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Glucocorticoid-mediated *Period2* induction delays the phase of circadian rhythm

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ABSTRACT

Glucocorticoid (GC) signaling synchronizes the circadian rhythm of individual peripheral cells and induces the expression of circadian genes, including *Period1* (*Per1*) and *Period2* (*Per2*). However, no GC response element (GRE) has been reported in the *Per2* promoter region. Here we report the molecular mechanisms of *Per2* induction by GC signaling and its relevance to the regulation of circadian timing. We found that GC prominently induced *Per2* expression and delayed the circadian phase. The overlapping GRE and E-box (GE2) region in the proximal *Per2* promoter was responsible for GC-mediated *Per2* induction. The GRE in the *Per2* promoter was unique in that brain and muscle ARNT-like protein-1 (BMAL1) was essential for GC-induced *Per2* expression, whereas other GRE-containing promoters, such as *Per1* and mouse mammary tumor virus, responded to dexamethasone in the absence of BMAL1. This specialized regulatory mechanism was mediated by BMAL1-dependent binding of the GC receptor to GRE in *Per2* promoter. When *Per2* induction was abrogated by the mutation of the GRE or E-box, the circadian oscillation phase failed to be delayed compared with that of the wild-type. Therefore, the current study demonstrates that the rapid *Per2* induction mediated by GC is crucial for delaying the circadian rhythm.

INTRODUCTION

The circadian clock is composed of an endogenous rhythm that provides approximate 24-h timing cues to various biological activities, including metabolism, physiological processes and behavior. In mammals, the master pacemaker resides in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN has self-sustainable oscillators and synchronizes the circadian timing of peripheral tissues by transmitting neuronal and humoral signals. Peripheral tissues also have endogenous clock machinery and are thus able to maintain the circadian rhythm without any external cues (1,2).

The endogenous circadian timing system consists of molecular feedback mechanisms, including the core and auxiliary loop. In the core loop, two basic helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS)-containing transcription factors, circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein-1 (BMAL1), bind to the E-box of clock-controlled genes, such as *Period (Per) 1/2*, *Cryptochrome (Cry) 1/2*, *Rev-erb α* and *ROR α*. The translated PERs and CRYs translocate to the nucleus and repress CLOCK:BMAL1 activity to return to the starting point. The auxiliary loop reinforces the circadian rhythm by regulating the rhythmic expression of BMAL1 through competitive binding of ROR α and REV-ERB α to RRE (Rev-erb/ROR binding element) on the *Bmal1* promoter (3,4).

The endogenous clock does not have an exact 24-h period and has the flexibility to adjust to the phases of the environmental cycle, especially the light/dark photocycle. Light exposure at the time of early/late subjective night results in a delay/advance of the next activity cycle, which is represented as a phase response curve. It has been widely accepted that rapid expression of *Per1* and *Per2* plays a crucial role in the light-dependent resetting process. In particular, *Per1* and *Per2* are thought to mainly play a role in phase advance and phase delay, respectively; however, their actual roles only in the resetting process remain to be elucidated (5–9). These molecular events also occur in peripheral tissues and immortalized cell lines through synchronizing signals (10,11).

Glucocorticoid (GC) is a multifunctional hormone that regulates glucose and lipid metabolism, immune activity, the stress response and learning and memory (12–14). The level of GC displays a robust circadian rhythm, and the administration can reset the rhythmic phase of

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peripheral tissues and immortalized cells (11). Furthermore, the expression of GC receptors (GRs) in most peripheral cells, not in the SCN, enables the entrainment of peripheral clocks without any interference of the master clock. Therefore, GC is considered to be the best candidate for the synchronizing signal between the SCN and peripheral tissues.

During the synchronization process, *Per1* and *Per2* are rapidly induced and oscillate in a circadian fashion. Whereas GC regulates *Per1* through the GC response element (GRE) in its promoter region, the molecular mechanisms of GC-mediated *Per2* expression have not been clearly elucidated (15). Chromatin immunoprecipitation (ChIP)-sequencing analysis revealed that three GR-binding sites exist near *Per2* gene (16). Several studies have shown that *Per2* promoter region, in which the canonical GRE has not been found, is enough for GC responsiveness to *Per2* (17,18). On the other hand, So *et al.* reported that the intronic GR-binding sequence (GBS) can confer GC responsiveness to *Per2* (19).

Per2-knockout mice show a significantly shorter circadian rhythm or arrhythmicity of locomotor activities and have defects in the anticipation of feeding (7–9,20,21). Per2 is not only a component of the circadian oscillator but also functions as a mediator for the timed regulation of many types of metabolism. Direct interactions between PER2 and various nuclear receptors, including HNF4α, REV-ERBα and PPARα, enable the circadian oscillation of glucose and lipid metabolism (22). Besides, *Per2*-knockout mice, which lack 9th intron containing GC-responsive region, exhibited altered GC-induced glucose intolerance and insulin resistance, partly owing to increased leptin levels (19). It is also related to the timing of sleep. *Per2*-knockout mice wake earlier than wild-type (WT) mice, and the human PER2 S662G mutation prevents the phosphorylation of PER2 by CKIε, resulting in rapid degradation and nuclear export, which is observed in patients with familial advanced sleep phase syndrome (23–25). Therefore, the exact timing of *Per2* expression may be critical for maintaining or restoring a physiology that is properly attuned to the environmental light–dark cycle.

In the present study, we investigated the molecular mechanisms underlying GC-mediated *Per2* induction and its functional relevance to the regulation of the circadian rhythm. We provide evidence that BMAL1-dependent binding of GR to the overlapping GRE/E-box in the 5′ upstream region of *Per2* gene induces the expression of *Per2*. Furthermore, we demonstrate that GC-mediated *Per2* induction by this BMAL1-dependent GR mechanism is responsible for the phase delay.

MATERIALS AND METHODS

Cell culture

WT, Per2::luc knock-in, *Per2*$^{-/-}$ and *Bmal1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were spontaneously immortalized as previously described (26–29). Primary or immortalized cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% carbon dioxide (CO_2).

Constructs

Per2 promoter region from −1671 to +26 from the transcription start site (TSS) was cloned into the GL3-basic vector (Promega, Madison, WI, USA), in which the PEST sequence was inserted into the C-terminal of the luciferase gene. The final construct was called Per2 (−1671)::dsluc. A series of 5′ deletion mutants were prepared from Per2 (−1671)::dsluc. E1, E2, GRE and palindromic GRE mutants were generated from Per2 (−271)::dsluc by the site-directed mutagenesis using the following primers: E1 mutant up: 5′-CGGGCTCAGCGCGCGGT**GCTAG**TTTCCACTATGTGACAGCGG-3′, E1 mutant dn: 5′-CCGCTGTCACATAGTGGAA**ACTAGC**ACCGCGCGCGCTGAGCCCG-3′; E2 mutant up: 5′-CGGCGAACATGGAGTTC**CATAGA**CGTCTTATGTAAAG-3′, E2 mutant dn: 5′-CTTTACATAAGACG**TCTATG**GAACTCCATGTTCGCCG-3′; GRE mutant up: 5′-GAGGAACCCGGGCGGC**TA**G**T**ATGG**ATA**TCCATGTGCGTCTTATG-3′, GRE mutant dn: 5′-CATAATACGCACATGGA**TAT**CCAT**AC**TAGCCGCCCGGGTTCCTC-3′; palindromic GRE up: 5′-GGAACCCGGGCGG**A**GAACATGG**T**GTTC**T**ATGTGCGTCTTATG-3′, palindromic GRE dn: 5′-CATAAGACGCACAT**A**GAAC**A**CCATGTTC**T**CCGCCCGGGTTCC-3′.

Luciferase assay

WT and *Bmal1*$^{-/-}$ MEFs were transfected using Lipofectamine PLUS reagents (Invitrogen). Cells were harvested after treatment with 0.1% ethanol or 1 μM dexamethasone, a synthetic GC (DEX; Sigma-Aldrich, St. Louis, MO, USA), for 10 h, which elicited the maximal induction. Luciferase activities were analyzed by the dual luciferase reporter assay system (Promega). Fold induction was calculated by dividing the luciferase activities in the DEX-treated group by those in the ethanol-treated group.

Recording of real-time luminescence

Per2::luc knock-in MEFs were cultured the day before the monitoring of luminescence. After treatment with the various compounds (0.1% ethanol, 1 μM DEX, 0.1% DMSO, 10 μM forskolin, 50% horse serum, 1 mM dibutyryl cyclic AMP [dbcAMP] and 1 μM ionomycin) for 2 h, and media were changed to normal culture media with 100 μM luciferin (Promega). Per2 (−271)::dsluc and its mutants were transfected into WT MEFs for 24 h. Bioluminescence was measured for 1 min for each dish at 10-min intervals with a real-time luminescence monitoring device (Kronos-Dio; ATTO Corporation, Tokyo, Japan) at 36°C in a humidified atmosphere containing 5% CO_2. Data were normalized by the average of the initial minimum value.

Real-time reverse transcriptase-polymerase chain reaction

MEFs were seeded in six-well plates and harvested at the indicated times after treatment with 0.1% ethanol or 1 μM

DEX (with or without 5 μM RU486; Sigma-Aldrich). Total RNA was isolated by the single-step acid guanidinium thiocyanate–phenol–chloroform method. Next, 2 μg of RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Promega). Real-time polymerase chain reaction was carried out in the presence of SYBR Green I. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. The primers used for real-time reverse transcriptase-polymerase chain reaction were as follows: *Per1* up, 5′-GTGTCGTGATTAAA TTAGTCAG-3′, *Per1* dn, 5′-ACCACTCATGTCTGGG CC-3′; *Per2* up, 5′-GCGGATGCTCGTGGAATCTT-3′, *Per2* dn, 5′-GCTCCTTCAGGGTCCTTATC-3′; *GAPDH* up, 5′-CATGGCCTTCCGTGTTCCTA-3′, *GAPDH* dn, 5′-CCTGCTTCACCACCTTCTTGA-3′.

Chromatin immunoprecipitation

WT and *Bmal1*$^{-/-}$ MEFs were treated with 0.1% ethanol or 1 μM DEX for 1 h and exposed to 1% formaldehyde for 10 min. Cells were collected and were made to swell with hypotonic buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Triton X-100, 1 mM Phenylmethanesulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail). After centrifugation, the nuclear pellet was lysed in nuclear lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitor cocktail). The chromatin was sheared off by sonication to <500 bp. Precleared samples were immunoprecipitated with normal rabbit serum and anti-GR (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunoprecipitated DNA was purified with phenol/chloroform. For PCR, the primers were as follows: *Per1* GRE up, 5′-AAGG CTGTGTGCATGTCCT-3′, *Per1* GRE dn, 5′-AGAGGG AGGTGACGTCAAAG-3′; *Per2* GRE up, 5′-GTGCCAG GTGAATGGAAGTC-3′, *Per2* GRE dn, 5′-AGCTACGC TCGTCAATTGGT-3′.

Adenoviral transduction

Per2 recovery constructs were designed to express PER2-LUCIFERASE fusion protein under WT or mutant *Per2* promoter (−271 to +26 from the TSS). Adenoviral constructs were generated according to the manufacturer's instructions (Invitrogen). To determine the effects of the WT or mutant viruses, *Per2*$^{-/-}$ MEFs were seeded in 35-mm culture dishes, and the adenoviruses were added after 24 h. To analyze the circadian patterns of PER2, we recorded luminescence at 36°C with 5% CO_2 using a real-time luminescence monitoring device after a 2-h DEX treatment.

Statistical analysis

Data were analyzed by 1-way analysis of variance with Tukey post hoc tests using GraphPad Prism software (GraphPad Prism Software, Inc., La Jolla, CA, USA). A p-value of <0.05 was considered to be significant.

RESULTS

GC signaling induces a prominent *Per2* expression and the delayed circadian phase

Many signaling pathways regulate clock gene expression (18,30). To examine the effects of GC on *Per2* expression, we treated Per2::luc knock-in MEFs with DEX, forskolin, dbcAMP, ionomycin or horse serum and compared the oscillation profiles by recording real-time bioluminescence. DEX significantly increased PER2 protein levels, with a peak at approximately 10 h after treatment, whereas the other stimuli did not elicit gene expression (Figure 1A). Moreover, the phase of PER2 oscillation was significantly delayed in the DEX-treated group (Figure 1B). To verify the functional role of GR on DEX-mediated *Per2* expression, Per2::luc knock-in MEFs were co-treated with RU486 (a GR antagonist). Both DEX-induced PER2 expression and the delay in phase were completely blocked by RU486 (Figure 1C and D). These results indicated that the intact GR activity was required for the regulation of *Per2* expression and delay in the circadian phase. Consistent with this, the induction of *Per1* and *Per2* mRNA expression was also blocked by RU486 treatment, indicating that those genes were regulated by GC at the transcriptional level (Figure 1E). Interestingly, we also found that the induction profile of *Per1* expression by DEX was more rapid than that of *Per2*. Moreover, *Per1* expression was elicited by other signals that did not increase *Per2* levels (Supplementary Figure S1) (15). Hence, although *Per1* and *Per2* are immediate early genes and have a certain redundancy of function in relation to circadian rhythm, they may produce different outputs depending on the combination of different signal transduction pathways activated. These results suggested that GC-induced *Per2* expression was responsible for the phase delay. Therefore, we investigated the molecular mechanism of GC-mediated *Per2* induction and its relevance to the regulation of circadian rhythms.

The GC-responsive region in the *Per2* promoter

To determine whether GC-induced *Per2* expression was regulated by the promoter region, we compared DEX-stimulated oscillation profiles of Per2::luc knock-in and *Per2* promoter-driven luciferase (Figure 2A). Although the overall profile of Per2::luc knock-in was delayed, compared with that of *Per2* promoter activity, the inductive and circadian oscillatory patterns exhibited similar profiles. Therefore, this result indicated that the 5′ upstream region of *Per2* gene was sufficient for DEX-mediated *Per2* induction and circadian oscillation. To narrow down the precise region required for *Per2* induction, we generated serially deleted promoter constructs (Figure 2B). Although the basal promoter activities of Per2 (−241)::dsluc and Per2 (−201)::dsluc were decreased, the fold induction by DEX treatment was maintained up to Per2 (−201)::dsluc. However, when the region from −201 to −171 was deleted, the fold induction was completely blocked (Figure 2C). These effects were also demonstrated by the recording of real-time bioluminescence. The induction and

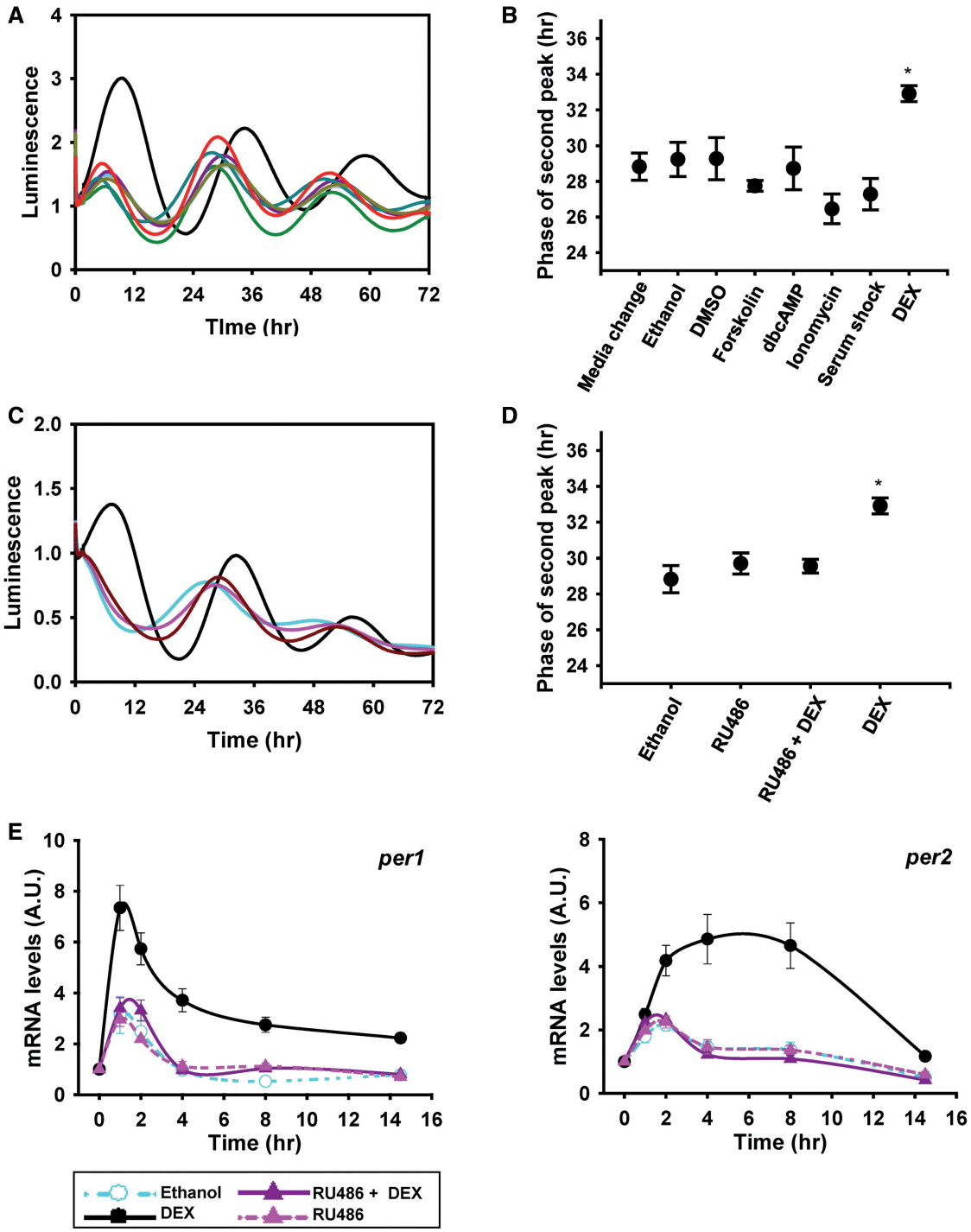

Figure 1. DEX-induced prominent *Per2* induction and the delayed phases of circadian rhythm. (**A**) Per2::luc knock-in MEFs were treated with the indicated synchronizing signals for 2 h and bioluminescence was measured. Ethanol (0.1%; sky line), DEX (1 μM; black line), DMSO (0.1%; gray line), forskolin (10 μM; green line), dbcAMP (1 mM; dark cyan line), ionomycin (1 μM; purple line), medium change (mustard-colored line) and serum shock (medium containing 50% horse serum; red line). (**B**) The phase of the second peak was measured for all stimuli in (A) ($*P < 0.05$; $n = 3$). (**C**) PER2::luc knock-in MEFs were treated with RU486 (5 μM) in combination with DEX for 2 h. Ethanol (sky line), DEX (black line), RU486 (pink line) and RU486 + DEX (dark red line). (**D**) The phase of the second peak was measured for all stimuli in (B) ($*P < 0.05$; $n = 4$). (**E**) WT MEFs were treated with ethanol or DEX for 2 h with or without RU486. Cells were harvested at the indicated times, and *Per1* and *Per2* mRNA levels were analyzed using real-time PCR. Each value was normalized to the GAPDH expression level. Values are the mean ± standard error of the mean (SEM) of three or four independent experiments performed in triplicates.

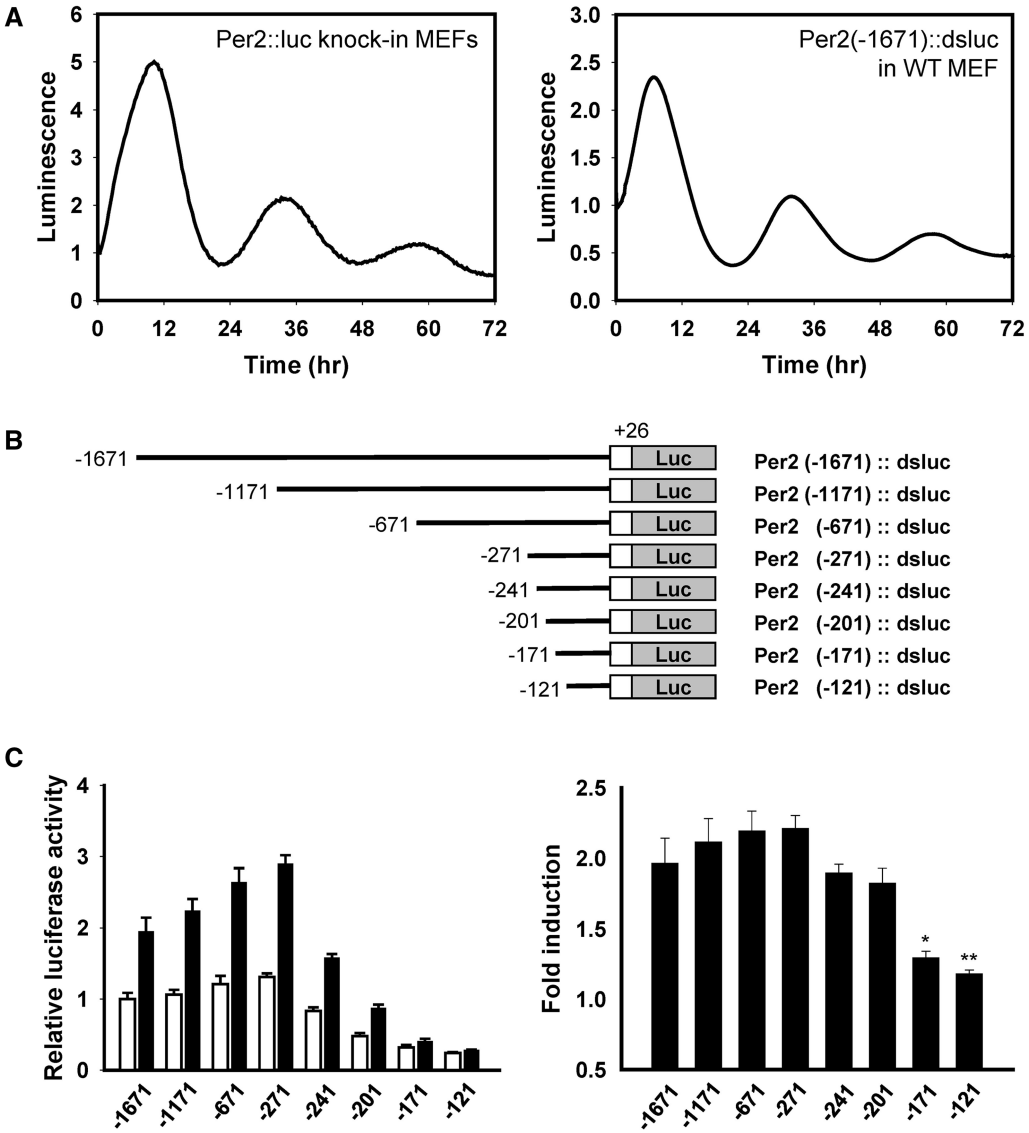

Figure 2. Serial deletion analysis of the mouse *Per2* promoter. (**A**) Comparison of the bioluminescence profiles after DEX treatment between the Per2::luc knock-in MEFs (left) and WT MEFs, which were transfected with *Per2* promoter driven-luciferase of approximately 1.7 kb (right). (**B**) Schematic diagram of the mouse *Per2* promoter serial deletion constructs. Nucleotides are numbered from the transcription start site. (**C**) DEX-responsiveness was analyzed in *Per2* serial deletion mutants. WT MEFs were transfected with the serial deletion mutants and treated with ethanol (0.1%; white bar) or DEX (1 μM; black bar) for 10 h, which elicited maximal *Per2* induction (left). Fold induction was calculated by dividing the luciferase activities in the DEX-treated group with those in the ethanol-treated group (right). Values are the mean ± SEM of three independent experiments performed in triplicates (*$P < 0.05$; **$P < 0.01$).

oscillation profiles of Per2 (−271)::dsluc were almost the same as those of the full-length promoter, i.e. Per2 (−1671)::dsluc, but Per2 (−201)::dsluc and Per2 (−171)::dsluc were not induced (Supplementary Figure S2). Interestingly, these deletion mutants maintained their circadian oscillations, but without *Per2* induction, implying that distinct mechanisms regulate the induction event and subsequent oscillations.

The conserved region containing the overlapping GRE and E-box is responsible for *Per2* induction

Circadian clock genes are highly conserved in mammals (31–33). To gain insight into the putative role of the GC-responsive region, we compared the 5′ upstream region of *Per2* gene in several mammalian species. The proximal region of *Per2* promoter was conserved in mice, humans, rats, cows and monkeys (Figure 3A). Sequence analysis revealed that the conserved regions contain a D-box, CCAAT-box, GC-box and a putative GRE, which differed slightly from the consensus sequence (5′-GGTACANNNTGT(T/C)CT-3′). We also found it to be overlapped with one of E-boxes (E2) by1-bp. To investigate the possibility of interaction between the two elements, we generated mutant constructs of Per2 (−271)::dsluc, the shortest construct exhibiting the same circadian oscillation and DEX responsiveness as the full-length promoter (Supplementary Figure S2).

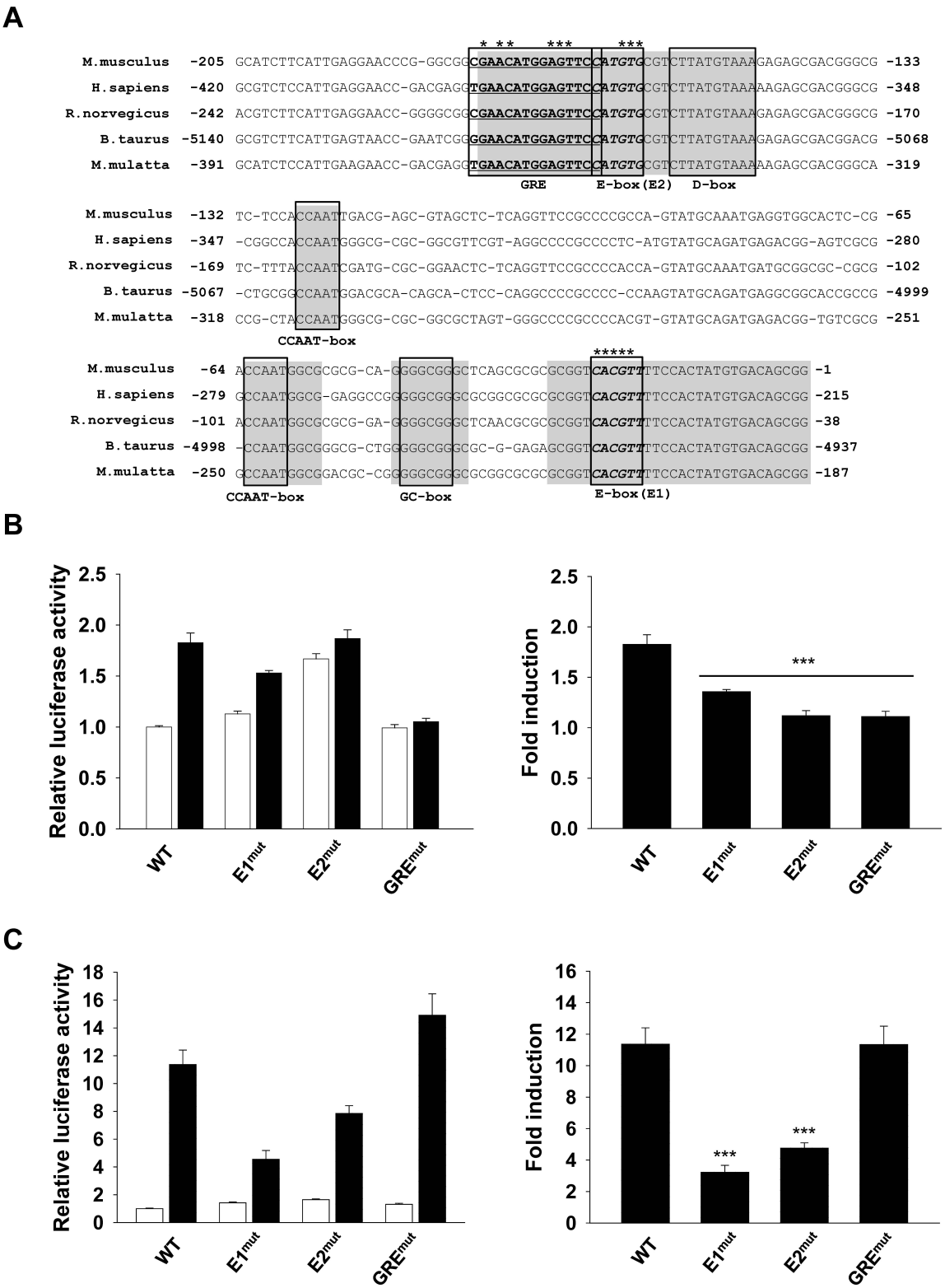

Figure 3. Both GRE and E-box were required for *Per2* induction. (**A**) Sequence alignment of the proximal *Per2* promoter region of some mammalian species, including mouse (*Mus musculus*), human (*Homo sapiens*), rat (*Rattus norvegicus*), cow (*Bos taurus*) and monkey (*Macaca mulatta*). The conserved regions are shaded gray. Several putative *cis*-elements are indicated, and two E-boxes are named as E1 and E2. The asterisk indicates the mutated base. (**B**) DEX-responsiveness of GRE and E-box mutants. WT MEFs were transfected with WT or mutant constructs and treated with ethanol (0.1%; white bar) or DEX (1 μM; black bar) for 10 h (left). Fold induction was calculated by dividing the luciferase activities in the DEX-treated group with those in the ethanol-treated group (right). (**C**) CLOCK:BMAL1-mediated transcriptional activation of GRE and E-box mutants. Fold induction was calculated by dividing the luciferase activities in CLOCK:BMAL1-transfected group (black bar) with those in pcDNA3-transfected group (white bar). Values are the mean ± SEM of three independent experiments performed in quadruplicates (***$P < 0.001$).

The basal levels of the GRE mutant (GRE^{mut}) and E1 mutant ($E1^{mut}$) were similar to the WT; however, the E2 mutant ($E2^{mut}$) exhibited increased basal activity. As expected, GRE^{mut} completely blocked responsiveness to DEX. Interestingly, DEX responsiveness was also blocked in $E2^{mut}$, whereas $E1^{mut}$ only partially decreased the fold induction (Figure 3B). Therefore, these data suggested that the overlapping GRE/E2 (GE2) was crucial for DEX-mediated *Per2* induction and E1 had only a moderate effect on this induction.

To elucidate whether the functional interaction between the GRE and E-box was generally found in GC signaling, we tested the effects of the GRE-E-box interaction on the mechanism of *Per1* induction. Whereas mutations in GREs completely blocked *Per1* induction, mutations in E-boxes did not affect this event (Supplementary Figure S3). These results indicated that the functional interaction between GRE and E-box, as shown in the *Per2* promoter, was not a general mechanism of GC signaling.

To confirm that E-boxes on the *Per2* promoter were responsible for the binding of the circadian clock machinery, we examined CLOCK:BMAL1-mediated transcriptional activities of these mutants. $E1^{mut}$ and $E2^{mut}$ partially impaired CLOCK:BMAL1 activities, whereas GRE^{mut} did not, despite its proximity to E2 (Figure 3C). From these results, we found that whereas both E-boxes had functional roles in CLOCK:BMAL1-mediated transcriptional activity, GRE was not involved in mediating this effect. These results implied that E-box-mediated transcriptional activity was closely related to GC-induced *Per2* expression.

BMAL1 is essential for GC-mediated *Per2* induction

The insights obtained from our determination of the functional interaction between the GRE and E-box suggested that BMAL1 was involved in GC-mediated *Per2* induction. To test this hypothesis, we generated Per2::luc knock-in MEFs of two genotypes (WT and $Bmal1^{-/-}$) and compared the circadian profiles of these MEFs after DEX treatment. As shown in Figure 4A, $Bmal1^{-/-}$ MEFs did not show circadian oscillation or responsiveness to DEX. Consistent with this result, *Per2* mRNA was not induced by DEX treatment in $Bmal1^{-/-}$ MEFs, whereas *Per1* mRNA was increased (Figure 4B). To determine whether the abrogation of *Per2* induction in $Bmal1^{-/-}$ MEFs may result from *Per2* promoter activities, we performed reporter assays in WT and $Bmal1^{-/-}$ MEFs (Figure 4C). Whereas *Per1* promoter-driven luciferase activities were still increased by DEX treatment in the absence of *Bmal1*, the induction of *Per2* promoter driven-luciferase activity was abolished in $Bmal1^{-/-}$ MEFs. To further examine these properties in relation to GRE-dependent mechanisms, we also tested mouse mammary tumor virus (MMTV) promoter activities in WT and $Bmal1^{-/-}$ MEFs. Similar to the result in the case of *Per1*, MMTV promoter activities were increased by DEX treatment in $Bmal1^{-/-}$ MEFs. These data indicated that BMAL1 was critical for DEX-induced *Per2* expression, and this was distinct from the general GRE mechanism of action.

BMAL1 regulates GR occupancy in the GRE of *Per2* promoter

To further elucidate the effects of GR on *Per2* promoter, we performed a reporter assay with the DNA binding mutant of GR (DBD^{mut}) (12,34). DBD^{mut} decreased DEX responsiveness of *Per2*, MMTV and *Per1* promoters (Figure 5A). To investigate the direct binding of GR to *Per2* promoter region and the role of BMAL1 in *Per2* induction, we performed ChIP assays in WT and $Bmal1^{-/-}$ MEFs. The recruitment of GR to the GRE in *Per2* promoter was increased by DEX treatment in WT MEFs but was completely absent in $Bmal1^{-/-}$ MEFs (Figure 5B). However, GR occupancy of *Per1* promoter was increased not only in WT MEFs but also in $Bmal1^{-/-}$ MEFs. We also analyzed GR occupancy of the GBS in the Per2 intron region (19). Similar to the effect observed in *Per1* gene, GR occupancy in the intronic GBS of *Per2* gene was increased by DEX treatment in WT and $Bmal1^{-/-}$ MEFs. Although the binding of GR to both *Per1* GRE and *Per2* GBS was increased by DEX treatment in both MEFs, the amount of immunoprecipitated DNA in $Bmal1^{-/-}$ MEFs was decreased than that in WT MEFs, possibly owing to low levels of GR in $Bmal1^{-/-}$ MEFs (Supplementary Figure S5). Consistent with the results of the ChIP assay, GBS-mediated luciferase activities were increased by DEX treatment in WT and $Bmal1^{-/-}$ MEFs (Supplementary Figure S4). These data suggested that BMAL1 was required for the binding of GR to the GRE in *Per2* promoter but was not critical for the binding of GR to the GBS in Per2 intronic region. Considering the complete absence of *Per2* induction in $Bmal1^{-/-}$ MEFs, GE2 in *Per2* promoter was epistatic to the intronic GBS in GC-mediated *Per2* induction.

An imperfect palindromic GRE confers the reliance on the overlapping E-box

The GRE in *Per2* promoter differs from the palindromic GRE sequence by 4 bp (5′-AGAACANNNTGTTCT-3′). It has been reported that GR has a higher binding affinity for the palindromic GRE than for the imperfect palindromic sequence and that the palindromic GRE decreases the need for the activities of accessory factors (35). To test the possibility that the imperfect palindromic sequence of *Per2* GRE leads to dependency on the E-box, we generated palindromic GRE mutants (GRE^{Pal}) with E1 or E2 mutations (Figure 6A). When the *Per2* GRE sequence was replaced with the palindromic sequence, DEX responsiveness was still maintained. Interestingly, additional mutations of E-boxes to GRE^{Pal} ($GRE^{Pal}E1^{mut}$ and $GRE^{Pal}E2^{mut}$) did not decrease the GC responsiveness of *Per2* promoter, although their basal promoter activities were reduced (Figure 6B). This is different from the original *Per2* promoter. Therefore, these results suggested that the imperfect GRE sequence increased the reliance on the transcription factor BMAL1.

In addition, we also swapped two E-boxes to analyze the role of the E-box in the function of the GRE (Figure 6A). The swapped construct showed increased

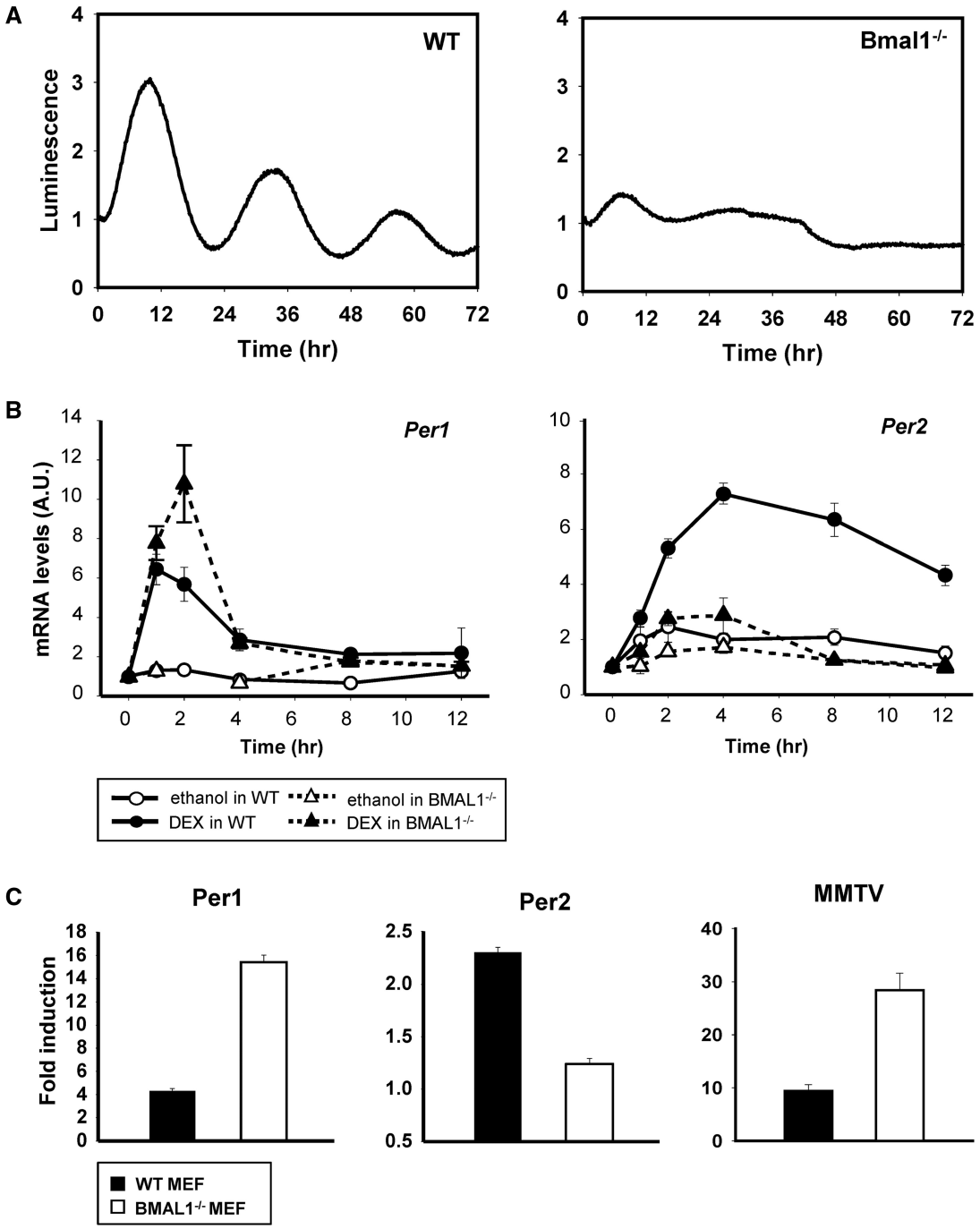

Figure 4. BMAL1 was necessary for *Per2* induction. (**A**) Per2::luc knock-in MEFs with a WT (left) or *Bmal1*$^{-/-}$ (right) genetic background. Bioluminescence was recorded after DEX (1 μM) treatment. (**B**) WT and *Bmal1*$^{-/-}$ MEFs were treated with ethanol (0.1%) or DEX (1 μM) for 2 h. Cells were harvested at the indicated times and *Per1* and *Per2* mRNA levels were analyzed by real-time PCR. Each value was normalized to the GAPDH expression level. Values are the mean ± SEM of three or four independent experiments. (**C**) Per1-luc, Per2-luc and MMTV-luc were transfected into WT (black bar) or *Bmal1*$^{-/-}$ MEFs (white bar), and cells were treated with ethanol (0.1%) or DEX (1 μM) for 10 h. Luciferase activities were normalized to the renilla luciferase activities, and the fold induction was calculated by dividing the luciferase activities in DEX-treated group with those in ethanol-treated group. Values are the mean ± SEM of three or four independent experiments performed in triplicates.

basal activities but maintained the fold induction (Figure 6C). Hence, the sequence of E-box only controlled the basal promoter activity and was not informative for GRE action. Rather, it is likely that the distance between GRE and E-box is an important factor for GE2 elements.

Impaired *Per2* induction cannot delay the circadian phase

To evaluate the physiological relevance of *Per2* induction, we conducted a rescue experiment. First, we examined the oscillation patterns of *Per2* promoter constructs. After stimulation with DEX, all the reporter constructs displayed circadian oscillation patterns; however, this

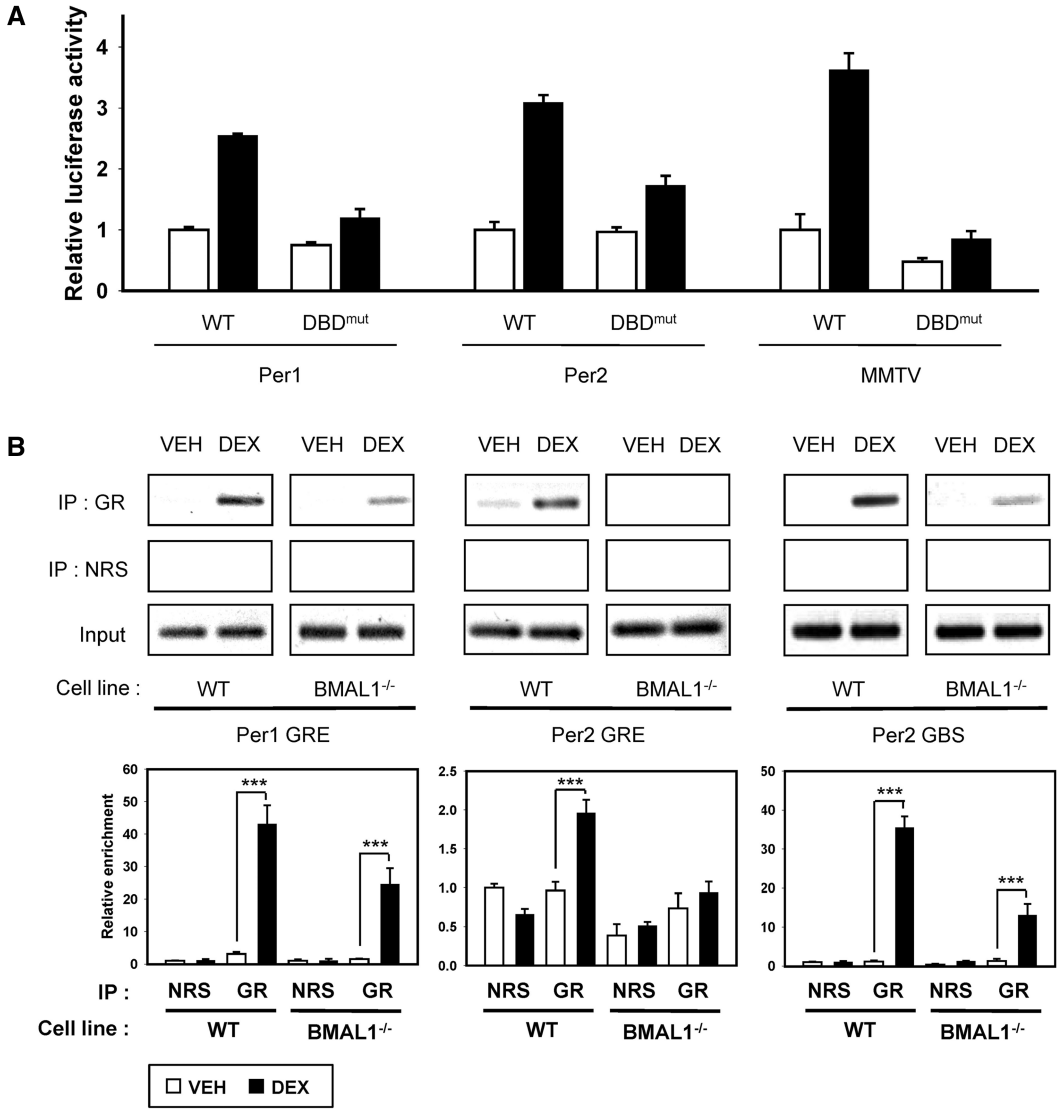

Figure 5. BMAL1-dependent binding of GR to *Per2* promoter. (**A**) WT GR or DBD^{mut} with MMTV-luc, Per1-luc and Per2-luc were transfected into WT MEFs. Cells were treated with ethanol (0.1%; white bar) or DEX (1 μM; black bar) for 10 h. Luciferase activities were normalized to renilla luciferase activities. Values are the mean ± SEM of three independent experiments performed in triplicates. (**B**) WT and *Bmal1*$^{-/-}$ MEFs were treated with ethanol (0.1%; VEH) or DEX (1 μM; DEX) for 1 h. Chromatin was extracted from the harvested cells, and chromatin immunoprecipitation assays were performed with normal rabbit serum (NRS) or anti-GR. Immunoprecipitated DNA was analyzed using the primer sets for Per1 GRE, Per2 GRE and Per2 GBS. Enrichment of GR binding was measured by the agarose gel electrophoresis (upper panel) and quantified by real-time PCR (lower panel). Values are the mean ± SEM of three independent experiments (***$P < 0.001$).

induction was not observed in GRE^{mut} or $E2^{mut}$ (Supplementary Figure S6A). The mutant reporters exhibited a slightly advanced phase compared with the WT reporter, as previously described (Supplementary Figure S6B) (36,37). Using these constructs, we generated adenoviruses expressing the PER2::LUC fusion protein (PER2 REC) driven by WT or mutant promoters (GRE^{mut} and $E2^{mut}$). *Per2*$^{-/-}$ MEFs were recovered by these viruses, and the bioluminescence was recorded after DEX treatment. The PER2 REC^{WT} responded to DEX treatment and exhibited similar profiles to PER2::luc knock-in MEFs. However, PER2 $REC^{GRE\ mut}$ or PER2 $REC^{E2\ mut}$ did not display inductive profiles and failed to exhibit a delay in the circadian phase compared with the WT (Figure 7A and B). Although mutant reporter activities were slightly advanced as compared with WT reporter activity, the functional recovery constructs displayed a more pronounced difference between WT and mutant reporters, indicating that the induction of the PER2 regulated the circadian phase (Supplementary Figure S6A and B). Therefore, these data suggested that DEX-mediated Per2 induction was a crucial step in determining the phase of the circadian rhythm.

DISCUSSION

The present study investigated the molecular mechanisms of *Per2* induction by GC signaling and its regulatory

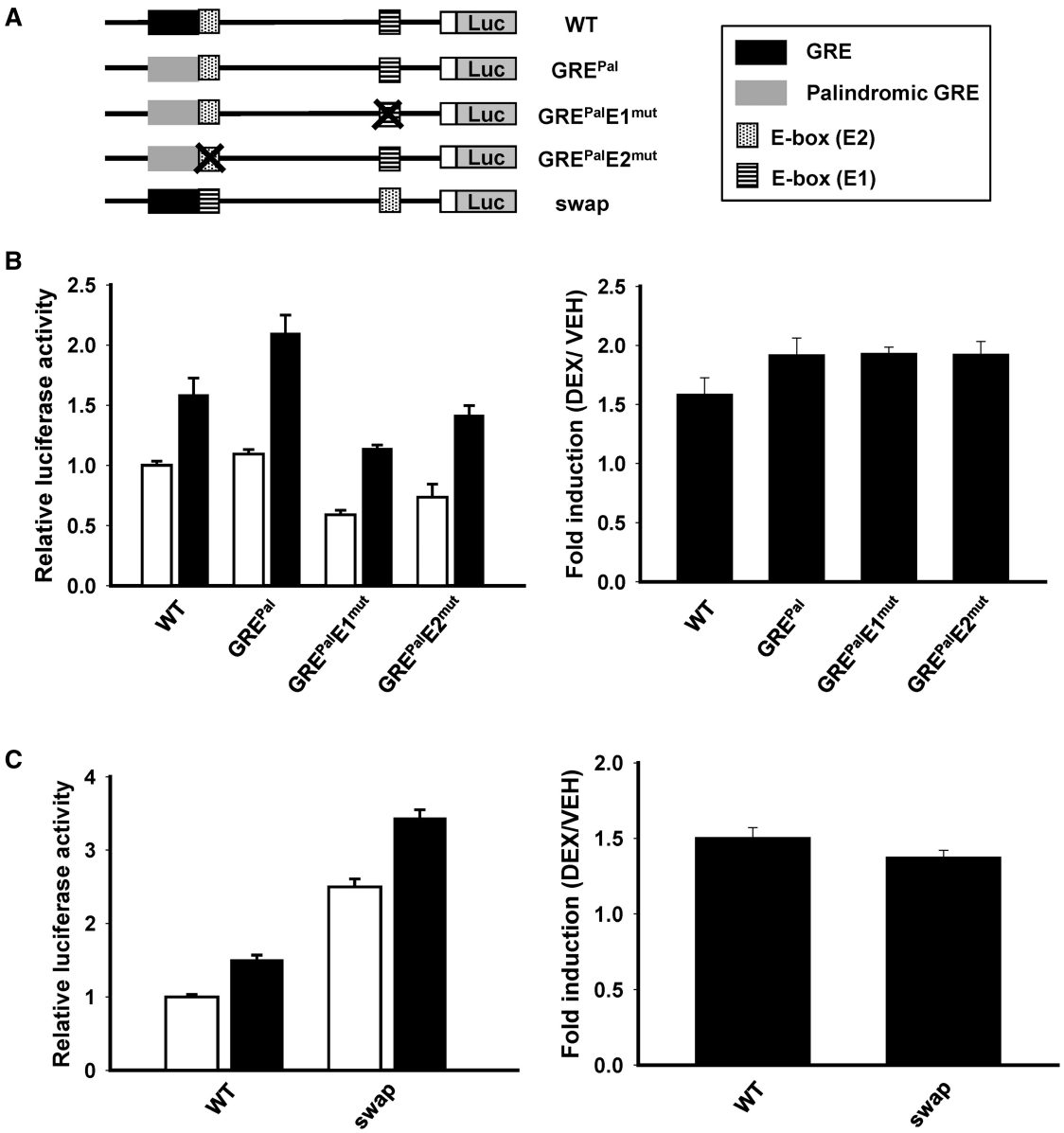

Figure 6. The effects of the palindromic GRE and swapped E-boxes on GC-mediated *Per2* induction. (**A**) Schematic diagram of *Per2* promoter mutants. (**B** and **C**) DEX-responsiveness of *Per2* promoter mutants indicated in (A). WT MEFs that were transfected with the reporters were treated with ethanol (0.1%) or DEX (1 μM) for 10 h. Fold induction was calculated by dividing the luciferase activities in the DEX-treated group with those in the ethanol-treated group. Data are represented as the mean ± SEM. Three independent experiments were done in triplicates for each condition.

effects on circadian rhythms. The induction of *Per2* expression by DEX was mainly mediated by the overlapping GRE and E-box (GE2). From our understanding of this molecular mechanism, we demonstrated that *Per2* induction was crucial for delaying the circadian phase.

Among the clock proteins, the fluctuation of PER expression is thought to be crucial for generating circadian oscillation (38–41). In this regard, it is expected that the resetting process requires an alteration in PER expression to generate new rhythms at the initial stage. In fact, various resetting stimuli increase *Per1* and *Per2* levels, whereas other resetting signals, such as glucose and exercise, lower their expression, respectively, both *in vitro* and *in vivo* (42–44). Several studies have endeavored to substantiate the importance of the induction of *Per1* and *Per2* in the light-induced phase resetting using *Per1*- or *Per2*-knockout mice, but the exact roles of *Per1* and *Per2* in the resetting process remain controversial. This inconsistency may arise from the fact that the mutant mice used by the research groups were different, and global *Per*-knockout mice have additional defects besides resetting function defects (5–8). Thus, to understand the functional importance of the rapid response of *Per1* and *Per2*, only the specific site responsible for the resetting signal should be mutated. In this respect,

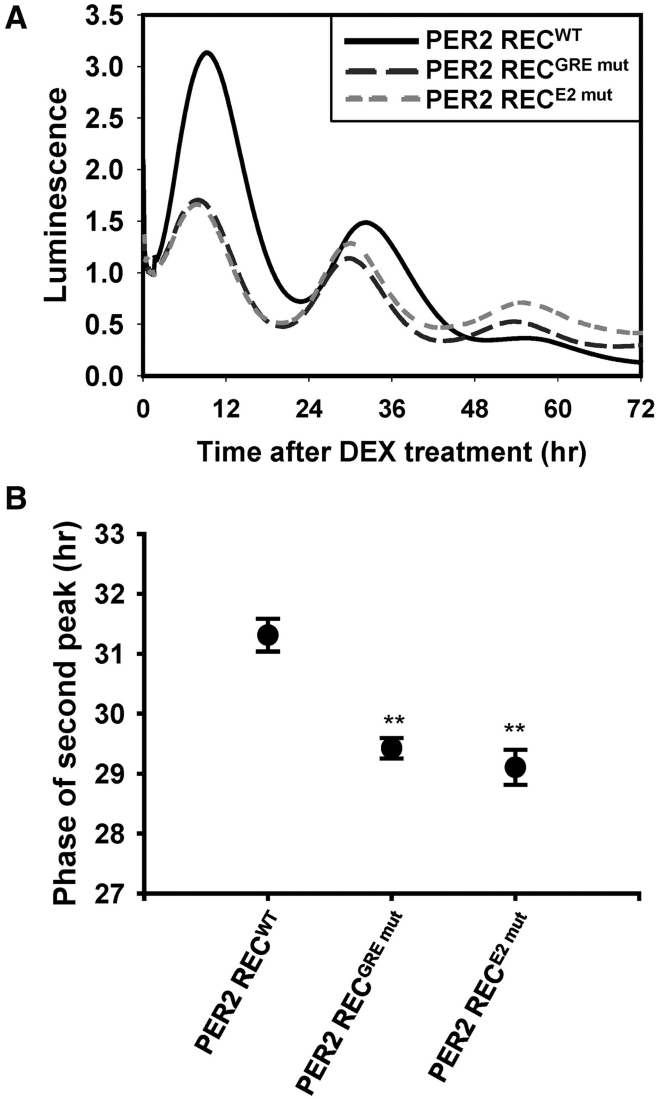

Figure 7. *Per2* induction mutants cannot delay the circadian rhythm (**A**) $Per2^{-/-}$ MEFs were recovered with Per2 adenoviruses, in which the expression of *Per2* was regulated by its own promoter. WT (Per2 REC^{WT}; solid line), GRE mutant (Per2 $REC^{GRE\ mut}$; long dashed line) and E2 mutant (Per2 $REC^{E2\ mut}$; short dashed line). Bioluminescence was recorded after 2 h of DEX treatment. (**B**) The phase of the second peak was measured ($^{**}P < 0.01$). Data are represented as the mean ± SEM from three independent experiments.

the present study attempted to clarify the molecular mechanisms of *Per2* induction and thereby investigate the precise roles of the initial response of *Per2* in the regulation of subsequent circadian rhythms.

GC-induced *Per2* expression was unique in that it required the additional transcription factor BMAL1 for the binding of the GR to the GE2 element. Previous studies revealed that GRE activity was interrupted by other overlapping transcription factor binding sites. For instance, the GRE in the *osteocalcin* gene promoter, which overlaps with all the sequences of the TATA boxes, blocks the binding of the general transcription factor IID and represses transcription (45). In a case similar to that of *Per2,* the cAMP-responsive element (CRE) and E-box (CRE/E-box) of the *cyclooxygenase-2* (*COX-2*) gene overlap by 2 bp. Endotoxin-induced *COX-2* gene expression accompanies the activity of the CRE/E-box, in which each element induces a higher level of gene expression than the overlapped sequence (46). In contrast, neither GRE nor E2 of *Per2* responded to DEX alone, and only their interaction induced *Per2* expression (Figure 3B). To the best of our knowledge, this is the first report of a positive regulatory mechanism, in which the GRE overlaps with other transcription factor binding elements. From a structural viewpoint, this mechanism suggests the cooperative binding of overlapping elements. A previous study showed that GR binds to CLOCK in a ligand-dependent manner, and we also observed direct binding of BMAL1 and GR (data not shown), indicating that the physical interaction between GR and CLOCK/BMAL1 heterodimer can occur (47). However, these data cannot answer the question of how this mechanism applied to GE2 on *Per2* promoter. Owing to the immediate vicinity of GRE and E2 elements, it is likely that BMAL1 and GR do not bind to GE2 at the same time, but instead do sequentially. Nevertheless, there still exists the possibility that the inherent GRE sequence in *Per2* promoter enables the transcription factors to bind at the same time (48). To clarify this structural issue, further studies are needed.

Moreover, this mechanism differs from the general GRE action. A previous report suggested that the canonical GRE activity is repressed by CLOCK:BMAL1 through the histone acetyl transferase activities of CLOCK, and consistent with this, we found that the MMTV or *Per1* promoter can respond to DEX, regardless of the activity of BMAL1 (Figure 4A) (47). In contrast, *Per2* cannot be induced in the absence of BMAL1, implying that this mechanism is clearly distinct from the canonical GRE mechanism. It is possible that the imperfect palindromic GRE sequence in *Per2* promoter increases the need for the involvement of other transcription factors (Figure 6B). In fact, many genes have GRE and E-box *in tandem* (49). This suggests that these genes are regulated in a gene-specific manner according to the fidelity of the sequences and the distance between the GRE and E-box.

We previously suggested that the activation of CLOCK:BMAL1 is involved in serum shock-induced *Per1* expression (50,51). Moreover, the present study revealed that BMAL1 was necessary in GC-mediated *Per2* induction. Therefore, the studies reported by our group suggest that the CLOCK:BMAL1 heterodimer regulates several pathways involved in the resetting process, although it is mainly shown to be a positive regulator of circadian clock genes. Furthermore, each of the resetting signals is likely to generate diverse phases of the circadian rhythm by modulating the expression levels of *Per1* and *Per2* according to the gene-specific functional interaction between the CLOCK:BMAL1 heterodimer and the specific mediator of the resetting signals, such as GR and CRE-binding protein (52).

Previous studies have reported that the regulation of circadian timing is achieved by a fine-tuning of circadian clock components at the transcriptional level. At least

three *cis*-elements, including the morning-time element (E-box), day-time element (D-box) and night-time elements (RRE), are thought to control this timing. For instance, the peak expression of *Cry1*, which is adjusted by the combinatorial regulation of D-box and RRE in addition to E-box, exhibits a certain delay relative to that of *Per2*, which is mainly controlled by E-box (53–55). Furthermore, we propose that GRE is another regulatory element involved in the modulation of circadian timing. It is thought that when the resetting stimuli, i.e. GC, is given, the cooperative interaction between GRE and E-box determines the phase of *Per2*. Indeed, the phase-delaying role of E2 has been previously suggested in several studies. Akashi *et al.* (36) reported that the proximal *Per2* promoter region consists of a phase-delaying region and an oscillation-driving region. Using a serial deletion analysis of the *Per2* promoter, they found that the phase-delaying region comprises from −386 to −106 from the TSS and that the oscillation-driving region comprises a region from −105 to +1. The oscillation-driving region contains a non-canonical E-box (E1), which is an essential and sufficient element for the generation of rhythm, whereas the phase-delaying region contains GE2 (56). Yamajuku *et al.* (37) demonstrated similar results in that the region from −161 to −143, which contains E2, was shown to be responsible for the phase delay. Although they used different stimuli (i.e. serum shock and dbcAMP), the E2-containing region was thought to be responsible for the phase delay. Consistent with this, we also found that $E2^{mut}$ advances the circadian rhythm of *Per2* in DEX-treated cells. Therefore, on the basis of our data and previous studies, DEX-dependent induction of *Per2* expression likely accompanies the long-lasting activation of GE2.

Although the mutated recovery constructs failed to delay the circadian rhythm, the phase difference between the WT and mutant constructs was not as large as that of Per2::luc knock-in cells, which were treated with DEX and other signaling molecules (Figures 1A and 7A). There are a number of plausible reasons for this. First, clock genes other than *Per2* can affect the resetting process. Previous studies reported that GC induces *Per1* expression and downregulates *Rev-erb α* (15,57). Although we found that *Per1* expression, which was stimulated by different signaling molecules, did not exhibit remarkable differences between treatments, there was a slight time lag between the stimuli, which may support the phase delaying effect (Supplementary Figure S1) (30). GC-induced downregulation of *Rev-erb α* can also directly or indirectly affect phase regulation. Second, it is likely that the excluded region in our recovery constructs was involved in this mechanism. A previous report showed that *Per2* induction does not occur in $Per2^{Brdm1}$ cells, which lack a genomic region of approximately 2 kb containing a GBS (19); however, our constructs did not contain a GBS. Therefore, it is feasible that *Per2* induction may be collectively regulated by the interaction between the two elements, GE2 and GBS. To accurately understand how GE2 and GBS regulate GC-mediated *Per2* induction, further studies need to be conducted using modified bacterial artificial chromosomes or the whole genome.

The proximal *Per2* promoter region is highly conserved in mammals and zebrafish (58). This region includes a non-canonical E-box (E1) that is sufficient for self-sustained circadian rhythm generation and a D-box that is implicated in higher amplitude generation (37,56). In addition, we found that GE2 in this region is also conserved in mammals, but only 5 bp (CATGG) in the middle of the GRE sequence is conserved in zebrafish (58). Although zebrafish has a hypothalamic–pituitary–inter-renal axis that regulates cortisol release in fish, its exact role in the circadian rhythm remains largely unknown. Considering that its peripheral cells can respond to the light directly and that the zebrafish *Per2* rhythm depends on the light-dark cycle, it is conceivable that the GC-regulated *Per2* induction mechanism evolved because peripheral tissues do not receive direct photic input (59).

Many people suffer from sleep disturbances as well as metabolic and cardiovascular disorders in relation to chronobiological problems that arise under various circumstances, including jet lag and shift work. These disturbances are related to phase misalignment in the master and/or peripheral clocks (60–62). GC is generally accepted as a strong synchronizer of the SCN and peripheral tissues, and an altered GC rhythm is closely related to a variety of circadian disorders. People suffering from Cushing syndrome, diabetes, depression, obesity, Alzheimer's disease and metabolic syndrome exhibit an altered GC rhythm and abnormal circadian physiology (14,63–66). This might be due to a dysregulation of GC-regulated clock genes, including *Per1* and *Per2* (67).

In conclusion, this study provides evidence that *Per2* induction is responsible for circadian phase delay through a novel regulatory mechanism. It is expected that these findings will help in the effort to achieve a better understanding of the physiological changes that occur in circadian rhythm disorders.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

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