Entrainment of a circadian clock *in vitro*

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Abstract: Circadian rhythms are the physiological oscillations with about 24 h periods that have period stability under various circumstances. While gene regulatory feedback loop has been proposed for the model of the origin of the circadian oscillation, my colleagues and I had proposed an alternative model. The cyanobacterial circadian clock can be reconstituted in vitro only by mixing the three clock proteins, KaiA, KaiB, and KaiC, with ATP. Namely, the ratio of phosphorylated KaiC oscillates every 24 h in the mixture. This simple biochemical reaction shows self-sustained oscillation like Belosov-Zhabotinsky reaction. Thus, KaiC phosphorylation rhythm can be core oscillator producing cyanobacterial circadian clock. In this paper, I will discuss entrainment of the "in vitro clock" by temperature cycles.
Keywords: Circadian clock, Cyanobacteria, Entrainment, in vitro clock, KaiC phosphorylation

I. INTRODUCTION

Circadian rhythms are the physiological oscillations with about 24 h periods; it can organize behavior to match the alternating day/night environment. All circadian clocks share three prominent characteristics. First, clocks generate self-sustained oscillations under constant conditions with ~24 hour period. Second, unlike typical biochemical processes, the period of circadian clocks is robust against environmental changes, such as temperature conditions or nutrient in medium. Third, circadian clocks can be entrained to external time cues derived from day/night alternation.

Cyanobacteria, a kind of prokaryote, is one of extensively studied model organism. Before 2005, transcription/translation feedback loop system had been proposed for the model of the origin of cyanobacterial circadian oscillation [1]. The mechanism was also proposed as a clock mechanism of the other model organisms, e.g. mammalian, fishes, flies and plants. However, we recently succeeded in reconstitution of minimal circadian oscillator without transcriptional processes. We incubated the three recombinant Kai proteins (KaiA, KaiB, KaiC) in vitro (in a test tube) with ATP at 30 ºC. Surprisingly, a self-sustained oscillation of KaiC phosphorylation with a 24 hours period was observed [2]. In other words, the ratio of phosphorylated KaiC to total KaiC autonomously oscillated. Furthermore, period of the oscillation was compensated under a variety of ambient temperature. Thus, the first and second characteristics of the in vitro clock had been already shown. In this paper, we will focus on the last property, entrainment of the in vitro clock.

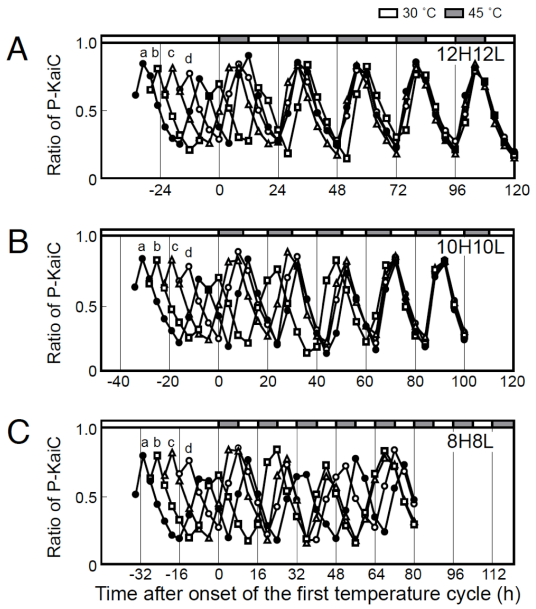

Fig.1 Temperature entrainment of *in vitro* clock

II. TEMPERATURE ENTRAINMENT OF IN VITRO OSCILLATOR

Daily alteration of both light and temperature are the main time cues that can entrain the circadian clocks. Here, we chose temperature cycles as an external cue because any Kai proteins are not concerned as photoreceptors. If a self-sustained rhythm is entrained by an external cycles, the period of the rhythm should adjust to the external cycle and the peak of the rhythm should be locked at a unique phase of the external cycle. To test whether the in vitro KaiC phosphorylation rhythm could be entrained by a temperature cycle, we prepared four mixtures of Kai proteins at 6 hours intervals. Under constant conditions (30 °C), the rhythm persisted with a period of 23.1 ± 0.23 h (n=4),

maintaining the phase angle differences determined by the time of mixing. Next, we exposed the four mixtures to temperature cycles of 12 h at 45 °C and 12 h at 30 °C (12H12L). As depicted in Fig. 1A, the peaks of the four KaiC phosphorylation rhythms were locked at the same phase and the periods of the four mixtures were extended to 24 h (23.9 ± 0.22 h, n=4). As shown in Fig. 1B, the 10H10L regimen also entrained the rhythm, as the period approached 20 h (19.9 ± 0.24 h, n=4) after the 4th cycle. In contrast, under 8H8L temperature cycles, the interval between peaks changed from cycle to cycle (18.4 to 24.4 h) and the peaks did not come together (Fig. 1C). Thus, in vitro clock could be entrained by 12H12L and 10H10L temperature cycles, but not by an 8H8L cycle.

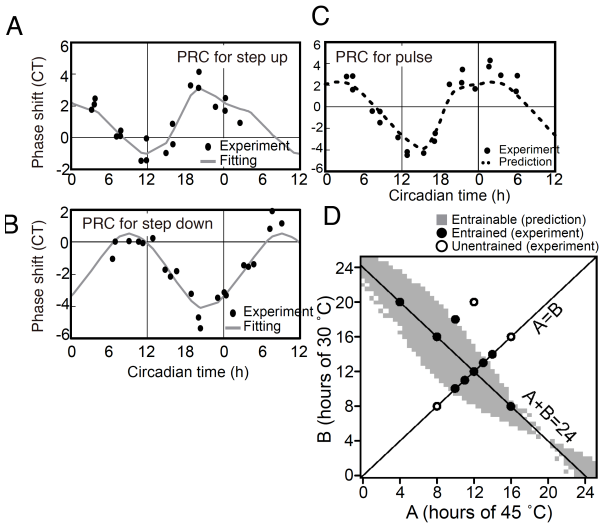

Fig.2 Phase Response curve (PRC) and Range of entrainment

III. PHASE RESPONSE AND RANGE OF ENTRAINMENT

Next we considered how the proteins sense the temperature cycles and adjust to them. We hypothesized that the temperature entrainment of in vitro rhythm might be due to discontinuous jumps in phase caused by temperature steps. To examine this possibility, we analyzed the effects of temperature shifts from 30 to 45 ^{o}C (step-up) and 30 to 45^{o}C (step-down) on the rhythm. As shown in Fig. 2AB, the rhythm was shifted by temperature step stimulus. Step-up and step-down tends to make the rhythm advanced and delayed, respectively.

If the rhythm can sense the step temperature stimulation, phase shifting by temperature pulse is assumed to be the sum of phase shifts caused by step-up and step-down. If a phase shift by temperature step-up is completed before step-down, the phase shift Δ(ϕ) of the high-temperature pulse at phase ϕ is calculated by the following equation:

$$\Delta(\phi) = f(\phi) + g(\phi + f(\phi) + 24A/\tau) \qquad (1)$$

where f(ϕ) and g(ϕ) respectively represent phase shift by step-up and step-down. We examined actual phase shifting by administering 4 h pulses at various times. Fig. 2C depicts phase shifts caused by 21 different temperature pulses ranging across the circadian cycle. On the other hand, we calculated phase shifting by 4 h high temperature pulses using equation (1) and overlaid the results with the observed phase shifts. As shown in Fig. 2C, the predicted PRC correlated well with the experimental results, suggesting that our hypothesis works in this biochemical oscillator.

Furthermore, we predicted a range of entrainment by cycles of various fractions of high and low temperature. When stable entrainment is attained under a cycle of A h at high temperature and B h at low temperature, equation (2) should be satisfied with a specific phase of ϕ_s (phase of onset of high temperature in entrained status), because under entrained conditions, the difference in free-running period of the rhythm and external cycle length should be compensated by two phase shifts accompanied by a temperature cycle.

$$\tau - (A+B) = \left(f(\phi_s) + g(\phi_s + f(\phi_s) + 24A/\tau)\right) \cdot (\tau/24) \qquad (2)$$

As shown in Fig. 2D, most of the temperature cycles that entrained the rhythm (closed circle) fell within the predicted range of entrainment. On the other hand, protocols that failed to entrain the rhythm (open circle) fell outside the range.

Temperature entrainment of circadian rhythms has been studied in many organisms as far. An extensive study of the eclosion rhythm of *Drosophila* demonstrated that temperature entrainment could be similarly ascribed to phase shifting by discontinuous temperature changes. Entrainment induced by discontinuous phase jump may be a common principle for temperature entrainment, because temperature can shift the phase of the rhythm but fails to alter period length due to temperature compensation of period.

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