Molecular cloning, mRNA expression, and immunocytochemical localization of a putative blue-light photoreceptor CRY4 in the chicken pineal gland

Yoko Kubo,*†1 Masashi Akiyama,*†1 Yoshitaka Fukada* and Toshiyuki Okano*†1

*Department of Biophysics and Biochemistry, Graduate School of Science, The University of Tokyo, Tokyo, Japan
†PRESTO, Japan Science and Technology Agency (JST), Kawaguchi, Saitama, Japan

Abstract
In non-mammalian vertebrates, the pineal gland contains an endogenous circadian oscillator and serves as a photosensitive neuroendocrine organ. To better understand the pineal phototransduction mechanism, we focused on the chicken putative blue-light photoreceptive molecule, Cryptochrome4 (cCRY4). Here we report the molecular cloning of pineal cCry4 cDNA, the in vivo expression of cCry4 mRNA, and the detection of cCRY4 protein. cCry4 is transcribed in a wide variety of chick tissues out of which the pineal gland and retina contain high levels of cCry4 mRNA. In the pineal gland, under 12 h light : 12 h dark cycles, the levels of both cCry4 mRNA and cCRY4 protein showed diurnal changes, and in cultured chick pineal cells, the cCRY4 mRNA level was not only up-regulated by light but also controlled by circadian signals. Immunoblot analysis with a cCRY4-specific antibody detected cCRY4 in a soluble fraction of the pineal lysate. Immunocytochemistry revealed that cCRY4 was expressed in many parenchymal cells and a limited number of stromal cells. These cCRY4 features strikingly contrast with those of the chick pineal photoreceptor pinopsin, suggesting a possible temporal and/or spatial duplicity of the pineal photoreceptive system, the opsin- and CRY-based mechanisms.

Keywords: chicken, circadian clock, cryptochrome, gene expression, photoreceptor, pineal gland.

The non-mammalian pineal gland is a photosensitive neuroendocrine organ. The pinealocytes synthesize and secrete melatonin depending on a light signal captured by endogenous photoreceptors, and also depending on time information generated by endogenous circadian clocks (Falcon et al. 2003; Okano and Fukada 2003). Light has two effects on melatonin synthesis in the chicken pinealocyte; it can produce an acute inhibitory effect during subjective night and it can photoentrain the circadian melatonin rhythm (Zatz and Mullen 1988; Takahashi et al. 1989). These effects are probably mediated by at least two independent phototransduction pathways in the pinealocyte. Zatz and colleagues have shown that the acute inhibitory pathway includes both a retinoid-based photoreceptor and a pertussis toxin-sensitive G-protein (Zatz and Mullen 1988; Zatz 1994). We have also identified the pineal-specific opsin pinopsin and pineal transducin (Gt1) that may be involved in the acute inhibitory pathway (Okano et al. 1994; Nakamura et al. 1999; Kasahara et al. 2000). Although we are gaining insight into the acute inhibitory effect light has in the pinealocyte, the photoentrainment pathway remains obscure. We previously identified the expression of G11, a Gq-type G-protein, in the pinealocyte, and demonstrated that transient activation of G11 induces light-like phase-shifts of the melatonin rhythm (Kasahara et al. 2002). Based on these findings, we suggested that the retinoid-based photoreceptor opsins couple with G11 to mediate photoentrainment; however, this idea was inconsistent with a previous observation that photoentrainment is not inhibited by the depletion of retinoids from chick
pineal cell culture (Zatz 1994). Thus, a non-opsin photopigment that does not rely on retinoids may operate as a circadian photoreceptor in parallel with the opsin-G11 pathway; or alternatively, such a pigment may activate G11 in a retinoid-independent manner.

Cryptochrome/DNA photolyase family proteins (CRYs/photolyases) that commonly use FAD as a chromophore served as our potential candidates for the non-opsin circadian photoreceptors in the pineal gland. This family is composed of several subfamilies with diverse functions such as circadian photoreception (invertebrate CRYs), negative regulation of the circadian clock (vertebrate CRYs), photorepair of the cyclobutane pyrimidine dimers and (6–4) photoproducts (photolyases, Cashmore et al. 1999; Sancar 2003).

In the chicken genomic database, we found three Cry genes, cCry1, cCry2 and cCry4, among which cCry1 and cCry2 are probable orthologs of mammal Cry1 and Cry2, respectively. cCry1 and cCry2 (cCry1/2) have been shown to repress E-box-mediated transactivation by cCLOCK:cBMAL, and cCRY1/2 are considered to work as negative regulators in the circadian clockwork of the pineal gland (Yamamoto et al. 2001). In addition, cCry1/2 are thought to play an important role in photoreception in the iris (Tu et al. 2004). Despite several findings related to cCry1/2, there were no reports on the function or in vivo expression of the cCry4 gene product. The only member of CRY4 described to date is the zebrafish CRY4 (zCRY4), the function of which is still unidentified (Kobayashi et al. 2000).

In this study, we cloned pineal cCry4 cDNA and investigated the temporal profiles of cCry4 mRNA levels in both chick tissues and cultured pineal cells. We detected cCry4-like protein in the pineal gland, providing the first demonstration of the in vivo expression of CRY4 protein.

Materials and methods

Animals

Animals were treated in accordance with the guidelines of The University of Tokyo. One-day-old male chicks were maintained at 28°C and entrained under 12 h light : 12 h dark (LD) cycles for ~2 weeks. Tissues were collected in the light (at Zeitgeber time 0 [ZT0], ZT6, and ZT12) or under a dim red light (> 640 nm; ~450 μW/cm² at ZT18) and kept in liquid nitrogen (for RNA isolation) or at ~80°C (for immunoblot analysis) until use.

RNA isolation, 5’-rapid amplification of cDNA ends (RACE), and cloning of cDNAs encoding the full-length cCRY4 coding sequence

Total RNA was isolated from the chick pineal gland using TRI Reagent (Invitrogen, Carlsbad, CA, USA). An oligo-dT (dT)₆ primer, was used for the first-strand cDNA synthesis with reverse transcriptase (SuperScript II, Invitrogen). The cDNA was then incubated at 37°C for 15 min in 100 μm dTTTP, 100 mM potassium cacodylate, 2 mM CoCl₂, 0.2 mM DTT, and 0.8 μg/μl terminal deoxynucleotidyl transferase (TaKaRa Bio, Otsu, Japan) to add poly (dT) at the 3’-terminus of each cDNA. In the second-strand cDNA synthesis, KSL(dA)₂₅ primer (5’-GGAGTGCCAGGTTATCGAATAAGCAAAAAA----------------A-3’) and LA Taq polymerase (1.25 U/μl, TaKaRa Bio) were mixed with the first-strand cDNAs and the mixture was incubated at 94°C for 1 min, 45°C for 5 min, 50°C for 5 min, 60°C for 5 min, 65°C for 5 min, 72°C for 4 min, and 94°C for 1 min. Subsequently, PCR was performed in the presence of the KSII primer (5’-GGAGTGCCAGGTTATCGAATAAGCA----------------A-3’) and the cCry4-R 5’-RACE primer (5’-AGAAGATCCGTGTATGGTG-3’; TG-3’) under the following conditions: 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min for 30 cycles. The cCry4-R 5’-RACE primer was designed based on the EST-sequence for an internal part of cCry4 (GenBank accession no. BU282778). Amplified products were cloned in TA-cloning vectors (Invitrogen) and sequenced. To isolate cDNA clones encoding the entire coding sequence of cCry4 (GenBank accession no. AY300013), cCry4-F-const primer (5’-GGAGGCGGGGAGCCTCAG-3’) and cCry4-R-const primer (5’-AGACACAGCTCTCTGTG-3’) were designed in 5’ and 3’ untranslated regions, respectively, and PCR was performed with the chick pineal cDNA template under the following conditions: 94°C for 30 s, 60°C for 60 s, and 72°C for 150 s for 18 cycles. The resultant PCR products were cloned in the TA-cloning vector, and the four independent clones were sequenced to exclude PCR errors.

Quantitative RT-PCR analysis

Total RNA was isolated from several chick tissues or cultured chick pineal cells (Hirota et al. 2001) using TRIZol Reagent (Invitrogen). Quantitative RT-PCR analyses were performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with the aid of the QuantiTect SYBR Green RT-PCR kit (Qiagen, Tokyo, Japan). Primers used in the analyses were as follows: for cCry1, 5’-ACTTAGTCTCGCATGCTGTG-3’ (cCry1SybrF1) and 5’-AAGAGCTCTCCTCCTCTCC-3’ (cCry1SybrR1); for cCry2, 5’-ACCTGGGAAGAAGAGGCGG-3’ (cCry2SybrF1) and 5’-AACGCCAAAGCAGGAGTACC-3’ (cCry2SybrR1); for cCry4, 5’-GGAGGCGGATGAAGGTGTTGA-3’ (cCry4SybrF1) and 5’-AACGGACCGGGGACAAATG-3’ (cCry4SybrR1); for cThp, 5’-CAACAGCAGTACCACAAAGATGAGTG-3’ (cThpSybrF1) and 5’-CCGTCAGGGGAATAGGCACTA-3’ (cThpSybrR1); and for cActin, 5’-GAGGAAATTTGTGTGACACATCA-3’ (cActinSybrF1) and 5’-CCTGAACCTTCTTATTGCA-3’ (cActinSybrR1). For relative quantification analysis, the fold change in expression was calculated by the 2⁻ΔΔCT method (Livak and Schmittgen 2001), see Fig. 1b) and

Statistical analysis

Data were analyzed with one-way ANOVA followed by post hoc comparisons (Tukey-Kramer test) for multiple groups using JMP5.1.1 software (SAS institute Japan, Tokyo, Japan).

Production of antibody to cCry4

Two kinds of fusion proteins were prepared: GST-DH and MBP-DH fusion proteins that are composed of the carboxyl-terminal region of cCry4 protein (DH region: Asp770-Thr792; see Fig. 1b) and
Fig. 1 cCry4 gene and cCRY4 protein. (a) Genomic organization of cCry4 gene. Boxes indicate exons. Open boxes indicate untranslated regions. Lengths of the exons (in bp) are shown above the boxes, and lengths of introns are shown below the horizontal line. Exon numbers are given in parentheses. (b) Alignment of zCRY4 and representative members of vertebrate CRYs and zebrafish (6–4) photolyase. Arrowheads depict the exon-intron junctions. Asterisks indicate the conserved three tryptophans possibly important for intramolecular electron transfer. Pluses indicate amino acid positions where amino acids act in the chromophore FAD-binding in Syn-echocystis CRY-DASH (Brudler et al. 2003). The α/β domain and helical domain deduced from the crystallographic structure of Syn-echocystis CRY-DASH (Brudler et al. 2003) are indicated. The 60 amino acid DH region used as an antigen for producing the anti-cCRY4 antibody is drawn in white on black. (c) A phylogenetic tree of CRY family proteins constructed by Neighbour-Joining method (Saitou and Nei 1987) using PHYLIP 3.572 software (Felsenstein 1996). Amino acid sequences in the conserved region of CRYs/photolyases (corresponding to Arg10-Ile427 in cCRY4) were aligned using Clustal W software at the DNA Data Bank of Japan. Bootstrap probabilities (p) are represented by closed circles on the nodes (p > 95%) or by values near the nodes.

© 2006 The Authors
glutathione S-transferase (GST) or maltose-binding protein (MBP), respectively. These fusion proteins were expressed in the Escherichia coli BL21 strain, and purified by affinity column chromatography as described (Okano and Fukada 2000). Eight-week-old female BALB/c mice were injected intraperitoneally with 50 μg of the GST-DH protein emulsified in a 1:1 mixture of complete Freund’s adjuvant (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and PBS [10 mM Na-phosphate (pH 7.4), 140 mM NaCl, 1 mM MgCl₂]. Seven weeks after injection, a boost was given with 50 μg of the purified GST-DH protein in PBS. Antibodies in the antisera were purified by affinity column chromatography using the HiTrap NHS-Sepharose column (Amersham Biosciences, Piscataway, NJ, USA), to which MBP-DH was immobilized.

Plasmid construction and transfection

cCry1, cCry2, and cCry4 cDNAs were subcloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen). A histidine tag and a 1D4 tag (the carboxyl terminal 8 amino acids of bovine rhodopsin; ETSQVAPA; MaKenzie et al. 1984) were added at the amino terminus and the carboxyl terminus of the CRYs, respectively, and the tagged proteins were termed His6-cCRY1–1D4, His6-cCRY2–1D4, and His6-cCRY4–1D4. HEK293 cells obtained from the RIKEN Cell Bank (RCB1637) were transfected with the expression plasmids using Lipofectamine and Plus Reagent (Invitrogen) according to the manufacturer’s instructions. The cells were collected 24 hours after transfection.

Immunoblot analysis

Chick pineal glands were homogenized using a Potter-type Teflon homogenizer with buffer A [10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, and one tablet per 50 mL of a protease inhibitor tablet (Complete EDTA-Free, Roche, Basel, Switzerland)], and the homogenate was used as ‘pineal homogenate’. For fractionation of the pineal proteins, freshly dissected chick pineal glands were homogenized with buffer A using a Dounce-type glass homogenizer, and centrifuged at 1000 C for 30 min at 4°C. The pellet was suspended in buffer A using a Dounce-type glass homogenizer, and centrifuged at 10 000 g for 10 min at 4°C. The supernatant was further centrifuged at 20 000 g for 30 min at 4°C, and the resultant supernatant was used as the ‘soluble fraction’. The pellet was suspended in buffer A (the ‘membrane fraction’). Samples were subjected to SDS-polyacrylamide (10%) gel electrophoresis, followed by electroblotting onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA). The membrane was incubated for 1 h in 1% Block Ace (Dainippon Pharmaceutical, Osaka, Japan), and incubated with a DH antibody diluted in 0.4% Block Ace for 24–36 h at 4°C. Subsequently, the membrane was washed with 0.4% Block Ace and incubated with an alkaline phosphatase-linked anti-mouse IgG antibody (1:1000 dilution, New England Biolabs, Beverly, MA, USA) in 0.4% Block Ace for 24 h. Signals were detected using CDP-Star Reagent (New England Biolabs).

Immunocytochemistry

The pineal glands were isolated from 2-week-old chickens that had been housed under an LD cycle for more than 10 days, and were fixed with Zamboni’s fixative at 4°C for 4 h. Ten-μm thick pineal sections were prepared as described previously (Kasahara et al. 2000). The sections were treated with a blocking solution (PBS containing 0.05% Tween 20 and 1.5% horse normal serum) for 2 h at room temperature, and then incubated for 48 h at 4°C with the primary antibody diluted in blocking solution. After washing sections with the blocking solution at room temperature, the sections were successively incubated with a biotinylated anti-mouse IgG and avidin-biotin complex solution using the Vectastain Elite ABC kit (Vector laboratory, Burlingame, CA, USA). Positive signals were visualized by incubating the slides for 5 min in 0.1 m Tris-HCl (pH 7.2) containing 0.1% diaminobenzidine and 0.02% H₂O₂.

Transcriptional assay

HEK293 cells were plated at 1.0 × 10⁵ cells per well in 24-well plates. Twenty-four hours later, the cells were transfected using Lipofectamine and Plus Reagent (Invitrogen) with various reporter and expression vectors. Forty-eight hours after transfection, cell extracts were subjected to dual-luciferase assays by luminometry (Promega) which were done according to the manufacturer’s protocol. Firefly luciferase activity was normalized by Renilla luciferase activity for each cell extract.

Results

Cloning of cCry4

During the 5’ RACE analysis of chicken pineal cDNAs, we isolated and sequenced several cDNA clones containing the 5’ untranslated region of cCry4. A comparison of these nucleotide sequences with the genomic sequence of the cCry4 gene in the Ensembl Chicken Genome Database enabled us to determine the putative translation initiation site of cCry4. We also cloned cDNAs covering the entire coding sequence of cCRY4 to deduce the amino acid sequence (see below) and genomic organization of the cCry4 gene. A possible translation initiation site was found in the second exon, and the coding sequence was divided into 10 exons (Fig. 1a and b). The polyadenylation site(s) for cCry4 transcripts could not be determined because in this study we did not clone cDNAs covering the 3′ untranslated region of cCry4.

Phylogenetic analysis

To elucidate the molecular identity and evolutionary background of cCRY4, we searched for Cry/photolyase genes in public databases but did not come up with any cCry4 orthologs in vertebrates other than zebrafish. We did, however, find novel mammalian Cry1/2 orthologs in insects (tentatively termed honeybee CRY-V [Ensembl honeybee genome database, ENSAPMP0000010049] and mosquito CRY-V [Ensembl mosquito genome database, ENSANGP0000013228] for their ‘vertebrate-type’ structures). By adding these sequences to the amino acid sequences of the conserved region, we constructed a phylogenetic tree of CRYs/photolyases using the Neighbor-Joining method (Fig. 1c). The tree shows how cCRY4 and zCRY4 constituted a single phylogenetic group (CRY4 containing...
group) that was of a different lineage than the other groups of CRYs/photolyases such as the negative regulators of the circadian clock (vertebrate CRY1/2), vertebrate-type cryptochromes of unknown function in insects (insect CRY-V) (6–4) photolyases, and circadian photoreceptors in insects (insect CRY).

cCry4 mRNA levels in various chick tissues
Initially, using RT-PCR, we investigated and compared temporal changes in the levels of cCry mRNAs in various chick tissues under LD conditions in order to gain a better understanding of the cCRY4 circadian properties (Fig. 2). cCry4 mRNA levels showed a statistically significant oscillation in the pineal gland (Fig. 2a; \( p < 0.001 \) in ANOVA) and the kidney (Fig. 2g; \( p = 0.012 \)), with a peak at ZT12 and ZT0, respectively. In the other tissues examined, cCry4 mRNA levels remained fairly constant (Figs 2b–f, h; \( p = 0.053, 0.314, 0.241, 0.541, 0.914, 0.134, \) and 0.693 in the retina, cerebrum, mesencephalon plus diencephalon, cerebellum, skin, liver, and heart, respectively), while cCry1 mRNA levels oscillated in most of the examined tissues (Fig. 2a–i; \( p < 0.001 \)), with peaks at ZT6 or ZT12, except for the cerebellum (\( p = 0.001 \)) and heart (\( p = 0.077 \)). cCry2 mRNA levels also significantly oscillated in several of the tissues examined (Fig. 2a–i; \( p < 0.001, p = 0.098, 0.022, 0.050, 0.725, 0.012, 0.008, 0.007 \) and 0.536 in the pineal gland, retina, cerebrum, mesencephalon plus diencephalon, cerebellum, skin, kidney, liver, and heart, respectively. We also measured levels of a housekeeping gene, chicken beta-actin (cActin; Fig. 2a–i; \( p < 0.001 \)), to confirm the tissue- and/or gene-specificity of the cCry mRNA oscillations. The ratios between mRNA levels of cActin and cTbp did not signify robust oscillation in all of the tissues examined, although variations of the cActin/cTbp ratios were statistically significant in some tissues (Fig. 2).

cCry4 mRNA levels in cultured chick pineal cells
Since cultured chicken pineal cells are known to retain clock function and photosensitivity (Deguchi 1979; Kasal et al. 1979), we investigated cCry4 mRNA levels in cultured chicken pineal cells under LD and constant dark (DD) conditions to determine whether the cCry4 mRNA level is controlled by a circadian clock and/or by light. In DD, the...

Fig. 2 Temporal changes in mRNA levels of Cry genes in nine chicken tissues as estimated by quantitative RT-PCR. Each tissue (\( n = 3 \)) was collected at ZT0, 6, 12 and 18. The mRNA level of each Cry was calculated as a value relative to that of cTbp in each sample (mean + standard error of mean [SEM]). MC + DC, mesencephalon plus diencephalon. By the Tukey-Kramer test, there was no significant difference in the mesencephalon plus diencephalon). \( * p < 0.05, **p < 0.01. \)
The mRNA level showed an approximately 2-fold circadian rhythmic change (p < 0.001), with a peak during early subjective day (Fig. 3, ◆), indicating that cCRY4 mRNA expression was driven by an endogenous circadian clock. In LD, the cCRY4 mRNA level gradually increased during the daytime, peaked during early night, and then decreased within 4 h (ZT18; Fig. 3, ○). A comparison of the cCRY4 mRNA profiles in LD and DD indicated that the cCRY4 mRNA level was up-regulated by light captured in the pineal cells in LD (p < 0.001, LD vs. DD in ANOVA).

Detection of cCRY4 protein

To examine cCRY4 protein expression in the chick pineal gland, an anti-cCRY4 antibody was raised against the carboxyl-terminal 60-amino acid region of cCRY4 (DH region, see Fig. 1b). The produced antibody (termed DH antibody) reacted with His- and 1D4-tagged cCRY4 (His6-cCRY4–1D4, Fig. 4a, lane 8 and supplementary Figure S1) and with native full-length cCRY4 (Fig. 4a, lane 9) expressed transiently in HEK293 cells. The molecular masses estimated from the main band mobility correlated well with the calculated molecular masses of His6-cCRY4–1D4 and cCRY4 (62.5 and 61.0 kDa, respectively). In addition, the DH antibody detected three minor bands, which were likely due to the degradation of cCRY4 in HEK293 cells. His6-cCRY1–1D4 and His6-cCRY2–1D4 were also expressed in HEK293 cells (Fig. 4a, lane 1 and 2) to confirm that the DH antibody showed no crossreactivity with cCRY1 or cCRY2 (Fig. 4a, lanes 6, 7 and supplementary Figure S1).

By using the anti-cCRY4 DH antibody, we could investigate the in vivo expression of cCRY4 through immunoblot analysis of the chick pineal proteins (Fig. 4b) and immunocytochemical analyses of the chick pineal gland (Figs 5 and 6, see below). Chick pineal glands collected both during the day (ZT6) and night (ZT18) were combined and subjected to fractionation into nuclear, membrane-bound, and soluble fractions. The DH antibody detected a strong immunoreactive signal in the soluble fraction (Fig. 4b, lane 8) and a very weak one in the nuclear fraction (lane 6). Mobility of these positive bands was very close to that of cCRY4 expressed in HEK293 cells (Fig. 4b, lane 9), although the protein bands derived from the pineal glands were apparently downshifted possibly due to protein overload.

Variation of cCRY4 protein levels in the chick pineal gland

To see whether the diurnal change in cCRY4 mRNA level (Fig. 2a) influenced its protein level, we quantified the levels of cCRY4 proteins in the total homogenate of the chick pineal gland every 6 h under LD conditions (Fig. 4c). There was a significant daily change (p = 0.003 in ANOVA), with a profile very similar to that of cCRY4 mRNA which also peaked at ZT12.

Localization of cCRY4-immunoreactivity in the chick pineal gland

A number of follicles exist in the chick pineal parenchyma that can be divided into two zones, the follicular and parafollicular zones (Boya and Calvo 1980). In the follicular zone, pinealocytes are radially arranged around each follicle so that outer segment-like structures of the pinealocytes are located in the luminal space of the follicle. On the other hand, in the parafollicular zone, pinealocytes show no clear polarization. Consistent with our previous report (Okano et al. 1997), pinopsin-immunopositive outer segment-like structures were observed in both the follicular and parafollicular zones (F and PF in Fig. 5b, respectively), while there was no pinopsin-immunopositive signal observed in the pineal stroma (interfollicular zone, IF in Fig. 5b). Immunostaining with the DH antibody revealed that cCRY4-immunopositive cells existed in all three zones. In the follicular and parafollicular zones, the cCRY4-immunopositive signals were widely spread across the parenchyma (Fig. 5a), and there was no difference in immunostaining between the follicular and parafollicular zones. As seen at higher magnification (Fig. 5d), distribution of the immunopositive structures was so variable that it was difficult to
determine intracellular localization of the cCRY4-immunopositive signals in the pineal parenchyma. In the interfollicular zone, cCRY4-immunopositive cells with intense signals were observed (Fig. 6), although the number of immunopositive cells was much fewer than the number of parenchymal immunopositive cells. Interestingly, the interfollicular immunopositive signals were visualized as small dot- and string-like structures of semi circular or circular arrangement in the pericellular region (Fig. 6a, arrowheads and 6d). In the chick pineal sections collected at ZT6 and shown in Figs 5 and 6, the distribution of cCRY4 immuno-reactivity was indistinguishable from the sections collected at other time points (ZT0, ZT12, ZT18; not shown).

**Effect of cCRY4 on cCLOCK : cBMAL-mediated transciptional activation**

We performed a transcriptional assay to examine the effect of cCRY4 on transcriptional activation from the cPer2 E-box element (Fig. 7). cCRY4 showed no inhibitory effect, while cCRY1 and cCRY2 displayed strong negative effects under the same conditions.

**Discussion**

The deduced amino acid sequence of cCRY4 is composed of 529 residues and has the highest sequence identity (62%) with zCRY4 and a relatively low sequence identity (44–46%) with mammalian CRY1 and CRY2 (Fig. 1b). Analyses of crystallographic structures of CRYs/photolyases (Brudler et al. 2003; Brautigam et al. 2004) reveal that CRYs/photolyases share conserved structures and that their polypeptides can be separated into four regions: the α/β domain, the interdomain region between the α/β domain and helical domain, the helical domain, and the carboxyl-terminal domain (Fig. 1b). Among...
In these domains, the α/β domain is predicted to contain α-helices and β-sheets, whereas the helical domain is likely enriched with α-helices without β-sheets (Brudler et al. 2003; Brautigam et al. 2004). The helical domain is considered to contribute to the binding of the chromophore FAD (Fig. 1b), and the amino acid sequences in this domain are highly conserved among CRYs/photolyases, including cCRY4. In the helical domain of cCRY4, the amino acid residues that possibly form the chromophore-binding site (pluses in Fig. 1b) and the three tryptophan residues for intraprotein electron transfer (asterisks in Fig. 1b; Brudler et al. 2003) are conserved. In the carboxyl-terminal region, CRYs/photolyases have highly diverged amino acid sequences, and this diversity has been mentioned in relation to subfamily-specific functions among the CRYs/photolyases (Yang et al. 2000; Busza et al. 2004). cCRY4 has a consistent, highly diverged carboxyl-terminal domain (Fig. 1b) suggestive of unique molecular function.

**Regulation of cCry4 gene expression**

The oscillation of cCry1 and cCry2 mRNA levels in most tissues (Fig. 2) is highly consistent in that not only central...
mRNA oscillation is essential for circadian clock might be transcriptionally regulated in a gene, numbered by a putative transcription start site

cPer2 mRNA expression. E-box elements. Fifty nanograms of a cCRY expression vector were mixed. Total amount of plasmids was then adjusted to a total of 450.2 ng per well by adding pcDNA3.1/V5/His empty vector (invitrogen). *p < 0.01 (Student’s t-test).

but also peripheral tissues possess circadian clocks where cCry1 and cCry2 play a key role as common negative regulators (Schibler 2000). Concerning cCry4, however, we found in this study that mRNA levels oscillated only in limited tissues (Fig. 2), which makes it difficult to assume that cCry4 mRNA oscillation is essential for circadian clock oscillation. As was seen in the pineal gland, cCry4 mRNA levels were also higher in the retina, the other photosensitive clock structure (Iuvone et al. 1997), implying a possible circadian role for cCry4 in the photoreceptive clock tissues.

We found certain similarities between cCry4 and the clock gene cPer2. The expression profile of cCry4 mRNA in cultured chick pineal cells maintained under DD conditions (Fig. 3) was similar to that of cPer2, which also peaks at CT2 (Okano et al. 2001), but different from other clock genes such as cCry1, cCry2, and cPer3, which peak at CT10, CT22, and CT22, respectively (Yamamoto et al. 2001). Therefore, cCry4 might be transcriptionally regulated in a manner similar to cPer2.

In the cPer2 promoter/enhancer region, we previously identified a CACGTG E-box element and an E4BP4-binding site through which the cCLOCK:cBMAL complex and cE4BP4 regulate cPer2 transcription, respectively (Doi et al. 2001; Okano et al. 2001). Searching E-box elements in the cCry4 gene highlighted an 8-bp E-box-containing sequence that is also conserved in the cPer2 promoter/enhancer region (ACACGTGA at +1223 to +1228 in the first intron of the cCry4 gene, numbered by a putative transcription start site as +1). A putative E4BP4-binding site was also found in the first intron (GTTATTAAT at +736 to +745). Notably, many copies of cyclic-AMP responsive element (CRE), which may act as a light responsive element in the mouse Per1 promoter (Travnickova-Bendova et al. 2002), exist near the putative transcription initiation site of cCry4. Future evaluation of these putative enhancer elements may help to further characterize the transcriptional control of cCry4.

In the kidney, the relative mRNA level of cCry4 was higher than in other peripheral tissues and oscillated with a phase quite different from that in the pineal gland. These properties imply that direct photoreception occurs in the kidney in chickens, as was previously found in zebrafish (Whitmore et al. 2000), and also suggest a kidney-specific mechanism that regulates cCry4 mRNA expression.

**Function of cCry4**

Phylogenetic analysis (Fig. 1c) indicated that cCry4 and zCry4 form a novel subfamily. Because the CRY/photolyase family includes subfamilies, each of which may have a subfamily specific function, it is difficult to speculate on what type of CRY4-specific function would be unique yet common to cCry4 and zCry4. zCry4 has been cloned from adult zebrafish RNA and its transcripts detected in the eye and brain by Northern blotting (Kobayashi et al. 2000). Functional analyses of zCry4 showed that there is no DNA photolyase activity nor is there a negative effect on E-box-mediated transactivation by zCLOCK:zBMAL (Kobayashi et al. 2000). Similar to the zCry4 results, we found that cCry4 had no effect on cCLOCK:cBMAL1-dependent transactivation from the cPer2-E-box element in HEK293 cells (Fig. 7). Thus, members of the CRY4 subfamily may have a unique function that does not include the circadian transcriptional repression of clock-controlled genes or the photorepair of UV-induced DNA damage. Molecularly, CRY4-subfamily members seem to retain the common structure required for blue-light photoreception, leading us to speculate that CRY4 might mediate photoreception for the circadian clock or an unknown mechanism.

**Light-dependent daily variation of cCry4 protein level in the chick pineal gland**

Immunoblot analysis of chick pineal proteins revealed a daily variation in cCry4 protein (Fig. 4c). This variation contrasts sharply with the nearly constant protein expression of pinopsin, a gene whose mRNA expression is also regulated by light (Takanaka et al. 1998, 2002). In this study, we found that the protein level of cCry4, as estimated by immunoblot analysis (1.5–4 ng/gland, Fig. 4c), was comparable to that of pinopsin (approximately 2 ng/gland, Takanaka et al. 1998). Thus, the pineal gland may have two photoreceptive
cascades for blue light reception that are mediated by cCRY4 and pinopsin, whose dominance might switch in a light- or time-dependent manner.

We also speculate that the oscillation of cCRY4 protein level (Fig. 4c) might reflect a possible phase-dependent variation in light sensitivity by the pineal circadian oscillator. Such a mechanism could act as a ‘Zeitnehmer’ (McWatters et al. 2000) in the circadian photoreception by contributing to a phase-dependent effect of light on the circadian clock. Alternatively, light-dependent cCRY4 protein accumulation might work as an hourglass timer to measure the duration of the light period.

**CRY4 and other circadian photoreceptor candidates**

Melanopsin/OPN4 has been identified as an important photoreceptive molecule for photoentrainment of the circadian oscillator and for the pupillary light response in mammals (Panda et al. 2002; Ruby et al. 2002; Hattar et al. 2003; Panda et al. 2003). For example, mammalian melanopsin/OPN4 was recently shown to activate the Gq/11 pathway (Panda et al. 2002; Qiu et al. 2005). Also, in the chicken, G11 was found to be involved in photoentrainment of the chicken pineal circadian oscillator (Kasahara et al. 2002), and the expression of melanopsin/OPN4 transcripts was detected in the chicken pineal gland and retina (Chaurasia et al. 2005; Holthues et al. 2005; Tomonari et al. 2005). These findings taken together strongly suggest that a melanopsin-G11 pathway is involved in pineal photoentrainment.

In addition, there are also reports of other vertebrate genes encoding putative opsins such as encephalopsin (Blackshaw and Snyder 1999), TMT opsin (Moutsaki et al. 2003), and Neuropsin/opn5 (Tarttelin et al. 2003), although nothing has been detected at protein levels. These less characterized opsins are additional candidates for circadian photoreceptor(s) in the chick pineal gland; however, the photoentrainment pathway in the pineal gland is resistant to the depletion of retinoids (Zatz 1994). Hence, CRY using a FAD chromophore would prove a more plausible candidate for a photoreceptor in the retinoid-resistant pathway [possibly in parallel with the opsin(s)-G11 pathway].

The present study revealed a soluble nature (Fig. 4b) and a diurnal expression of cCRY4 protein in the pineal gland (Fig. 4c), features that differ from those of pinopsin, a constantly expressed membrane protein (Okano et al. 1997; Takanaka et al. 1998). cCRY4 and pinopsin also showed quite different distribution patterns in the pineal gland (Figs 5 and 6). Such a striking contrast seems to suggest a possible temporal and/or spatial duplicity of the pineal photoreceptive system, the opsin- and CRY-based mechanisms.

To date, phototransduction mechanisms via CRY have been studied extensively in Arabidopsis and Drosophila (Yang et al. 2000; Wang et al. 2001; Sancar 2003; Busza et al. 2004). Recently, CRY1 and CRY2 have been suggested to play a role in the pupillary light response in mice and chicks (van Gelder et al. 2003; Tu et al. 2004), although there is no direct evidence for the photosensitivity of vertebrate CRYs. The present identification and initial characterization of CRY4 in the chicken pineal gland may enable the development of not only a more detailed characterization of this photoreceptor candidate, but also an approach to a novel CRY-mediated phototransduction pathway in vertebrates.

**Acknowledgements**

We are grateful to Dr Robert S. Molday (University British Columbia) for providing 1D4 monoclonal antibody. This work was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Science, Sports and Culture.

**Supplementary material**

The following supplementary material is available for this article online.

Figure S1. Fluorescent immunostaining of CRY-expressing cells. This material is available as part of the online article from http://www.blackwell-synergy.com

**References**


© 2006 The Authors


