JB Special Review—Circadian Clock Systems in Vertebrates *J. Biochem.* **134**, 791–797 (2003)
DOI: 10.1093/jb/mvg221

Chicktacking Pineal Clock

Toshiyuki Okano[1,2] and Yoshitaka Fukada[*,1]

[1]Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033; and [2]PRESTO, Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama

Received November 12, 2002; accepted October 21, 2003

Many tissues in non-mammalian vertebrates contain both photoreceptors and circadian clock systems. Among these photosensitive clock structures, the chick pineal gland has been characterized in detail from cellular and molecular aspects of the clock oscillation and entrainment. Analyses of the pineal photic-input pathway revealed a phase-shifting mechanism mediated by activation of G11, one of the Gq-type G-proteins. A major photoreceptive molecule, pinopsin, likely triggers this pathway by transmitting the light signal to the circadian oscillator. In the chick pineal oscillator, the transcription/translation-based autoregulatory feedback loop is composed of positive and negative elements (clock gene products) that are homologous to those identified in mammals. In the molecular cycling, a CACGTG E-box located in the promoter region of the negative element genes plays a central role in the transcriptional regulation. The phase of the molecular cycling is modulated by many regulatory components, among which E4BP4 and extracellular signal-regulated kinase (ERK) are closely associated with the photic entrainment. A light-responsive element was found in the promoter region of the *Pinopsin* gene, and the element included a CACGTG E-box, suggesting a novel role of the E-box as a point of convergence of light and circadian signals. These observations together point to general and unique features of the chick pineal circadian system among animal clocks.

Key words: circadian clock, E4BP4, mitogen-activated protein kinase, pineal gland, pinopsin.

The circadian clock regulates a variety of activities in animal physiology and behavior, such as the sleep-wake cycle, body temperature, production of hormones, and locomotor activities. Circadian clock systems generally contain three components: (i) input pathway(s) for resetting the oscillator, (ii) a self-sustained oscillator, and (iii) output pathway(s) for transmission of time signals to downstream effectors (reviewed in Ref. *1*). Light plays a major role as a time cue that synchronizes the oscillator with the ambient 24-h light/dark cycle, and therefore the light-input pathway to the oscillator has been studied to understand the mechanism of the circadian-clock resetting.

In mammals, the "central oscillator" regulating the locomotor activity resides in the suprachiasmatic nucleus (SCN) in the hypothalamus (2; reviewed in Ref. *3*). The phase of the central oscillator is reset by the light signal transmitted from the retina through the retinohypothalamic tract, as the SCN is not itself photosensitive. A photoreceptive molecule responsible for the resetting is called a "circadian photoreceptor," and the retinal opsins including rhodopsin, cone opsins and melanopsin appear to operate together as the circadian photoreceptors activating the resetting pathway in a redundant manner (*4*, *5*). In addition to the SCN, a variety of peripheral tissues contain the circadian oscillator (*6*). When isolated, mammalian peripheral tissues display self-sustained oscillation of *Per1* expression (*7*), but these oscillators, termed "peripheral clocks," are not reset by light due to the light-insensitive nature of these tissues. Instead, they are reset by humoral factors such as glucocorticoid (*8*), retinoid (*9*), and glucose (*10*) that are probably regulated by the central clock in the SCN or by feeding-associated events (*11–13*).

In non-mammalian vertebrates, the distinction between the "central clock" and the "peripheral clock" is less clear than in mammals, in which most tissues are blind. In the zebrafish, peripheral tissues such as heart and kidney retain circadian clocks that are light-entrainable in isolated culture, indicating the presence of an intrinsic light-input pathway to the oscillator (*14*). A typical photosensitive clock structure present outside the hypothalamus is the pineal gland (*15–19*), which plays a major role in the circadian production of melatonin, a well-known mediator of the circadian physiology in animals. This review summarizes recent studies on this small photosensitive clock structure in the chick. The finding of the chick pineal photosensitivity by Deguchi (*17*, *19*) was a landmark in this research field.

Pinopsin as a circadian photoreceptor in chick pineal gland

The pineal gland of non-mammalian vertebrates is tightly linked with a light-input pathway(s) inherent in the gland (reviewed in Refs. *20–22*). This forms a clear contrast to the mammalian pineal gland, which is a light-

[*]To whom correspondence should be addressed: Tel/Fax: +81-3-5802-8871, E-mail: sfukada@mail.ecc.u-tokyo.ac.jp

insensitive neuroendocrinal organ synthesizing melatonin in a circadian rhythmic manner under the control of the SCN. More than 20 years ago, Deguchi demonstrated that the chick pineal gland is light-sensitive and has the ability to produce melatonin in a circadian rhythmic and light-dependent manner in culture (*16*, *17*, *19*). Since then, the chick pineal gland has been widely used as one of the best experimental models for the study of the animal circadian clock (*20–22*). The photosensitivity and the clock function of chick pineal cells are well-maintained in dissociated cell culture, and this great advantage allows several experiments to be performed that are difficult in other systems.

Light has two distinct effects on the chick pineal melatonin rhythm. One is an acute inhibitory effect on melatonin synthesis (acute effect), and the other is a phase-shifting effect on the circadian oscillator. In 1981, Deguchi reported an action spectrum for the acute effect of light and suggested the involvement of a rhodopsin-like molecule (*19*). Consistent with this prediction, an opsin-like molecule was identified in the chick pineal gland in 1994 and named pinopsin (*23*). Pinopsin showed nearly equal similarities in amino acid sequence to chicken retinal rod and cone visual pigments (*24*, *25*), indicating that the *pinopsin* gene is a distant relative of the retinal opsin genes. Based on knowledge of the retinal phototransduction mechanism, pinopsin was considered to couple with a heterotrimeric G-protein to trigger the downstream biochemical cascade (*21–23*).

Immunocytochemical analysis of chick pineal sections and molecular screening of a pineal cDNA library revealed the expression of several kinds of heterotrimeric G-protein α-subunits, among which $Gt_1\alpha$ (rod transducin α-subunit) and G11α (α-subunit of Gq-subtype G-protein) were colocalized with pinopsin in the outer segment-like membrane structures of the chick pinealocytes (*26–30*). To determine whether these G-proteins are involved in the photic-input pathway, m1 or m2 subtype of muscarinic acetylcholine receptor (mAChR) was transiently expressed in cultured pineal cells, to which the AChR agonist carbachol was administered to activate endogenous G11 (*via* m1 receptor stimulation) or Gt_1 (*via* m2 receptor stimulation). The carbachol treatment of m1 receptor-expressing cells induced a phase-dependent phase-shift of the melatonin rhythm, and the effect was indistinguishable from the effect of light (*30*). On the other hand, the carbachol treatment of m2 receptor-expressing cells had no effect on the rhythm. This study was unique in that the phase-shifting light-signaling pathway was explored in the dark by administering "non-photic" pharmacological stimuli to the clock cells expressing a foreign receptor mimicking the endogeneous photoreceptor(s). This strategy allows the G11 cascade to be triggered without activating other possible photic-input pathways that might operate in a redundant manner, and the results demonstrate the contribution of G11-mediated signaling pathway to the photic-input mechanism. This is consistent with the pioneering study showing that pertussis toxin blocks the acute effect of light but not the phase-shifting effect (*31*).

It is likely that pinopsin couples with two G-proteins, G11 and Gt1, in the gland, and that the pinopsin-G11 cascade inputs to the oscillator. Besides pinopsin, the putative photoreceptive molecule melanopsin in the chick pineal gland (Provencio *et al.*, Genbank accession no. AY036061) may couple with G11 to activate the photic-input pathway, although further biochemical and immunohistochemical characterization is necessary in order to define the contribution of this less-characterized opsin and its signaling pathway to the photic-entrainment.

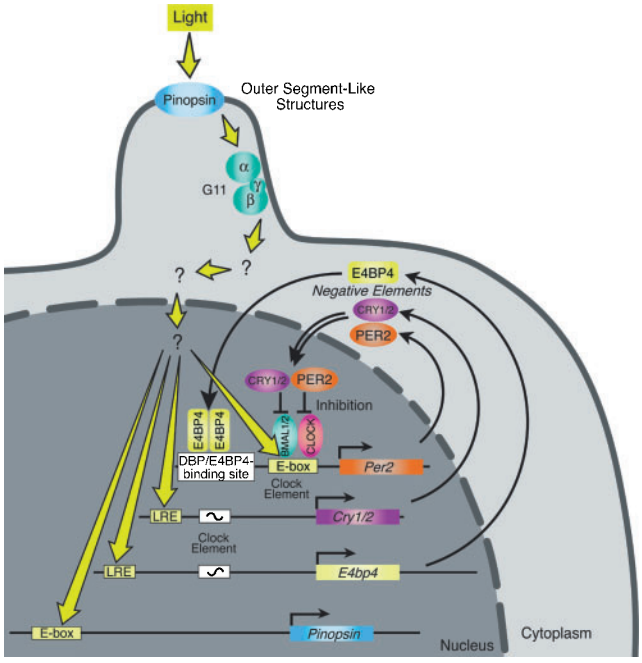

Fig. 1. **A model for the role of clock-related molecules in circadian oscillation and light entrainment of the chick pineal clock.** LRE, a putative light-responsive element. This model was drawn with reference to mammalian models for the transcription/translation-based autoregulatory negative feedback loop (*70*).

Core clock elements in the chick pineal clock

The clock gene period was first identified in *Drosophila*, and later in mouse (*mPer1-3*). Since the identification of *mPers* during 1997–1998, extensive studies have identified CLOCK, BMAL1, and CRY1/2 in several vertebrate species, and these proteins have been shown to constitute the core feedback loop of the circadian oscillator (reviewed in Refs. *22* and *32*). Recently BMAL2 (MOP9 or CLIF) was identified in human and in some other vertebrates (*33–37*), but it is not yet known whether the *Bmal2* gene is essential for the clock oscillation. In chicken, a series of orthologues of the mammalian clock genes, chicken *Per2* (*cPer2*) (*37*), *cPer3* (*38*), *cBmal1* (*37*, *39*), *cBmal2* (*37*), *cCry1* (*38*), *cCry2* (*38*), and cClock (*40*), have been identified to date (Fig. 1). Birds appear to lack a Per1 gene.

Recent dramatic advances in the field of the circadian biology have facilitated the construction of a model for the molecular oscillation based on the transcription/translation-based autoregulatory negative feedback loop (Fig. 1). In the chick pineal clock system, a CACGTG E-box element exists in the *cPer2* promoter region, and cCLOCK-cBMAL1/2 multimer shows significant transactivation ability through this element. Interestingly, cBMAL2 shows an inhibitory effect depending on its

dose, implying its unique modulatory role in the E-box-mediated transactivation (*37*). In the chick pineal gland, the mRNA level of each clock gene oscillates in a circadian manner with a peak at its unique phase (*37*), and the transcript levels of *cPer2*, *cCry1*, and *cCry2* are up-regulated by light captured by the endogenous photoreceptor in the pineal cells (Fig. 1) (*38*). The molecular oscillatory mechanism of the chick pineal clock seems very similar to that of the mammalian clock in the SCN (Figs. 1 and 2), but the chick pineal clock may be more susceptible to light than the mammalian SCN clock in terms of light-responsiveness of the clock (related) genes.

E4BP4 as a key molecule for phase-delaying process

Light-responsiveness of the clock (related) genes is important in regulating the phase of the molecular clock (*1*, *3*, *22*). To identify genes whose expression is regulated by light in a phase-dependent manner, more than 5,000 transcripts expressed in the chick pineal gland were screened by differential display analysis (*41*). This screening revealed only a few transcripts that are subject to regulation by both the circadian clock and light, implying the physiological importance of the transcripts. One of these transcripts encoded cE4BP4, a member of PAR family transcription factors, having a basic leucine zipper motif for its DNA-binding (*41*). Interestingly, a cE4BP4-binding site was found in *cPer2* promoter (Figs. 1 and 2), and cE4BP4 repressed the transcription of a reporter gene from *cPer2* promoter through the binding site (*41*). The temporal expression pattern of *cE4bp4* had its peak at ZT10 (ZT, Zeitgeber time) and CT12 (CT, circadian time) under LD cycles and DD condition, respectively, and this is almost antiphasic to that of *cPer2* peaking at ZT2 and CT4 (*41*). Because the change in E4BP4 protein level rapidly follows that in mRNA level (*42*), it is possible that cE4BP4 may be involved in the down-regulation of *cPer2* mRNA level occurring from midday to evening in the chick pineal gland (Fig. 2).

More importantly, the light-dependent up-regulation of *cE4bp4* mRNA level suggests that cE4BP4 plays a critical role in the phase-shifting mechanism of the pineal clock. The prolongation of the light period to the early night shifts backward the clock phase by delaying the rising phase of *cPer2* in the next morning, and this phase-shift is not accompanied by light-dependent up-regulation of *cPer2* expression (*41*). Such behavior of *cPer2* expression is well explained by the light-dependent induction of cE4BP4, which would suppress the *cPer2* expression from evening to midnight (Fig. 2). It is most probable that cE4BP4 is a key mediator of the evening light signal that causes the phase-delay (Fig. 1) (*41*).

The binding site of E4BP4 is shared with another member of the PAR family, albumin D-site binding protein (DBP) (*43*, *44*). DBP and E4BP4 act as a positive and a negative regulator, respectively, both acting on the same DNA-binding site (*44*). mDBP (*mDbp*) shows robust circadian oscillation in the mRNA and protein levels in the mouse SCN (*45*, *46*), and activates transcription of *mPer1* gene through a DBP/E4BP4-binding site in *mPer1* promoter (*46*). In addition, the temporal expression pattern of *mDbp* in the SCN is a few hours earlier than that

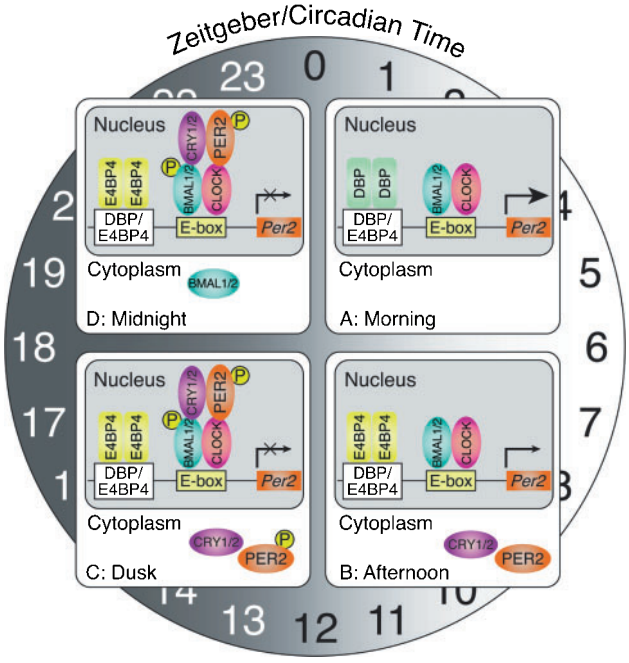

Fig. 2. **Ticktacking of the circadian oscillator in the chick pineal gland.** (A) Early morning to midday: Transcription of *cPer2* genes is activated through the CACGTG E-box element by an activator complex composed of cCLOCK and cBMAL1/2. cDBP has not been identified in the chicken, yet it may bind DBP/E4BP4-binding site to enhance the *cPer2* transcription. (B) Midday to evening (Afternoon): Stimulation of *cPer2* transcription leads to cytosolic accumulation of cPER2 protein, which then associates with cCRY1/2 (chicken Cryptochrome 1/2). Transcription of *cE4bp4* leads to accumulation of cE4BP4, which then inhibits the *cPer2* transcription by binding to DBP/E4BP4-binding site. (C) Evening to midnight (Dusk): The cPER2-cCRY1/2 complex enters the nucleus to inhibit the cCLOCK-cBMAL1/2 complex, resulting in suppression of *cPer2* transcription. (D) Midnight: The cPER2-cCRY1/2 inhibitor complex is gradually down-regulated through post-translational steps such as phosphorylation and destruction, with cytoplasmic cPER and cCRY1/2 exhausted. BMAL1/2 proteins accumulate before restarting the *cPer2* transcription (A). PER and BMAL1 may be phosphorylated by casein kinases (CKIε and CKIδ) and MAPK, respectively (*50–53*, *62*).

of mPer1 and is nearly antiphasic to that of *mE4bp4*, suggesting agonistic and antagonistic roles of *mDbp* and *mE4bp4* in the transcriptional regulation of *mPer1* (*44*). Therefore, the circadian oscillation of the balance between DBP and E4BP4 activities may contribute to the stabilization of the core negative feedback loop. It would be interesting to investigate a similar role of *cDbp* in the chick pineal clockwork and to ask whether a light-signal might also affect cDBP expression.

Role of protein kinases in the pineal clock

Not only the transcriptional control but also posttranslational regulations such as protein phosphorylation, nuclear entry and degradation contribute to maintenance of stable 24-h oscillation by driving or delaying the molecular cycling. Recent studies on the animal clock systems have demonstrated that casein kinase I (CKIε/CKIδ), *Shaggy* kinase (GSK3β) and two members of the mitogen-activated protein (MAP) kinase family (ERK and p38) are involved in the circadian clockwork. The

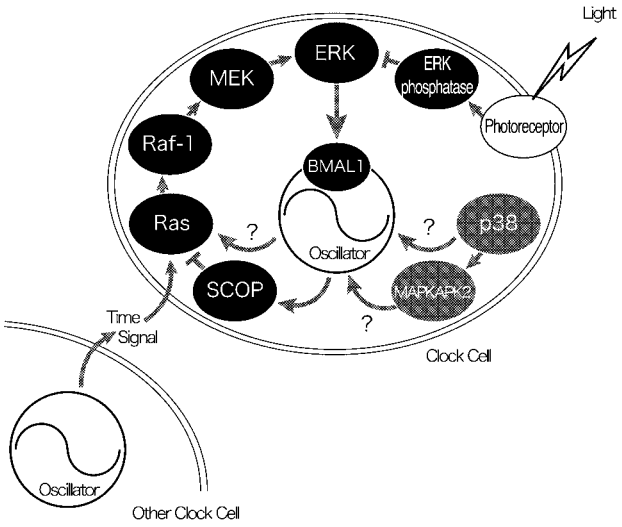

Fig. 3. **A model for the roles of MAP kinases, ERK and p38, in circadian oscillation and light entrainment of the chick pineal clock system.** SCN circadian oscillatory protein (SCOP) found in the rat SCN is a negative regulator of K-Ras (*61*) and tentatively included in this model, although its expression has not been examined in the chick pineal cells.

clock-associated function of CKIε was first identified in *Drosophila doubletime* (*dbt*) mutants having mutations in the dbt gene, which encodes a protein kinase highly related to mammalian CKIε (*47*, *48*). The period length of the circadian rhythmicity of these mutants is lengthened (in dbt^L mutant) or shortened (in dbt^S mutant) (*47*, *48*), and later *Cklε* was shown to be responsible for *tau* mutant phenotype in hamster (*49*). CKIε (or DBT) and CKIδ directly phosphorylate PERs and consequently alter the stability or subcellular localization of PER proteins (*50–52*). Although the functions of CKIε and CKIδ have not been examined in non-mammalian clock structures, potential sites for CKIε phosphorylation (Ser residues in Ser-X-X-Ser-X-X-Ser-X-X-Ser-X-X-Ser) identified in hPER2 (*53*) are conserved in the corresponding region of cPER2.

The protein level of CKIε is constant over the day in the mouse liver (*54*), whereas the regulation of CKI activity is not fully understood in clock structures. On the other hand, it was clearly shown that phosphorylation and activity of ERK is circadian-regulated in a number of clock structures such as the mouse SCN (*55*, *56*), chick pineal gland (*57*), bullfrog retina (*58*), and chick retina (*59*). In the bullfrog retina and chick pineal gland, ERK activity is low during the (subjective) day and peaks at midnight. ERK oscillation is likely regulated *via* a classical Ras and ERK cascade (*60*), and a novel K-Ras modulator SCOP (SCN circadian oscillatory protein) might be one of the upstream regulators of circadian activation of Ras (*61*; Fig. 3). It should be noted that the phase of the pineal clock is affected by inhibiting MEK (ERK kinase) activity during the subjective night, indicating that the ERK activation signal inputs to the oscillator. Therefore, the ERK cascade is likely to form a secondary loop that may contribute to stabilization of the core feedback loop (*57*). Just like ERK in the mammalian SCN, the chick pineal ERK activity is regulated not only by the circadian clock but also by light. A light stimulus given during the subjective night leads to rapid dephosphorylation (and inactivation) of ERK in the pineal gland without affecting the activity of upstream MEK (*57*, *60*). This indicates that the pineal phototransduction pathway activates a phosphatase that dephosphorylates ERK. Thus, in the chick pineal clock, ERK seems to represent a converging point of two signals, time and light (Fig. 3).

Identification of *in vivo* substrates of ERK is an important outstanding issue for understanding the physiological role of the circadian-regulated ERK. Among the chicken clock-related gene products, BMAL1 directly associated with ERK, and was efficiently phosphorylated by activated ERK *in vitro* (*62*). In the transcriptional assay in 293EBNA cells, activation of ERK by expression of constitutive active MEK resulted in partial suppression of the transactivation from the CACGTG E-box element by CLOCK and BMAL1 (*62*). This suppressive effect was abrogated when one of the ERK phophorylation sites Thr 534 in BMAL1 was mutated to alanine (*62*). ERK has the ability to negatively regulate the transactivation from the E-box element through the phosphorylation of BMAL1 at Thr 534.

Considering the physiological role of the ERK cascade in the clockwork, an interesting question is whether the circadian regulation is an intracellular event or involves intercellular signal transmission. In the former case, ERK may contribute to a circadian time-keeping mechanism by generating an appropriate time delay in the E-box-dependent transcriptional activation. In the latter case, the ERK cascade would be activated by a signal generated by neighboring pineal clock cells, and hence it may act as a synchronizer among the individual clock cells (Fig. 3).

p38, another member of the MAP kinase family, is also present in the chick pineal gland (*63*). Both the p38 protein level and its phosphorylation (activation) were almost constant over the day in the chick pineal gland, but interestingly, chronic application of p38 inhibitor delayed the phase of the pineal melatonin rhythm in a time-of-day specific manner (*63*). These observations suggest that p38 plays a time-of-day-specific role in the pineal clock oscillation. Although the downstream cascade of p38 in the pineal gland is not clear, MAP kinase-activated protein kinase 2 (MAPKAPK2) has been suggested as a downstream target of p38 (*63*; Fig. 3).

Light-responsive element indispensable for the light-dependent gene expression

Transcription of *mPer1* is induced by light in the mouse SCN *in vivo*, and this seems to be associated with the light-dependent phase-shift of the locomotor activity rhythm (*64*). As described, transcripts of *cPer2*, *cCry1/2*, and *cE4bp4* are up-regulated by light in the chick pineal gland in culture (*38*, *41*). These observations suggest an idea that the light-dependent transcription of the clock (related) gene(s) is essential for the phase-shift. The light-dependent gene expression regulated by light-responsive elements (LREs) has been been extensively studied in plants (reviewed in Ref. *65*). In contrast, LREs and LRE-containing promoters have been less well characterized in animal cells (*66*), probably due to the lack of an appropriate assay system to evaluate enhancer activi-

ties under the light and dark conditions. The transcriptional analysis established in the primary culture of the chick pineal cells has made it possible to measure the light-dependent enhancer activity. This was employed to search the promoter region of the *pinopsin* gene for an LRE, because the mRNA level of *pinopsin* is positively regulated by light in the chick pineal gland *in vivo* and in tissue culture (*67*, *68*). Pinopsin LRE was found in an 18-bp region (TGGCACGTGGGGTTCCTC) that included a CACGTG E-box element (*68*). Mutation of the E-box sequence abolished the light-responsiveness, indicating the functional importance of the E-box sequence (*68*). Interestingly, the CACGTGG sequence, termed the G-box, is known to operate as an LRE in plants (*69*), and this implies a light-dependent mechanism common to animals and plants. A putative LRE-binding factor was detected in the chick pineal nuclear extract by electrophoretic mobility shift assay (EMSA). EMSA using double-stranded oligonucleotide competitors for *cPer2* or *arginine vasopressin* E-box sequence indicated that the pinopsin LRE-binding factor has an affinity to these E-box sequences as well (*69*). Therefore, not only the circadian time signal but also the light signal may input to the CACGTG E-box element found in the promoter region of several clock (related) genes (Fig. 2).

Final Comments

The chick pineal gland and the pineal cells in culture are good models for the study of the animal circadian clock and its photic-input pathway. Characterization of the light-dependent regulatory mechanism of gene expression is feasible only in the light-sensitive clock cells, which would help to clarify in detail the phase-resetting mechanism of the clock. Comparative studies on the clock system of mammalian SCN and avian pineal gland are of great importance in addressing how the clock system has developed. Elucidation of a core mechanism conserved among a wide variety of living organisms should lead to understanding of the origin of the circadian clock.

Supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Culture, Education, Sports, Science and Technology.

REFERENCES

1. Williams, J.A. and Sehgal, A. (2001) Molecular components of the circadian system in Drosophila. *Annu. Rev. Physiol.* **63**, 729–755
2. Inouye, S.T. and Kawamura, H. (1979) Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc. Natl Acad. Sci. USA* **76**, 5962–5966
3. Weaver, D.R. (1998) The suprachiasmatic nucleus: a 25-year retrospective. *J. Biol. Rhythms* **13**, 100–112
4. Hatter, S., Lucas, R.J., Mrosovsky, N., Thompson, S., Douglas, R.H., Hankins, M.W., Lem, J., Biel, M., Hofmann, F., Foster, R.G., and Yau, K.-W. (2003) Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature* **424**, 76–81
5. Panda, S., Provencio, I., Tu, D.C., Pires, S.S., Rollag, M.D., Castrucii, A.M., Pletcher, M.T., Sato, T.K., Wiltshire, T., Andahazy, M., Kay, S.A., Van Gelder, R.N., and Hogenesch, J.B. (2003) Melanopsin is required for non-image-forming photic responses in blind mice. *Science* **301**, 525–527
6. Balsalobre, A., Damiola, F., and Schibler, U. (1998) A serum shoch induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**, 929–937
7. Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., Block, G.D., Sakaki, Y., Menaker, M., Tei, H. (2000) Peripheral circadian oscillators in transgenic rats. *Science* **288**, 682–685
8. Balsalobre, A., Brown, S.A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H.M., Schutz, G., and Schibler, U. (2000) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* **289**, 2344–2347
9. McNamara, P., Seo, S.P., Rudic, R.D., Sehgal, A., Chakravarti, D., FitzGerald, G.A. (2001) Regulation of CLOCK and MOP4 by nuclear hormone receptors in the vasculature: a humoral mechanism to reset a peripheral clock. *Cell* **105**, 877–889
10. Hirota, T., Okano, T., Kokame, K., Shirotani-Ikejima, H., Miyata, T., and Fukada, Y. (2002) Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured rat-1 fibroblasts. *J. Biol. Chem.* **277**, 44244–44251
11. Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., and Schibler, U. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.* **14**, 2950–2961
12. Stokkan, K.A., Yamazaki, S., Tei, H., Sakaki, Y., and Manaker, M. (2000) Entrainment of the circadian clock in the liver by feeding. *Science* **291**, 490–493
13. Hara, R., Wan, K., Wakamatsu, H., Aida, R., Moriya, T., Akiyama, M., and Shibata, S. (2001) Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. *Genes Cells* **6**, 269–278
14. Whitmore, D., Foulkes, N.S., and Sassone-Corsi, P. (2000) Light acts directly on organs and cells in culture to set the vertebrate circadian clock. *Nature* **404**, 87–91
15. Binkley, S.A., Riebman, J.B., and Reilly, K.B. (1978) The pineal gland: a biological clock *in vitro*. *Science* **202**, 1198–1201
16. Deguchi, T. (1979) A circadian oscillator in cultured cells of chicken pineal gland. *Nature* **282**, 94–96
17. Deguchi, T. (1979) Circadian rhythm of serotonin N-acetyltransferase activity in organ culture of chicken pineal gland. *Science* **203**, 1245–1247
18. Kasal, C.H., Menaker, M., and Perez-Polo, J.R. (1979) Circadian clock in culture: N-acetyltransferase activity of chick pineal glands oscillates *in vitro*. *Science* **203**, 656–658
19. Deguchi, T. (1981) Rhodopsin-like photosensitivity of isolated chicken pineal gland. *Nature* **290**, 706–707
20. Takahashi, J.S., Murakami, N., Nikaido, S.S., Pratt, B.L., and Robertson, L.M. (1989) The avian pineal, a vertebrate model system of the circadian oscillator: cellular regulation of circadian rhythms by light, second messengers, and macromolecular synthesis. *Rec. Prog. Horm. Res.* **45**, 279–352
21. Okano, T. and Fukada, Y. (1997) Phototransduction cascade and circadian oscillator in chicken pineal gland. *J. Pineal Res.* **22**, 145–151
22. Fukada, Y. and Okano, T. (2002) Circadian clock system in the pineal gland. *Mol. Neurobiol.* **25**, 19–30
23. Okano, T., Yoshizawa, T., and Fukada, Y. (1994) Pinopsin is a chicken pineal photoreceptive molecule. *Nature* **372**, 94–97
24. Okano, T., Kojima, D., Fukada, Y., Shichida, Y., and Yoshizawa, T. (1992) Primary structures of chicken cone visual pigments: Vertebrate rhodopsins have evolved out of cone visual pigments. *Proc. Natl Acad. Sci. USA* **89**, 5932–5936
25. Okano, T. and Fukada, Y. (2000) Photoreceptors in pineal gland and brain: Cloning, localization, and overexpression. *Methods Enzymol.* **316**, 278–291
26. Okano, T., Takanaka, Y., Nakamura, A., Hirunagi, K., Adachi, A., Ebihara, S., and Fukada Y. (1997) Immunocytochemical identification of pinopsin in pineal glands of chicken and pigeon. *Mol. Brain Res.* **50**, 190–196

27. Okano, T., Yamazaki, K., Kasahara, T., and Fukada, Y. (1997) Molecular cloning of heterotrimeric G-protein α-subunits in chicken pineal gland. *J. Mol. Evol.* **44**, S91–97
28. Kasahara, T., Okano, T., Yoshikawa, T., Yamazaki, K., and Fukada, Y. (2000) Rod-type transducin α-subunit mediates a phototransduction pathway in the chicken pineal gland. *J. Neurochem.* **75**, 217–224
29. Matsushita, A., Yoshikawa, T., Okano, T., Kasahara, T., and Fukada, Y. (2000) Colocalization of pinopsin with two types of G-protein α-subunits in the chicken pineal gland. *Cell Tissue Res.* **299**, 245–251
30. Kasahara, T., Okano, T., Haga, T., and Fukada, Y. (2002) Opsin-G11-mediated signaling pathway for photoc entrainment of the chicken pineal circadian clock. *J. Neurosci.* **22**, 7321–7325
31. Zatz, M. and Mullen, D.A. (1988) Two mechanisms of photoendocrine transduction in cultured chick pineal cells: Pertussis toxin blocks the acute but not the phase-shifting effects of light on the melatonin rhythm. *Brain Res.* **453**, 63–71
32. King, D.P. and Takahashi, J.S. (2000) Molecular genetics of circadian rhythms in mammals. *Annu. Rev. Neurosci.* **23**, 713–742
33. Cermakian, N., Whitmore, D., Foulkes, N.S., and Sassone-Corsi, P. (2000). Asynchronous oscillations of two zebrafish CLOCK partners reveal differential clock control and function. *Proc. Natl Acad. Sci. USA* **97**, 4339–4344
34. Hogenesch, J.B., Gu, Y.Z., Moran, S.M., Shimomura, K., Radcliffe, L.A., Takahashi, J.S., and Bradfield, C.A. (2000) The basic helix-loop-helix-PAS protein MOP9 is a brain-specific heterodimeric partner of circadian and hypoxia factors. *J. Neurosci.* **20**, RC83
35. Maemura, K., de la Monte, S.M., Chin, M.T., Layne, M.D., Hsieh, C.M., Yet, S.F., Perrella, M.A., and Lee, M.E. (2000) CLIF, a novel cycle-like factor, regulates the circadian oscillation of plasminogen activator inhibitor-1 gene expression. *J. Biol. Chem.* **275**, 36847–36851
36. Okano, T., Sasaki, M., and Fukada, Y. (2001) Cloning of mouse BMAL2 and its daily expression profile in the suprachiasmatic nucleus: a remarkable acceleration of Bmal2 sequence divergence after Bmal gene duplication. *Neurosci. Lett.* **300**, 111–114
37. Okano, T., Yamamoto, K., Okano, K., Hirota, T., Kasahara, T., Sasaki, M., Takanaka, Y., and Fukada, Y. (2001) Chicken pineal clock genes: Implication of BMAL2 as a bidirectional regulator in circadian clock oscillation. *Genes Cells* **6**, 825–836
38. Yamamoto, K., Okano, T., and Fukada, Y. (2001) Chicken pineal Cry genes: Light-dependent up-regulation of cCry1 and cCry2 transcripts. *Neurosci. Lett.* **313**, 13–16
39. Chong, N.W., Bernard, M., and Klein, D.C. (2000) Characterization of the chicken serotonin N-acetyltransferase gene. Activation *via* clock gene heterodimer/E box interaction. *J. Biol. Chem.* **275**, 32991–32998
40. Larkin, P., Baehr, W., and Semple-Rowland, S.L. (1999) Circadian regulation of iodopsin and clock is altered in the retinal degeneration chicken retina. *Mol. Brain Res.* **70**, 253–263
41. Doi, M., Nakajima, Y., Okano, T., and Fukada, Y. (2001) Light-induced phase-delay of the chicken pineal circadian clock is associated with the induction of cE4bp4, a potential transcriptional repressor of cPer2 gene. *Proc. Natl Acad. Sci. USA* **98**, 8089–8094
42. Ikushima, S., Inukai, T., Inaba, T., Nimer, S.D., Cleveland, J.L., and Look, A.T. (1997) Pivotal role for the NFIL3/E4BP4 transcription factor in interleukin 3-mediated survival of pro-B lymphocytes. *Proc. Natl Acad. Sci. USA* **94**, 2609–2614
43. Lopez-Molina, L., Conquet, F., Dubois-Dauphin, M., and Schibler, U. (1997) The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J.* **16**, 6762–6771
44. Mitsui, S., Yamaguchi, S., Matsuo, T., Ishida, Y., and Okamura, H. (2001) Antagonistic role of E4BP4 and PAR proteins in the circadian oscillatory mechanism. *Genes Dev.* **15**, 995–1006
45. Ripperger, J.A., Shearman, L.P., Reppert, S.M., and Schibler, U. (2000) CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev.* **14**, 679–689
46. Yamaguchi, S., Mitsui, S., Yan, L., Yagita, K., Miyake, S., and Okamura, H. (2000) Role of DBP in the circadian oscillatory mechanism. *Mol. Cell. Biol.* **13**, 4773–4781
47. Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998) The Drosophila clock gene double-time encodes a protein closely related to human casein-kinase Iε. *Cell* **94**, 97–107
48. Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M.W. (1998) Double-time is a novel Drosophila clock gene that regulates PERIOD protein accumulation. *Cell* **94**, 83–95
49. Lowrey, P.L., Shimomura, K., Antoch, M.P., Yamazaki, S., Zemenides, P.D., Ralph, M.R., Menaker, M., and Takahashi, J.S. (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* **288**, 483–492
50. Takano, A., Shimizu, K., Kani, S., Buijs, R.M., Okada, M., and Nagai, K. (2000) Cloning and characterization of rat casein kinase 1ε. *FEBS Lett.* **477**, 106–112
51. Vielhaber, E., Eide, E., Rivers, A., Gao, Z.H., and Virshup, D.M. (2000) Nuclear entry of the circadian regulator mPer1 is controlled by mammalian casein kinase Iε. *Mol. Cell. Biol.* **20**, 4888–4899
52. Akashi, M., Tsuchiya, Y., Yoshino, T., and Nishida, E. (2002) Control of intracellular dynamics of mammalian period proteins by casein kinase Iε (CKIε) and CKIδ in cultured cells. *Mol. Cell. Biol.* **22**, 1693–1703
53. Toh, K.L., Jones, C.R., He, Y., Eide, E.J., Hinz, W.A., Virshup, D.M., Ptacek, L.J., and Fu, Y.H. (2001) An hPer2 phosphorylation site mutation in familial advanced sleep phase syndrome. *Science* **291**, 1040–1043
54. Lee, C., Etchegaray, J.-P., Cagampang, F.R.A., Loudon, A.S.I., and Reppert, S.M. (2001) Posttranslational mechanisms regulate the mammalian circadian clock. *Cell* **107**, 855–867
55. Obrietan, K., Impey, S., and Storm, D.R. (1998) Light and circadian rhythmicity regulate MAP kinase activation in the suprachiasmatic nuclei. *Nat. Neurosci.* **1**, 693–700
56. Nakaya, M., Sanada, Y., and Fukada, Y. (2003) Spatial and temporal regulation of mitogen-activated protein kinase phosphorylation in the mouse suprachiasmatic nucleus. *Biochem. Biophys. Res. Commun.* **305**, 494–501
57. Sanada, K., Hayashi, Y., Harada, Y., Okano, T., and Fukada, Y. (2000) Role of circadian activation of mitogen-activated protein kinase in chick pineal clock oscillation. *J. Neurosci.* **20**, 986–991
58. Harada, Y., Sanada, K., and Fukada Y. (2001) Circadian activation of bullfrog retinal mitogen-activated protein kinase associates with oscillator function. *J. Biol. Chem.* **275**, 37078–37085
59. Ko, G.Y., Ko, M.L., and Dryer, S.E. (2001) Circadian regulation of cGMP-gated cationic channels of chick retinal cones. Erk MAP kinase and Ca^{2+}/calmodulin-dependent protein kinase II. *Neuron* **29**, 255–266
60. Hayashi, Y., Sanada, K., and Fukada, Y. (2001) Circadian and photic regulation of MAP kinase by Ras- and protein phosphatase-dependent pathway in the chicken pineal gland. *FEBS Lett.* **491**, 71–75
61. Shimizu, K., Okada, M., Nagai, K., and Fukada, Y. (2003) Suprachiasmatic nucleus circadian oscillatory protein, a novel binding partner of K-Ras in the membrane rafts, negatively regulates MAPK pathway. *J. Biol. Chem.* **278**, 14920–14925
62. Sanada, K., Okano, T., and Fukada, Y. (2002) Mitogen-activated proteinkinase phosphorylates and negatively regulates basic helix-loop-helix-PAS transcription factor BMAL1. *J. Biol. Chem.* **277**, 267–271
63. Hayashi, Y., Sanada, K., Hirota, T., Shimizu, F., and Fukada, Y. (2003) p38 mitogen-activated protein kinase regulatesand

oscillation of chick pineal circadian clock. *J. Biol. Chem.* **278**, 25166–25171
64. Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J.J., Dunlap, J.C., and Okamura, H. (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. *Cell* **91**, 1043–1053
65. Terzaghi, W.B. and Cashmore, A.R. (1995) Light-regulated transcription. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* **5**, 445–475
66. Travnickova-Bendova, Z., Cermakian, N., Reppert, S.M., and Sassone-Corsi, P. (2002) Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity. *Proc. Natl Acad. Sci. USA* **99**, 7728–7733
67. Takanaka, Y., Okano, T., Iigo, M., and Fukada, Y. (1998) Light-dependent expression of pinopsin gene in chicken pineal gland. *J. Neurochem.* **70**, 908–913
68. Takanaka, Y., Okano, T., Yamamoto, K., and Fukada, Y. (2002) A negative regulatory element required for light-dependent pinopsin gene expression. *J. Neurosci.* **22**, 4357–4363
69. Donald, R.G. and Cashmore, A.R. (1990) Mutation of either G box or I box sequences profoundly affects expression from the Arabidopsis rbcS-1A promoter. *EMBO J.* **9**, 1717–1726
70. Reppert, S.M. and Weaver, D.R. (2001) Molecular analysis of mammalian circadian rhythms. *Annu. Rev. Physiol.* **63**, 647–676