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Molecular Oscillation Behind the Clockface

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The earth rotates on its own axis while orbiting around the sun. This regular movement of the solar system results in cyclic changes of the light condition of the earth with a period of 24 h, although the lengths of daytime and nighttime depend on the latitude. The organisms living on the earth have evolved an internal time-measuring system called the "circadian clock," which ticks with a period of approximately 24 h in order to adapt to the environment and to anticipate the next cycle. The fact that most of existing organisms retain the circadian clock suggests that the clock-ownership must have been advantageous over non-ownership during their evolution. Here I will introduce the background of the research field of circadian rhythm and present an outline of this Special Review series, which is composed of three articles that review recent research into the molecular mechanisms of the three types of circadian clock systems in vertebrates.

Key words: circadian clock, negative feedback loop, post-translational regulation, transcriptional and translational regulation.

Characteristics of circadian clock

One of the fundamental properties of the circadian clock is that it oscillates autonomously within a cell, even in the absence of external time cues (*1*, *2*). Under constant environmental conditions, the circadian rhythms "free-run" with period lengths of 24 h plus or minus a few hours; and this deviation of the free-running period from 24 h is the origin of the term *circadian* (Latin, *circa* = about; *dies* = a day).

A second feature of the circadian clock is that, despite its self-sustaining oscillation, it adjusts its phase to the local time by responding to changes in environmental conditions, thereby compensating for slight deviations of the period from 24 h. The adjustment of the phase, termed "entrainment," is most evident in the response to light. Most living organisms appears to have evolved a light-entrainable clock, probably because the light-dark cycles have been the most reliable time cue (Zeitgeber) on the earth. It is conceivable that the occurrence of the circadian clock in an ancient unicellular organism was associated with the acquisition of intrinsic photosensitivity owing to a photosensitive molecule. Although some of existing clock cells are not photosensitive by themselves, they are considered to have lost their photosensitivity during their evolution.

The third feature of the circadian clock is that its period is not greatly affected by changes in the ambient temperature. This property, called "temperature compensation," is very important for organisms living at higher latitudes. Given that the molecular oscillation is based on biochemical reactions, we have to postulate a mechanism that actively cancels or reduces the effect of temperature on the reactions determining the period length. Theoretical models have been proposed, but substantially no experimental data are available that fully account for the temperature compensation.

Molecular clock

Currently the circadian clock system represents one of the few brain functions that we can understand in terms of the connection between genes and behavior. The clock genes are responsible for the normal oscillation of the circadian clock, and the investigation of the molecular mechanism of the animal clock oscillation started with the isolation of a *per* mutant of the fruit fly *Drosophila melanogaster* exhibiting abnormalities in the rhythms of both eclosion and activity. Parallel studies on the clock mechanisms of *Drosophila* and rodents have contributed significantly to an explosive increase in our knowledge. Extensive studies on various organisms including mouse, *Drosophila*, plants, fungi and cyanobacteria (reviewed in Ref. *3*) have shown that the basic frameworks of the oscillation are very similar to each other, but the molecular structures are conserved only marginally. This Special Review will introduce the clock mechanism by focusing on vertebrate clock systems.

The current model of the clock oscillation mechanism in vertebrates is composed of negative and positive element feedback loops that interact with each other and with the other regulatory loops (Fig. 1). First, a transcriptional negative feedback loop was proposed, in which *Per* gene products (mPER1, mPER2, mPER3 in the mouse) were postulated to play central roles as negative elements. Later, *Cry* gene products (mCRY1 and mCRY2 in the mouse) were found to be the stronger negative elements, and more recently *Dec* genes have been nominated as potential negative element genes. All these genes are subject to transcriptional activation by basic helix-loop-helix (bHLH)-PAS (Period, Arnt and Single-minded) domain-containing transcriptional factors,

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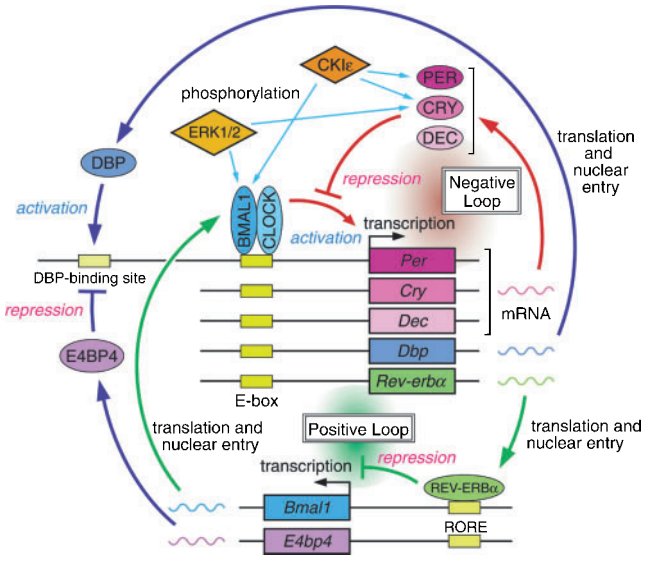

Fig. 1. **A model of the molecular oscillation in the circadian clock.** The feedback loops of the negative elements (PERs and CRYs) and positive element (BMAL1) are connected with each other to constitute the circadian clock oscillation, and these loops are regulated by the other components. The core molecular mechanism of the clock oscillation in the central tissue of the rodents appears to be basically conserved not only in the peripheral tissues but also in the central and peripheral tissues of other vertebrate species (chicken, zebrafish, *Xenopus, etc.*). However, the numbers of orthologous genes differ among species, and the regulation of the clock genes (*e.g.*, light responsiveness of the gene expression) may also differ. [Modified from Hirota and Fukada, to be published elsewhere.]

CLOCK and BMAL1, which associate with each other to bind to a CACGTG E-box enhancer located in the promoter/enhancer regions of the negative element genes. The translated negative elements in turn inhibit expression of their own genes by repressing the transactivation exerted by the CLOCK-BMAL1 complex, and hence these interacting elements form a negative element feedback loop (Fig. 1, red lines). The negative elements work cooperatively by forming a multimeric large complex when acting on the CLOCK-BMAL1 complex (*1–4*).

On the other hand, a central part of the positive element feedback loop (Fig. 1, green lines) is the rhythmic transcription of the positive element gene, *Bmal1*, whose mRNA level is almost antiphasic to those of the negative elements. This reversal is attributable to the E-box-dependent rhythmic expression of the orphan nuclear receptor gene, *Rev-erbα*, because the translated product REV-ERBα represses *Bmal1* transcription by binding to REV-ERB/ROR response element (RORE) in the *Bmal1* promoter. Thus, the negative element PERs and CRYs repress the transactivation of their own genes on one hand (Fig. 1, Negative Loop), and on the other hand they up-regulate transcription of the positive element gene through the down-regulation of its repressor (Fig. 1, Positive Loop). The DNA elements such as E-box and RORE are distributed in the promoters of various genes that are regulated by clock signals. These "clock-regulated genes" play a major role of transmitting the clock signals to cellular responses as outputs.

The core molecular oscillation driven by mutually interacting negative/positive feedback loops appears to become more stable and to cycle closer to 24 h by virtue of additional loops that connect protein products of circadian oscillatory genes (clock-controlled genes) with regulatory DNA elements within the clock genes. For example, expression of the *Dbp* gene (encoding a transcription factor that binds to the D site of the albumin gene) is circadian-regulated through the E-box, and the gene was assumed to be an output gene. Later, the gene product DBP was found to regulate the *Per* gene by binding to the D site (DBP-binding site, Fig. 1) located upstream of the *mPer1* gene and to enhance its expression by producing an additive effect to the E-box-mediated transactivation. In parallel, the DBP-binding site is regulated negatively by a bZIP transcription factor, E4BP4. Expression of the *E4bp4* gene is almost antiphasic (probably due to the regulation of RORE) to that of *Dbp* gene, allowing stabilized oscillation through these agonistic and antagonistic regulations of the DBP-binding site of the *mPer1* gene (Fig. 1, dark blue lines in the outer part; Ref. *4*). Transcription of the *E4bp4* gene is up-regulated by light in the chick pineal clock cells, and E4BP4 plays a pivotal role in the light-entrainment, especially for the phase-delay upon light-period prolongation, as reviewed in Ref. *5*.

It may appear strange to see in Fig. 1 that the gene expressions of the positive regulator DBP and the negative elements PERs and CRYs are controlled by the same DNA element, the E-box. Despite this apparent anomaly, the peak times of the effective protein levels of these elements within cellular nuclei are widely separated from each other by translational and post-translational mechanisms that delay the nuclear accumulation of a certain subset of proteins, primarily of the negative elements.

In particular, the post-translational controls of proteins are of critical importance for the circadian time-keeping mechanism that generates a stable molecular oscillation with a long period of 24 h. These include regulatory processes of clock gene products such as protein phosphorylation, nuclear entry/export, redox, and degradation, molecular events that are closely interrelated. Some protein kinases such as casein kinase Iε (CKIε) and mitogen-activated protein kinases ERK1/2 have been shown to phosphorylate the clock gene products (Fig. 1) for regulation of their function and degradation. Other potein kinases such as GSK3β, p38 kinase and CaMKII are also important for circadian time-keeping and for light-entrainment. These issues are emphasized in every article of this review series (*5–7*). It should be pointed out, however, that protein-level studies on central clock neurons in mammals are currently difficult to perform due to the limited availability of cells, so that peripheral tissues such as the liver and cultured cells or central clock cells from a larger organ, such as the chick pineal gland, are used for analysis.

Vertebrate clocks in central, peripheral, and photosensitive tissues

The molecular mechanisms of the vertebrate clock systems in central, peripheral, and photosensitive tissues are the major topics of the three articles contributed by Isojima *et al.* (*6*), Tsuchiya and Nishida (*7*), and Okano and Fukada (*5*), respectively, in this Special Review

series. The concepts of central and peripheral clocks were established in recent years (*8*). Previously, it was believed that the clocks were localized in certain neuronal cells, for example, in mammals, the suprachasmatic nucleus (SCN) in the hypothalamus, and that the peripheral tissues and cells were amenable to the control of the central clock located in the brain. Now that almost all cells have been shown to have clock functions, an important issue is the hierarchical regulation of the peripheral clocks within the whole body by the central clock. These issues are reviewed by Isojima *et al.* (*6*) in this Special Review series.

A major difference between the central and peripheral clocks could be the sensitivity to the ambient light conditions (with a few exceptions of the light-entrainable clocks in the peripheral organs of the zebrafish). The central clock is light-entrainable even if it is itself insensitive to light; for example, the clock in the mammalian SCN is regulated by light captured in the retinas through neural connections of the retinohypothalamic tract (*9*). On the other hand, most peripheral clocks are not directly affected by light. Instead, they are entrainable to various humoral and neural signals arising from the central clock or generated by intentional or forced behaviors such as food intake. Therefore, the molecular mechanism for the phase-shift of the peripheral clocks in response to various external signals is of interest in a wide range of research fields of intracellular signal transduction (*8*). A new approach to these studies was opened by the finding that rat-1 fibroblasts in culture exhibit the circadian rhythm in expression of the clock genes after pulse-treatment with a high concentration of serum. Since then, several cell lines have been used as cellular models for the studies on the peripheral clocks, and recent progress made with this approach is reviewed by Tsuchiya and Nishida (*7*) in this review series.

Central clocks are generally light-entrainable, and the photoreceptive molecule that regulates the phase of the clock is called a "circadian photoreceptor." In mammals, the eye is required for the light-entrainment of the central clock in the SCN. The retinal opsins, including rhodopsin, cone opsins and the newly found melanopsin, appear to serve as circadian photoreceptors through a redundant mechanism (*9*). In vertebrates, the three organs related to the circadian rhythm function, the retina, the SCN and the pineal gland, are all of diencephalic origin, and it is conceivable that they are derived from an ancient organ that was a photosensitive clock structure with a secretory function. During the course of evolution, this putative diencephalic organ is thought to have duplicated, and the resultant organs to have taken charge of one of the three functions: light perception (in the retina), the clock function (in the SCN) and melatonin production (in the pineal gland) as are established in mammals. This idea is supported by the fact that these organs in non-mammalian species still retain two or three of these functions. A typical example is the chicken pineal gland, which produces melatonin in circadian and light-sensitive manners. The circadian clock regulating rhythmic production of melatonin is also light-entrainable due to the intrinsic photosensitivity, and therefore the pineal cell appears to represent a "prototype" of the cellular clocks in vertebrates. Okano and Fukada (*5*) summarize the molecular mechanisms of the clock oscillation and intracellular phase-shifting pathway downstream of the intrinsic photosensitive molecule(s), pineal opsin(s).

Conclusion

This Special Review series aims to summarize recent progress in research into the molecular mechanisms underlying the circadian rhythms of vertebrates. We usually see the hands of the clock, but we are beginning to discern the intricate molecular cyclings behind the clockface and to watch the gears moving downstream of the knob that adjusts the molecular clock.

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