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Molecular Mechanism of Photic-Entrainment of Chicken Pineal Circadian Clock

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The chicken pineal gland has been used for studies on the circadian clock, because it retains an intracellular phototransduction pathway regulating the phase of the intrinsic clock oscillator. Previously, we identified chicken clock genes expressed in the gland (*cPer2*, *cPer3*, *cBmal1*, *cBmal2*, *cCry1*, *cCry2*, and *cClock*), and showed that a cBMAL1/2-cCLOCK heteromer acts as a regulator transactivating *cPer2* gene through the CACGTG E-box element found in its promoter. Notably, mRNA expression of *cPer2* gene is up-regulated by light as well as is driven by the circadian clock, implying that light-dependent clock resetting may involve the up-regulation of *cPer2* gene. To explore the mechanism of light-dependent gene expression unidentified in animals, we first focused on pinopsin gene whose mRNA level is also up-regulated by light. A pinopsin promoter was isolated and analyzed by transcriptional assays using cultured chicken pineal cells, resulting in identification of an 18-bp light-responsive element that includes a CACGTG E-box sequence. We also investigated a role of mitogen-activated protein kinase (MAPK) in the clock resetting, especially in the E-box-dependent transcriptional regulation, because MAPK is phospholylated (activated) in a circadian manner and is rapidly dephosphorylated by light in the gland. Both pulldown analysis and kinase assay revealed that MAPK directly associates with BMAL1 to phosphorylate it at several Ser/Thr residues. Transcriptional analyses implied that the MAPK-mediated phosphorylation may negatively regulate the BMAL-CLOCK-dependent transactivation through the E-box. These results suggest that the CACGTG E-box serves not only as a clock-controlled element but also as a light-responsive element.

Key words: pinopsin, pineal gland, chicken, BMAL, E-box, mitogen-activated protein kinase, transcriptional regulation, circadian clock

INTRODUCTION

Circadian rhythms in physiology and behavior are observed in a variety of living organisms, and are regulated by endogenous clocks [1]. These clocks autonomously oscillate with a period length close to 24 hr under constant conditions, and they can be entrained (synchronized) by environmental stimuli, most commonly by light [2]. Similar to the suprachiasmatic nucleus (SCN) governing the circadian functions in mammals, the pineal gland plays a pivotal role in regulation of circadian physiology in many non-mammalian vertebrates, and it produces melatonin in a circadian and light-dependent manner under the control of the endogenous oscillator [3]. Among others, the chicken pineal gland has been a prominent model for the study of the vertebrate circadian clock systems at the cellular level, because the chicken pinealocyte retains the circadian oscillator, photic-input pathway and melatonin-output pathway even under the cultured conditions [3-5].

Previously, we identified expression of chicken clock genes, chicken *Per2* (*cPer2*), *cPer3*, *cBmal1*, *cBmal2*, *cCry1*, *cCry2*, and *Clock* genes in the pineal gland [6,7]. Functional characterization of these molecules supports their key roles contributing to the E-box-dependent transcriptional regulation in the circadian clock system [6, 7]. Importantly the mRNA expression of *cPer2*, *cCry1*, and *cCry2* genes is up-regulated by light as well as is driven by the circadian clock, implying that light-dependent clock resetting may involve the up-regulation of these clock genes. To understand the mechanism underlying the light-dependent gene expression in the photosensory cells, we paid special attention to gene regulation of pinopsin that was identified in the chicken pineal gland as a pineal-specific photoreceptive molecule [5]. This is becasue mRNA

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levels of pinopsin are kept low in the dark and increase about 6-fold upon 6 hr light-exposure of chicks [8]. Even in the isolated pineal organ culture, the expression of pinopsin gene is up-regulated by light albeit with less pronounced degree (about 1.5-fold) than *in vivo*.

In addition to the transcriptional regulation, posttranslational modifications such as phosphorylation of clock gene products seem to regulate the stability and period length of the circadian cycle by generating an appropriate time-lag. We previously demonstrated that a transient inhibition of circadian activation of mitogen-activated protein kinase (MAPK) induced a phase-shift of the oscillator in the chick pineal gland [9]. Several lines of evidence indicate that MAPK activity is regulated by the clock and photic signals via independent pathways, and suggest a pivotal role of MAPK in maintenance of the circadian rhythm and its photic-entrainment.

In the present study, we show that (1) an 18-bp element at positions -1103 to -1086 in the pinopsin upstream region plays a key role in the light responsiveness [10] and that (2) MAPK directly interacts with the clock component BMAL1 for negative regulation of BMAL1:CLOCK-induced transcription via BMAL1 phosphorylation.

MATERIALS AND METHODS

A light responsive element (LRE) in the pinopsin promoter was identified by transcriptional analysis of pinopsin promoter using dispersed chick pineal cells as described [9]. A nuclear factor binding to the light-responsive element in the pinopsin promoter was analyzed by electrophoretic mobility shift assay (EMSA) as described [9].

Characterization of phosphorylation of BMAL1 by MAPK was performed as described [10].

RESULTS AND DISCUSSION

Light-Responsive Element in the Pinopsin Promoter

We cloned a 2.5-kb chick genomic fragment including the pinopsin gene upstream region, in which we found three copies of CACGTG E-boxes at positions -1100 to -1095, -2009 to -2004 and -2023 to -2018. The *cis*-DNA element(s) responsible for the light-dependent gene expression was searched by transcriptional analyses and at least one of LREs indispensable for the light-dependent pinopsin gene expression was localized in the vicinity of the position -1122/-1097 [10]. To determine critical sequence for the light-dependent transactivation, mutations were introduced into the construct -1156/+31 at a region between position -1121 and -1082 (Fig. 1A, M1-M8). M1, M2, M3 and M8 displayed light-sensitive reporter activities, whereas activities of M4-M7 were insensitive to light (Fig. 1B). M1-M3 showed reporter activities markedly higher than wild-type -1156/+31 construct (WT) in the light and dark, suggesting that a transcriptional inhibitor(s) binds to the region between -1121 and -1104. The light-insensitive reporter activities of the mutants M4-M7 were more comparable to that of the wild-type detected in the light than that in the dark (B), and hence the 18-bp element at

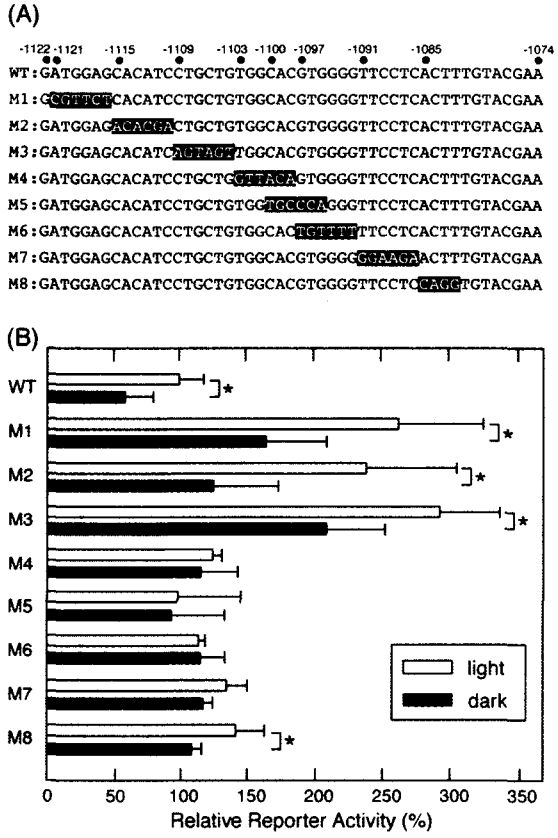

Fig. 1. Scanning of critical sequences for light responsive gene expression. (A) Positions of mutations introduced into -1156/+31 construct. The numbers on the top of the figure refer to base position relative to the transcription initiation site. (B) Luciferase assays performed with constructs harboring various mutations of the pinopsin promoter region. The cells were transfected with each construct and harvested in the light (open bars) or in the dark (solid bars). Shown in each panel is typical set of data out of 3 independent experiments. The bars represent mean (–) SD of 4 replicated cell culture. $*p < 0.05$, Student s *t* test.

positions -1103 to -1086 (TGGCACGTGGGGTTCCTC) seems to elicit transcriptional repression in the dark.

By using a probe of the 49-bp DNA fragment (-1122/-1074, termed WT49) including the 18-bp LRE, EMSA was performed to explore a specific DNA-binding protein(s) in the chick pineal nuclear extract. The DNA sequence specificity of the interaction was examined by assessing the competitive effects of mutated WT49 fragments, and it turned out that the binding patterns are in good agreement with the effects of mutations on the light-responsiveness of the transcription evaluated by the luciferase assay [10](not shown). Notably, E-box (CACGTG)-containing oligonucleotides corresponding to regions upstream of mouse arginine vasopressin gene and *cPer2* gene had competitive effects completely dependent on the CACGTG sequence [10](not shown), although the flanking sequences of these E-boxes showed only weak similarities to each other.

Interestingly, CACGTG sequence found in the pinopsin LRE completely matches G-box (CACGTGG), one of the LREs identified in plants, in which the element is not effective by itself and a combination with its specific minimal promoter is indispensable for expression of light-responsiveness. In animals, a CACGTG E-box has been identified to play a key role in transcription/translation-based autoregulatory feedback loop of the circadian oscillator [2]. At least a single copy of E-box with similar function is present in the promoter region of *cPer2* expressed in the pineal gland [6]. However, only a little information is available about the *Per* gene transcriptional regulation contributing to its light-dependent expression. Our present results raised a possibility that CACGTG E-box(es) in the promoter region of clock-related genes such as *Per*s may contribute to a light-regulatory mechanism similar to that underlying the light-dependent regulation of pinopsin gene expression through its LRE.

Phosphorylation of BMAL1 by MAPK

Bacause MAPK in some cases forms a complex with its substrate nuclear proteins including transcription factors to regulate their activities, we performed a yeast two-hybrid assay to examine possible interaction of MAPK (kinase-dead MAPK as a bait) with a couple of bHLH-PAS transcription factors, chicken BMAL1 and CLOCK. MAPK interacted with BMAL1 but not with CLOCK (data not shown). We further confirmed this interaction by GST pull-down assay [11]. GST-BMAL1 preferentially associated with phosphorylated MAPK rather than with myc-MAPK [11]. Deletion of BMAL1 implied that the C-terminal activation domain is not required for the interaction with MAPK (not shown). These two analyses suggest that BMAL1 could be one of the *in vivo* targets of phosphorylated and hence activated MAPK.

We then tested whether BMAL1 is phosphorylated by activated MAPK. In the presence of radiolabeled ATP, GST-BMAL1 was efficiently phosphorylated by phosphorylated-MAPK but not by MAPK [11]. Quantitative analysis of time-dependent change of the phosphorylated band revealed that BMAL1 incorporated 6-7 pmol of ^{32}Pi per pmol of protein, indicating that MAPK phosphorylates BMAL1 at multiple sites. Noticeably, deletion of the C-terminal 140 amino acids of BMAL1 resulted in substantial loss of the MAPK-dependent BMAL1 phosphorylation.

To determine the phosphorylation site(s), GST-BMAL1 was treated with phosphorylated or nonphosphorylated MAPK, digested by lysylendopeptidase and V8 protease, and subjected to reversed-phase HPLC for isolating the proteolytic fragments. Based on peptide analyses of these proteolytic fragments by using MALDI-TOF/MS, we identified three sites for *in vitro* MAPK phosphorylation, Ser527, Thr534 and Ser599 in BMAL1 (Fig. 2).

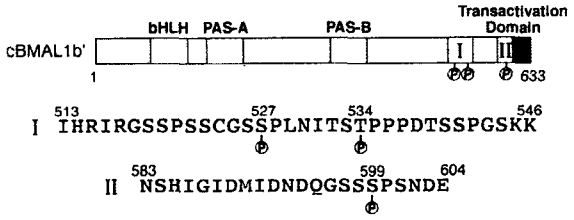

Fig. 2. Three residues in BMAL1 were identified to be phosphorylated by MAPK *in vitro*. P s in circles denote phosphate groups. I and II indicate phosphorylated peptides identified by using MALDI-TOF/MS.

A functional role of MAPK-catalyzed phosphorylation of BMAL1 was examined by transcriptional assay in 293 cells, in which coexpression of BMAL1 and CLOCK stimulated E-box element-dependent transcription of a luciferase reporter gene [6]. Under the conditions, coexpression of DE-MEK (a constitutive active form of MEK) suppressed BMAL1:CLOCK-induced transcription, and the maximal inhibition reached 23%-30%. This inhibitory effect of DE-MEK was completely reversed by additional coexpression of KR-MAPK (a kinase-dead mutant of MAPK), suggesting that activated MAPK mediates the

inhibition of BMAL1:CLOCK-induced transcription. To evaluate contribution of BMAL1 phosphorylation to the inhibition, effects of BMAL1 mutations at the phosphorylation sites (S527A, T534A and S599A) were examined in the transcription assay. Like wild-type BMAL1, every mutant induced a large increase in E-box element-dependent transcription together with CLOCK in 293 cells. And, as is observed for wild-type BMAL1:CLOCK, DE-MEK-dependent suppression of the transactivation was observed for BMAL1(S527A):CLOCK and BMAL1(S599A):CLOCK (inhibition by 21% and 25%, respectively). On the other hand, the transactivation induced by BMAL1(T534A):CLOCK was not affected by DE-MEK. These results indicate that MAPK negatively regulates E-box-dependent transactivation induced by BMAL1:CLOCK heteromers via phosphorylation of BMAL1 at Thr534.

MAPK activity exhibits a circadian rhythm with a peak at mid to late subjective night in various clock structures. During the nighttime, protein levels of negative regulatory elements in the circadian feedback loop (mPERs and mCRYs) are in declining phase, but their mRNA levels do not start to increase until these protein levels reach to their circadian trough at late subjective night. BMAL1:CLOCK-mediated transcription of *mPer/mCry* is kept suppressed during mid to late subjective night in spite of very low protein levels of negative elements. Therefore the negative elements-independent inhibition of BMAL1:CLOCK heteromer seems to delay the restart of the *mPer/mCRY* cycle, generating an appropriate time-lag required for the circadian rhythmicity of the oscillator. The MAPK-mediated inhibition of BMAL1 activity observed in this study can explain such a time-lag for activation of E-box element-dependent transcription during the nighttime, and therefore MAPK is likely involved in the time-keeping mechanism of the circadian oscillation.

The phosphorylation of MAPK in the chicken pineal gland shows not only circadian rhythm but also light-dependent acute dephosphorylation in the (subjective) night [9]. Thus MAPK would also participate in the light-dependent transcription mechanism. The light-dependent downregulation of MAPK and the resulting activation of BMAL1 might contribute to the regulation of E-box-containing LRE(s) such as pinopsin LRE.

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