Direct Regulation of \textit{CLOCK} Expression by REV-ERB

Christine Crumbley, Thomas P. Burris*

The Scripps Research Institute, Jupiter, Florida, United States of America

Abstract

Circadian rhythms are regulated at the cellular level by transcriptional feedback loops leading to oscillations in expression of key proteins including \textit{CLOCK}, BMAL1, \textit{PERIOD} (PER), and \textit{CRYPTOCHROME} (CRY). The \textit{CLOCK} and BMAL1 proteins are members of the bHLH class of transcription factors and form a heterodimer that regulates the expression of the \textit{PER} and \textit{CRY} genes. The nuclear receptor REV-ERB\textsubscript{\alpha} plays a key role in regulation of oscillations in BMAL1 expression by directly binding to the BMAL1 promoter and suppressing its expression at certain times of day when REV-ERB\textsubscript{\alpha} expression levels are elevated. We recently demonstrated that REV-ERB\textsubscript{\alpha} also regulates the expression of NPAS2, a heterodimer partner of BMAL1. Here, we show that REV-ERB\textsubscript{\alpha} also regulates the expression another heterodimer partner of BMAL1, \textit{CLOCK}. We identified a REV-ERB\textsubscript{\alpha} binding site within the 1\textsuperscript{st} intron of the \textit{CLOCK} gene using a chromatin immunoprecipitation – microarray screen. Suppression of REV-ERB\textsubscript{\alpha} expression resulted in elevated \textit{CLOCK} mRNA expression consistent with REV-ERB\textsubscript{\alpha}'s role as a transcriptional repressor. A REV-ERB response element (RevRE) was identified within this region of the \textit{CLOCK} gene and was conserved between humans and mice. Additionally, the \textit{CLOCK} RevRE conferred REV-ERB\textsubscript{\alpha} responsiveness to a heterologous reporter gene. Our data suggests that REV-ERB\textsubscript{\alpha} plays a dual role in regulation of the activity of the BMAL1/CLOCK heterodimer by regulation of expression of both the BMAL1 and \textit{CLOCK} genes.

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Materials and Methods

ChIP-on-chip

HepG2 cells (ATCC, Manassas, VA) were infected with REV-ERB\textsubscript{\alpha} adenovirus and harvested for use in ChIP/microarray as previously described by our laboratory [9,10,11,12].

Chromatin Immunoprecipitation (ChIP)

HepG2 cells were transfected with 50 nM control or REV-ERB\textsubscript{\alpha} siRNA (Dharmacon) using Lipofectamine RNAiMax (Invitrogen) according to manufacturer’s instructions. Media
was changed 24 hrs after transfection. Cells were fixed using formaldehyde 72 hrs after transfection. The ChiP-IT Express kit from Active Motif was used. Cells were lysed and then sonicated to shear the chromatin. Immunoprecipitations were incubated overnight at 4°C. The ChiP reactions contained 5 μg of the following antibodies: IgG (Active Motif), anti-RNA Pol II (Active Motif), anti-hREV-ERBα (Cell Signaling), and anti-hNCoR (Santa Cruz sc-8994). The ChiP reactions were washed and chromatin was eluted, according to manufacturer’s instructions. PCR reactions were performed using 50 μL PCR Supermix High Fidelity (Invitrogen), 1.5 μL of each primer (10 μM), and 10 μL of eluted chromatin. The IgG and anti-RNA Pol II were provided in the ChiP-IT human control kit (Active Motif). The thermostycler program was 94°C for 3 mins; 40 cycles of 94°C for 20 s, 65°C for 30 s, 72°C for 30 s, 72°C for 2 min. PCR products were visualized using ethidium bromide gel electrophoresis.

Electrophoretic Mobility Shift Assay (EMSA)

The REV-ERBα coding sequence was excised from p3xFLAG-REV-ERBα using BamH1 and HindIII. The vector pcDNA3.1+ (Invitrogen) was digested with BamH1 and HindIII. All fragments were gel purified, then ligated using T4 DNA Ligase (Promega). The constructs were verified by sequencing. The constructs contain a T7 promoter for in vitro transcription and translation and were used to generate protein for EMSA using the TNT T7 kit (Promega). The putative CLOCK RevRE and the BMAL1 RevRE were annealed and labeled with α32P dATP using Klenow polymerase (Promega). Binding reactions contained binding buffer (Promega), labeled probe, and REV-ERBα protein. The resulting complexes were loaded onto 5% TBE gels (Biorad) and analyzed by autoradiography. For competition experiments, unabled CLOCK RevRE (wt or mt) was added at 10-, 50- or 100-fold molar excess. The sequences of the probes utilized in the EMSA are indicated below:

- hCLOCK_ROREwt_F: TTGGAATTTAACGGGTGACACAA-GGC
- hCLOCK_ROREwt_R: TTGCTTTTGTGACGCCTTTAT-TCC
- hCLOCK_RORemut_F: TTGGAATTTAACGGGTGACACAA-GGC
- hCLOCK_RORemut_R: TTGCTTTTGTGACGCCTTTAT-TCC
- hBmal1_ROREwt_F: TTGAAGGCAGAAAGTAGGTCAGG-GACGGACGGAG
- hBmal1_ROREwt_R: TTCTCCGTCCCTGACCTACTTCTTCC

Cell Culture and Cotransfection Assay

The putative CLOCK RevRE and a mutated RevRE were synthesized as a three-times repeat (3×RevRE) with XhoI and MluI restriction sites on the ends (IDT). The wild-type and mutant CLOCK 3×RORE oligos were digested with XhoI and MluI (Promega). The pTL-Luciferase vector was also digested with XhoI and MluI (Promega). All fragments were gel purified and ligated using T4 DNA Ligase (Promega). The constructs were verified by sequencing. The p3x-FLAG-REV-ERBα has already been described previously [13]. Human HepG2 cells

Figure 1. Identification of a REV-ERBα binding site within the CLOCK gene. (a) Significant REV-ERBα occupancy was observed within the CLOCK gene within the 1st intron. The genomic structure of CLOCK is shown with REV-ERBα occupancy illustrated as the gray line. The arrow indicates the direction of transcription. The raw ChIP/chip data is shown in a window beneath the gene as is a screen shot from the integrative genome browser. (b) The region of REV-ERBα occupancy was scanned for conserved RevRE using the Evolutionarily Conserved Region Browser and MatInspector. A RevRE was found to be conserved between mice and humans, the alignment of the RevRE is shown. (c) Alignment of the CLOCK RevRE to a characterized RevRE in the Bmal1 promoter.

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were maintained and propagated in minimum essential medium supplemented with 10% fetal bovine serum at 37°C under 5% CO₂. For transfections, HepG2 cells were plated in 96-well plates at a density of 15 × 10⁴ cells/well 24 h prior to transfection. Transfections were performed using Lipofectamine 2000, according to manufacturer’s instructions (Invitrogen). Per well, the transfection mixture included 50 ng Renilla luciferase as an internal control, 100 ng of the appropriate CLOCK luciferase construct, and 100 ng of the p3x-FLAG-REV-ERBα expression construct. The luciferase activity was measured using the Dual-Glo luciferase assay system 24 h after transfection (Promega). The luciferase readings were normalized by well to the Renilla readings. For treatment with GSK4112, HepG2 cells were plated into 12-well plates. Cells were then treated with 10 μM GSK4112 or equivalent volume of vehicle. Treatment lasted for 24 hrs. Cells were harvested for RT-PCR determination of CLOCK expression.

siRNA Transfection
The siRNAs targeting RORα, RORγ, and REV-ERBα were purchased from Dharmacon (Thermo Fischer). The siRNA was transfected into HepG2 cells using Lipofectamine RNAiMax, according to manufacturer’s instructions (Invitrogen). The siRNA-treated HepG2 cells were incubated for 24 hours before being harvested for mRNA isolation.

RT-PCR
Extraction of mRNA, synthesis of cDNA, and quantitative PCR were performed as described previously [14]. The cyclophilin B (M60857) gene was used as the control. All primers were designed for human genes. Primer sequences are listed below.

Cyclophilin B forward: 5’- GGAGATGGCACAGGAGGAAA -3’
Cyclophilin B reverse: 5’- CGTAGTGCTTCAGTTTGAA-GTTCCTCA -3’
REV-ERBα forward: 5’- TTCCGCTTCGTTGAGCAAGC -3’
REV-ERBα reverse: 5’- CCGGTTTCTCAGCACCAGAG -3’
CLOCK forward: 5’- TGGGAATCCCTCAAATGCTGAGGAGGTTCTCA -3’
CLOCK reverse: 5’- GACTGAGGAAGGTGCTCTG -3’

Statistical Analysis
In the co-transfection assays, the values indicated represent the means ± S.E. from eight independently transfected wells. In the RT-PCR assays, the values indicated represent the means ± S.E. from four independently transfected wells. The experiments were repeated at least three times.

Results
A ChIP/chip screen was performed to determine regions where REV-ERBα is bound within the genome as previously described [9]. Significant REV-ERBα occupancy was observed with the

Figure 2. REV-ERBα is required for normal expression of CLOCK. HepG2 cells were transfected with siRNAs targeting RORα (78% reduction), RORγ (50% reduction) and REV-ERBα (57% reduction) to reduce their expression. CLOCK expression was then examined by RT-PCR. CLOCK expression was elevated when REV-ERBα expression was reduced (a), but unaffected by alteration of RORα (a) or RORγ (b). Data shown is mean ± SEM where n = 4. (d) ChIP assay illustrating REV-ERBα and NCoR occupancy of the 1st intron of the CLOCK gene. (e) The synthetic REV-ERBα agonist GSK4112 suppresses CLOCK gene expression in HepG2 cells. *, p<0.05.
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REV-ERB DNA can compete for binding to REV-ERB. GSK4112 (SR6452) binds directly to the ligand binding domain of REV-ERB. We next assessed the ability of a synthetic REV-ERB agonist to modulate the expression of CLOCK expression in HepG2 cells. GSK4112 (SR6452) binds directly to the ligand binding domain of REV-ERB and increases the recruitment of NCoR leading to increased repression of target genes [17,18,19,20]. As shown in Figure 2c, treatment of the HepG2 cells with GSK4112 results in repression (~55%) of CLOCK gene expression. Interestingly, overexpression of REV-ERBα in HepG2 cells did not result in any change in CLOCK gene expression (data not shown), suggesting that the endogenous REV-ERBα levels may be saturating.

The putative RevRE was examined for REV-ERBα binding using an EMSA. Synthetic oligonucleotides encoding the putative CLOCK RevRE were radiolabeled, and then incubated with REV-ERBα protein produced in vitro. The sequences of the oligonucleotides are shown in Figure 3a. Direct binding of REV-ERBα to the wild-type CLOCK RevRE is shown in Figure 3b. Addition of unlabeled wild-type CLOCK RevRE probe was able to displace the radiolabeled CLOCK RevRE from REV-ERBα, as shown in Figure 3b while an excess of unlabeled mutant CLOCK RevRE probe was unable to displace the radiolabeled CLOCK RevRE probe from the REV-ERBα protein demonstrating specificity. We also assessed the ability of the CLOCK RevRE probe to compete with the well-characterized BMAL1 RevRE. The BMAL1 RevRE was radiolabeled and then 100-fold molar excess of unlabeled CLOCK RevRE was included as a competitor. As illustrated in Figure 3c, the CLOCK RevRE was able to compete for binding of REV-ERBα to the BMAL1 RevRE.

Due to the location of the putative RevRE within the 1st intron of the CLOCK gene, we generated a three-times repeat of the putative RevRE into HepG2 cells did not result in increased repression of target genes [17,18,19,20]. As shown in Figure 4a, 3×RevRE reporter construct was transfected into HepG2 cells, the co-transfection of REV-ERBα suppressed luciferase expression relative to reporter alone (Figure 4b).
expression of luciferase from the mutant 3×RevRE reporter construct was unaffected by co-transfection of REV-ERBα when transfected into HepG2 cells. Similar results were observed in HEK293 cells (data not shown).

Discussion

CLOCK heterodimerizes with BMAL1 and maintains the circadian expression of target genes containing E-boxes in their promoters. BMAL1 is a known direct target gene of RORα and REV-ERBβ, whose circadian expression influences the expression of BMAL1. However, little is known about how the dimerization partner of BMAL1, CLOCK, is regulated at the transcriptional level. We recently demonstrated that another BMAL1 heterodimerization partner, NPAS2, is regulated by RORα and REV-ERBα providing for a potential mechanism for coordinated regulation of BMAL1/CLOCK heterodimers.

Figure 4. Identification of a functional RevRE within the CLOCK gene using reporter-luciferase assays. (a) The reporter constructs contain a three-times repeat of the putative CLOCK RevRE cloned upstream of the firefly luciferase gene. (b) REV-ERBα was co-transfected with a luciferase reporter containing the wild-type 3×RevRE leading to reduced expression of luciferase relative to the reporter alone. The expression of luciferase from the mutant 3×RORE was unaffected by REV-ERBα co-transfection. Data shown is mean ± SEM, n = 8; * p<0.05. (c) Proposed model for coordinated regulation of BMAL1/CLOCK heterodimers.

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Given the similarity between the BMAL1 and CLOCK RevREs this is surprising and it is currently unclear what differences may be driving the selectivity. It is possible that the REV-ERBα selectivity for regulating the CLOCK gene may be a function of the HepG2 cell line used in this study and in other cell types RORα may play a significant role. Alternatively, other BMAL1 partners such as NPAS2 may play a significant role in maintaining the proper ratio of BMAL1 to heterodimerization partner. It is interesting to note that in the HepG2 cells we previously observed circadian oscillations in the expression of BMAL1, RORα and NPAS2 but not REV-ERBα following a serum shock [9]. We also observed that CLOCK does not oscillate in the HepG2 cells (data not shown), which is consistent with the lack of oscillation in REV-ERBβ and the lack of responsiveness to the oscillating RORα. Again, this pattern of regulation may be specific for HepG2 cells or in cells or tissues where CLOCK does not exhibit a strong circadian pattern of expression. Clearly, REV-ERBβ plays an important role in the circadian pattern of expression of CLOCK in some circumstances since the REV-ERBβ null mouse exhibits a loss in the pattern [8]. Our data suggests that at least a component of the effect may be mediated by a direct effect of REV-ERBβ on expression of the CLOCK gene. REV-ERBβ mediated regulation of CLOCK adds to the complexity of the feedback loop that maintains circadian rhythms. The BMAL1/CLOCK dimer is capable of up-regulating REV-ERBβ, which will then repress BMAL1 and CLOCK expression. This coordinate regulation of BMAL1 and
CLOCK by REV-ERBα may help maintain the circadian oscillations of various genes, including REV-ERBα itself (Figure 4c).

Author Contributions
Conceived and designed the experiments: TPB CC. Performed the experiments: CC. Analyzed the data: TPB CC. Contributed reagents/materials/analysis tools: TPB CC. Wrote the paper: TPB CC.

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