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Circadian Clock System in the Pineal Gland

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Abstract

The pineal gland is a neuroendocrine organ that functions as a central circadian oscillator in a variety of nonmammalian vertebrates. In many cases, the pineal gland retains photic input and endocrinal-output pathways both linked tightly to the oscillator. This contrasts well with the mammalian pineal gland equipped only with the output of melatonin production that is subject to neuronal regulation by central circadian oscillator located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Molecular studies on animal clock genes were performed first in *Drosophila* and later developed in rodents. More recently, clock genes such as *Per, Cry, Clock,* and *Bmal* have been found in a variety of vertebrate clock structures including the avian pineal gland. The profiles of the temporal change of the clock gene expression in the avian pineal gland are more similar to those in the mammalian SCN rather than to those in the mammalian pineal gland. Avian pineal gland and mammalian SCN seem to share a fundamental molecular framework of the clock oscillator composed of a transcription/translation-based autoregulatory feedback loop. The circadian time-keeping mechanism also requires several post-translational events, such as protein translocation and degradation processes, in which protein phosphorylation plays a very important role for the stable 24-h cycling of the oscillator and/or the photic-input pathway for entrainment of the clock.

Index Entries: Biological rhythm; circadian clock; clock gene; pineal gland; suprachiasmatic nucleus; photic input; melatonin; MAP kinase; chicken.

Introduction

The central oscillator of the circadian clock regulating animal behavior and physiology is located in the suprachiasmatic nucleus (SCN) in mammals *(1).* In contrast, nonmammalian vertebrates retain the central oscillator in multiple tissues such as the pineal gland, retina, and SCN *(2–8),* which are anatomically and functionally associated with the photoreception. In the case of birds, hypothalamic photosensory cells may also contain the circadian oscillator function, although they are probably

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involved in the photoperiodic response rather than in the circadian regulation *(9)*. The photosensitive clock cells in the retina, the pineal gland and the deep brain have opsin-type photoreceptive molecules *(10–17b)* that trigger G-protein-mediated phototransduction pathways *(18,19)*.

The role of the pineal gland has been changed in the course of vertebrate evolution, from photosensory organ in the lowest vertebrates such as lamprey *(20)* to photoendocrinal organ in the lower vertebrates *(21,22)*, and eventually to neuroendocrinal organ in mammals *(23)*. The avian pineal gland seems to have the intermediate properties between the latter two as it retains photic-input pathway and melatonin-output pathway that is regulated by both sympathetic innervation *(2,24)* and the circadian oscillator present within the individual pineal cells *(25,26)*. The relative importance of these regulations varies among avian species, but in the case of the chick pineal gland, the photic-input and melatonin-output pathways are tightly associated with the intrinsic circadian oscillator, and hence the pinealocyte has been one of the best models for the studies on the circadian clock system in higher vertebrates *(6,25,27)*. Recent studies have revealed several clock genes important for the circadian clock function in the avian pineal gland *(28–30)*. In this review we mainly focus on the avian pineal clock system, of which the oscillator mechanism is less understood than that in the rodent SCN, but studies on the photosensitive clock structure should provide fruitful information about the intracellular link of the oscillator with the photic-input pathway.

General Features of Autoregulatory Feedback Loop

Extensive studies on the molecular mechanism of the circadian clock oscillator have demonstrated that the framework of the molecular oscillation is generally based on a feedback loop(s) composed of the transcriptional and translational regulation of the clock genes *(1,31)*. In animals, central oscillators in *Drosophila* and mice share similar clock genes with functions slightly diverged from each other *(32,33)*. In a model for the molecular oscillation in the mouse SCN, the transcription of genes for negative regulatory components, PER and CRY, is stimulated by a positive regulatory complex composed of CLOCK and BMAL through E-box enhancer elements in the promoter or intronic region(s) of *Per* and *Cry* genes *(1,34)*. The E-box-mediated transactivation of *Per* and *Cry* genes is strongly inhibited by their own products, PER and CRY proteins, which allow molecular cycles of circadian activation and inactivation of their transcription. The components in the core feedback loop regulate transcription of several clock-controlled genes as well via the same E-box-mediated mechanism *(35–37)*. The overall framework of the core feedback loop proposed for the rodent SCN clock system seems applicable to that in the avian pineal gland (Fig. 1; *28–30*).

More recently, another stream of *Bmal*-based loop is proposed in addition to the *Per*-based core loop described earlier, and these "interacting molecular loops" *(38)* are included in Fig. 1. This idea is not yet supported fully by experimental observations, but it is mainly based on the facts that: 1) the mRNA level of *Bmal1* shows circadian oscillation with a phase opposite to that of *Per (39–41)*, and 2) the canonical negative regulator PER seems to stimulate (perhaps indirectly) expression of *Bmal* gene *(38)*. Another aspect of recent interests is the presence of several paralogs of *Per, Cry,* and *Bmal* genes in vertebrates (Table 1; *see* below), and this contrasts well with clock genes in *Drosophila* having only one functional ortholog for each of the genes. This suggests a more complex regulation in the vertebrate oscillator feedback loop than that in *Drosophila*. The molecular frameworks of mammalian and *Drosophila* clock systems and their comparison are well-summarized in recent reviews *(1,31–33,42)*.

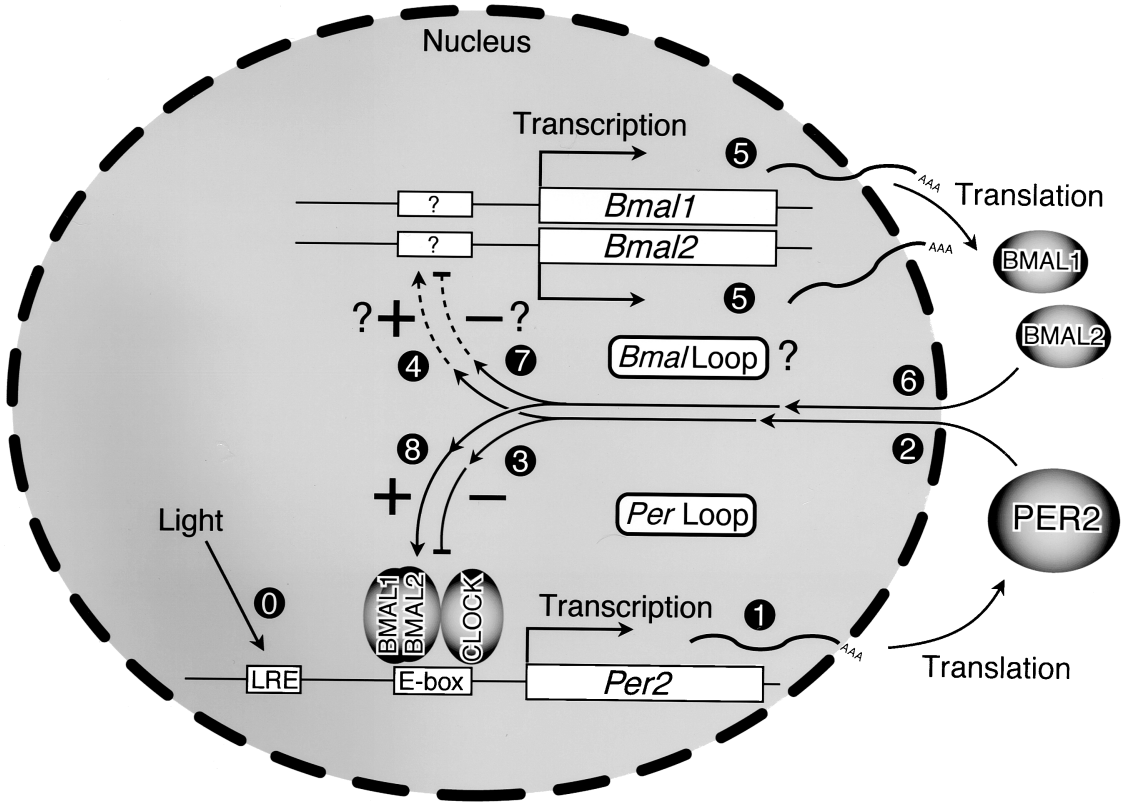

Fig. 1. A model for the molecular circadian oscillator composed of chicken pineal clock genes forming a transcription/translation-based autoregulatory feedback loop. In this model, the *Per* loop and a hypothetical *Bmal* loop are postulated to form interacting molecular loops *(32,38).* Light induces the transcription of *Per* gene (step 0; a number on a solid circle) through a putative light responsive element (LRE). The elevation of transcription of *Per* gene (step 1) leads to accumulation of PER protein, which then translocates to the nucleus (step 2) to inhibit transcriptional activation exerted by a BMAL-CLOCK heteromer (step 3). These steps (1–3) form a *Per* loop for clock oscillation. PER protein might also transactivate *Bmal* gene expression (step 4: hypothetical). The elevation of *Bmal* mRNA levels (step 5) results in accumulation of BMAL proteins, which then enter the nucleus (step 6) to inhibit its own transcription (step 7: hypothetical *Bmal* loop) and to activate the transcription of *Per* gene through the E-box (step 8). This enables the molecular cycle to start again from step 1.

Negative Regulators

Three *Per* paralogs were identified in rodents *(43–49),* whereas two paralogs in the quail (*qPer2* and *qPer3; 28*) and in the chicken (*cPer2* and *cPer3; 29,30*) have been cloned and shown to be expressed in their pineal glands. Recent identification of several *Per* paralogs in other vertebrates (Table 1; *50,51*) together with the fourth paralog in human *(hPer4; 42)* suggests that the multiple *Per* genes exist in a wide variety of vertebrate species.

Although the physiological importance and functional divergence of the *Per* paralogs are less understood, the temporal changes in mRNA levels of all the three *Per* genes *(Per1–3)* show similar but distinctive profiles in the mouse SCN *(48).* That is, the mRNA levels have peaks during the daytime under 12 h-light and 12 h-dark cycle condition (LD, in a simple

Table 1
Clock Genes and Their Candidates in Vertebrates

Mammals	Chicken (c) quail (q)	Frog (*Xenopus*, x)	Zebrafish (z)
Negative regulators			
Per1 (45,46)		*xPer1 (51)*	
Per2 (43,44,47)	*cPer2 (29), qPer2 (28)*	*xPer2 (51)*	
Per3 (48,49)	*cPer3 (30), qPer3 (28)*		*zPer3 (50)*
(Per4) (42)			
Cry1 (59,61)	*cCry1 (30)*		*zCry1a, 1b (60)*
Cry2 (59,61)	*cCry2 (30)*		*zCry2a, 2b (60)*
			zCry3, zCry4 (60)
Positive regulators			
Clock (63,64)	*cClock (68b)*	*xClock (93)*	*zClock (94)*
Bmal1 (69,70)	*cBmal1 (29,36)*		*zBmal1 (71)*
Bmal2 (73)	*cBmal2 (29)*		*zBmal2 (71)*
Other regulators			
Dbp (37,76)			
CKIε (82)			
E4bp4 (78)	*cE4bp4* (77)		

abbreviation) or during the subjective day in constant darkness (DD), but their peak times are slightly different between *Per1* (in the morning) and *Per2* (in the evening). In contrast, the temporal profiles in mRNA levels of *Per1* and *Per2* in the rodent pineal gland show their peaks at (subjective) night *(52,53),* being remarkably differnet from those in the SCN as summarized in Table 2. Such a phase-delay of the peripheral clocks relative to the central clock has been observed in other tissues *(49,54).*

The circadian rhythm of the pineal melatonin production has a peak at nighttime, and this largely depends on the rhythmic change in the mRNA level of arylalkylamine *N*-acetyltransferase *(NAT)* gene *(6).* In the rat, the mRNA levels of *NAT* and *Per1* genes in the pineal gland show quite similar temporal profiles with their peaks at nighttime under LD and DD conditions *(53)* and this is opposite to the phase of *Per1* rhythm in the SCN (Table 2). Furthermore, expression of both of the two genes in the rat pineal gland is regulated by β–adrenergic signal that originates from the SCN *(53)* and it is downregulated by light *(52). Per1* expression in the mammalian pineal gland seems to be mainly regulated by a cAMP-dependent mechanism common to the regulation of *NAT* gene *(6).*

Contrary to the light-dependent downregulation of *Per1* and *Per2* mRNA expression in the rat pineal gland (a peripheral clock) *(52),* light upregulates the mRNA levels of *Per* genes not only in the mouse SCN *(43,55)* but also in the chicken pineal gland, a central clock tissue *(29).* In addition, the temporal expression profile of *Per2* in the chicken pineal gland shows a peak in the morning and a trough in the night as is observed for *Per1* in the rodent SCN, a central clock tissue (Table 2). This profile is quite different from that of *NAT* gene with a peak of the mRNA level at nighttime and trough during daytime *(6,56),* and therefore chick pineal *Per2* and *NAT* genes are likely transcribed by way of regulation quite different from each other. This is apparently inconsistent with recent observations for the

Table 2
Peak and Trough in *Per, Cry, Clock, Bmal*, and *E4bp4* mRNA Levels in SCN and Pineal Gland

Gene	Light Condition	Mammal SCN		Mammal pineal gland		Chicken pineal gland	
		Peak	Trough	Peak	Trough	Peak	Trough
Per1	in LD	ZT4 *(46)*	ZT16 *(46)*	ZT16 *(53)*	ZT0-12 *(53)*	–	–
	in DD	CT4 *(46)*	CT16 *(46)*	CT16 *(53)*	CT0–8 *(53)*	–	–
Per2	in LD	ZT12 *(47)*	ZT20–0 *(47)*	ZT16–20 *(53)*	ZT4–8 *(53)*	ZT2 *(29)*	ZT14–18 *(29)*
	in DD	CT8 *(47)*	CT20–0 *(47)*	CT16–20 *(53)*	CT4–8 *(53)*	CT2 *(29)*	CT10–14 *(29)*
Per3	in LD	ZT4–8 *(48)*	ZT16–20 *(48)*	–	–	ZT22 *(30)*	ZT10–18 *(30)*
	in DD	CT4–8 *(48)*	CT20 *(48)*	–	–	CT22 *(30)*	CT10–14 *(30)*
Cry1	in LD	ZT12 *(62)*	ZT20–0 *(62)*	–	–	ZT10 *(30)*	ZT22 *(30)*
	in DD	CT12 *(62)*	CT20–0 *(62)*	–	–	CT10 *(30)*	CT22 *(30)*
Cry2	in LD	ZT8–16 *(62)*	ZT0 *(62)*	–	–	ZT22–10 *(30)*	ZT14 *(30)*
	in DD	CT8–16 *(62)*	CT20–0 *(62)*	–	–	CT22 *(30)*	CT10 *(30)*
Clock	in LD	ZT6 *(68a)*	ZT18–22 *(68a)*	ZT18* *(41)*	ZT6* *(41)*	ZT10–18 *(29)*	ZT22–2 *(29)*
	in DD	–	–	–	–	CT10–18 *(29)*	CT22–2 *(29)*
Bmal1	in LD	ZT18 *(39)*	ZT2–6 *(39)*	ZT6* *(41)*	ZT18* *(41)*	ZT10 *(29)*	ZT22 *(29)*
	in DD	CT18 *(39)*	CT2–10 *(39)*	–	–	CT10–14 *(29)*	CT22–2 *(29)*
Bmal2	in LD	const *(73)*	const *(73)*	–	–	ZT14 *(29)*	ZT22 *(29)*
	in DD	–	–	–	–	CT14 *(29)*	CT22–2 *(29)*
E4bp4	in LD	ZT16 *(78)*	ZT8 *(78)*	–	–	ZT10 *(77)*	ZT20–0 *(77)*
	in DD	CT12 *(78)*	CT4–8 *(78)*	–	–	CT12 *(77)*	CT20–0 *(77)*

–; Not examined or not expressed, const; constant level or very weak rhythm *; Data from comparison between ZT6 and ZT18.

involvement of the E-box element in the transcription of both chicken *Per2 (29)* and *NAT* genes *(36)*. In addition to common E-box-dependent mechanism, there might be transcriptional regulation different between clock genes *(Per2)* and clock-controlled genes *(NAT)*.

Another important negative regulator in vertebrate-clock oscillation loop is CRY. CRY was originally found as an animal homolog of plant blue-light photoreceptor cryptochromes, and later it was identified as a circadian photoreceptor in *Drosophila (57)*. On the other hand, mammalian CRYs (CRY1 and CRY2) are negative regulators in the E-box-mediated autoregulatory feedback loop *(58)* and they are indispensable for the clock oscillation *(59)*. The chicken has also two *Cry* paralogs, *Cry1* and *Cry2 (30)*, whereas in the zebrafish, five paralogs are found but a gene orthologous to *mCry2* is not identified *(60)*. The *Cry* transcripts are present in various tissues of mice, among which the retina and SCN show higher expression levels *(61)*, but there is no report so far for *Cry* expression in the mammalian pineal gland. The mRNA levels of *Cry1* and *Cry2* genes show robust circadian oscillations with their peaks at ZT8 (under LD condition) in the rodent SCN *(61,62)*, and similar oscillations are observed in the chick pineal cells in culture *(30)*. It should be emphasized, however, that *Cry1* mRNA level is markedly upregulated by light captured by endogenous photoreceptor in the chicken pineal cells *(30)*. This suggests a unique light-signaling process regulating the phase of the chicken pineal clock, contrasting to that in the mammalian SCN expressing *Per* genes as the major light-responsive genes.

At present, it is still an open question whether or not CRYs are photosensors to be involved in the photic-input pathway of the vertebrate circadian clock systems. Clearly, molecular analysis of CRYs' functions, espe-

cially their contribution to the intrinsic photic-input pathway in the avian pineal clock system, is one of the important issues to be elucidated in future studies.

Positive Regulators

Clock gene was originally identified as a gene responsible for *Clock* mutant mice phenotype *(63,64),* and its ortholog in *Drosophila (dClock)* was found later as a clock component. In addition to *Clock,* vertebrates have its paralog termed *Mop4* or *Npas2* (neuronal PAS domain protein-2; *65,66*). Like CLOCK, MOP4/NPAS2 protein has an ability to dimerize with BMAL to stimulate the transcription through the E-box element *(34,66,67),* but CLOCK seems to play a relatively more important role for circadian regulation in the SCN *(34,67). Clock* mRNA levels do not show a robust circadian rhythm in various clock tissues such as the rodent SCN *(68a),* chicken pineal gland *(29),* and chicken retina *(68a).*

BMAL1 (MOP3) was first reported as an orphan bHLH-PAS protein *(69),* and later it was shown to be an essential component for the circadian oscillator as a functional partner of CLOCK *(34,66,70).* Recently, a paralog of BMAL1 was reported by several groups and termed as BMAL2 (Table 1; *(29,71–73),* CLIF *(74),* or MOP9 *(75).* Interestingly, BMAL2 proteins appear to be diverged with a higher rate in amino acid substitutions than BMAL 1 proteins, and a phylogenetic analysis of BMALs strongly suggests that the selective pressure on the amino acid substitutions in BMAL2 remarkably decreased after the divergence of *Bmal* gene duplication *(73).* Nonetheless, BMAL1 and BMAL2 show similar profiles in selectivity for dimerization partners *(29,74,75),* implying a yet-to-be-discovered function of BMAL1. In the rodent SCN, the level of the *Bmal1* mRNA shows an oscillation with a phase opposite to those of *Per* genes (Table 2), and this suggests mutual interactions between *Per/Bmal* transcription and their protein products (Fig. 1). *Bmal2* gene is, however, constitutively expressed like *Clock* in the mouse hypothalamus containing the SCN *(73),* indicating differential regulation of transcription between these *Bmal* genes. Interestingly, *Bmal1* and *Bmal2* mRNA levels show robust rhythms with the phases slightly different from each other in the zebrafish tissues *(71)* and in the chicken pineal gland (Table 2; *29*).

Other Regulators and Photic-Input Pathway

In addition to the positive and negative regulators involved in the core feedback loop of the oscillator, several components seem to contribute to amplification or stabilization of the oscillatory loop. DBP, a PAR domain-containing bZip transcription factor, may positively regulate the transcription of *Per* gene in the SCN *(76),* though its physiological importance in the pineal clock system is not examined. On the other hand, another bZip transcriptional factor E4BP4 *(77,78),* a counterpart of a *Drosophila* clock component VRI encoded by *vrille* gene *(79),* may negatively regulate the transcription of *Per* gene. E4BP4 (repressor) and DBP (activator) show circadian rhythmic expression in their protein levels with opposite phases to each other, and they potentially bind to a common binding site found in *Per1* promoter in a competitive manner *(78).* In the chicken pineal gland, mRNA levels of *E4bp4* is regulated not only by the circadian clock but also by a photic signal *(77).* Interestingly, a phase-delay induced by the elongation of the light period to the early night is associated with light-dependent high-level expression of *E4bp4,* which seems responsible for the delay of the onset of *Per2* transcription in the next morning. These results, along with the presence of a functional E4BP4 binding site in *Per2* promoter *(77)* suggest that E4BP4 is an important regulator serving not only as a clock component for generating the stable cycling of *Per* transcription but also as a pineal clock modifier for the phase-delay upon the light stimulation early in the night.

As well as the essential regulation at the transcriptional level, recent studies have shown an important role of post-translational events such as phosphorylation, nuclear entry, and degradation of proteins for maintenance of the time-keeping mechanism. In *Drosophila*, *double-time* (*dbt*) gene encodes a protein kinase responsible for PER phosphorylation, and its mutations result in lengthening or shortening of the circadian rhythmicity *(80,81).* Similarly, casein kinase Iε (CKIε), a mammalian counterpart of DBT, was shown to be responsible for the period shortening in locomotor activity of *tau* mutant hamster *(82)* probably by regulating PER protein stability and/or its subcellular localization *(83,84).* Physiological significance of casein kinase lε in the chick pineal gland and in other clock structures remains to be elucidated.

An interesting nature of another protein kinase to be described is the circadian activation/deactivation cycles of mitogen-activated protein kinase (MAPK) observed in several clock structures such as the mouse SCN *(85),* the chicken pineal gland *(86),* the bullfrog retina *(87),* and the chicken retina *(88).* The rhythmicity of the chicken pineal MAPK (ERK2) activity is synchronized with rhythmic activities of components in the classical Ras-MAPK cascade, Ras, Raf-1 and MEK1, all of which are activated during the nighttime *(89).* This suggests that MAPK activity is regulated by a clock output via the Ras-MAPK cascade. Interestingly, a transient inhibition of MEK1 activity during the subjective night delayed the phase of the oscillator both in the chick pineal gland *(86)* and in the bullfrog retina *(87).* It is thus likely that MAPK plays a role not only in the output pathway *(85)* but also in the input pathway toward the circadian oscillator (*90*). Just like a recent observation that clock outputs feedback to the core oscillator *(76),* MAPK cascade may form a secondary feedback loop as well and hence contribute to stabilization of the core loop of the circadian oscillator.

The photic induction of *Per1* gene is likely a key step in the phase-shifting process of the clock system in the rodent SCN *(55,91),* and the *Per1* induction is mediated by glutaminergic innervation *via* retinohypothalamic tract from the retina. Similarly, expression of chicken pineal *Per2* gene is enhanced by light, but in this case, the photic induction is observed even in the isolated culture *(29),* indicating that the photic-input pathway intrinsic to the pinealocyte contributes to the upregulation. A retinal opsin-related molecule, pinopsin, has been identified as a chick pineal-specific photoreceptor *(10)* and light-activated pinopsin couples with heterotrimeric G-proteins such as rod-type transducin (Gt1) and G11 in the chicken pinealocytes (*18,92;* to be published elsewhere). Another pineal-specific opsin, exo-rhodopsin, and Gt1 are co-expressed in the zebrafish pineal gland *(16),* which retains a robust clock function even in culture and thereby represents an excellent experimental model for the clock studies *(22).* Thus, one can approach the phase-shifting mechanism by investigating the intracellular light-signaling pathway in the pinealocyte (Fig. 2). MAPK may also play an important role in the photic entrainment, because MAPK is rapidly dephosphorylated/deactivated by light pulse given at night when it is highly phosphorylated in the chick pineal gland. The phase-dependent dephosphorylation of chicken pineal MAPK occurs within 10 min of light exposure *(86,89),* suggesting that it precedes the induction of any known clock genes. Notably the MAPK dephosphorylation is attributable to the light-dependent activation of protein phosphatase (tyrosine-specific or dual-specificity protein phosphatase), and the upstream Ras, Raf-1 and MEK activities are apparently insensitive to light *(88).* Taken together, MAPK seems to serve as one of the converging points of photic and circadian signals and to play a pivotal role in the maintenance and entrainment of the circadian oscillator (Fig. 2).

Conclusion

Several positive and negative regulators in the circadian feedback loop show rhythmic expression in mRNA levels, but their peaks are

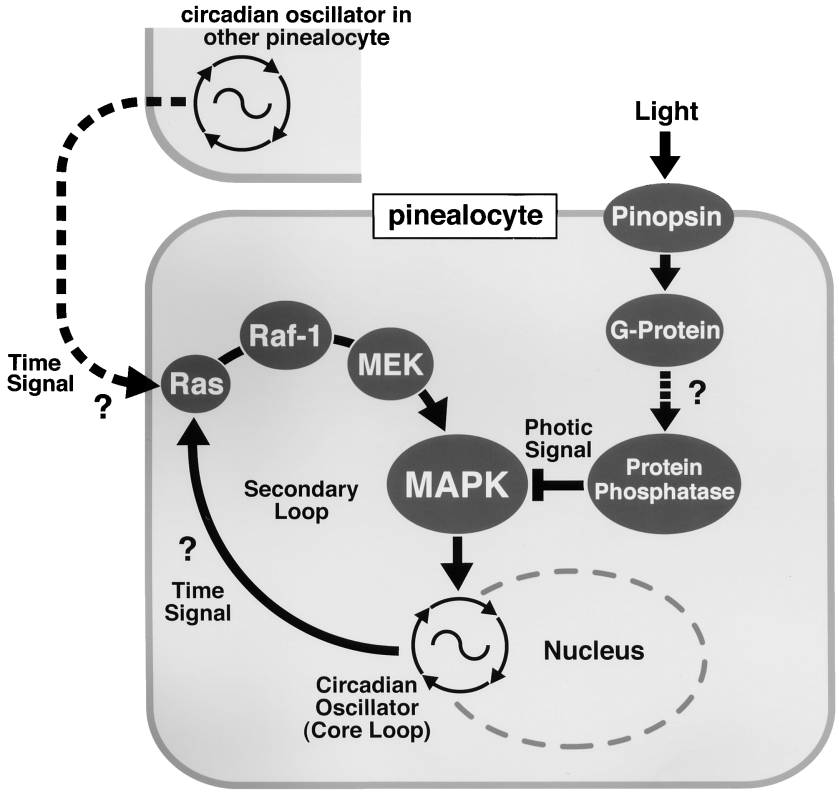

Fig. 2. A model for the role of MAPK cascade in the circadian clock system in the chicken pinealocyte.

different among tissues and species for each gene. The temporal profiles of these mRNA levels in the chick pineal gland are similar to those in the rodent SCN rather than to those in the rodent pineal gland, suggesting that the chicken pineal gland and mammalian SCN share a fundamental molecular framework of the circadian clock systems. This is consistent with the role of the chicken pineal gland as a central clock, which contrasts with the mammalian pineal gland governed by the SCN. Comparative analyses of the functions of the positive and negative regulators in addition to several circadian regulators such as CKIε and MAPK among a variety of species would make clearer the general molecular mechanism underlying the clock oscillation and how the vertebrate circadian clock system has evolved out of an ancestral clock system.

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