Mechanism of circadian oscillation of the mammalian core clock gene *Per2*

（時計遺伝子 *Per2* の発現制御機構）

2014

跡部　祐太

Contents

Abstract

Daily rhythms in behavior and physiology are controlled by an internal self-sustained molecular oscillator, referred to as the circadian clock. Circadian rhythms are generated in a cell-autonomous manner through transcription/translation-based autoregulatory feedback loops, wherein protein products of three *Period* genes (*Per1*, *Per2* and *Per3*) periodically suppress their own transcription. Genetic studies have suggested that, of the three *Period* genes, *Per2* has a dominant role in both humans and mice. However, little is known about the regulatory mechanism underlying expression of this gene. In this thesis, I investigated the molecular mechanism driving the circadian transcription of the mouse *Per2* gene. I find that the *Per2* promoter contains a circadian *cis*-element, termed Rhythm-box (R-box). Furthermore, I generated novel mutant mice carrying a point mutation in the R-box sequence of the *Per2* promoter. Analysis of the mutant mice revealed a previously unknown *in vivo* function of this element in regulation of circadian transcription of the *Per2* gene.

Introduction

Daily rhythms in behavior and physiology are controlled by an internal self-sustained molecular oscillator, referred to as the circadian clock[1-5]. Circadian rhythms are generated in a cell-autonomous manner through transcription/translation-based autoregulatory feedback loops[5-8], wherein protein products of three *Period* genes (*Per1*, *Per2* and *Per3*) periodically suppress their own transcription (**Figure 1**). In this general model, the circadian positive regulators, CLOCK and BMAL1, promote transcription of the *Period* (*Per1*, *Per2*, and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) genes through E-box *cis*-elements (5′-CACGT[G/T]-3′) located in their promoter regions. Once the repressor proteins, PER and CRY, reach a critical concentration, they form a complex and inhibit CLOCK:BMAL1-mediated transcription.

Genetic studies have suggested that, of the three *Period* genes, *Per2* has a dominant role in both humans and mice[9-11]. However, little is known about the regulatory machinery of *Per2*, although there are many studies describing *Per1* expression mechanism[12-15]. In this thesis, I investigated the molecular mechanism driving the circadian transcription of the mouse *Per2*

gene. I found that *Per2* promoter contains a circadian *cis*-element, termed Rhythm-box (R-box) (**Chapter 1**). Furthermore, I generated novel mutant mice carrying a point mutation in the R-box sequence of the *Per2* promoter (**Chapter 2**). Analysis of the mutant mice revealed a previously unknown *in vivo* function of this element in regulation of circadian transcription of the *Per2* gene (**Chapter 3**).

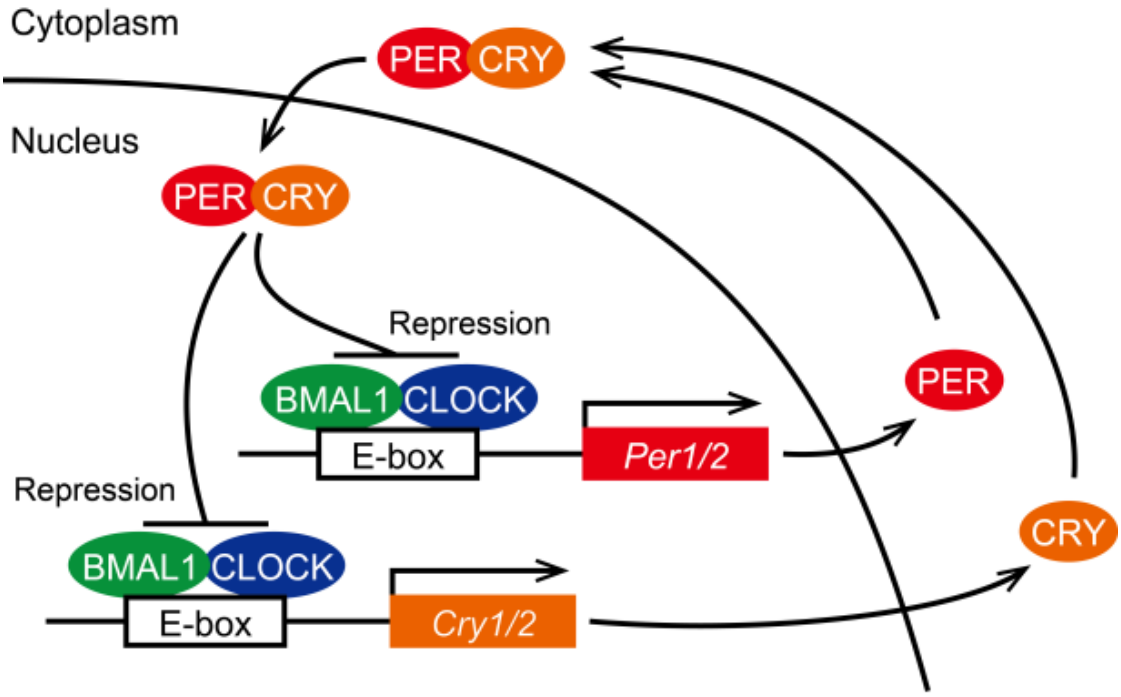

Figure 1. Autoregulatory feedback model of the mammalian cellular clock. The circadian clock mechanism involves transcription-ranslation feedback loops comprised of a set of core clock genes (see main text for details).

Results and Discussions

Chapter 1: Autoregulatory feedback model of *Per2* transcription through R-box

Firstly, I sought to determine the circadian oscillation profiles of *Per2* mRNA and protein in the mouse liver. Mice were sacrificed at 4 h intervals over a 24-h cycle. Quantitative RT-PCR and Western blot analyses revealed that the levels of *Per2* mRNA and protein exhibit an overt circadian oscillation with a typical phase-relationship. The PER2 protein began to increase 4 hours behind mRNA expression and reached its peak when the mRNA levels were decreasing. These observations corroborate the current negative feedback model, in which PER2 represses its own transcription. In an attempt to further test this model, I next explored the regulatory *cis*-element on the *Per2* promoter. Mouse embryonic fibroblasts were transfected with a luciferase reporter vector containing a 1.7-kb fragment of the 5'-flanking region of *Per2*, and its promoter activity was monitored using a realtime luminometer system. I observed a robust circadian oscillation of the *Per2* promoter activity over three cycles. Furthermore, I

demonstrated that a single point mutation that was introduced into the R-box sequence located in the vicinity of the transcription start site of *Per2*[16, 17] resulted in a complete abrogation of this rhythmicity. These *in vitro* data strongly suggest that this single R-box element is indispensable for the circadian activation and repression of *Per2* gene.

Chapter 2: Generation of mice carrying a point mutation of the *Per2* R-box

To investigate the role of R-box in regulation of *Per2* oscillation *in vivo*, I generated *Per2* R-box mutant mice. To introduce a point mutation into this element, I employed a *piggyBac* (*PB*) transposon system. *PB* is a moth-derived DNA transposon[18] whose functionality is retained in mammals[19, 20]. Unlike the well-established methods relying on the Cre/*loxP* and Flp/*FRT*-based recombination systems[21, 22], PB transposase (PBase)/*PB*-based system achieves a 'seamless' excision of a *PB*-flanked target sequence without any residual 'footprint'[18, 20]. This allows, for example, 'complete' removal of a neomycin resistant gene-coding cassette from a mutant mouse genome without leaving any unwanted residual sequence[23, 24]. I therefore constructed a targeting vector, carrying a mutant R-box sequence and a neomycin resistant cassette flanked by *PB* terminal repeats. Targeted mouse embryonic stem cell clones were injected into blastocysts to generate chimeric mice. To remove the *PB*-flanked neomycin cassette from the genome, F1 heterozygotes were intercrossed with ROSA26-PBase

mice that carry a ubiquitously expressed PBase gene at the ROSA26 locus[25]. I performed Southern blot analysis and confirmed that the neomycin cassette was deleted without reintegration into the host genome. Finally, a seamless excision of the cassette was confirmed by DNA sequencing. The sequence analysis also verified that a point mutation was indeed introduced into the R-box. Genotypes were also determined by allele-specific TaqMan qPCR. Moreover, the established mutant mice were backcrossed to the C57BL/6J background, and at the end of backcrossing, I confirmed that 64 microsatellite markers covering all individual mouse chromosomes were replaced with those of the C57BL/6J mouse strain.

Chapter 3: Characterization of *in vivo* role of the *Per2* R-box

To evaluate whether the R-box mutation *in vivo* abrogates rhythmicity of *Per2* expression, I measured circadian profiles of *Per2* mRNA in the mouse liver samples collected every 4 hours in constant darkness. Quantitative RT-PCR analysis revealed that the point mutation of the *Per2* R-box caused alteration of the circadian rhythm of *Per2*. This mutation impairs circadian repression of *Per2* expression and thereby elevates the levels of the baseline. Similar results were obtained for the suprachiasmatic nucleus (SCN) in the brain, which serves as the mammalian circadian center. Thus, the R-box appears to be important for repression of *Per2* transcription *in vivo*. The current feedback model expects the repressor proteins PER and CRY to be recruited to the E-box elements in their target gene promoters[2]. I therefore hypothesized that the *Per2* R-box might play an important role in recruitment of the circadian transcriptional repressors *in vivo*. To test this hypothesis, I performed chromatin immunoprecipitation (ChIP) assays. To detect chromatin binding of PER and CRY, I employed a dual crosslinking ChIP method[26, 27], which uses disuccinimidyl glutarate

(DSG) prior to formaldehyde crosslinking. DSG has a longer spacer arm than formaldehyde and has been used to crosslink transcription factor (cofactor) protein-protein interactions on DNA[28]. Firstly, to validate the specificity of the method, ChIP assays for PER1, CRY1, and CRY2 were performed with the mouse liver samples from wild-type (WT) and respective mutant mice (*Per1*$^{-/-}$, *Cry1*$^{-/-}$, and *Cry2*$^{-/-}$). The results demonstrate that the antibodies I used for PER1, CRY1, and CRY2 immunoprecipitate the *Per2* R-box fragment from WT sample more efficiently than the corresponding mutant. Then, to determine circadian binding profiles of the negative regulators to the *Per2* promoter R-box, WT mice were sacrificed every 4 hours over a 24-hr cycle in constant darkness (DD) and their liver samples were subjected to ChIP assays for PER1, CRY1, and CRY2. The results revealed that PER1, CRY1, and CRY2 were all recruited to the *Per2* R-box in a circadian time specific manner in the wildtype mouse liver. The recruitment of PER1 predominantly occurred at CT12 (CT represents circadian time; CT0 denotes the beginning of the subjective day and CT12 the beginning of the subjective night). In comparison, CRY2 binding was observed from CT12 to CT20.

On the other hand, CRY1 binding started to increase at CT20 with a peak at CT4. Considering that *Per2* transcription reaches its maximum and minimum at CT16 and CT4, respectively, the negative regulators, PER1, CRY1, and CRY2, might play distinct roles in circadian transcriptional repression of *Per2*.

Finally, I tested whether the R-box mutation abrogates the recruitment of PER1, CRY1 and CRY2 to the *Per2* promoter. I found that ChIP values of PER1, CRY1 and CRY2 were all reduced to near basal levels throughout the circadian cycle by the R-box mutation, demonstrating that the R-box is required for binding of the negative regulators. Based on these results, I conclude that the R-box is responsible for circadian repression, rather than activation, of *Per2* transcription *in vivo*.

Reference

1. Wijnen, H. & Young, M.W. Interplay of circadian clocks and metabolic rhythms. *Annual review of genetics* **40**, 409-448 (2006).
2. Takahashi, J.S., Hong, H.K., Ko, C.H. & McDearmon, E.L. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nature reviews. Genetics* **9**, 764-775 (2008).
3. Dunlap, J.C. Molecular bases for circadian clocks. *Cell* **96**, 271-290 (1999).
4. Schibler, U. & Sassone-Corsi, P. A web of circadian pacemakers. *Cell* **111**, 919-922 (2002).
5. Reppert, S.M. & Weaver, D.R. Coordination of circadian timing in mammals. *Nature* **418**, 935-941 (2002).
6. Liu, A.C., Lewis, W.G. & Kay, S.A. Mammalian circadian signaling networks and therapeutic targets. *Nature chemical biology* **3**, 630-639 (2007).
7. Hastings, M.H., Maywood, E.S. & O'Neill, J.S. Cellular circadian pacemaking and the role of cytosolic rhythms. *Curr Biol* **18**, R805-R815 (2008).
8. Okamura, H., Yamaguchi, S. & Yagita, K. Molecular machinery of the circadian clock in mammals. *Cell Tissue Res* **309**, 47-56 (2002).
9. Zheng, B. et al. The mPer2 gene encodes a functional component of the mammalian circadian clock. *Nature* **400**, 169-173 (1999).
10. Toh, K.L. et al. An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* **291**, 1040-1043 (2001).

11. Fu, L., Pelicano, H., Liu, J., Huang, P. & Lee, C. The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* **111**, 41-50 (2002).
12. Albrecht, U., Sun, Z.S., Eichele, G. & Lee, C.C. A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. *Cell* **91**, 1055-1064 (1997).
13. Takumi, T. et al. A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. *EMBO J* **17**, 4753-4759 (1998).
14. Yamaguchi, S. et al. The 5' upstream region of mPer1 gene contains two promoters and is responsible for circadian oscillation. *Curr Biol* **10**, 873-876 (2000).
15. Yamamoto, T. et al. Acute physical stress elevates mouse period1 mRNA expression in mouse peripheral tissues via a glucocorticoid-responsive element. *The Journal of biological chemistry* **280**, 42036-42043 (2005).
16. Yoo, S.H. et al. A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. *Proc Natl Acad Sci U S A* **102**, 2608-2613 (2005).
17. Akashi, M., Ichise, T., Mamine, T. & Takumi, T. Molecular mechanism of cell-autonomous circadian gene expression of Period2, a crucial regulator of the mammalian circadian clock. *Mol Biol Cell* **17**, 555-565 (2006).
18. Fraser, M.J., Ciszczon, T., Elick, T. & Bauser, C. Precise excision of TTAA-specific lepidopteran transposons piggyBac (IFP2) and tagalong (TFP3) from the baculovirus genome in cell lines from two species of Lepidoptera. *Insect Mol Biol* **5**, 141-151 (1996).
19. Ding, S. et al. Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell* **122**, 473-483 (2005).

20. Wang, W. et al. Chromosomal transposition of PiggyBac in mouse embryonic stem cells. *Proc Natl Acad Sci U S A* **105**, 9290-9295 (2008).
21. van der Weyden, L., Adams, D.J. & Bradley, A. Tools for targeted manipulation of the mouse genome. *Physiol Genomics* **11**, 133-164 (2002).
22. Hockemeyer, D. et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol* **27**, 851-857 (2009).
23. Yusa, K., Rad, R., Takeda, J. & Bradley, A. Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* **6**, 363-369 (2009).
24. Yusa, K. et al. Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells. *Nature* **478**, 391-394 (2011).
25. Rad, R. et al. PiggyBac transposon mutagenesis: a tool for cancer gene discovery in mice. *Science* **330**, 1104-1107 (2010).
26. Ota, T. et al. Angiotensin II triggers expression of the adrenal gland zona glomerulosa-specific 3β-hydroxysteroid dehydrogenase isoenzyme through de novo protein synthesis of the orphan nuclear receptors NGFIB and NURR1. *Mol Cell Biol* **34**, 3880-3894 (2014).
27. Zeng, P.Y., Vakoc, C.R., Chen, Z.C., Blobel, G.A. & Berger, S.L. In vivo dual cross-linking for identification of indirect DNA-associated proteins by chromatin immunoprecipitation. *BioTechniques* **41**, 694, 696, 698 (2006).
28. Tian, B., Yang, J. & Brasier, A.R. Two-step cross-linking for analysis of protein-chromatin interactions. *Methods Mol Biol* **809**, 105-120 (2012).

Acknowledgements

First of all, I wish to express sincere thanks to Prof. H. Okamura and Dr. M. Doi for their direction and kind encouragement throughout my graduate study.

I thank Prof. M. Hashida (Kyoto University, Kyoto), who kindly provided the DNA construct encoding a *piggyBac* terminal repeat, Prof. A. Bradley (Wellcome Trust Sanger Institute, UK) for providing the ROSA26-PBase mice, Dr. C. Lee (Florida State University, USA) for providing anti-CRY1 and anti-CRY2 antibodies, and Dr. N. Koike (Kyoto Prefectural University of Medicine, Kyoto) for technical advice in the ChIP assay.

I am grateful to all the member of Okamura's lab for their advice and help. In particular, I appreciate the technical assistance and contributions of Mr. H. Hayashi.

I thank my parents for their support and encouragement. Without their support I would not have accomplished this thesis.