

# Identification of functional clock-controlled elements involved in differential timing of *Per1* and *Per2* transcription

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## ABSTRACT

It has been proposed that robust rhythmic gene expression requires clock-controlled elements (CCEs). Transcription of *Per1* was reported to be regulated by the E-box and D-box in conventional reporter assays. However, such experiments are inconclusive in terms of how the CCEs and their combinations determine the phase of the *Per1* gene. Whereas the phase of *Per2* oscillation was found to be the most delayed among the three *Period* genes, the phase-delaying regions of the *Per2* promoter remain to be determined. We therefore investigated the regulatory mechanism of circadian *Per1* and *Per2* transcription using an *in vitro* rhythm oscillation-monitoring system. We found that the copy number of the E-box might play an important role in determining the phase of *Per1* oscillation. Based on real-time bioluminescence assays with various promoter constructs, we provide evidence that the non-canonical E-box is involved in the phase delay of *Per2* oscillation. Transfection experiments confirmed that the non-canonical E-box could be activated by CLOCK/BMAL1. We also show that the D-box in the third conserved segment of the *Per2* promoter generated high amplitude. Our experiments demonstrate that the copy number and various combinations of functional CCEs ultimately led to different circadian phases and amplitudes.

## INTRODUCTION

The system regulating mammalian circadian timing is organized in a hierarchical manner, in which a master

pacemaker in the suprachiasmatic nucleus (SCN) controls peripheral oscillators (1). The clock mechanism in the SCN and the peripheral oscillators are known to be similar at the molecular level (2–5), and comprise a network of transcriptional/translational feedback loops involving a set of clock genes (1,6).

In the primary feedback loop, the positive components include members of the basic helix–loop–helix-PAS transcription factor family, CLOCK and BMAL1. These heterodimerize and initiate the transcription of target genes containing E-box *cis*-regulatory elements, including *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) (7–11). The translated PER and CRY proteins translocate into the nucleus to interfere with the CLOCK and BMAL1 heterodimer complex, thus blocking their transcription (6,12–14). This heterodimer also induces the transcription of *Rev-erbA* and *Ror*, which interact with Rev-erbA/ROR-binding elements (RREs) in the promoter of *Bmal1*, repressing or driving its transcription, respectively (15–19). DBP and E4BP4 are able to activate or suppress transcriptional activity, respectively, through the same sequence called as D-box (20).

It has been proposed that circadian timing of clock and clock-controlled genes may be dependent on conserved and functional clock-controlled elements (CCEs) during the morning (E/E'-boxes), daytime (D-boxes) and night (RREs) (21,22). For example, RREs play an important role in generating circadian night expression in phase with *Bmal1* expression (19). On the other hand, circadian regulation of *Per2* appears to be more complex, because multiple CCEs are involved in its regulation. Recent studies have shown that the E'-box located 20 bp upstream from the *Per2* transcription start site (TSS) is essential to generate *Per2* oscillation *in vitro* (23) and *in vivo* (24). In addition, the D-box (+197) located downstream from the *Per2* TSS is also required for robust

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circadian expression of *Per2* (25). In contrast to our understanding of rhythm generation, it is not sufficient to verify the phase-control elements. In particular, the phase-delaying regions of the *Per2* promoter remain to be elucidated.

Transcription of *mPer1* is regulated by the canonical E-box and D-box (9,26,27). However, it is important to note that circadian rhythm generation cannot be verified using a conventional transient reporter assay. A conventional reporter assay, although useful for the characterization of promoter activity and protein–DNA interactions, has not revealed the phase and amplitude of circadian transcription. In contrast, a previous study verified that the D-box of the *Per3* promoter was sufficient to generate the phase of *Per3* circadian oscillation using an *in vitro* rhythm oscillation-monitoring system (21). Therefore, we investigated the regulatory mechanism of circadian *Per1* and *Per2* transcription in detail.

The current report shows that the number of functional CCEs might play a significant role in determining the phase of the clock gene. Furthermore, we provide the evidence that the non-canonical E-box is responsible for delaying the phase of the clock gene.

## MATERIALS AND METHODS

### Plasmid construction

The mouse *Per2* gene promoter region [chr1 (–): 93289505 bp–chr1: 93293019 bp in the Build 36 Assembly of the UCSC Genome Browser; <http://genome.ucsc.edu/>], which is essential for oscillation, was linked with destabilized firefly luciferase (dLuc; luciferase bound to the ornithine decarboxylase PEST sequence) based on the pGL3-basic vector (Promega, Madison, WI, USA), as described elsewhere (21). Site-directed mutagenesis of the reporter construct was performed with a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The wild-type sequences were as follows: E'-box (CACGTT), E''-box (CATGTG) and D-box (TTATGTAA). The mutation sequences were as follows: mutated E'-box (ACCGGT), mutated E''-box (ACCGGT) and mutated D-box (CACCCGGC). Construction of the *mPer1*-dLuc reporter vector was as described elsewhere (21).

### Animals

Mice were housed under 12:12 h light/dark or constant dark conditions. Food and water were given *ad libitum*. SCN, livers and white adipose tissues (WATs) were obtained every 4 h over 2 days. Upon removal, tissues were immediately frozen in liquid nitrogen and stored at –80°C until they were processed for RNA analysis. We extracted total RNA from the 50 pooled SCNs, 4 pooled livers and 4 pooled WATs at each time point. All protocols for experiments using animals in this study were approved by the Yamanouchi and Astellas Pharma Inc., Animal Research Committee.

### Real-time monitoring of bioluminescence and data analysis

Rat-1 cells were cultured as described in previous studies (21), transfected with several mutant promoter vectors of *mPer1*-dLuc and *mPer2*-dLuc and incubated for 72 h. Then, dibutyl cyclic AMP (dbcAMP; Wako Pure Chemical Industries, Ltd.) was added to a final concentration of 1 mM. In the presence of 0.1 mM luciferin (Promega), light emission was measured and integrated for 1 min at intervals of 15 min with the PMT detector assembly (Hamamatsu Photonics), and the luminescence was observed continuously for ~5 days at 30°C. The phase and amplitude were calculated as described in previous studies (28–31), using the Origin 6.1 program (Origin Lab, Corporation Northampton, MA, USA). Briefly, bioluminescence records were detrended by subtraction of the 24 h running average from the raw data, then smoothed with the 2 h running average. The phases were measured starting with the third peak after stimulation. Relative amplitude data were calculated from the third to the eighth peak of oscillation.

### Luciferase assay

NIH3T3 cells were cultured as described in previous studies (21), and plated in 24-well plates at  $4 \times 10^4$  cells/well the day before transfection. The NIH3T3 cells were transfected using Lipofectamine 2000 reagent (Invitrogen) with an internal control (pRL-TK; 2 ng) and the SV40-dLuc vector containing the wild-type or mutant CCE (10 ng), in the presence or absence of pCI-*Bmal1* (100 ng), pCI-*Clock* (100 ng), pCI-*Cry1* (100 ng) and pDNA3.1-*Dec1* (100 ng) (32). A pCI-neo or pCMV-Sport6 plasmid was used to adjust the amount of DNA (412 ng). After 24 h transfection, the cells were harvested and assayed with the Dual-Luciferase Reporter Assay System (Promega).

### Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (Q-PCR) was performed as described previously (21). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were quantified and used as an internal control. The oligonucleotide DNA primers for Q-PCR were as follows: *Per1* (F: 5'-CGTCCTACCTCCTTTATCCAGA-3', R: 5'-TGT TTGCATCAGTGTGCATCAGC-3'); *Per2* (F: 5'-CATTG AACTTGAGACTGAGGT-3', R: 5'-AAGGGAACACA CTGAGAGGAT-3'); *Per3* (F: 5'-GAAGCGAGAGGCA GAAGCACAA-3', R: 5'-GAAAAGAGGGGAGGAGA TAAGG-3'); and *Gapdh* (F: 5'-CAAAATGGTGAAGGT CCGTGTG-3', R: 5'-ATTTGATGTTAGTGGGGTCT CG-3').

### Statistical analysis

Multiple comparisons among group mean differences were checked using Dunnett's test. A  $P < 0.05$  (one asterisk) was considered to be statistically significant.

### Peak time analysis

Estimation of molecular peak time was conducted as described by Ueda *et al.* (33). We prepared cosine curves

of 24 h periodicity with peaks from 0 to 24 h in increments of 10 min, yielding a total of 144 test cosine curves, and calculated the correlation value of the best-fitted cosine curve for each probe set. We estimated the peak time of each cycling gene from the peak time of the best-fitted cosine curve and defined it as the molecular peak time.

## RESULTS AND DISCUSSION

### Temporal expression profiles of mouse *Per1*, *Per2* and *Per3* in central and peripheral tissues

We confirmed the circadian expression profiles of *mPer1*, *mPer2* and *mPer3* in the SCN, liver and WAT under constant dark conditions. The expression levels of *mPer1*, *mPer2* and *mPer3* were normalized by *Gapdh*, as a house-keeping gene (Figure 1). The phases of *mPer1* were 5.2 h (SCN), 11.3 h (liver) and 9.7 h (WAT). The phases of *mPer2* were 8.7 h (SCN), 14.7 h (liver) and 12.8 h (WAT). The phases of *mPer3* were 8.7 h (SCN), 11.3 h (liver) and 11.3 h (WAT).

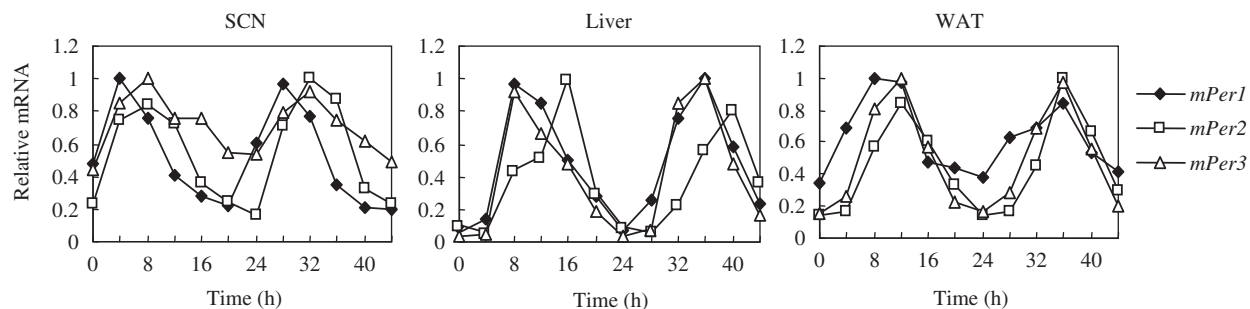
Clock and clock-controlled genes have numerous conserved and functional CCEs for morning (E/E'-boxes), daytime (D-boxes) and night (RREs) (21). These CCEs play key roles in the circadian timing of clock genes (21,22). Our previous study revealed that *mPer1* contains five E-boxes and one D-box in the promoter region, whereas *mPer2* contains one E'-box and one D-box (21). In contrast, *mPer3* has two D-boxes (21). These elements were conserved between human and mouse genomic sequences in regions 10 kb upstream and 2 kb downstream from the TSS. If the phase of clock genes is determined by three major CCEs (that is, the E-box, D-box and RRE) and their combinations, we predicted that *mPer1* then *mPer2* and *mPer3* would have successively later phases. However, the phase of *mPer2* oscillation was found to be the latest among the three *Period* genes in central and peripheral tissues. The phases of the *mPer1* and *mPer3* messenger RNA (mRNA) rhythms were largely in accordance with this hypothesis, but that of *mPer2* was later than expected. Here, wave peak point is used interchangeably with the term 'phase'. These observations led us to hypothesize a different mechanism in which

other unknown elements were involved in generating the variety of phases of clock genes. Therefore, it was necessary to investigate each promoter in greater detail.

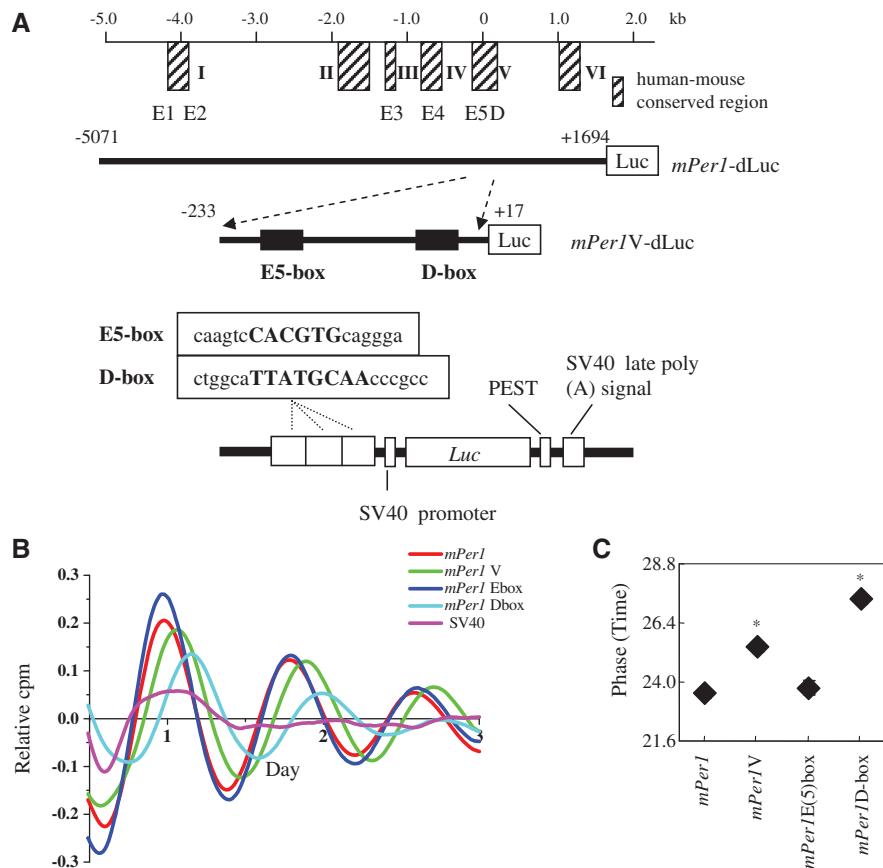
### Phase control of *mPer1* oscillation was governed by canonical E-boxes

It has been reported that the transcription of *mPer1* was regulated by the canonical E-box and D-box using a conventional transient reporter assay (9,26,27). However, that type of experiment is not conclusive in terms of how the CCEs and their combinations determine the phase of the *Per1* gene. We constructed several mutant promoter vectors of the *Per1* genes. Their roles were verified using an *in vitro* rhythm oscillation-monitoring system, in which cultured fibroblasts that were transiently transfected with reporter vectors exhibited robust circadian bioluminescence. Using a real-time bioluminescence system, we were able to investigate the relationship between phase control and CCE. The region of the inserted fragment in *mPer1*-dLuc (26) was shown to be sufficient to drive rhythmic expression *in vivo* (34). We constructed *mPer1V*-dLuc (from -233 to +17) containing one E-box and one D-box. We also constructed SV40-driven dLuc reporters containing three tandem repeats of either the E(5)box or the D-box, designated *mPer1E(5)box* SV40-dLuc or *mPer1 D-box* SV40-dLuc, respectively (Figure 2A). In our preceding work (21), we observed that reporter vectors with one CCE did not exhibit high-amplitude oscillations, while those with two and three CCEs exhibited circadian oscillations in comparable phases. Therefore, bioluminescence from three CCEs was used for the intrinsic phase.

Rat-1 cells were transfected with the constructs and stimulated with dbcAMP. We then measured the luminescence from these cells. To better calculate phase differences, data sets were detrended by subtracting the 24 h running average from the raw data (Figure 2B). The phases (measured from the third peak after stimulation) of *mPer1*-dLuc, *mPer1V*-dLuc, *mPer1 E(5)box* and *mPer1 D-box* were  $23.5 \pm 0.1$  h,  $25.4 \pm 0.2$  h,  $23.8 \pm 0.3$  h and  $27.3 \pm 0.2$  h (means  $\pm$  SE,  $n = 3$ ), respectively. The phase of *mPer1*-dLuc was similar to that of *mPer1 E(5)box* SV40-dLuc (Figure 2C). It was noteworthy



**Figure 1.** The expression rhythm of mouse *Per1*, *Per2* and *Per3* mRNAs. Total RNA was extracted from SCN, liver and WAT, and Q-PCR was performed to examine *mPer1*, *mPer2* and *mPer3* mRNA expression. The relative level of each mRNA was normalized to the corresponding *Gapdh* mRNA level. The maximal amount of mRNA was set to 1. Filled square, open square and open triangle lines represent *mPer1*, *mPer2* and *mPer3*, respectively.



**Figure 2.** Real-time analysis of circadian expression of luciferase driven by the mouse *Per1* promoter. (A) Schematic representation of reporter vectors for the *mPer1* promoter. +1 corresponds to the TSS. (B) Transcriptional oscillation of *mPer1* was monitored by a real-time monitoring system. Rat-1 cells were transfected with the *mPer1* reporter constructs and stimulated with 1 mM dbcAMP. Bioluminescence records were detrended by subtraction of the 24 h running average from the raw data. (C) Phase data for *mPer1*, *mPer1 V*, *mPer1 E(5)box* and *mPer1 D-box*. The phases were measured from the third peak after stimulation; \* $P < 0.05$ .

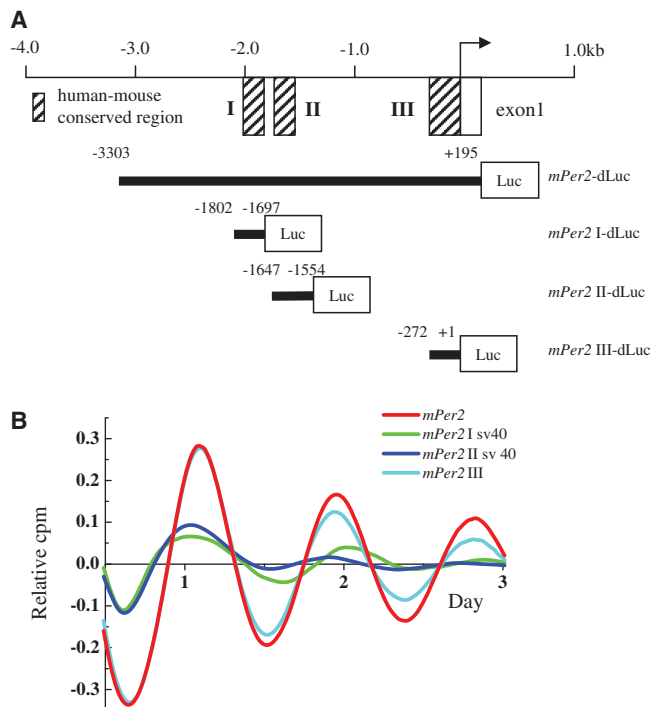
that the phase of *mPer1 V*-dLuc, containing one E-box and one D-box, was in the middle of the expected phase of the E-box and D-box (Figure 2C). One possible explanation for these observations is that combinations of different types of CCEs might generate a new phase of circadian gene expression.

The role of each E-box in the *mPer1* promoter was evaluated by a conventional reporter assay (26). This study revealed that each of the E-boxes functions as an enhancer for the transactivation of *mPer1* by CLOCK/BMAL1. We confirmed that the phase of SV40-driven dLuc reporters containing first E-box  $\times$  3 and second E-box  $\times$  3 was similar to that of *mPer1 E(5)box* SV40-dLuc (fifth E-box  $\times$  3) (Supplementary Figure). Here, we compared four conditions (that is, *mPer1*-dLuc, *mPer1 V*-dLuc, *mPer1 E(5)box* and *mPer1 D-box*). As a whole, the phase of the *Per1* gene rhythm was reflected by the E-box, despite the fact that it was adjusted by both the E-box and the D-box. Our finding led us to speculate that five E-boxes rather than one D-box might play a dominant role in controlling the phase of *Per1* gene oscillation. These observations suggested that the number of functional CCEs might play a significant role in determining the phase of clock genes.

### The third conserved region in *mPer2* is responsible for *mPer2* oscillation

To analyze the mechanism controlling *Per2* rhythm, we created a construct in which the *mPer2* promoter reporter (*mPer2*-dLuc) was inserted into the -3303 to +195 region from the TSS fused to a dLuc reporter gene (Figure 3A). This *mPer2*-dLuc promoter reporter construct is sufficient to make circadian oscillation *in vitro* (19) and *in vivo* (35,36).

The *Per2* promoter reporter (-3303 to +195) consists of segments I (-1802 to -1697), II (-1647 to -1554) and III (-254 to +41) that are conserved between humans and mice. For the functional analysis of the *mPer2* promoter, we generated deletion-mutant constructs (Figure 3A). Although segments I and II are highly conserved in the *mPer2* promoter, these regions did not show basal promoter activities (data not shown). Therefore, *mPer2* I-dLuc and *mPer2* II-dLuc were subcloned into the SV40-driven dLuc reporter. Cells were transfected with these constructs, stimulated with dbcAMP and the bioluminescence was measured. Clearly, *mPer2* III-dLuc showed oscillation similar to that of *mPer2*-dLuc, whereas *mPer2* I-SV40-dLuc and *mPer2* II-SV40-dLuc did not show such an oscillation



**Figure 3.** Segment III, conserved between human and mouse *Per2*, is responsible for *Per2* expression. (A) Schematic representation of deletion mutants of the *mPer2* promoter. *mPer2*-dLuc and *mPer2* III-dLuc were subcloned into the dLuc vector, and *mPer2* I-dLuc and *mPer2* II-dLuc were subcloned into the SV40-driven dLuc reporter. I, II and III are indicated in the conserved regions shared by human and mouse in the *Per2* promoter. +1 corresponds to the TSS. (B) Transcriptional oscillation of *mPer2* was monitored. Rat-1 cells were transfected with the *mPer2* construct and stimulated with 1 mM dbcAMP. Bioluminescence records were detrended as in Figure 2.

(Figure 3B). The relative amplitudes of *mPer2* I-SV40-dLuc, *mPer2* II-SV40-dLuc and *mPer2* III-dLuc compared to *mPer2*-dLuc were  $9.8 \pm 3.1\%$ ,  $8.3 \pm 2.1\%$  and  $75.3 \pm 4.5\%$  (means  $\pm$  SE,  $n = 3$ ), respectively. These results suggested that the third conserved region in *mPer2* was responsible for *mPer2* oscillation.

#### Characterization of the third conserved segment of mouse and human *Per2* genes

The third conserved segment of 218 bp was positioned immediately upstream from the first exon and had 79% identity (Figure 4). This segment included an E-box like element, E'-box (CATGTG), E'-box (CACGTT), D-box (TTATGTAA) and two putative SP1 binding sites, but no canonical TATA-box (Figure 4). Recent studies have shown that the E'-box, which is activated by CLOCK/BMAL1, is responsible for generating *Per2* oscillation *in vitro* (23) and *in vivo* (24). The E''-box (CATGTG) is classified as a CANNTG-type non-canonical E-box (a novel E-box-like element). However, whether this element is involved in the gene expression of *Per2* remains unclear.

#### The 20-bp region containing the E''-box is responsible for the phase delay of *mPer2* oscillation

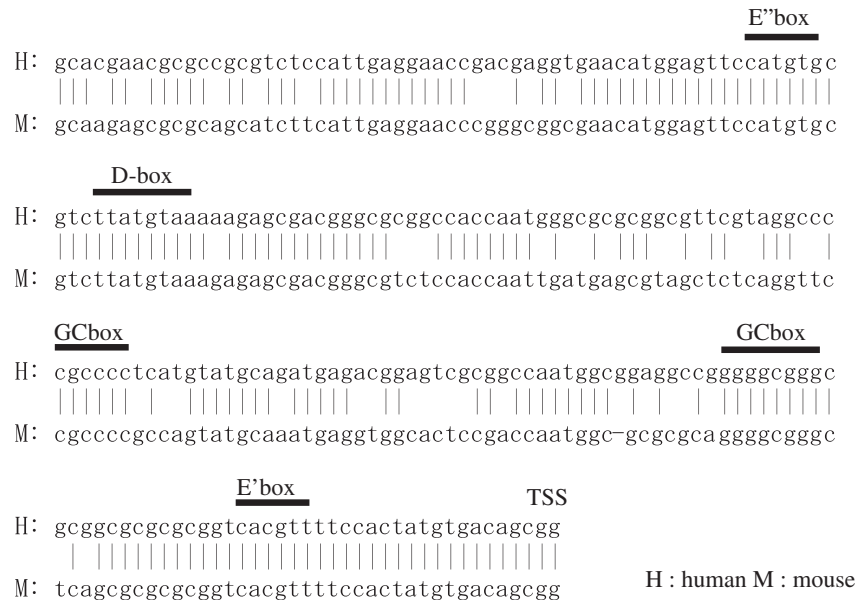
To analyze the function of these *cis*-elements in the third conserved segment, we generated various deletion-mutant reporters in the third conserved segment of *mPer2* (Figure 5A). Rat-1 cells were transfected with these constructs, then stimulated with dbcAMP and the bioluminescence was measured. *mPer2* III (-254 to +41)-dLuc and *mPer2* III\_1 (-181 to +1)-dLuc were observed to be in the same phase, whereas *mPer2* III\_2 (-161 to +1)-dLuc, and *mPer2* III\_3 (-142 to +1)-dLuc showed a phase advance against *mPer2*\_III-dLuc (Figure 5B). The phases of *mPer2* III-dLuc, *mPer2* III\_1-dLuc, *mPer2* III\_2-dLuc and *mPer2* III\_3-dLuc were  $26.5 \pm 0.2$  h,  $26.3 \pm 0.3$  h,  $24.8 \pm 0.2$  h, and  $24.6 \pm 0.3$  h (means  $\pm$  SE,  $n = 4$ ), respectively. *mPer2* III\_1 (-181 to +1)-dLuc constructs showed a 1.5 h phase delay compared with *mPer2* III\_2 (-161 to +1)-dLuc (Figure 5C). From these results, we conclude that the 20-bp region including the E''-box is responsible for the phase-delaying region.

#### The E''-box contributes to delaying the phase of *mPer2* oscillation

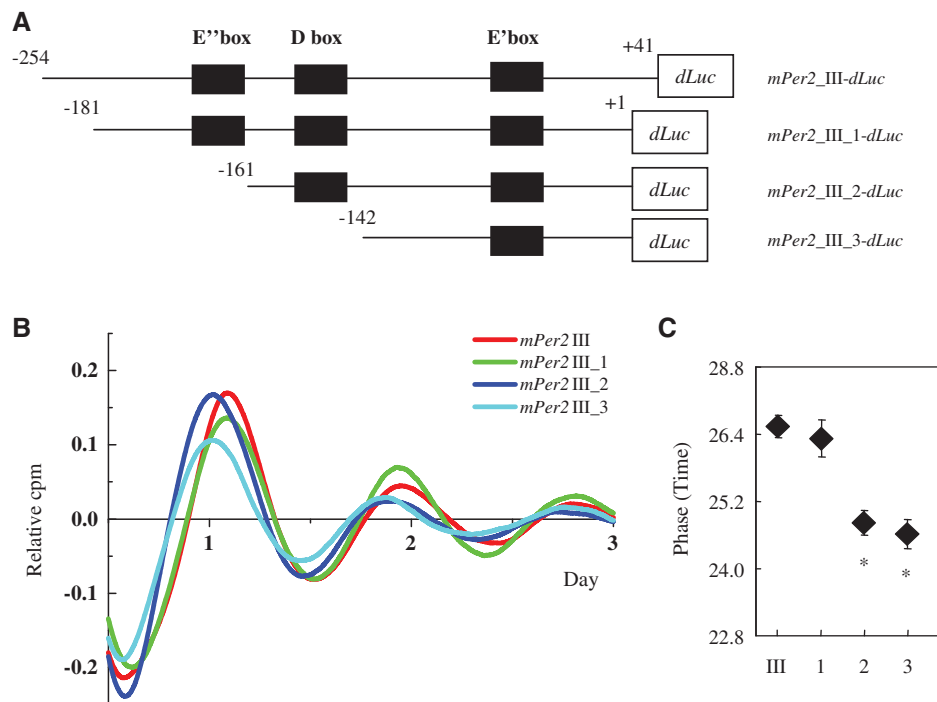
To analyze further the phase-delaying region, we constructed mutant reporter vectors of *mPer2* III-dLuc by site-directed mutagenesis (Figure 6A). Rat-1 cells were transfected with these constructs, stimulated with dbcAMP, and the bioluminescence was measured. Similar phases were observed with the E'-box mutant (*mPer2* III E'MT-dLuc) and the D-box mutant (*mPer2* III DMT-dLuc), whereas a phase advance (1.5 h earlier) was detected only when using the E''-box mutant (*mPer2* III E''MT-dLuc) construct (Figure 6B). The phases of *mPer2* III-dLuc, *mPer2* III E'MT-dLuc, *mPer2* III DMT-dLuc and *mPer2* III E''MT-dLuc were  $25.9 \pm 0.1$  h,  $24.4 \pm 0.3$  h,  $26.0 \pm 0.1$  h and  $26.3 \pm 0.1$  h (means  $\pm$  SE,  $n = 3$ ), respectively (Figure 6C). We therefore concluded that the E''-box plays a pivotal role in the phase delay of *mPer2* oscillation.

CLOCK, BMAL1, CRY1 and DEC1 are transcription factors that regulate the E-box (21). CRY1 interacts with CLOCK/BMAL1, thus blocking their transcription. DEC1 serves as a transcriptional repressor for CLOCK/BMAL1-enhanced promoter activity, through binding competition for the E-box (22).

We compared the effects of CRY1 and DEC1 on transcriptional activities from E'-box and E''-box-carrying promoters. CRY1 abolished the CLOCK/BMAL1-enhanced transcriptional activities of both the E'-box and the E''-box (Figure 6E). In contrast, DEC1 abolished the CLOCK/BMAL1-enhanced transcriptional activities of the E'-box, whereas the E''-box-containing construct was weakly affected by DEC1 (Figure 6E). It is known that DEC1 directly binds to canonical E-box of clock genes and modulates the circadian phase, whereas it weakly affects clock genes containing the non-canonical E-box because of weak binding to it (37). While the mechanism by which the E''-box delays the phase of the *Per2* oscillation has not been fully clarified, impaired



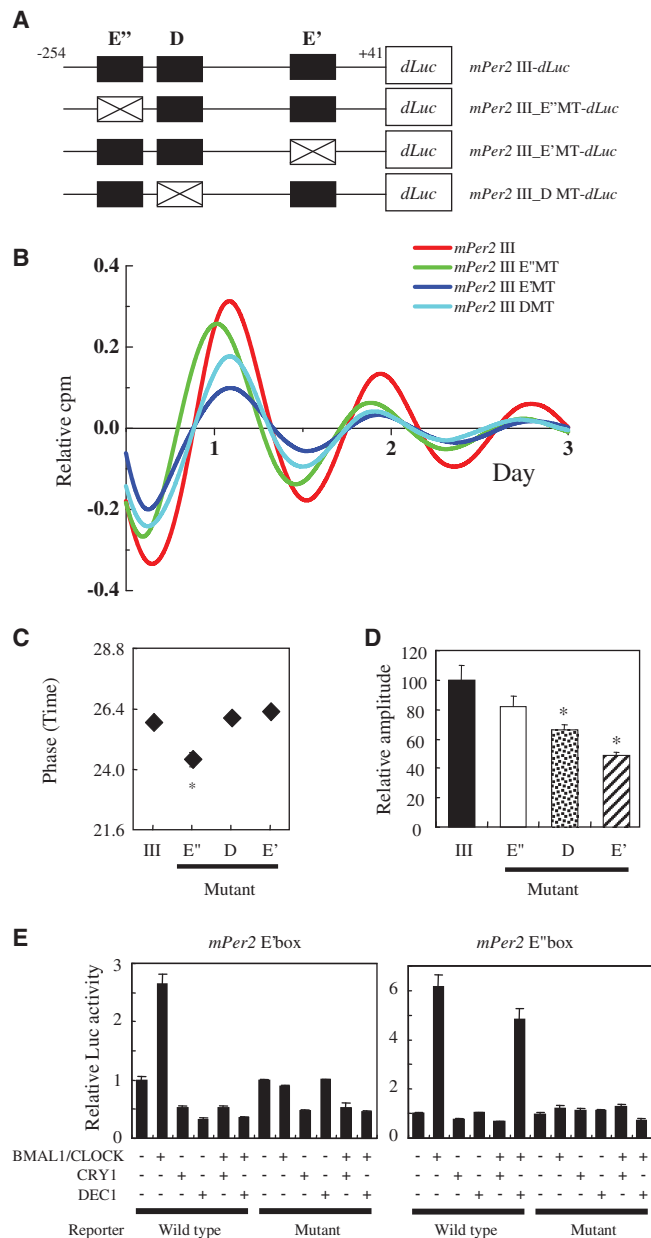
**Figure 4.** Nucleotide sequence alignments of conserved segment III shared by human and mouse *Per2*. H and M represent human and mouse sequences, respectively. The colored letters represent the putative consensus sequence for transcription factors. A nucleotide sequence alignment of the 5'-proximal promoter of mouse *Per2* is presented with putative *cis*-regulatory elements of the E''-box (from -162 to -157), D-box (-152 to -145), Sp1 (-100 to -92 and -47 to -40) and E' box (-24 to -19). Promoter analysis was performed by TFSEARCH. Numbers indicate nucleotide distances from the predicted TSS.



**Figure 5.** Deletion analysis of the third conserved region in the *mPer2* gene. (A) Schematic diagrams of various *mPer2* promoter constructs are shown and the names of the plasmids are listed. (B) De-trended bioluminescence data of *mPer2* III and its deletion mutants fused to a dLuc reporter gene. Rat-1 cells were transfected with these constructs and stimulated with 1 mM dbcAMP. Bioluminescence records were detrended as in Figure 2. (C) Phase data for *mPer2* III-dLuc, *mPer2* III\_1-dLuc, *mPer2* III\_2-dLuc and *mPer2* III\_3-dLuc. The phases were measured from the third peak after stimulation; \**P* < 0.05.

transcriptional regulation of DEC1 at the E''-box might play a pivotal role in this system. Recently, the pulses of prolactin promoter activity were shown to depend on the noncanonical E-box (CATTG) that binds to the

CLOCK/BMAL1 complex (38). Our data, together with previous reports, suggest that non-canonical E-boxes might not only play a role in circadian enhancers, but might also generate various phases.



**Figure 6.** The E'-box (a novel E-box-like element) and the D-box in the third conserved segment of the *Per2* promoter are functional *cis*-elements. (A) Schematic representation of point mutagenesis of putative *cis*-elements in the conserved segment III (*mPer2* III-*dLuc*). The wild-type is indicated by a closed box and the mutant type is indicated by a white box (christcross). The mutations were as follows: mutated E'-box (ACCGGT), and mutated E''-box (ACCGGT), and mutated D-box (CACCCGCG). (B) Detrended bioluminescence data of *mPer2* III-*dLuc* and its point mutants. Rat-1 cells were transfected with the *mPer2* III-*dLuc* and its point mutants, and stimulated with 1mM dbcAMP. Bioluminescence records were detrended as in Figure 2. (C) Phase data for *mPer2* III, *mPer2* III E''MT, *mPer2* III DMT and *mPer2* III E'MT. The phases were measured from the third peak after stimulation; \* $P < 0.05$ . (D) Relative amplitude data were calculated from the third to the eighth peak of oscillation. Relative amplitudes of *mPer2* III\_E''MT, *mPer2* III\_DMT and *mPer2* III\_E'MT to *mPer2* III are shown; \* $P < 0.05$ . (E) Transcriptional activation by CLOCK and BMAL1, and transcriptional repression by CRY1 or DEC1 of the reporters containing three tandem repeats of either the wild-type or the mutants. The presence (+) or absence (-) of the expression plasmid is denoted. Each value is the mean  $\pm$  SE of three replicates for a single assay.

### The D-box in the third conserved segment of *mPer2* promoter is a functional *cis*-element that increases amplitude

The relative amplitudes of *mPer2* III E''MT-*dLuc*, *mPer2* III DMT-*dLuc* and *mPer2* III E'MT-*dLuc* compared to *mPer2*-*dLuc* were  $82.2 \pm 6.8$  %,  $66.0 \pm 3.4$  % and  $49.1 \pm 1.6$  % (means  $\pm$  SE,  $n = 3$ ), respectively (Figure 6D). We confirmed that the E'-box was essential to generate *mPer2* oscillation, which is consistent with the observation by Akashi *et al.* (23) and Yoo *et al.* (24). We also showed, for the first time, that the D-box in the third conserved segment generated high amplitude.

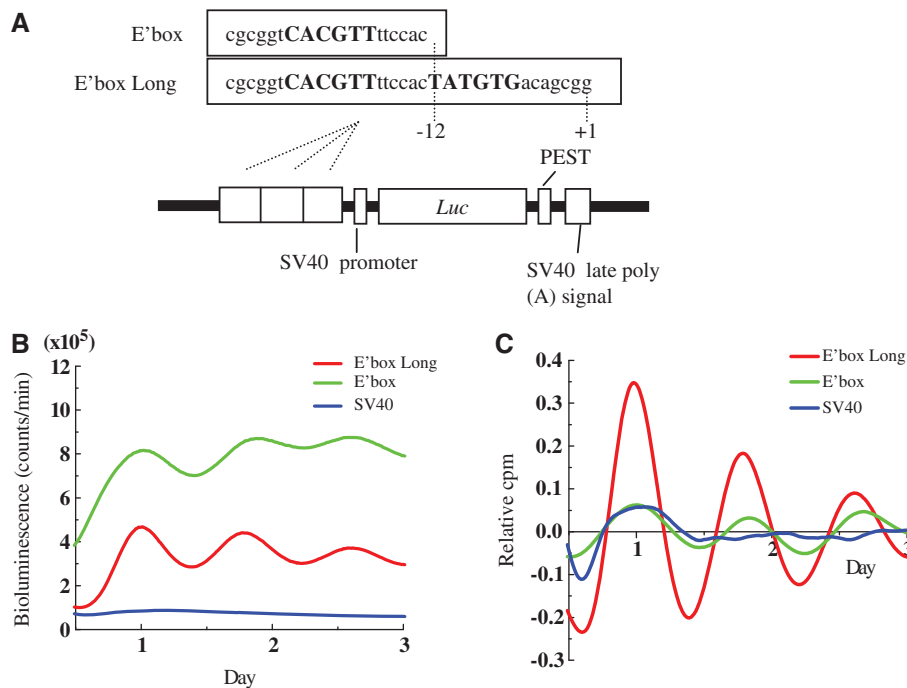
Ohno *et al.* (25) reported that the D-box in the third conserved region is not a prerequisite for the circadian expression of *Per2*. Nevertheless, we have shown that the D-box in the third conserved segment of the *Per2* promoter is a functional *cis*-element that increases amplitude. The circadian oscillations of *Per2* expression exhibited stable during Day 3 in the current study (Figure 6B). On the other hand, the circadian oscillations of *Per2* expression persisted but attenuated during this period in the published work (25). Therefore, sustained and longer circadian oscillation might help to generate the high amplitude in the D-box of the third conserved region of the *Per2* promoter.

### The region between the E'-box and the TSS (-12 to +1) tightly regulates *mPer2* oscillation

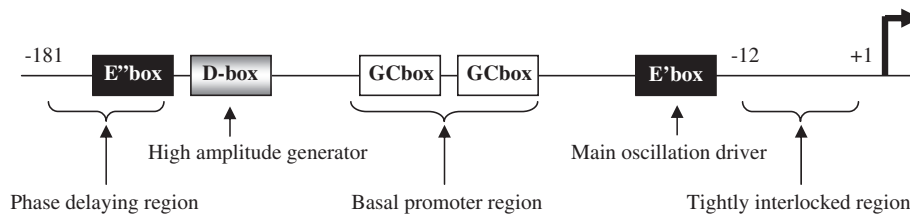
To better understand the mechanism driving oscillation of *mPer2*, we constructed deletion mutant reporters including E'-box, which is essential to generate *mPer2* oscillation (Figure 7A). We transfected the deletion mutant vectors into Rat-1 cells, then stimulated them with dbcAMP, and measured the bioluminescence. The E'-box SV40-*dLuc* displayed a higher bioluminescence than did the E'-box Long SV40-*dLuc* (Figure 7B). However, when these data were de-trended by subtracting the 24h running average from the raw data, the E'-box Long SV40-*dLuc* showed higher amplitude than the E'-box SV40-*dLuc* (Figure 7C). These results indicate that the -12 to +1 region enhances the amplitude of *mPer2* oscillation.

Recent studies have shown that the direct repeat of the E-box-like elements is the minimal required element for the generation of cell-autonomous transcriptional oscillation (39). In the present study, we also showed that the region (-12 to +1) adjacent to the E'-box containing the direct repeat of the E-box-like elements (39) serves to generate more robust oscillation in the *Per2* gene.

Kumaki *et al.* (40) revealed that the importance of the affinity balance between transactivators and transrepressors in generating high-amplitude circadian transcriptional output. In the case of E-box, lowest affinity for CLOCK/BMAL1 was those giving the highest amplitude of oscillation. Additionally, we showed that the bioluminescence of the E'-box region itself was greater than that displayed by the E'-box long regions containing a direct repeat of the E-box-like elements. However, based on the de-trended data, we demonstrated that the E'-box long regions displayed a



**Figure 7.** Analysis of the *mPer2* tightly-interlocked region. (A) Schematic diagrams of various *mPer2* promoter constructs are shown and the names of the plasmids are listed. The E'box Long regions contains a direct repeat of the E-box-like elements, which is described previously (39). (B) Transcriptional oscillations of E'box and E'box long-SV40-dLuc were monitored. Rat-1 cells were transfected with these constructs and stimulated with 1 mM dbcAMP. (C) The signals obtained in (B) were detrended.



**Figure 8.** Schematic representation of molecular mechanisms of *mPer2* circadian transcription.

higher amplitude than the E'-box regions. These observations suggested that the E'-box long regions displayed a lower bioluminescence were attributable to the lowest affinity for CLOCK/BMAL1, as well as generating high amplitude.

## CONCLUSIONS

A key issue concerning the logic of the mammalian circadian clock is how the expression peaks of circadian oscillating genes are determined. It has recently been proposed that the robust rhythmic expression requires CCE (E-box, D-box and RRE). While three major CCE might be capable of controlling circadian timing, not all phase have so far proved amenable. For example, although the recent application of a 'synthetic molecular biology' approach successfully generated many fundamental circadian phases, it has not yet been able to generate the basic morning phase (22). Therefore, it is necessary to investigate each promoter in further detail using an *in vitro* rhythm oscillation-monitoring system.

Here, we focused on the differential circadian timing of *Per1* and *Per2* transcription. These genes are E-box and D-box regulated genes, but in central and peripheral tissues peak *Per2* mRNA expression is delayed by several hours relative to that of *Per1*. In the case of the *Per1* gene, the copy number of the E-box might play an important role in determining the phase of *Per1* oscillation. Deletion and point mutation analyses led to the identification of critical sequences responsible for the phase delay of *Per2* oscillation. This sequence was classified as a CANNTG-type non-canonical E-box. Transfection experiments confirmed that the non-canonical E-box could be activated by CLOCK/BMAL1 and repressed by CRY1, whereas it was weakly affected by DEC1. Our observations revealed that CLOCK/BMAL1-mediated transcriptional regulation might not only play a role in circadian enhancers, but also generate various phases. We also showed that the D-box in the third conserved segment of the *Per2* promoter generated high amplitude. Figure 8 summarizes the schematic models of the molecular mechanisms of *Per2* transcription. Our study demonstrated that the copy number and various



combinations of functional CCEs ultimately led to different circadian phase and amplitude.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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