Real-Time Analysis of the Circadian Oscillation of the Rev-Erbβ Promoter

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Aim: The Rev-Erbβ gene plays crucial roles in circadian rhythm, lipid and glucose metabolism, and several diseases. The molecular mechanisms of the transcriptional regulation of Rev-Erbβ that generate and determine the phase of the circadian oscillation remain unclear.

Methods: We analyzed the Rev-Erbβ promoter by luciferase reporter assays, real-time bioluminescence monitoring assays and electrophoretic mobility shift assays.

Results: Luciferase reporter assays indicated that only the 5´ region and exon 1 have obvious promoter activity. Real-time bioluminescence monitoring assays revealed that E1, E2, E3, D boxes are important for maintenance of the amplitude of Rev-Erbβ oscillation. Based on EMSA results, REV-ERBβ binds ROREs in the Bmal1 promoter region and inhibits Bmal1 promoter activity.

Conclusion: We provide direct evidence that three E-boxes and one D-box located in the first intron are crucial for the phase of circadian oscillation in Rev-Erbβ expression and that the sequences upstream from its transcription start site function as a promoter with no circadian regulation. We also found that the E1 box affects the Rev-Erbβ oscillation phase. Our results offer new insight into the role of Rev-Erbβ in the circadian rhythm system.


Key words; Rev-Erbβ, Circadian Oscillation, Bmal1, Promoter, Lipid metabolism

Introduction

Mammalian circadian oscillations are generated by the rhythmic transcription of central and peripheral clock output genes, which is controlled by a molecular oscillator consisting of interlocked positive and negative transcription/translation feedback loops of a set of clock genes1, 2. Genetic analyses have shown that circadian regulation of gene expression occurs through the E-box3, D-box4, and Rev-erba/ROR-binding elements (ROREs)5 and that the combination of these binding sites is a critical determinant for oscillation phases6.

REV-ERBα (Nr1d1) and β (Nr1d2) are transcriptional repressors that regulate several important biological processes associated with circadian rhythm and lipid and glucose metabolism7, 8. REV-ERBs play an important role in normal hepatic lipid homeostasis. Disruption of Rev-erbs causes hepatic steatosis in mouse liver9. They have also been implicated in diseases such as diabetes, inflammation, atherosclerosis, and cancer10-13. Bmal1, a circadian clock component, has a nocturnal peak oscillation14, 15. The oscillatory mechanism of Bmal1 involves the retinoic acid-related orphan receptor (ROR) α and REV-ERBα via ROR response elements (RORE) located in the Bmal1 promoter region16-18. RORα, RORβ, and RORγ positively regulated Bmal1 transcription, whereas REV-
ERBβ repressed transcription by competing for the same ROR-binding sites \(^{17,19,20}\). The expression level of RORα was reported to be nearly constant in the mouse liver and to contribute to the Bmal1 amplitude, but to be dispensable for Bmal1 rhythm \(^{20}\). Some evidence suggests that REV-ERBα and β are required for Bmal1 rhythm in fibroblasts and that REV-ERBα plays a more prominent role than the RORs in the basic clock mechanism \(^{10}\). Rev-Erbα and Rev-Erbβ displayed robust oscillation in wild-type (WT) animals, but were nearly undetectable at all time points in Bmal1 mutants \(^{21}\). These results suggest that Bmal1 may influence the rhythmic expression of Rev-Erbα and Rev-Erbβ.

Recently, the Rev-Erbβ gene was proposed to be regulated by BMAL1/CLOCK and DBP \(^{4,22}\) via an E-box and D-box, respectively, in its promoter-enhancer region \(^{6,10,23}\). In addition, it was postulated that the rhythmic expression of Rev-Erbβ as well as Rev-Erbα is likely to be a key event generating the Bmal1 oscillation \(^{10,19}\). Therefore, understanding the mechanism of the transcriptional regulation of Rev-Erbβ in the generation and determination of the phase in circadian oscillation is essential. To elucidate the precise regulatory mechanism, we cloned a 2.2-kilobase pair promoter-enhancer region of the mouse Rev-Erbβ gene and identified a promoter and regulatory sites that generate its robust oscillation.

**Materials and Methods**

**Mouse Rev-Erbβ Promoter Cloning, Mutagenesis, and Sequence Analysis**

The Rev-Erbβ promoter fragment was amplified by polymerase chain reaction using mouse genomic DNA (Novagen, Madison, WI) and subcloned into a customized ELuc-PEST-T vector (Toyobo, Osaka, Japan) \(^{24}\). The E-box and D-box mutants were created using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Four mutated primers were used in this study: Rev-Erbβ-E1mut: 5’-CCTGCGCACTGGCTTCGTGCACGGTGTG-3’; Rev-Erbβ-E2mut: 5’-GTGGCGGGCGGAGATTCGTGAGG-3’; Rev-Erbβ-E3mut: 5’-CATTACATGGGGCGAGCTGGGTGGTG-3’; Rev-Erbβ-Dmut: 5’-GTGA CGCAGCAT -3’. The substituted bases are underlined. Sequence analyses were performed with an automated 377 DNA Sequencing System (Applied Biosystems, Foster City, CA).

**Transient Transfections and Reporter Assays in NIH3T3 Cells**

The luciferase assay was performed as described previously \(^{25}\). Transfected NIH3T3 cells were used. One day before transfection, the cells were seeded (5 × 10^4 per well) on 24-well plates containing Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Life Technologies), penicillin (24 U/mL), and streptomycin (25 μg/mL). The cells were transfected using lipofectamine (Life Technologies). For each sample, transfected DNA (260 ng) was added to each well. Each transfection mixture contained 60 ng of the Rev-Erbβ promoter reporter, 50 ng of the indicated reporter, and 6 ng of the phRG-TK vector (Promega, Madison, WI), which provides a constitutive expression of Renilla luciferase and served as an internal control. The amount of added DNA per well was kept constant by adding the pcDNA3 vector. Twenty-four hours after transfection, the cells were washed in PBS and disrupted with 100 μL passive lysis buffer (Promega). Luciferase activity was determined using a Dual-Luciferase Reporter Assay System (Promega) and an Ascent FS II luminometer (Thermo Scientific).

**Real-Time Monitoring of Circadian Transcriptional Dynamics and Analysis of Circadian Rhythms in Bioluminescence**

The real-time bioluminescence monitoring assay was conducted using an AB-2500 Kronos (ATTO, Tokyo, Japan) as described previously \(^{25}\). NIH3T3 cells were seeded in a 35-mm dish, two days before transfection, and 500 ng of the reporter plasmid was transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer’s instructions. One day before transfection, the cells were treated with 100 nM dexamethasone (Nacalai Tesque, Kyoto, Japan) for 2 hr, and the medium was replaced with DMEM in the absence of phenol red supplemented with 10% FBS and 100 μM D-luciferin (Toyobo), and overlaid with mineral oil (Sigma-Aldrich, St. Louis, MO) to prevent evaporation. Bioluminescence was measured at 37°C under 5% CO₂ atmosphere and integrated for 1 min at intervals of 10 min. Bioluminescence activity was expressed as relative light units (RLU).

**Analysis of Circadian Rhythms in Bioluminescence**

The cells were cultured in the luminometer for more than 4 days, measuring their bioluminescence. To compare the phase and amplitude of WT and mutant Rev-Erbβ promoters, the obtained crude data (10 min bins) were smoothed by a 10-point moving average method and detrended by subtracting 12 hr
moving average from smoothed data.

**Electrophoretic Mobility Shift Assays (EMSA)**

Electrophoretic mobility shift assays (EMSA) were also performed as indicated in a previous study\(^25\). REV-ERBβ, DBP, and E4BP4 proteins were synthesized using a TNT T7-coupled Reticulocyte Lysate System (Promega). The oligonucleotide probe sequences used for this analysis were as follows: Bmal1-RORE1: 5’-ACGCTTGGACATCAAGAGA-A-3’; Bmal1-RORE1mut: 5’-ACGCTTGGACATCAGAGAACAA-3’; Bmal1-ROR E2:5’-CGGAAGTAGGTATAGGTACATTAGTGTTGCG-3’; Bmal1-RORE2mut: 5’-CGGAAA-GTAATTAGTGTTGCG-3’; Bmal1-RORE3: 5’-CAGAAATAGTCAGGGACGGA-3’; Bmal1-RORE3mut: 5’-CAGAAATTACATACGGGACGGA-3’. The underlined portion indicates the WT and mutant binding sequences, respectively.

**Western Blotting Analysis**

Western blot analysis was conducted as previously described\(^26\). Blots were probed with anti-REV-ERBβ, (Perseus Proteomics, Tokyo), anti-DBP (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-GAPDH (Sigma-Aldrich) antibodies.

**Statistical Analysis**

Time series data were analyzed using one-way analysis of variance (one-way ANOVA) for rhythm. The significance of the differences between the two groups was analyzed by Student’s \(t\)-test. Analysis programs (cosinor periodogram and Lomb-Scargle periodogram by R. Refinetti: http://www.circadian.org/software.html) were used to compare the circadian periods. To compute the acrophase (time at which the peak of the rhythm is reached) and the amplitude, we used a fitted cosine wave procedure (Acro program by R. Refinetti: http://www.circadian.org/software.html)\(^27\). Comparisons at \(p<0.05\) were considered significant.

**Results**

**Genomic Structure of mRev-Erbβ**

The Database of Transcriptional Start Sites (DBTSS) was used to determine the transcriptional start site (TSS) of the mRev-Erbβ gene. We discovered a gap in the promoter-enhancer site extracted from the mouse genome database (NCBI Build 36.1), specifically in the region of exon 1 and intron 1. An extensive search for Rev-Erbβ sites that could bind clock gene products revealed an additional E-box, which was called E3 (Fig. 1A), located in the downstream sequence of previously reported E-boxes and a D-box [5]. These results support the presence of two E-boxes and one D-box in the intron 1 region and demonstrate that Rev-Erbβ regulatory regions are highly conserved between humans and mice (data are not shown).

**Identification of Promoter/Enhancer Region of mRev-Erbβ Gene by Deletion Study**

To determine which regions function as a promoter, the expression of deletion constructs of the promoter-enhancer region fused with the luciferase gene was studied in NIH3T3 cells using a dual luciferase system. Deletion of the 5′ region upstream from the TSS resulted in about 60% reduction of luciferase activity (Del-1103), compared with that of the full-length reporter construct (Rev-Erbβ-F-L). Furthermore, complete deletion of both the 5′ region of the TSS and exon 1 showed nearly basal levels of luciferase activity, indicating cryptic promoter activity (Del-673; Del-570; Del-496). These results indicate that only the 5′ region and exon 1 have obviously promoter activity. Deletion of the E-boxes and D-box had no effect on the basal level of the reporter gene, as compared with other constructs (Del-570 and Del-496), suggesting that endogenous factors that interact with these regions have minimal effects on the regulation of Rev-Erbβ (Fig. 1B).

**Identification of the Rhythmic Expression Region of mRev-Erbβ Gene by a Real-Time Monitoring Assay**

Real-time monitoring analysis of mRev-Erbβ promoter-enhancer activities was also performed (Fig. 1C and 1D). The mRev-Erbβ-F-L construct (a; red line) clearly showed rhythmic expression, with a period of approximately 24 h. The first peak appeared 27 h after dexamethasone stimulation. The reporter construct consisting of intron 1 (c; green line) also showed circadian oscillation but not full amplitude. On the other hand, the promoter region containing only the 5′ sequence of the TSS (b; blue line) showed no obvious circadian expression. These results suggest that the 5′ sequence of the TSS not only promotes expression but also plays an important role in influencing the amplitude of circadian oscillation, whereas intron 1 has binding sites for DNA-binding elements that are sufficient to generate circadian oscillation and maintain some oscillation amplitude (Fig. 1C).
Fig. 1. Schematic of the mouse Rev-Erbβ promoter, its activity in the 5’-flanking region, and identification of the region promoting rhythmic oscillation.

(A) Genomic structure of the mouse Rev-Erbβ gene surrounding exon 1, which contains the initiation codon. Exons are represented as filled boxes. The promoter and intron 1 are represented by horizontal lines. Unidentified sequences are shown as open gaps. The transcription start site begins at +1. Fragment sizes are indicated by arrows. (B) Terminally truncated Rev-Erbβ promoter-luciferase constructs were assayed in transiently transfected NIH3T3 cells. The activities of these constructs are relative to that of Rev-Erbβ-F-L, which was designated as 100%. All values are shown as fold increases (mean ± SEM, n=4) compared to the control (reporter plasmid without regulatory region). E1-E3 indicates the position of the putative E-boxes. Asterisks represent p<0.05 as compared with the empty plasmid. (C) Rev-Erbβ promoter-luciferase reporter activities in NIH3T3 cells. Rev-Erbβ-F-L (a: red line), Rev-Erbβ-1.3k (b: blue line), and Rev-Erbβ-del-673 (c: green line). The acrophase of Rev-Erbβ-F-L oscillation was about 3.7 and 0.5 h earlier than that of Rev-Erbβ-1.3k and Rev-Erbβ-del-673, respectively. The amplitudes for Rev-Erbβ-1.3k and Rev-Erbβ-del-673 were lower than that for Rev-Erbβ-F-L. (D) Relative activities and bioluminescence signals of Rev-Erbβ promoter-luciferase reporters. Data were detrended as described in methods. Rev-Erbβ-F-L (a: red line), Rev-Erbβ-1.3k (b: blue line), and Rev-Erbβ-del-673 (c: green line). (E) Structure comparisons with the reporter constructs used in this study.5
Fig. 2. E-box and D-box response elements control circadian expression of Rev-Erbβ, and E1 specifically influences the oscillation phase of Rev-Erbβ.

Relative activities and bioluminescence signals were measured in three independent experiments using a real-time bioluminescence monitoring assay. The amplitudes for the constructs with mutated E-boxes (E1E2E3, E1, and E3) (Fig. 2A-a; 2B-a; 2B-c) or D-box (Fig. 2A-b) were lower than that for the WT construct, whereas the E2 mutant did not cause any change (Fig. 2B-b). E1 specifically influences the phase of oscillation in Rev-Erbβ. The relative activities are shown on the y-axis and the measurement of time after starting bioluminescence is shown on the x-axis. The method of analyzing circadian rhythms via bioluminescence is described in the experimental procedures.
E1, E2, E3, D boxes are Important for Maintenance of the Amplitude of Rev-Erbβ Oscillation

The regulatory roles of the E-boxes and D-box in the generation of Rev-Erbβ rhythmic expression were investigated using mutated promoter reporter constructs. Superimposed oscillation profiles of the WT and mutant constructs revealed that the amplitudes for the constructs with mutated E-boxes (E1, E2, and E3) (Fig. 2A-a) or D-box (Fig. 2A-b) were lower than that for the WT construct. Furthermore, the amplitude of the E1E2E3Dbox mutant was almost eliminated (Fig. 2A-c). Thus, the E1-, E2-, E3-, and D-boxes are important for maintaining the oscillation amplitude of Rev-Erbβ.

E1 Influences the Phase of Oscillation in Rev-Erbβ

As the E1E2E3 mutant had an effect on the oscillation phase of Rev-Erbβ (Fig. 2A-a), identification of the putative E-box that directly affects Rev-Erbβ was determined. A real-time monitoring assay demonstrated that the E1 mutant altered the oscillation phase of Rev-Erbβ (Fig. 2A-a; 2B-a), whereas the E2 and E3 mutants did not cause obvious change (Fig. 2B-b; 2B-c). The acrophase of the WT oscillation was about 2h earlier than that of the E1 and E1E1E3 mutants (Fig. 2A-a; 2B-a). These results suggest that the E1-box participates in the regulation of the Rev-Erbβ oscillation phase.

BMAL1/CLOCK and DBP Enhanced mRev-Erbβ Promoter/Enhancer Activity and Increased REV-ERBβ Expression, Whereas CRY1 Repressed it

To test whether DNA-binding elements are functionally important for the regulation of Rev-Erbβ, we analyzed the effect of clock gene products on mRev-Erbβ promoter-enhancer activity using a dual luciferase assay system. Reporter activity was induced 1.8-fold and 2.4-fold by BMAL1/CLOCK and DBP, respectively (Fig. 3A, C). CRY1 repressed the BMAL1/CLOCK-induced activation (Fig. 3A). Western blot
analysis of REV-ERBβ protein in NIH3T3 cells transfected with a control vector or vector containing BMAL1, CLOCK, CRY, or DBP for 24 h indicated that BMAL1/CLOCK and DBP increased the expression of REV-ERBβ, whereas CRY1 repressed it (Fig. 3B, D). Based on EMSA results, the Rev-Erbβ D-box was a direct target of both DBP and E4BP4 (Fig. 4). These results support a previous report that Rev-Erbβ is regulated by clock genes via E-box- and D-box-binding proteins.

REV-ERBβ Binds ROREs in the Bmal1 Promoter Region and Inhibits Bmal1 Promoter Activity

Previous studies have revealed that the mouse Bmal1 gene contained two ROREs in its promoter region, near the TSS. Analysis of DNA binding sites using the MatInspector program (Genomatix, Munich, Germany) demonstrated another possible binding site for RORα and REV-ERBα at about 800 bp upstream from the TSS. An EMSA using probes was synthesized for the three ROREs in the Bmal1 promoter, designated RORE1, RORE2, and RORE3, from the 5’ sequence to the TSS (Fig. 5A). We used labeled ROREs and unlabeled ROREs or unlabeled RORE mutant probes. The shifted bands for the RORE oligonucleotide were specifically detected in the presence of REV-ERBβ and all bands disappeared in the presence of unlabeled RORE probe competitors, but not in the presence of unlabeled ROREs mutant probes. The results revealed that REV-ERBβ binds specifically to all three ROREs in the mBmal1 promoter-enhancer region, although RORE1 binding is not markedly stronger than that of RORE2 and RORE3 (Fig. 5C). To study the effect of REV-ERBβ on Bmal1 transcription, a luciferase assay was performed on NIH3T3 cells co-transfected with expression plasmids containing a Bp/915-LUC Bmal1 reporter. REV-ERBβ caused a substantial, dose-dependent reduction in Bmal1 transcription (Fig. 5B).

Differences in Peak Phase and Period Length between Rev-Erbβ, Per2 and Bmal1 Gene Reporters Detected in the Real-Time Monitoring System

We observed apparent peak phase differences among the circadian rhythms of Rev-Erbβ, Per2 and Bmal1 in NIH3T3 cells using a real-time monitoring system. The acrophase of the Rev-Erbβ oscillation was about 2.6 and 10.4 h earlier than that of Per2 and Bmal1, respectively. The circadian period of Rev-Erbβ, Per2, and Bmal1 in this experiment was 24.4, 23.4
tors and that the combination of these binding sites is the critical determinant of oscillation phases.\textsuperscript{5, 6, 32-34} However, the regulation machinery of gene transcription is more complex than simply the existence of consensus binding sites for transcription factors, and thus more information is necessary in order to understand the transcriptional regulation of the circadian system. We suggest promoter analysis using the promoter-enhancer region in a reporter construct, as generated in the present study. The regulatory units of clock genes involved in oscillation have been categorized into five groups based on a combination of E/E' boxes, DBP/E4BP4-binding elements, and Rev-ErbA/ROR-binding elements.\textsuperscript{35} This includes genes regulated solely by E/E' boxes or D-boxes, by both E/E' boxes and D-boxes, or by both E/E' boxes and ROREs.

\textbf{Rev-Erb}β belongs to the clock genes regulated by both E/E' boxes and D-boxes.\textsuperscript{4, 6, 36, 37} Recently, Ueda and coworkers reported the importance of core and flanking sequences as regulatory elements of clock genes for the generation and amplification of circadian oscillation.\textsuperscript{6} Phase differences among the five groups of clock genes have been determined by measuring the bioluminescence produced from and 21.6 h, respectively. Amplitude comparisons of these clock reporter genes showed that \textit{Bmal1} had the highest amplitude, followed by \textit{Per2} and \textit{Rev-Erb}β. The oscillation amplitude may be affected by multiple factors such as the condition and type of cells, length of the promoter-enhancer region, efficiency of transfection, and interactions between cells (Fig. 6). Previous studies have shown that the E'-box and D-box structure of \textit{Per2} gene is very similar to \textit{Rev-Erb}β gene, which may be one of the reasons why the phase of \textit{Rev-Erb}β gene oscillation looks similar to that of \textit{Per2} gene.\textsuperscript{28}

\section*{Discussion}

The oscillation amplitude can be affected by multiple factors such as the type and condition of the cells, the length of the promoter-enhancer region, the efficiency of transfection, and the interactions between the cells. Bioinformatics provides powerful tools for investigating biological systems such as rhythms, cell cycles, and cellular networks.\textsuperscript{31} Bioinformatics studies on the circadian system led to the findings that clock and clock-controlled genes are regulated by E-box-, D-box-, and RORE-binding transcription factors and that the combination of these binding sites is critical for the determination of oscillation phases.\textsuperscript{5, 6, 32-34} However, the regulation machinery of gene transcription is more complex than simply the existence of consensus binding sites for transcription factors, and thus more information is necessary in order to understand the transcriptional regulation of the circadian system. We suggest promoter analysis using the promoter-enhancer region in a reporter construct, as generated in the present study. The regulatory units of clock genes involved in oscillation have been categorized into five groups based on a combination of E/ E'-boxes, DBP/E4BP4-binding elements, and Rev-ErbA/ROR-binding elements.\textsuperscript{35} This includes genes regulated solely by E/E'-boxes or D-boxes, by both E/ E' boxes and D-boxes, or by both E/E'-boxes and ROREs. \textit{Rev-Erb}β belongs to the clock genes regulated by both E/E'-boxes and D-boxes.\textsuperscript{4, 6, 36, 37} Recently, Ueda and coworkers reported the importance of core and flanking sequences as regulatory elements of clock genes for the generation and amplification of circadian oscillation.\textsuperscript{6} Phase differences among the five groups of clock genes have been determined by measuring the bioluminescence produced from
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Fig. 6. Real-time monitoring of Rev-Erbβ-, Bmal1-, and Per2-driven rhythmic expression in NIH3T3 cells.

Relative activities and bioluminescence signals were measured in four independent experiments using a real-time bioluminescence monitoring assay. ELuc-PEST reporter plasmids (500 ng) harboring the Rev-Erbβ, Bmal1 (~428 to +99), or Per2 (~279 to +112) promoter were transfected into NIH3T3 cells, followed by treatment with dexamethasone. Luciferase activities are shown on the y-axis and the measurement of time after starting bioluminescence is shown on the x-axis. Rev-Erbβ promoter (red line), Per2 promoter (purple line), and Bmal1 promoter (blue line). All bioluminescence were normalized by maximum count. The method of analyzing circadian rhythms via bioluminescence is described in the experimental procedures.

Acknowledgements

We thank Dr. M. Nomura for helpful discussions and Dr. M. Noshiro (Hiroshima University) for the generous gift of the mouse DBP expression construct. We would also like to thank Dr. Haiyuan Xu and Cheng Piao for assistance with our experiments. We also thank the Research Center for Genomic Medicine, Saitama Medical University for the use of their facilities. FY was supported by the Uehara Memorial Foundation. This research was supported by the Japan Private School Promotion Foundation.

Conflicts of Interest

None.

References

8) Burris TP: Nuclear hormone receptors for heme: REV-ERBalpa and REV-ERBBeta are ligand-regulated components of the mammalian clock. Mol Endocrinol, 2008; 22: 1509-1520
histone deacetylase 3 controls hepatic lipid metabolism. Science, 2011; 331: 1315-1319


12) Vasu VT, Cross CE, Gohil K: Nr1d1, an important circadian pathway regulatory gene, is suppressed by cigarette smoke in murine lungs. Integr Cancer Ther, 2009; 8: 321-328


15) Yu W, Nomura M, Ikeda M: Interactivating feedback loops within the mammalian clock: BMAL1 is negatively autoregulated and upregulated by CRY1, CRY2, and PER2. Biochem Biophys Res Commun, 2002; 290, 933-941


27) Reffert R: Laboratory instrumentation and computing: comparison of six methods for the determination of the period of circadian rhythms. Physiol Behav, 1993; 54: 869-875


