whether the regulation is direct or indirect. Overexpression-based transient reporter assay has shown that transcription of the \textit{Per2} gene, a key component for rhythm generation, is also upregulated by coexpression of circadian transcription factors (BMAL1, CLOCK and NPAS2), as shown for the \textit{Per1} gene (Travnickova-Bendova \textit{et al.}, 2002; Kaasik and Lee, 2004). Yet, while \textit{Per1} contains 5 classical circadian E-boxes (Hida \textit{et al.}, 2000; Yamamoto \textit{et al.}, 2004), no evolutionarily conserved E-box (the classical circadian E-box) has been identified in the upstream sequence of \textit{Per2}. 

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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 approximately 20-bp 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). 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 hours. 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 photomulti-plier tube (Hamamatsu Photonics) 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 hr 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 hour 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.

**Pull-down experiment**

Mouse liver extracts were prepared at 4-hr intervals by homogenizing the tissue in ice-cold incubation buffer (Akashi *et al.*, 2002), and then the extracts were incubated with an 80-bp double-stranded biotinylated oligonucleotide that had been immobilized on streptavidin-Sepharose beads (Amersham). 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 \((mPer2, -105\sim+15)\):

\[
\begin{align*}
5' & \text{CTCAGGTTCGCCCCGCCAGTATGCAATGAGGTGGCACTCCGACCAATGG} \\
& \text{CGCGCGCAGGGCGGCTCAGCGCGCGGTCACGTTTTCGACCAATGG} \\
& \text{GCGGAGGGCGACGCGGC3'}
\end{align*}
\]

mutant \((mPer2, -105\sim+15)\):

\[
\begin{align*}
5' & \text{CTCAGGTTCGCCCCGCCAGTATGCAATGAGGTGGCACTCCGACCAATGG} \\
& \text{CGCGCGCAGGGCGGCTCAGCGCGCGGTCACGTTTTCGACCAATGG} \\
& \text{GAGGGCGACGCGGC3'}
\end{align*}
\]

wild-type \((mPer1, 3E-box)\):

\[
\begin{align*}
5' & \text{GAAAGCTTTAGCCACGTGACAGTGGAGGGACCCCTTAAACGACGCGTG} \\
& \text{ACAGTGAGGGGCACCCCTTAACGACGCGTG} \\
& \text{CCCTCAATTGAGCACCACCAAGTCCACGTGCCAGGGATGTGTGGGGCGGCAGGGCC} \\
& \text{TGGCATTAGCAACCCCGCCTCCAGCCTC3'}
\end{align*}
\]

mutant \((mPer1, 3E-box)\):

\[
\begin{align*}
5' & \text{GAAAGCTTTAGCCACGTGACAGTGGAGGGACCCCTTAAACGACGCGTG} \\
& \text{ACAGTGAGGGGCACCCCTTAACGACGCGTG} \\
& \text{TGAGCACCACCAAGTCCACGTGCCAGGGATGTGTGGGGCGGCAGGGCC} \\
& \text{GCCTCCAGCCTC3'}
\end{align*}
\]
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). The cDNA equivalent to 20 ng total RNA was PCR-amplified in an ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Forward primers and reverse primers were as follow:

Gapdh forward: 5’-CATCCACTGGTGCTGCAAGGCTGT-3’;

Gapdh reverse: 5’-ACAACCTGGTCCTCAGTGTAGCCCA-3’

mPer1 forward: 5’-CAGGCTAACCAGGAATATTACCAGC-3’;

mPer1 reverse: 5’-CACAGCCACAGAGAAGGTGTCCTGG-3’

mPer2 forward: 5’-GGCTTCACCATGCCTGTTGT-3’;

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 (Fig. 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 and amplitudes were observed in the
cells transfected with the constructs from −2811 to −386, whereas a phase advance (approximately 2 hours earlier) was detected only when using the Per2 (-105) construct (Fig. 1B). To better calculate phase differences, data sets were detrended, and the time of the first peak was used as a phase marker (Fig. 1C). Again the Per2 (-105) construct showed a 2-h phase advance compared to the other constructs, as expected in Fig. 1B. Thus, these data suggest that a phase delaying element is located between −386 and −106 bp upstream from the TSS(s) and that a rhythm-generating element exists between −105 and +1 bp upstream from the TSS(s) (Fig. 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. has very recently reported that a 210-bp fragment upstream from the TSS(s) drives Per2 circadian oscillation in vivo (Yoo et al., 2005).

An approximately 20-bp 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 (Fig. 2A and 2C). When 40-bp fragments overlapping by 20 bp were used (Fig. 2A), the Per2 (-45--6)-SV40-luc construct and the Per2 (-25--+15)-SV40-luc construct clearly showed Per2-controlled fluctuations of luminescence as compared with the other constructs (Fig. 2B). The detrended data suggests that the SV40 promoter activity exhibits not a drastic but significant circadian fluctuation (Supplemental Fig. 1A), indicating that this promoter contains some circadian enhancer elements. The detrended bioluminescence datasets make clear that only the Per2 (-45--6)-SV40-luc and Per2 (-25--+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 (Supplemental Fig. 1B). The higher amplitude of oscillation was maintained over the cycles in Per2 (-45--6)-SV40-luc. The Per2 (-45--6)-SV40-luc/Per2 (-25--+15)-SV40-luc amplitude ratio was obtained by dividing the Per2 (-45--6)-SV40-luc wave amplitude by the Per2 (-25--+15)-SV40-luc wave amplitude in each cycle (Supplemental Fig. 1C), demonstrating that the amplitude of Per2 (-45--6)-SV40-luc gradually grew to be 2-fold higher than that of Per2.
(-25~+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-bp fragments overlapping by 15 bp (Fig. 2C). The results show that the Per2 (-25~6) fragment possessed the most potent ability to oscillate SV40 promoter activity (Fig. 2D). The detrended data highlighted the Per2 (-25~6) and Per2 (-30~11) fragments’ ability to drive oscillation (Supplemental Fig. 1D). The Per2 (-25~6)-SV40-luc maintained a higher amplitude in each cycle than that of Per2 (-30~11)-SV40-luc (Supplemental Fig. 1E). Additionally, the Per2 (-25~6)-SV40-luc/Per2 (-30~11)-SV40-luc amplitude ratio in each cycle demonstrated that the amplitude of Per2 (-25~6)-SV40-luc was also considered, being 2-fold higher over cycles than that of Per2 (-30~11)-SV40-luc (Supplemental Fig. 1F). The merge of Per2 (-25~6)-SV40-luc (Supplemental Fig. 1D) and Per2 (-45~6)-SV40-luc (Supplemental Fig. 1A) illustrated almost the same pattern of oscillation, confirming that the Per2 (-25~6) region is necessary and sufficient to drive Per2 (-45~6)-SV40-luc oscillation (Supplemental Fig. 1G). This region (-25~6), therefore, contains the core element for rhythmic transcription of Per2.

We also constructed mutants lacking 10 bp in the –105~+6 region, and monitored transcriptional fluctuation of Per2 in real-time (Fig. 3A). The amplitude of
circadian transcription of Per2 was strongly diminished in mPer2-luc Δ (-25~−16) and mPer2-luc Δ (-15~−6). Furthermore, both of these two constructs exhibited a phase delay, illustrating that both 10-bp regions contribute to maintain the original phase. Other regions were required for amplification, whereas these 2 regions were essential for driving rhythmic transcription of Per2. After removal of baseline changes (Supplemental Fig. 2A), the period was obtained from regression analysis of a circadian marker (trough) in Supplemental Figure 2B. As compared to wild-type Per2-luc, mPer2-luc Δ (-95~−86), mPer2-luc Δ (-85~−76), mPer2-luc Δ (-75~−66), mPer2-luc Δ (-65~−56), mPer2-luc Δ (-55~−46) and mPer2-luc Δ (-45~−36) showed a 20 to 40-min shorter period. Notably, the -75~−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~−76), mPer2-luc Δ (65~−56), mPer2-luc Δ (-45~−36) or mPer2-luc Δ (-35~−26) was significantly smaller over cycles, compared to wild-type Per2-luc (Supplemental Fig. 2C), and deletion of -15~−6 or -25~−16 caused a remarkable inhibition of circadian fluctuation, as shown in
Figure 3A. The others showed only a slight decrease of the initial amplitude. In order to compare the damping pattern in the constructs, the amplitude in the initial cycle was set to 100 (Supplemental Fig. 2D). While mPer2-luc Δ (-25~16) showed a slightly irregular damping pattern, the others had almost the same damping pattern as that of wild-type Per2-luc. Taken together, the -85~76, 65~56, -45~36 and -35~26 regions work to enhance the amplitude of transcriptional oscillation, whereas the -15~6 and -25~16 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 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 (Supplemental Fig. 3). These results are consistent with those in Figure 2B and 2D. 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 (Fig. 3B).

Endogenous circadian transcription factors bind site-specifically to the Per2
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→+15) double-stranded DNA fragments immobilized on streptavidin beads (Fig. 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-hr 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 ~ ZT12. By pull-down 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 (lower 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 co-dependent 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 (data not shown). As a positive control, when a DNA fragment containing 3 different Per1 E-boxes was used for pull-down assays, a similar pattern of BMAL1 binding was detected. Mutation of these Per1 E-boxes completely inhibited BMAL1 binding. As observed in the Per2 pull-down assays, the peak of phosphorylated BMAL1 bound to the Per1 E-boxes correlated with that of Per1 mRNA expression (lower 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 (Fig. 5A), whereas the amplitude of Per1 mRNA rhythms was significantly lower than that of Per2 mRNA rhythms in serum-stimulated NIH3T3 cells (Fig. 5B). This observation was reproducible when Rat-1 fibroblasts were used (data not shown; 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 recognised; 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 (Fig. 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 m*Per1*-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 bp 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 (data not shown). Interestingly, this 1-bp substitution resulted in small amplitude of circadian gene expression of *Per2* (Fig. 5C, left panel), as observed in *Per1*-luc (Fig. 5C, right panel). The detrended bioluminescence datasets made it clear that compared to *Per2*-luc, *Per2*-luc (E-box) showed a small amplitude, and additionally that its period time-dependently became longer (Fig. 5D). Interestingly, *Per2*-luc (E-box) had a very similar pattern of amplitude, period and damping rate to those of *Per1*-luc (Fig. 5E). Thus, our data suggest that the *Per2* E-box-like sequence generates high amplitude of circadian gene expression through cooperation between these 2 distinct elements (the –25 to –16 region and the –15 to –6 region) and that the 1-bp 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~+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~+15) containing the putative E4BP4 consensus (Fig. 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 (Fig. 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 Fig. 3B and 5C, Per2 Δ (-15~6)-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 paper was submitted demonstrated that a 210-bp 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 cell-autonomous circadian gene expression of Per2. Not only the E-box described by Yoo et al. and Ueda et al. (Ueda et al., 2005; Yoo et al., 2005) but also the 10-bp 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 (Fig. 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. In order 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 6 bp 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, due to 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-bp 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-bp difference (CACGTG) 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 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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Figure Legends

Figure 1. A phase-delaying element and a rhythm-generating element are required
for robust cell-autonomous circadian gene expression of \textit{Per2}

(A) Schematic representation of deletion mutants of the \textit{mPer2} promoter. +1 corresponds to the transcription start site. (B) Transcriptional oscillation of \textit{mPer2} was monitored by using the cell culture-based luminescent reporter assay. NIH3T3 cells were transfected with the \textit{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 counts per minute, horizontal scale: 1440 minutes = 1 day). 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 \( \pm \) SEM; \( n = 4 \)). (D) Schematic representation of the results obtained from the deletion analysis.

\textbf{Figure 2. Identification of the region responsible for driving cell-autonomous circadian oscillation of \textit{Per2} transcription}

(A) Schematic representation of a set of constructs in which a 40-bp fragment from \textit{Per2} (\(-105\sim+15\)) was inserted into the upstream of the SV40 promoter attached to the luciferase gene. These fragments overlap by 20 bp. (B) Transcriptional oscillation of \textit{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 counts per minute, horizontal scale: 1440 minutes = 1 day). 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-bp fragment from Per2 (-45~+15) was inserted into the upstream of the SV40 promoter attached to the luciferase gene. These fragments overlap by 15 bp. (D) Transcriptional oscillation of mPer2-SV40-luc was monitored. Peak values of the curves were set to 1 (horizontal scale: 1440 minutes = 1 day). A representative result of three independent experiments is shown. For accurate comparison, thin lines show the curve for “SV40-luc”.

Figure 3. Mapping of the rhythm-generating element by using deletion mutants of Per2

(A) Transcriptional oscillation of a series of mPer2-luc 10-bp 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 counts per minute, horizontal scale: 1440 minutes = 1 day). 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-bp 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.

**Figure 4. Temporal patterns of the site-specific binding of endogenous circadian transcription factors to the Per2 E-box-like sequence**

(Upper and middle panels) Mouse liver extracts were harvested at 4-h intervals, and then subjected to immunoblot analysis with anti-BMAL1 antibody (upper 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 3 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 (upper panels) or anti-CLOCK antibody (middle panels). (Lower panels) Temporal expression patterns of the mPer2 and mPer1 genes in mouse liver were assayed by real-time quantitative RT-PCR. Each value represents the average of 3 independent RT-PCR experiments. The relative levels were normalized to the corresponding Gapdh RNA
levels. Peak values of the \textit{mPer2} and \textit{mPer1} curves were set to 1.

\textbf{Figure 5. A potential mechanism by which the cell-autonomous core clock generates more overt circadian oscillations in \textit{Per2} transcription than in \textit{Per1} transcription}

(A and B) Temporal expression patterns of the \textit{mPer1} and \textit{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 on 8 a.m.; lights off 8 p.m.) for 2 weeks to establish entrainment. Three animals were sacrificed at the times given on the abscissae of the diagrams. Each value represents the average of 3 independent RT-PCR experiments. The relative levels of each RNA were normalized to the corresponding \textit{Gapdh} RNA levels. Peak values of the \textit{mPer1} and \textit{mPer2} curves were set to 1. (C) Transcriptional oscillation of \textit{mPer2-luc}, \textit{mPer2-luc} (E-box), and \textit{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 counts per minute, horizontal scale: 1440 minutes = 1 day). 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 \textit{Per2} (-45~+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 \textit{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 \textit{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 \textit{Per1} and \textit{Per2} oscillation. A combination of the E-box-like sequence and a cooperating element increases the amplitude of rhythmic transcription of \textit{Per2}, and consequently the amplitude of \textit{Per2} mRNA rhythms is significantly higher than that of \textit{Per1} mRNA rhythms. The 1-bp difference (CACGTT) from the classical circadian E-box may be indispensable for this combination. The cell-autonomous core clock generates overt circadian oscillations in \textit{Per2} transcription, whereas the high amplitude of \textit{Per1} oscillation \textit{in vivo} largely depends on the extracellular environment, which changes cyclically around the clock.
rather than on the core clock. Thus, the \textit{Per1} gene might not be tightly incorporated into the cell-autonomous core clock mechanism.
The image shows a gel-based pull-down assay with samples labeled as Lysate (Liver) with various treatments: WT: mPer2 (-105→+15), MT: mPer2 (-105→+15), No ODN, WT: mPer1 (3E-box), and MT: mPer1 (3E-box). The gels are probed with anti-BMAL1 and anti-CLOCK antibodies. The graphs below the gel images show the relative mRNA abundance of mPer2 and mPer1 over time (ZT: 0, 4, 8, 12, 16, 20), indicating a cyclical pattern.