Different Circadian Expression Profiles of Clock Genes in the Mouse Suprachiasmatic Nucleus

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ABSTRACT

The mammalian clock genes *clock*, *BMAL1*, and *Periods*, were cloned and demonstrated to localize in the suprachiasmatic nucleus (SCN), the mammalian circadian center. In the present study, we examined the daily mRNA expression profiles of these genes by quantitative *in situ* hybridization. *Clock* was constantly expressed in the mouse SCN in any circadian time, although mammalian *Periods* and *BMAL1* were expressed in time-dependent manner: peaks of *Periods* appeared in the daytime but those of *BMAL1* appeared in the nighttime. Digoxigenin-*in situ* hybridization demonstrated that all of these clock genes were expressed in most SCN cells. These findings suggest that clock genes were expressed in the SCN with the different expression profiles.

Daily oscillations in behavioral and physiological phenomena are governed by an endogenous circadian pacemaker. In spite of definite localization of mammalian central clock in the suprachiasmatic nucleus (SCN), the core molecules of mammalian circadian time-keeping system had been hidden in a veil of mystery for a long time. The molecular dissection of these endogenous oscillatory mechanisms has been achieved in cyanobacteria (8), *Neurospora* (3), *Drosophila* (5),

and mice (14) as models. In all four species, the core oscillator consists of a transcription / translation feedback loop in which expressed clock gene products negatively regulate their own transcription (see 3 for review).

In mice, the transcription of *mPer1* gene is activated by the binding of the CLOCK-BMAL1 complex to the E-boxes (CACGTG) in the promoter region of the *mPer1* gene (4, 17) and is believed that this activation is specifically inhibited by cryptochromes and their own products (7, 9).

Here we tried to delineate the expression properties of these mammalian clock genes by *in situ* hybridization in the mouse SCN. The cellular level expression profiles were also examined by digoxigenin-labeled *in situ* hybridization. These findings suggest that clock genes were expressed with the different expression profiles in the central pacemaking cells in the SCN.

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Abbreviations:

CT, circadian time; DTT, dithiothreitol; EDTA, ethylen diamine tetra ammonium; hr, hour; mRNA, messenger RNA; oc, optic chiasma; PB, phosphate buffer; PBS, phosphate-buffered saline; SCN, suprachiasmatic nucleus; SSC, sodium chloride and sodium citrate; tRNA, transfer RNA; V, third ventricle.

MATERIALS AND METHODS

Probes.

We made two probes for *clock* mRNA detection without overlapping (963–1475 / 1543–2308), and also a specific probe for *BMAL1* (1656–2259). cDNA was subcloned into BSSK (+) and PGEM T-easy vector, respectively. Probes for *mPer1* (538–1752), *mPer2* (1–638), and *mPer3* were detailed in the previous report (13). Antisense and sense probe for *in situ* hybridization was transcribed by using T3/T7/SP6 RNA polymerases and labeled by using ³³P-UTP (New England Nuclear) with a standard protocol for cRNA synthesis. Since digoxigenin-labeled probes allow a better resolution than isotope probes for analyzing the cellular distribution of mRNA, we made digoxigenin-labeled antisense cRNA probes using digoxigenin-UTP (Boehringer Mannheim) following a standard protocols of cRNA synthesis by Boehringer Mannheim.

Animals.

Male *Bulb/c* mice purchased five weeks postpartum, were exposed to 2–4 weeks of complete light-dark (LD) cycles (300 lux, fluorescent light) and then kept in 2 days of complete darkness as a continuation of the dark phase of the last LD cycle. We sampled at every four hours beginning at ZT 0 (ZT = Zeitgeber time used for assessing biological time in an LD cycle; ZT0 is light-on and ZT12 is light-off), and at CT 0 (CT = circadian time used for assessing biological time in an DD cycle; CT0 is subjective light-on and ZT12 is light-off). Mice were deeply anesthetized with pentobarbital and intracardially perfused with autoclaved 100 mL of ice-cold saline and 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, postfixed for 24 h at 4°C, and placed in 0.1 M PB with 20% sucrose for 48 h. These brains were frozen using dry ice and stored at –80°C until use. The number of animals for the quantification study was $n=4$ at each time point in each clock gene. The experimental protocol of the current research was approved by the Committee for Animal Research of the Kobe University Graduate School of Medicine.

Quantitative in situ hybridization.

For quantitative *in situ* hybridization, serial fron-

tal sections (40 μ m thick) were made from the rostral end to the caudal end of the SCN by a cryostat (Reichert). To minimize technical variations throughout the hybridization procedure, sections from different experimental conditions were gathered into one group and processed simultaneously. *In situ* hybridization histochemistry was performed as described previously (12). Briefly, tissue sections were processed with proteinase K (1 μ g/mL, 0.1 M Tris buffer pH 8.0; 50 mM EDTA; 10 min) at 37°C and 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. The sections were then incubated in the hybridization buffer [55% formamide, 10% dextran sulfate, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.6 M NaCl, 0.2% *N*-laurylsarcosine, 500 μ g/mL tRNA, 1 $\times$ Denhardt's, 0.25% SDS and 10 mM dithiothreitol (DTT)] containing the radio-labeled *mPer1* cRNA for 16 h at 60°C. After high stringency posthybridization wash, sections were treated with RNase A. Air-dried sections with ¹⁴C-acrylic standards (Amersham, UK) were exposed to Biomax film (Kodak) for 5 days, and moved to the imaging analyzing process. The radioactivity of each SCN on BioMax film was analyzed using a microcomputer interfaced to an image analyzing system (MCID, Imaging Research Inc., Canada) after conversion into the relative optical densities produced by the ¹⁴C-acrylic standards (Amersham). Data were normalized with respect to the difference between signal intensities in equal areas of the SCN and the corpus callosum. The intensities of the optical density of the sections from the rostralmost to the caudalmost of the SCN (10 sections per one mouse) were then summed; the sum was considered a measure of the amount of specific peptide mRNA in this region. The value was expressed as mean $\pm$ SEM. For statistical analysis, one-way ANOVA followed by Sheffe's multiple comparisons were applied. We use "relative RNA abundance", which refers to the peak value was adjusted to 100.

In situ hybridization with digoxigenin probe.

Digoxigenin *in situ* hybridization was performed to answer the question in what percentage of SCN cells expressed the clock genes. We examined the clock gene expressed cells at their maximum expression time point. CT4 for *mPer1* and *mPer3*, CT8 for *mPer2*, CT12 for *clock* and *BMAL1*. The tissue preparation, prehybridiza-

tion, hybridization and posthybridization-washing steps were the same as the protocol for hybridization of isotope-probes. For digoxigenin-labeled probes, sections were processed for immunocytochemistry with the nucleic acid detection kit (Boehringer Mannheim, Germany). Signals were visualized blue in the solution containing nitroblue tetrazolium salt (0.34 mg/mL) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (0.18 mg/mL) (Boehringer Mannheim, Germany) for 16 h. To confirm the specificity of the *in situ* hybridization signals visualized by the isotope and the digoxigenin-labeled cRNA probes, we performed a control study using a sense digoxigenin-labeled cRNA probe or RNase-predigested sections including SCN prepared from various circadian times. The

use of digoxigenin-labeled sense and antisense probes in RNase-treated sections revealed no specific hybridization signals.

RESULTS

In situ hybridization study showed the specific daily expression pattern profiles of clock genes (*Clock*, *BMAL1*, *mPer1*, *mPer2*, and *mPer3*) in the SCN. Fig. 1 showed representative sections of the SCN at each time point in LD conditions. In any these clock genes, the signal levels of the SCN were much stronger than those of the surrounding preoptic area.

We first examined the temporal expression of positive clock components (*clock* and *BMAL1*) in the SCN, where the master oscillatory clock is

Fig. 1 *In situ* hybridization demonstrating *clock*, *BMAL1*, *mPer1*, *mPer2* and *mPer3* mRNAs in the suprachiasmatic nucleus (SCN) in LD conditions. Arrows indicate SCN at the peak time at each clock genes. Bar = 1 mm.

localized, by the quantitative *in situ* hybridization. On the film autograph, *clock* mRNA signal was moderate level in the SCN, but the signal was distinct among the preoptic region. Although the expression of *clock* mRNA was higher at ZT/CT12 than at ZT/CT0 in SCN, there was not statistically significant (ANOVA) (Fig. 2A). In both LD and DD conditions, *clock* showed a weak peak at dusk, but their peak / trough ratios of *clock* mRNA were only about 125%.

In contrast to *clock*, *BMAL1* mRNA showed a clear light-dark cycle (Fig. 2B). The trough time of *BMAL1* in LD was ZT4, and thereafter rapidly increased, and reached to the peak at ZT16. In DD conditions, *BMAL1* remained low during CT0 to CT8, and increased to the peak at CT12. In both LD and DD conditions, the peak / trough ratio of *BMAL1* mRNA reached about 250%.

These expression profiles of above genes were

Fig. 2 The expression profiles of *clock* (A) and *BMAL1* (B) mRNAs in the SCN in LD and in DD conditions. For the *clock* and *BMAL1* genes, the relative RNA abundance was determined by quantitative *in situ* hybridization with the peak value adjusted to 100%. Values are expressed as means $\pm$ SEM (n=4).

compared to those of *mPers* (*mPer1*, *mPer2* and *mPer3*). In DD, *mPer1* mRNA showed a peak at CT4, *mPer2* at CT8, and *mPer3* at CT4 - 8 (Fig. 3). In LD, the expression profiles of *mPer1* and *mPer3* mRNAs were virtually the same with those in DD. However, *mPer2* mRNA continued to increase until ZT12, with the oscillatory phase about 4 hours delay compared to DD. The peak / trough ratios of *mPer1*, *mPer2* and *mPer3* in DD conditions were about 960%, 910% and 210%, respectively.

Fig. 3 The expression profiles of *mPer1* (A), *mPer2* (B) and *mPer3* (C) mRNAs in the SCN in LD and in DD conditions. The relative RNA abundance was determined by quantitative *in situ* hybridization with the peak value adjusted to 100%. Values are expressed as means $\pm$ SEM (n=4).

To get the better cellular localization of *mPer1*, *mPer2*, *mPer3*, *BMAL1* and *clock* mRNA in the SCN than the autoradiographic methods, we performed the *in situ* hybridization using the digoxigenin-labeled cRNA probes. As shown in Fig. 4, most of SCN cell bodies show the positive digoxigenin products when their expressions are maximum (CT4 for *mPer1*, CT8 for *mPer2* and *mPer3*, CT12 for *clock* and *BMAL1*).

DISCUSSION

The circadian profiles of *clock* and *BMAL1* mRNA in the SCN showed clear contrast to those of *mPer1*, *mPer2* and *mPer3*. As shown in the present study, *mPer1* and *mPer2*, the mammalian oscillator genes (19), showed a robust circadian oscillation peaked at daytime in the SCN. On the other hand, *clock* mRNA showed flat expression profile; at any time point of sampling we detected the constant level of signals in the SCN. No or very weak rhythm was also reported in rats (1),

but in *Drosophila*, a homologue of mammalian clock gene showed a clear circadian rhythm (2). It is noteworthy that the positive rhythmic regulation is not indispensable in the negative feedback theory of clock oscillation. Another positive oscillatory factor *BMAL1* showed a completely inverted pattern of expression compared to *mPer* genes. This is in harmony with the concept of positive oscillatory loop proposed by Shearman *et al.* (11), but the details should be clarified in future studies.

Above clock proteins, CLOCK, BMAL1, mPER1, mPER2 and mPER3, have some similarities in their motifs. The most conspicuous one is that all of them have PAS domain. PAS domain is a protein sequence motif common to three proteins (PER, ARNT and SIM), which enables the formation of heterodimer and homodimer (6, 10). It is known that CLOCK and BMAL1 are bHLH proteins, and after forming the heterodimer to each other, they bind to E-box of the promoter region of *mPer1* and increases the tran-

Fig. 4 Digoxigenin-*in situ* hybridization of *mPer1* at CT4 (A), *mPer2* at CT8 (B), *mPer3* at CT8 (C), *clock* at CT12 (D), *BMAL1* at CT12 (E) in the mice SCN. Note mammalian clock genes were expressed in most SCN cells at moderate to high levels. oc, optic chiasma; v, third ventricle. Bar = 100 μ m.

scription (4). The heterodimeric and homodimeric complexes made by above PAS proteins may function as the key regulator of the machinery of circadian clock.

The cellular analysis using digoxigenin-probe revealed that the clock genes are expressed in most of the SCN cells, although the expression time point is different in circadian cycle. This supports the concept that the clock oscillation occurs in SCN cells (15). Our previous study revealed that *mPer1* and *mPer2* are co-localized in most of neurons in the SCN (13). Although the circadian profiles were different, these clock genes would make diverse combination of complexes depend on the phase of circadian clock in each SCN cells.

Flat circadian profile of mRNA expression demonstrated by quantitative *in situ* hybridization indicates that clock is a constitutional molecule in the SCN. These data, with other reports characterizing *clock*, *BMAL1*, *mPer1*, *mPer2* and *mPer3* and the model of *Drosophila*, provides the possible picture of mammalian endogenous time-keeping system (4, 9, 16, 17, 18): CLOCK, which heterodimerizes with BMAL1, upregulates the expression of *mPer1* and *mPer2* gene by interacting their promoter with bHLH region, and produced mPER protein in conjunction with cryptochromes and mPER3 suppresses the transcriptional activity of CLOCK-BMAL1 heterodimer.

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