Molecular Oscillation of \textit{Per1} and \textit{Per2} Genes in the Rodent Brain: An \textit{In Situ} Hybridization and Molecular Biological Study

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The circadian rhythm is originally generated by a transcription-translation based oscillatory loop where \textit{Per1} and \textit{Per2} genes locate in its central. In the rat brain, rhythmic expressions of \textit{Per1} and \textit{Per2} were observed not only in neurons of the hypothalamic suprachiasmatic nucleus (SCN) but also in those of non-SCN regions including the cerebral cortex. The E-box enhancer elements possible to regulate transcription of \textit{Per1} and \textit{Per2} genes were highly conserved in rats and mice. When E-box-activating transcription factors, CLOCK and BMAL1, were coexpressed, each of both proteins showed two molecular forms. The presence of these higher molecular weight forms seems to be correlated with the E-box mediated transcription activation. This mechanism might not be involved in the \textit{PER2} mediated suppression of E-box, since adding \textit{PER2} did not change the content of the higher molecular forms of CLOCK and BMAL1.

Circadian core oscillator is thought to be composed of an autoregulatory transcription-(post) translation-based feedback loop involving a set of clock genes (3, 4, 10, 16). In this loop, \textit{Per1} and \textit{Per2} genes are located in the center of this loop, and the transcriptional oscillation of these genes reflects rhythms at cells, tissues, and system levels (10, 16). The crucial role of these genes are verified by the finding that the double knockout mice of \textit{Per1} and \textit{Per2} genes show the immediate stop of their circadian behavioral rhythms (2, 26).

From the cloning of \textit{Per1} and \textit{Per2} genes in rodents, their expression profiles were thoroughly investigated in the suprachiasmatic nucleus (SCN), the center of the biological clock in rodents (12, 14, 15, 17, 18, 19, 24). After the discovery of clock genes in many visceral organs, many studies have revealed their expression in a variety of visceral organs (9, 11). In the non-SCN brain, some studies have been reported (1, 8, 13, 20), but the detailed studies have not been performed yet.

In the present study, we examined the daily expression of \textit{Per1} and \textit{Per2} in various brain regions including the cerebral cortex in the rat brain. Since it is speculated that CLOCK/BMAL1 heterodimer binding to E-box of the promoter regions of the \textit{Per1} and \textit{Per2} genes plays the initial step of the transcription (5, 6, 22, 25), we further examined the possible E-box sites of \textit{Per1} and \textit{Per2} in the rat genome comparing those of well characterized mice \textit{Per1} and \textit{Per2} genes, and found that E-box sites were well conserved between mice and rats. Lastly, we present some molecular biological data on the activation mechanism of CLOCK and BMAL1 on E-box sites, including the appearance of higher molecular weight forms of both CLOCK and BMAL1.
MATERIALS AND METHODS

Animals

Wistar strain albino male rats (200-250 g body weight; JAC, Osaka, Japan) were used in this study. They were housed under 12 h : 12 h light-dark (LD) cycles for at least for 1 week in a temperature-controlled (22 ± 2 °C) environment and were given food and water ad libitum. They were then kept in complete darkness (DD) for 2 days as a continuation of the dark phase of the last LD cycle.

For the study of the day-night difference in various brain regions from the olfactory bulb to the medulla oblongata, we sacrificed 6 animals at CT4 (CT = circadian time used for assessing biological time without any time cues; CT4 means 4 hours after the subjective dawn) and CT16 (4 hours after the subjective dusk). For the quantitative study, the expression of Per1 and Per2 mRNAs was examined in the second DD cycle, every 4 h (n = 5 at each time point for each experiment), starting at CT0 (CT0 is subjective dawn and CT12 is subjective dusk). The number of experimental animals was minimized as possible, and the experimental protocol of the current research was approved by the Committee for Animal Research at Kobe University Graduate School of Medicine.

In situ hybridization

In situ hybridization method using the free-floating sections was performed according to the method detailed previously (24). We used 33P-radiolabeled complementary RNA (cRNA) probes for rat Per1 and Per2 for the in situ hybridization studies (24). For the quantitative in situ hybridization, signal from somatosensory cortex on BiMax film (Kodak, Rochester, NY) was analyzed using a microcomputer interfaced to an image-analyzing system after conversion into the relative optical densities produced by the [14C] acrylic standards (Amersham, Buckinghamshire, UK). The intensities of the optical density of the 10 sections of the somatosensory cortex were then summed. The results are expressed as means ± standard error of the mean (SEM). The peak value of rats was adjusted to 100 for each probe, and relative RNA abundance was used. For the statistical analysis, one-way analyses of variance (ANOVA) followed by Sheffe’s multiple comparisons were applied.

Cell culture and Transcriptional assay

NIH3T3 or COS7 cells were maintained in the culture medium (Dulbecco’s modified eagle medium (nacalai tesque, Kyoto, Japan) with 10% fetal bovine serum, 4.5g/L glucose, and antibiotics). NIH3T3 cells were plated in 12-well plates 24 hours before transfection. Cells were transfected by LipofectAmine-Plus reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. In this study, we used the Dual Luciferase Reporter system (Promega, WI, USA). Unless otherwise noted, the cells in each well were transfected with 700 ng (total) of the following plasmids; 2xE-box(5'-CACGTG-3')-pGL3Promoter for reporter, pRL-SV40 (Promega, WI, USA) for internal control, pcDNA3-flag-mClock, pcDNA3-flag-mBmal1, pcDNA3-flag-mPer2. The total amount of DNA per well was adjusted by adding pcDNA3 vector. After 2 days, the cells in each well were extracted with passive lysis buffer (Promega, WI, USA), and the extracts were taken for assays of Firefly luciferase and Renilla luciferase by TD-20/20 luminometer (Turner, Sunnyvale, CA, USA).

Immunoprecipitation and Western blotting

Immunoprecipitation was performed by using of whole-cell lysates. Subconfluent COS7 cells in 6 cm dishes were transfected with total 7 micrograms of the plasmids with lipofect amine 2000 (Invitrogen, CA, USA). The cells were harvested with 0.5 ml of the lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, and Complete Mini Protease Inhibitor (Roche, Mannheim, Germany)). Each total cell lysate was incubated on ice for 30 min, and centrifuged at top speed for 30 min at 4 °C. Anti-Flag-tag antibody (Sigma, MO, USA) was
added in the resultant supernatant, and incubated for 2 hours at 4 °C with mild agitation. After adding 30 microliter of protein A-agarose beads equilibrated with the lysis buffer, the beads were collected and washed fifth with the lysis buffer. The proteins bound to the beads were eluted in SDS sample buffer (50 mM Tris-HCl pH 6.8, 100 mM DTT, 2 % SDS, 10 % glycerol, 0.1 % BPB) and analyzed by SDS-PAGE. Western blotting were carried out by standard procedures, and Chemiluminescence was performed by using western blotting Luminol reagent (Santa Cruz, CA, USA) and analyzed by LAS1000 (Fuji Film, Tokyo, Japan).

RESULTS AND DISCUSSION

The day-night expression profile of Per1 and Per2 of non-SCN brain regions is reversed to that of the SCN in the rat

Time-dependent difference of the expression of Per1 and Per2 mRNA in the brain was examined at the subjective day (CT4; 4 hours after the subjective dawn) and at the subjective night (CT16, 4 hours after the subjective dusk). To exclude the possible environmental light effect, we performed the experience in constant dark conditions. At CT4, Per1 mRNA expression is high in the internal granular layer of the olfactory bulb, granular layer of the hippocampal gyrus dentatus, and SCN (Fig. 1A), of which distribution well corresponds to that of Per1 in the mouse brain (23). Moderate level of Per1 signals were found in the tuberculum olfactorium, piriform cortex, supraoptic nucleus, hypothalamic ventromedial nucleus, arcuate nucleus, stratum pyramidal of the Ammon’s horn, and the granular layer of the cerebellar cortex. At CT16, Per1 mRNA signals were enhanced except for olfactory bulb, hippocampus, and SCN. Among these, the signal level in SCN was very high at CT4, which contrasted with that of very low level at CT16 (Fig. 1A).

At CT4, Per2 expression was very high in SCN, but the signal levels in other brain regions were low (Fig. 1B). At CT16, the signal intensity of the SCN was reduced, but that in other brain regions was increased. These findings clearly demonstrate that Per1 and Per2 genes expressed in many brain regions show the clear day-night difference, but the expression profile of non-SCN regions are reversed to that of SCN.

Early night peak and early morning trough are the circadian expression profiles of Per1 and Per2 in the cerebral cortex

To examine the more precise daily expression profiles in non-SCN regions, we analyzed Per1 and Per2 mRNA levels in the somatosensory cortex by the quantitative in situ hybridization (Fig. 1C). For this purpose, we used 30 animals at 4 hours intervals housed in the constant dark conditions beginning at CT0 (n=5 at each time point). Per1 mRNA showed the clear circadian rhythm (one-way ANOVA, P<0.01) with the highest at CT16 and the lowest at CT4. Per2 mRNA showed the moderate circadian rhythm (one-way ANOVA, P<0.01) with the peak at CT12 and the trough at CT4. The night-peak-with-morning-trough pattern of both genes in the cerebral cortex is completely inverted to the expression profile of those genes in the SCN (24).

E-boxes in 5' upstream region of Per1 and Per2 deduced from the genome database in mice and rats

The above rat data of Per1 and Per2 oscillation strongly support that the molecular feedback loop of clock genes is working in many brain neurons of rats similar to mice. In mice, it is believed that CLOCK/BMAL1 heterodimer plays the crucial role to initiate the transcription of Per1 and Per2 genes (5, 6, 22, 25). As proposed first by Gekakis et al. (5) for Per1 gene, recent studies have established that the key transcription regulatory sites of Per1
Fig. 1. Rhythmic expression of *Per1* and *Per2* mRNAs in the rat brain in constant dark conditions. (A) Representative *in situ* hybridization autoradiograms showing *Per1* and *Per2* mRNA in various regions of the rat brain at CT4 and CT16. Abbreviations: II/III CCx, layer II/III of the cerebral cortex; Arc, arcuate nucleus; GrCerebellCx, granular layer of the cerebellar cortex; GrGD, granular layer of the hippocampal gyrus dentatus; HippPyr, stratum pyramidale of the Ammon’s horn; IGrOlB, the internal granular layer of the olfactory bulb; PirCx, piriform cortex; PVN, hypothalamic paraventricular nucleus; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; TuO, tuberculum olfactorium; VMH, hypothalamic ventromedial nucleus. (B) Quantitative analyses of *Per1* and *Per2* mRNAs in constant dark conditions using 5 rats at each time point (mean ± SEM; n=5). The mean peak values at CT20 are adjusted to 100 for each probe. Representative *Per1* and *Per2* *in situ* hybridization autoradiograms is shown in the left side. Numbers on each autoradiogram indicate the sampling circadian time (hours).
and *Per2* are E-boxes located in the upstream of the transcription initiation sites (5'-CACGTG-3' or 5'-CACGTT-3') (6, 22, 25) (Fig. 2). Importantly, these 5' upstream regions are sufficient to drive the self-sustained circadian rhythm of the luciferase activity in *Per1* and *Per2* promoter-luciferase transgenic mice (22, 25). To elucidate whether these E-box sequences are conserved also in rats, we compared the genome sequences of rats to those of mice from GenBank database.

Two alternative spliced promoters (Exon1A and Exon1B) are reported in mice *Per1* (22), and there are two E-boxes (E1 and E2) just above Exon1A, and three E-boxes (E3, E4 and E5) just above Exon1B. The promoter region (~8kb from the translation initiation site) of *Per1* gene was highly conserved in both species, and particularly, 4 out of 5 E-boxes (E1, E2, E3 and E5) were completely identical (Fig. 2A). In the rat, E4 (5'-CACGTG-3') was not conserved, but there was 5'-CAGGTG-3', which was also one of E-box in a broad sense (5'-CANNTN-3').

Mice *Per2* has only one noncanonical 5'-CACGTT-3' E-box located 20 bp upstream of the *Per2* transcription start site (Fig. 2B). In the rat, this noncanonical sequence was completely conserved.

These findings show that 5'upstream regions, particularly E-boxes, are well conserved in mice and rats. Since E-boxes which are critical for the start of the transcription in mice, circadian rhythms of *Per1* and *Per2* in rats are also generated by the similar E-box mediated CLOCK/BMAL1 transcription activation mechanisms.

Fig. 2. Comparison of the deduced sequences of *Per1* (A) and *Per2* (B) genomes of rats to those of mice from GenBank database. GenBank accession numbers: mouse *Per1* (NT_096135), rat *Per1* (NW_047334), mouse *Per2* (NT_039173), rat *Per2* (NW_047817).

Naming of two alternative spliced promoters (Exon1A and Exon1B) of *Per1* and *Per1* for *Per2* are followed by the previous reports in mice (22, 25).

Note that the E-box names of rat sequences are determined from the sequence similarity of corresponding mouse data which are well characterized in previous reports (6, 22, 25).
Appearance of the higher molecular forms of CLOCK and BMAL1 correlates with the increase of the E-box mediated transcription activation

To elucidate how CLOCK and BMAL1 can activate E-box, we first examined their molecular forms by transfecting tagged full length cDNA to COS7 cells, followed by the immunoprecipitation with antibody against Flag-tag (Fig. 3). CLOCK protein formed a single band when solely expressed. BMAL1 also showed a single band when expressed alone. However, when CLOCK and BMAL1 were coexpressed, higher molecular weight forms of both proteins appeared (Fig. 3A).

Then we examined the influence of these compounds by the E-box mediated luciferase reporter system in clock oscillating NIH3T3 cells (21). The E-box mediated luciferase reporter activity was completely absent when expressed solely for CLOCK or BMAL1, but it was highly enhanced when CLOCK and BMAL1 were coexpressed (Fig. 3B). Thus, it is interesting that the appearance of the higher molecular weight forms of the CLOCK and BMAL1 are related to the transcription activation. The correlation of the transcription activation of E-box and the higher molecular weight form of BMAL1 is already reported in other cell lines (HEK293 cells) (7). PER2, a possible negative feedback element of clock molecular oscillation, did not influence the molecular forms of CLOCK and BMAL1 (Fig. 3A). In contrast, the coexpression of PER2 with CLOCK and BMAL1 suppressed the E-box mediated luciferase activity (Fig. 3B). Thus it is speculated that the transcription suppression of PER2 is not mediated via the change of the content of higher molecular forms of CLOCK and BMAL1.

![Fig. 3. Molecular forms of CLOCK and BMAL1 and their ability to E-box mediated transcription activation.](image)

(A) Immunoprecipitation of Flag-tagged proteins expressed by the plasmids (pcDNA3-flag-mPer2, pcDNA3-flag-mClock, and pcDNA3-flag-mBmal1) into COS7 cells. Whole cell extract from COS7 cells transfected following each plasmid was immunoprecipitated with antibody against Flag-tag. Each protein was detected with antibody against Flag-tag.

(B) Luciferase reporter activity by the transfection of Clock, Bmal1 and Per2 plasmids into NIH3T3 cells. NIH3T3 cells were transfected with indicated expression and reporter plasmids, and luciferase activity was measured after 2 days. Luciferase reporter plasmid including the two intact E-boxes was used in all lanes. Each value represents the mean ± SEM of three replicates for a single assay. This result shown is representative of three independent experiments.
CONCLUSION

The circadian rhythm is originally generated by a transcription-translation based oscillatory loop composed of a set of clock genes. We can consider the cellular circadian oscillation in mammals to start with the transcription of two genes: Per1 and Per2. In the present study, we demonstrated that Per1 and Per2 oscillated in many cells outside the SCN in the rat brain. The E-box sequences possible to regulate transcription of Per1 and Per2 genes were highly conserved in rats and mice. The existence of higher molecular weight forms of CLOCK and BMAL1 seems to activate the E-box mediated transcription, but the transcription suppression of PER2 is not mediated via the change of these forms.

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