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Phase Shifts of Circadian Transcripts in Rat Suprachiasmatic Nucleus

Ryoko Morioka[1,2] Masanori Arita[1,2,3] Katsuhiko Sakamoto[4]
Soshi Kawaguchi[4] Hajime Tei[4] Katsuhisa Horimoto [4,*]

[1]Computational Biology Research Center, Advanced Industrial Science and Technology, Koto, Tokyo, Japan
[2]Plant Science Center, RIKEN, Yokohama, Kanagawa, Japan
[3]Department of Computational Biology, University of Tokyo, Kashiwa, Chiba, Japan
[4]Mitsubishi Kagaku Institute of Life Science, Machida, Tokyo, Japan

Abstract We analyzed the phase shifts of oscillated transcripts relative to the timing of phase-reset stimuli. Oscillations in gene expression profiles were extracted using the fast Fourier transform and fitted by sine curve with random periods. The phase differences among multiple phase-reset conditions were analyzed to elucidate the mechanism of the core circadian clock. For the expression profiles, cultured cells of rat suprachiasmatic nucleus were measured by Affimetrix GeneChip system. Folskolin stimulus was used as a phase-reset agent, causing irregular shift characteristic to oscillatory transcripts. The results suggest that the fluctuations of gene expressions in the core clock fall into two major categories and can be shifted by folskolin. Other clock related genes might adjust their oscillation by counter-steering each other in the feed-back regulation system.

1 Introduction

The master pacemaker of circadian rhythm in mammalians resides in suprachiasmatic nucleus (SCN), where a transcriptional-translational autoregulatory loop generates molecular oscillations of the "central clock" [1]. The variations of the oscillating expression profiles of clock genes tell the regulatory motion of the transcriptional-translational feedback loop of the clock system caused by external stimuli.

In rat circadian system, the free-running period is 24.5 hours. It is adjusted to 24 hours by entraining agents like light and temperature [2][3][4]. In *vivo*, the average of free-running period of SCN cells is 24 hours and the average period is kept by interaction among SCN cells [5]. In cultivated cells of SCN, on the other hand, the circadian period is 27 hours, and forskolin stimuli can reset the clock of the cultivated cells [2][6][7][8][9]. In this study, we analyzed the periodic fluctuation of SCN cultivated cells under different timing of forskolin stimuli.

*To whom correspondence should be addressed.

2 Related Models and Analyses

Only a few studies are available for the phase shift of gene expression profiles. There is a study that phase shifts of circadian gene expression were modeled as a mixture of two von Mises distributions corresponding to two gene clusters, tissue-dependent phase cluster and tissue-independent synchronized phase cluster [10].

In biology, phase advance and phase delay phenomena were thought to be related with different stress [11]. In this study, three kinds of phase shift experiments were examined induced by the timing of phase-reset stimuli by drug. First experiment is called CT6, and the master clock gene *per1* is considered to keep its phase unchanged as in the control condition. Second experiment is called CT14, and the clock gene *per1* shows phase delay against the control condition. The last experiment is called CT22, and the *per1* shows phase advance in comparison with the control condition. Not all circadian-related genes are synchronized like *per1* in phase, and phase-difference distributions seem to have unidentified complex structure. A part of the mechanism will be elucidated in Data section.

About the modeling and approximation for circadian data analyses, many conventional studies use cosine fitting with minimum square method. For example, Fast Fourier Transform (FFT) was used for Arabidopsis circadian rhythm [12]. Another trigonometrical function analysis was used for sleep analysis [13]. Cluster analysis based on the cosine correlation was applied for mouse circadian clock [14]. Lomb-Scargle periodograms were also applied [15][16][17].

In this study, we focused on the phase-shift phenomena caused by drug stimuli for SCN cultured cells. A few similar study exists [10][18], in which only known clock genes were observed in mice. In contrast, we observed all oscillating genes including known clock genes to explore the characteristics of phase-difference distribution among three phase-shift experiments.

3 Materials and Methods

3.1 Phase detection

Each time series of control and three conditions was first normalized assuming a normal distribution whose mean is zero and the variance is 1 for each experiment. Fast Fourier transform was formulated for each normalized time series. Because the variance of power spectra of each gene determines whether typical oscillations exist or not, we identified only those genes as oscillating whose spectral variance are significantly large. In our case, around 300 oscillating genes, about 1 percent of the whole genes, were extracted, considering the previous reports that the number of oscillatory genes is from several percents to ten percents of expressed genes in each organ [14][19][20][21].

Random period fitting was formulated based on the following formula.

$$y = a\sin(\frac{2\pi x}{p} - \theta) + b$$

y is the expression time series of oscillating genes. x is time. a and b are constant parameters. θ is phase parameter. p is period variable sampled from a normal distribution whose mean is 27 and variance is 1.

The phase-difference distributions were generated by calculating the phase difference between control phase and experimental phase for each gene assuming that the control oscillations keep their periodicity until the same time with experimental conditions. Also the relationship between the random periods and phase difference was explored.

3.2 Data

We used rat cultured cells sampled from SCN, and measured gene expression profiles with Affimetrix microarray (Genechip Rat Genome 230 2.0). The oscillation period was set to about 27 h because our previous report explored and found the circadian period around 27h.

4 Results and Discussion

4.1 Random Period Model

We follow the random period model for the approximation of each gene time-series [18], because genes apparently fluctuate in various scale for each cycle, to adjust to light conditions or other environmental factors. However, our model is much simpler than the model by Liu *et al.* [18] in order to check the phase-difference distribution under drug stimuli. The phase difference was calculated between the control data and three phase-shifted experimental data.

Figure 1 shows the distributions of phase difference, consisting of 300 oscillatory genes between the phase under control and three different conditions: a) In the experimental condition called CT6 phase-reset stimulus was supplied at the time of 18 hours from the time of exchanging culture medium for the cultured cells; b) In the experimental condition called CT14, the stimulus was supplied at the time of 27 hours; and c) In the experimental condition called CT22, the stimulus was added at 36 hours.

4.2 Existence of Two Major Periodic Groups

The experimental condition CT6 has been considered that the phase-reset stimuli does not cause phase shift. However, Figure 1a shows that there are many phase-shifted oscillatory genes. CT14 has been considered as the phase delay condition. Figure 1b shows, however, the result including dual phase differences, around 27 degree and 207 degree. CT22 has been considered as the phase advanced condition. Figure 1c shows the result including dual phase differences, around 99 degree and 279 degree.

These results indicate that the phase shift characteristics depend on each gene and phase-reset timing, even though the conditions are roughly called “phase stable” or “phase-advanced/delay” [3]. The results of CT14 and CT22 imply the existence of dual phase-fluctuation structure which may be achieved the role difference in circadian clock system [1]. Note that the dual deviations are consistent and shifted about 60 degree between CT14 and CT22.

The phase-difference distributions were different from the report of the past study [10], which extrapolated the mixture of only two von Mises distributions for known circadian genes in mouse. Unlike the past study [10], “unchanged phase cluster” was not identified in our study. Moreover, CT22 data exhibited multiple phase differences, although the other two (CT6 and CT14) showed only two major phase differences. There

are two reasons why the distributions were so different from the past study. First is the difference of biological target. The past study examined the phase difference among tissues, but our study examined different timings of phase-reset stimuli. The other reason is the coverage of genes. Since we examined all genes that seem to be oscillating, the number was well over 300.

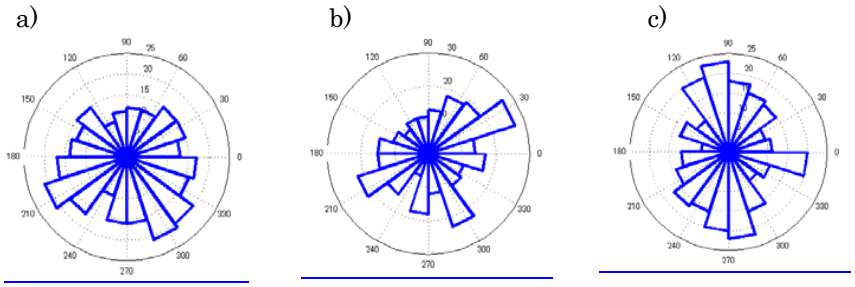

Figure 1: The phase-difference distribution of a) CT6 b) CT14 and c) CT22 among oscillating 300 genes

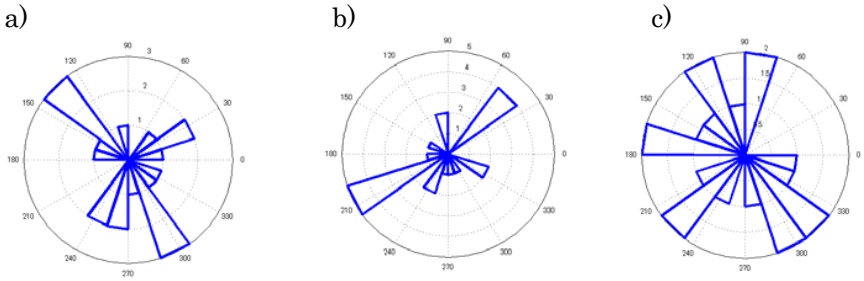

Figure 2: Phase-difference distribution of known clock genes under the condition of a) CT6 b) CT14 and c) CT22

Figure 2 shows the phase-difference distribution among known clock genes [1]. As in Figure 1, the results in Figure 2 show dual phase differences. The clock related genes, *Per1, Per3* and *NPAS2*, were included in the 135 degree, and *CK1*ε/δ are in the 297 degree in Figure 2a. *Clock, Per1* and *Ror*$\beta$and *CK1*$\sigma$are included in the 207 degree, and *Cry2, Nr1d1* and *CK1*$\varepsilon/\delta$are included in the 45 degree in Figure 2b. Both Figure 2a and Figure 2b show dual shifts, and the phase angles of clock related genes are different by 180 degree. A negative-feedback regulation [1] may exist in its background to adjust the cycle at transcription level and enzyme level (*CK1* in this case). Between Figure 2a and 2b, the dual structure is shifted by 90 degree. Figure 3c is quite different from the other two: clock related genes were fluctuated strongly, and various phase shifts were observed . These results indicate that turbulence of phase syntony depends on the timing of stimuli.

In summary, genetic interactions among oscillating genes were kept under the control, CT6 and CT14 conditions. The only change was the phase differences. On the other hand, the condition CT22 affected the synchronization mechanism and generated strong fluctu-

ation of the oscillatory system. We can hypothesize the existence of unknown regulations that cause the difference between CT22 and the other conditions.

4.3 Dispersion of Clock Genes

Two representative clock genes, *per1* and *CK1,* were synchronized in all phase shifts regardless of different phase-reset stimuli (Figure 2a and 2b). We consider that observations only on such genes have led to the false assumption of CT6, CT14, and CT22 as phase-stable, advance, and delay, respectively. There were also genes scattering in phase-difference distribution (Figure 2c). Biological reason for this large variance of CT22 is unknown. One of our assumption for this dispersion phenomenon is the timing of forskolin stimuli CT22 is close to the border between the phase advance and phase delay, and the timing closed to the border might cause the fluctuation of phase shift mechanism [3].

5 Conclusion

We extracted over 300 oscillatory genes, including known clock-related genes, from the expression data of over 30,000 genes in mouse. By fitting their oscillation to sine curve, their distribution of phase differences was obtained. The distribution had a novel complex structure. Two large gene clusters showed a phase difference of 180 degree in all three experiments with stimuli, indicating the hierarchical role in circadian system. Two experiments showed a clear 90 degree shift, which was almost consistent with the time of stimuli (the time difference of stimuli is 8 hours in 27 hour-cycle and the phase shift is 90 degree.) The last experiment (CT22), however, showed scattered phase differences. It suggested the possibility that there is a particular timing of stimuli which causes large fluctuations of phase synchronization in circadian system.

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