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

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Abstract

In non-mammalian vertebrates, the pineal gland contains an endogenous circadian oscillator and serves as a photosensitive neuroendocrinal 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.
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The non-mammalian pineal gland is a photosensitive neuroendocrinal 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

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Abbreviations used: CRY, cryptochrome; CT, circadian time; DD, constant dark; GST, glutathione *S*-transferase; LD, 12 h light : 12 h dark; MBP, maltose-binding protein; ZT, Zeitgeber time.

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; ~450μW/cm^2 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 TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). An oligo-dT $(dT)_{16}$ 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 mM dTTP, 100 mM potassium cacodylate, 2 mM $CoCl_2$, 0.2 mM DTT, and 0.8 U/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, KSII$(dA)_{25}$ primer (5′-GAGGTCGACGGTATCGATAAGCAAAAAAAAAAAAAAAAAAAAAAAA-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, 55°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′-GAGGTCGACGGTATCGATAAGC-3′) and the cCry4-R 5′-RACE primer (5′-AGAAGATCCGTGTGTAGTGG 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′-GGAGCCGGGAGCTCAG-3′) and cCry4-R-const primer (5′-AGCACAGCTCCTCCTGCTC-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′-ACTTAGCTCGGCATGCTGTG-3′ (cCry1SybrF1) and 5′-CAAAGACCTTCATTCCTTCTTCC-3′ (cCry1SybrR1); for *cCry2*, 5′-ACACTTGGAAAGAAAGGCATGG-3′ (cCry2SybrF1) and 5′-AACAGCCAAAACGCAGGTAGG-3′ (cCry2SybrR1); for *cCry4*, 5′-GGAGGGCATGAAGGTGTTTGA-3′ (cCry4SybrF1) and 5′-AACGGACCGGGCAGAAGAT-3′ (cCry4SybrR1); for *cTbp*, 5′-GCACAGCAGTAACAAGAGATGGA-3′ (cTBP-TF) and 5′-GCGTCAGGGAAATAGGCACTAA-3′ (cTBP-TR); for *cActin,* 5′- GAGAAATTGTGCGTGACATCA-3′ (ActinF1) and 5′-CCTGAACCTCTCATTGCCA-3′ (ActinR1); for *cGapdh*, 5′-ACCACTGTCCATGCCATCAC-3′ (cGapdhF1) and 5′-TCCAC AACACGGTTGCTGTA-3′ (cGapdhR1).

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: Asp^{470}-Thr^{529}, see Fig. 1b) and

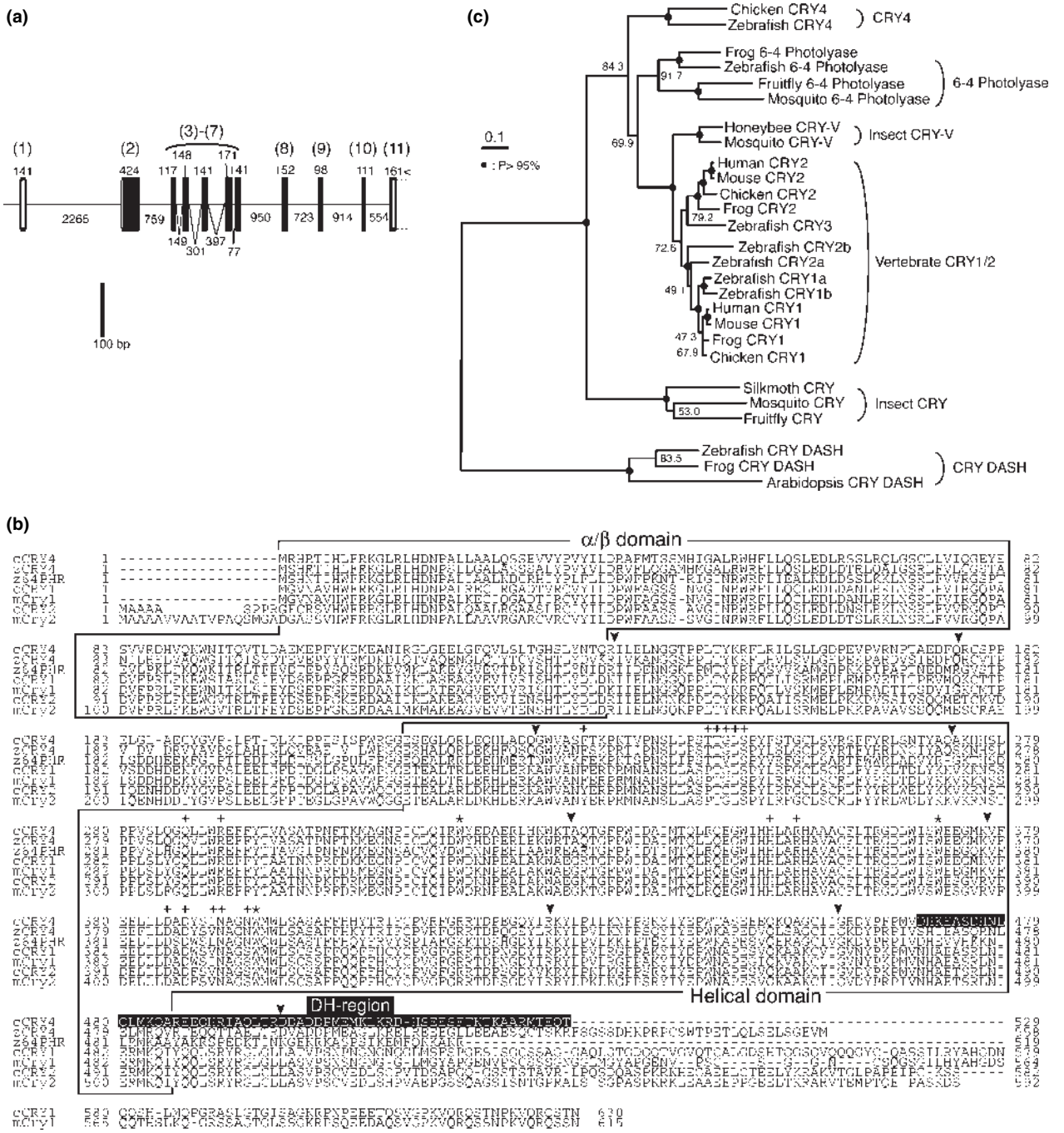

Fig. 1 *cCry4* gene and cCRY4 protein. (a) Genomic organization of *cCry4* gene. Boxes indicate exons. Open boxes indicate untranslated regions. Lengths of the exons (in bp) are shown above the boxes, and lengths of introns are shown below the horizontal line. Exon numbers are given in parentheses. (b) 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 *Synechocystis* CRY-DASH (Brudler *et al.* 2003). The α/β domain and helical domain deduced from the crystallographic structure of *Synechocystis* 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 Arg^{10}-Ile^{427} 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.

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 $MgCl_2$]. 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; MacKenzie *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 $MgCl_2$, 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 ***g*** for 10 min at 4°C. The pellet was then suspended in buffer A and used as the 'nuclear fraction'. 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 4% 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% H_2O_2.

Transcriptional assay

HEK293 cells were plated at 1.0×10^5 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, ENSAPMP00000010049] and mosquito CRY-V [Ensembl mosquito genome database, ENSANGP00000013228] 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 Neighbour-Joining method (Fig. 1c). The tree shows how cCRY4 and zCRY4 constituted a single phylogenetic group (CRY4

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, i; $p =$ 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‴), 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

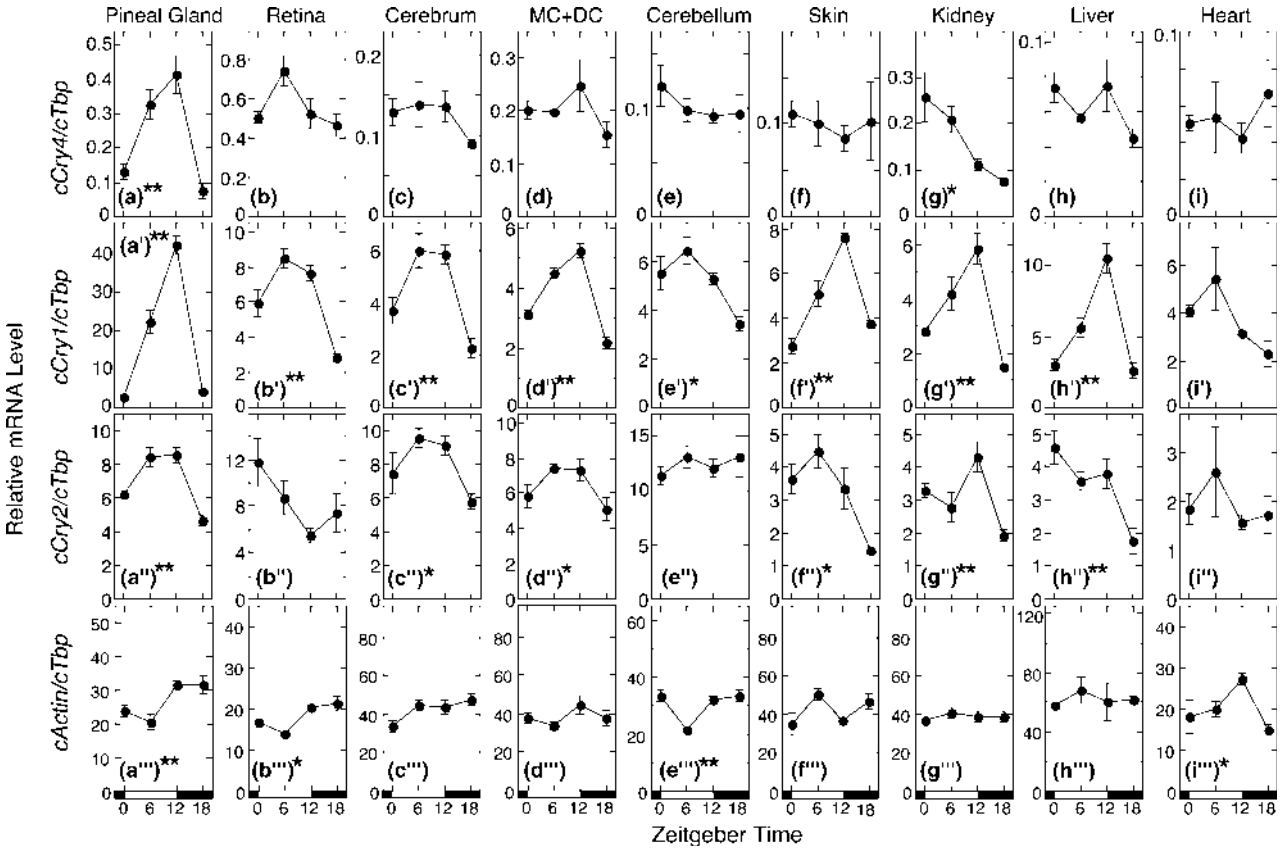

Fig. 2 Temporal changes in mRNA levels of *Cry* genes in nine chicken tissues as estimated by quantitative RT-PCR. Each tissue ($n = 3$) was collected at ZT0, 6, 12 and 18. The mRNA level of each *Cry* was calculated as a value relative to that of *cTbp* in each sample (mean + standard error of mean [SEM]). MC + DC, mesencephalon plus diencephalon. By the Tukey-Kramer test, there was no significant difference in the mesencephalon plus diencephalon). $^{*}p < 0.05$, $^{**}p < 0.01$.

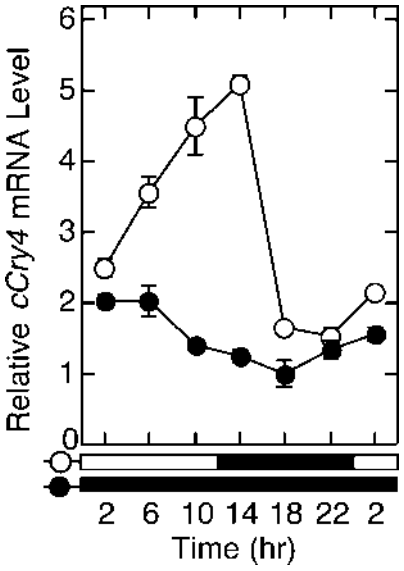

Fig. 3 Temporal variation of *cCry4* mRNA levels in cultured chicken pineal cells. The pineal cells isolated from 1-day-old male chicks were plated on a 12-well plate-dish on day 1, then cultured under LD conditions for 5 days (two glands per dish). On day 6, one group of the culture plates was transferred to constant darkness (closed circles), while the other was kept in LD (open circles). The cells were harvested every 4 h on days 6–7. The mRNA levels of *cCry4* and *cGapdh* were estimated by quantitative RT-PCR, and mRNA levels of *cCry4* were calculated as the value relative to that of *cGapdh* in each sample [mean + SEM from two (LD) or three (DD) independent cultures]. The minimum value for LD and DD was set to 1.

cCry4 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

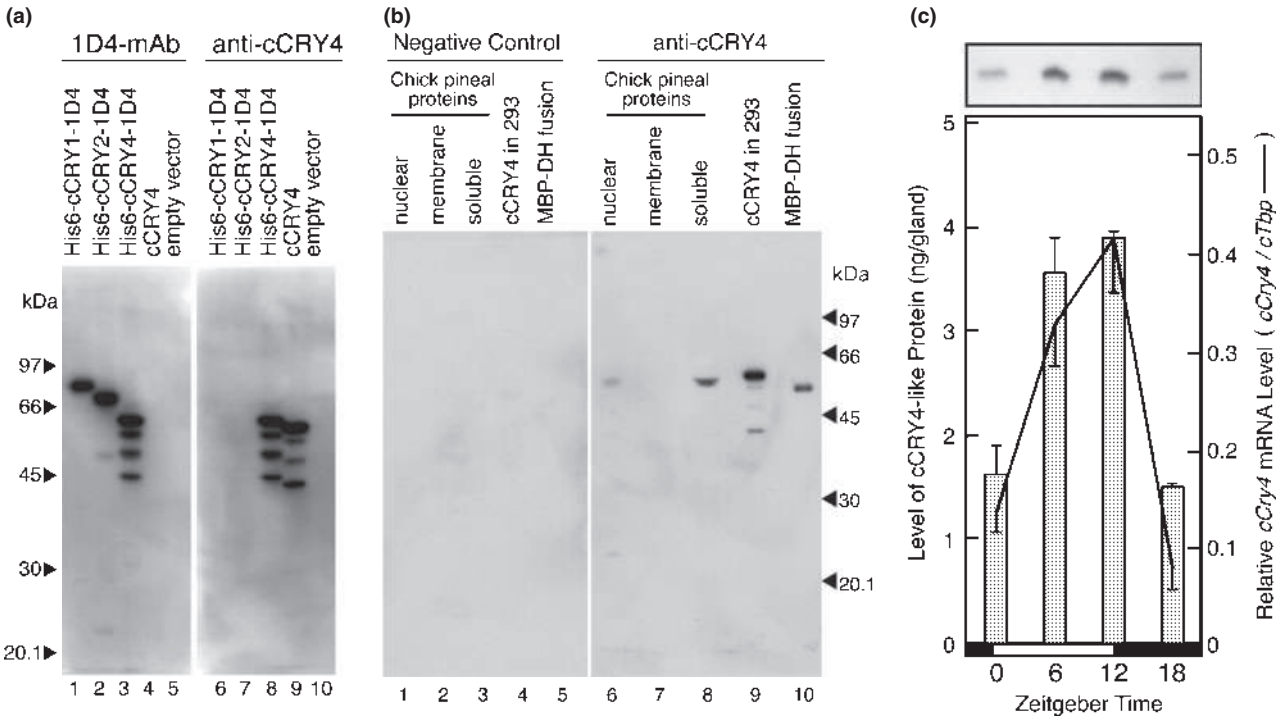

Fig. 4 Immunoblot analysis of cCRY4. (a) Immunoblot detection of cCRY4 expressed in HEK293 cells. The DH antibody recognized cCRY4 but not cCRY1 or cCRY2. HEK293 cells were transfected with an expression vector for tagged cCRY1/2/4 or native full-length cCRY4, or by an empty vector (pcDNA3.1). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with a DH antibody (0.04 μg/mL) or 1D4 antibody (1.4 μg/mL). (b) Immunoblot analysis of chick pineal proteins using an anti-cCRY4 antibody. Chick pineal proteins were fractionated into nuclear, membrane, and cytoplasm soluble fractions. Then the fractions were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with a DH antibody (0.08 μg/mL) or mouse immunoglobulin (0.08 μg/mL). Each lane contains fractionated proteins equivalent to 0.4 in the pineal gland. (c) Temporal variation of levels of cCRY4-like immunoreactivity in the pineal gland in LD. Chick pineal homogenates were subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting with a DH antibody. A representative blotting image is shown in the upper panel. Bars in the lower panel represent levels of cCRY4-like immunoreactivity (mean + SEM, $n = 3$) estimated by comparing their signals with those of various amounts of MBP-DH fusion proteins (0.5–8.0 ng, not shown). Proteins equivalent to those from one pineal gland were loaded in each lane. For comparison, the cCRY4 mRNA profile in LD was reproduced from Fig. 2(a). The protein level of cCRY4 in the pineal gland oscillated significantly ($p = 0.003$).

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 immunoreactivity was indistinguishable from the sections collected at other time points (ZT0, ZT12, ZT18; not shown).

Effect of cCRY4 on cCLOCK : cBMAL-mediated transcriptional 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

Structure of cCRY4

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

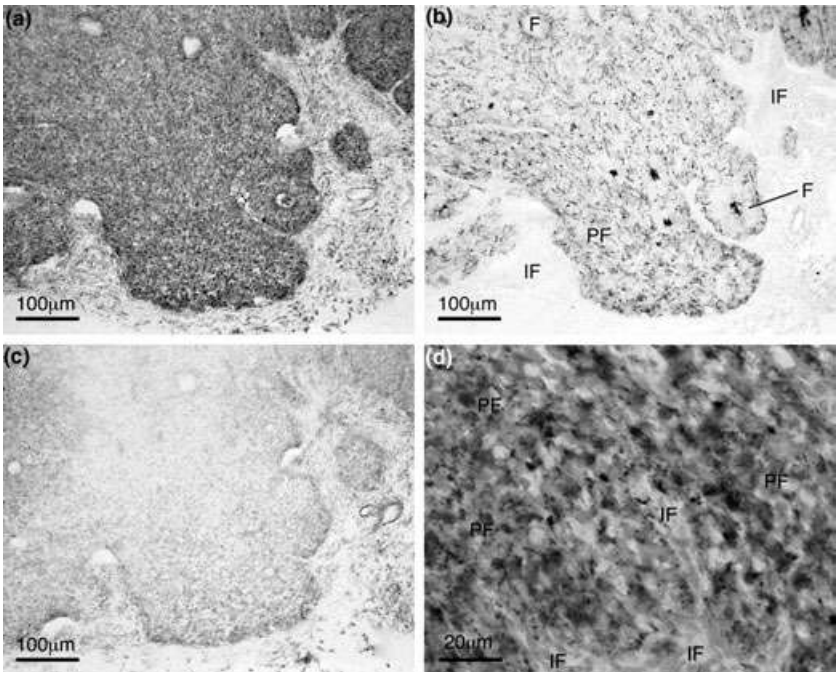

Fig. 5 cCRY4 immunoreactivities in the follicular and parafollicular zones of the chicken pineal gland. (a) cCRY4 immunoreactivities detected by a DH antibody (0.16 μg/mL). (b) Pinopsin immunoreactivities detected by a P1 anti-pinopsin antibody (2 μg/mL; Okano and Fukada 2001) in the section adjacent to that shown in (a). (c) A negative control section processed in the same way as in (a) except that mouse immunoglobulin (0.16 μg/mL) was used instead of a DH antibody. (d) A highly magnified view of the parafollicular and interfollicular zones. F, follicular zone; PF, parafollicular zone; IF, interfollicular zone.

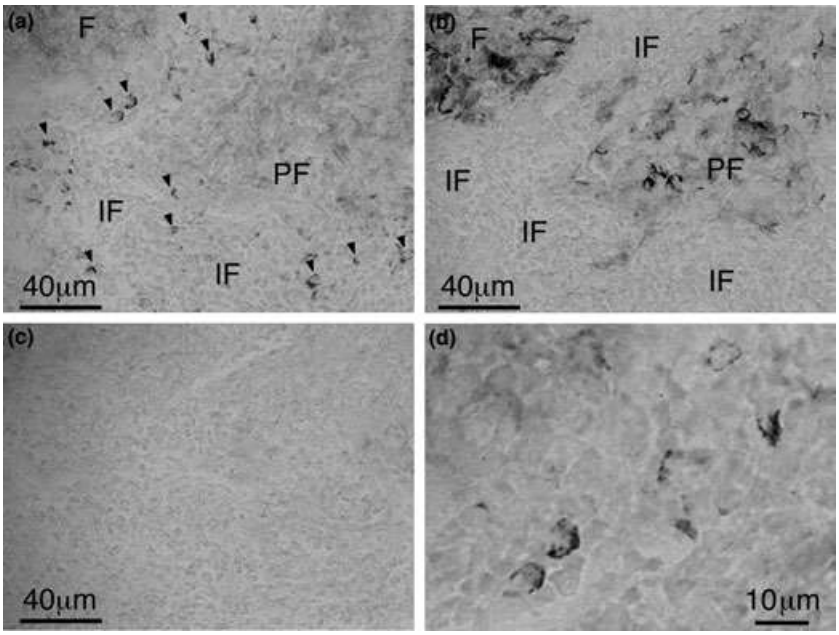

Fig. 6 cCRY4 immunoreactivities in the interfollicular zone of the chicken pineal gland. (a) cCRY4-immunoreactive cells (arrowheads) detected by a DH antibody (0.16 μg/mL). (b) Pinopsin immunoreactivities detected by P1 an anti-pinopsin antibody (2 μg/mL) in the section adjacent to that shown in (a). No pinopsin immunoreactivity was found in the interfollicular zone. (c) A negative control section processed in the same way as in (a) except that mouse immunoglobulin (0.16 μg/mL) was used instead of a DH antibody. (d) High magnification of the cCRY4-immunoreactive cells shown in (a). PF, parafollicular zone; IF, interfollicular zone.

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

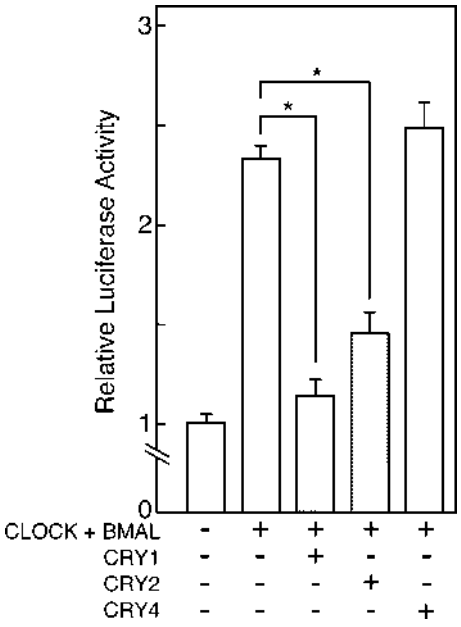

Fig. 7 Effects of cCRY on the CLOCK : BMAL1-mediated transcriptional activation from *cPer2* E-box elements. Fifty nanograms of a firefly luciferase reporter, *cPer2* E-box3-SV40 promoter (Doi *et al.* 2001), 0.2 ng of *Renilla* luciferase reporter, pRL-CMV as an internal control (Promega), 100 ng of cCLOCK expression vector (Okano *et al.* 2001), 10 ng of cBMAL1 vector (Okano *et al.* 2001), and 0 or 5 ng of 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∼ + 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 (GTTATTTAAT at + 736∼ + 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*. 2005; 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.

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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

Blackshaw S. and Snyder S. H. (1999) Encephalopsin: a novel mammalian extraretinal opsin discretely localized in the brain. *J. Neurosci.* **19**, 3681–3690.

Boya J. and Calvo J. (1980) Ultrastructural study of the post-hatching evolution of the pineal gland of the chicken *(Gallus Gallus). Acta Anat.* **107**, 143–168.

Brautigam C. A., Smith B. S., Ma Z., Palnitkar M., Tomchick D. R., Machius M. and Deisenhofer J. (2004) Structure of the photolyase-like domain of cryptochrome 1 from *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* **101**, 12142–12147.

Brudler R., Hitomi K., Daiyasu H. *et al.* (2003) Identification of a new cryptochrome class: structure, function, and evolution. *Mol. Cell* **11**, 59–67.

Busza A., Emery-Le M., Rosbash M. and Emery P. (2004) Roles of the two *Drosophila* CRYPTOCHROME structural domains in circadian photoreception. *Science* **304**, 1503–1505.

Cashmore A. R., Jarillo J. A., Wu Y. J. and Liu D. (1999) Cryptochromes: blue light receptors for plants and animals. *Science* **284**, 760–765.

Chaurasia S. S., Rollag M. D., Jiang G. *et al.* (2005) Molecular cloning, localization and circadian expression of chicken melanopsin (*Opn4)*: differential regulation of expression in pineal and retinal cell types. *J. Neurochem.* **92**, 158–170.

Deguchi T. (1979) A circadian oscillator in cultured cells of chicken pineal gland. *Nature* **282**, 94–96.

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.

Falcon J., Gothilf Y., Coon S. L., Boeuf G. and Klein D. C. (2003) Genetic, temporal and developmental differences between melatonin rhythm generating systems in the teleost fish pineal organ and retina. *J. Neuroendocrinol.* **15**, 378–382.

Felsenstein J. (1996) *Phylip*, Version 3.572. University of Washington, Seattle, WA, USA.

van Gelder R. N., Wee R., Lee J. A. and Tu D. C. (2003) Reduced pupillary light responses in mice lacking cryptochromes. *Science* **299**, 222.

Hattar S., Lucas R. J., Mrosovsky N. *et al.* (2003) Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. *Nature* **424**, 76–81.

Hirota T., Kagiwada S., Kasahara T., Okano T., Murata M. and Fukada Y. (2001) Effect of brefeldin A on melatonin secretion of chick pineal cells. *J. Biochem. (Tokyo)* **129**, 51–59.

Holthues H., Engel L., Spessert R. and Vollrath L. (2005) Circadian gene expression patterns of melanopsin and pinopsin in the chicken pineal gland. *Biochem. Biophys. Res. Commun.* **326**, 160–165.

Iuvone P. M., Bernard M., Alonso-Gomez. A., Greve P., Cassone V. M. and Klein D. C. (1997) Cellular and molecular regulation of serotonin N-acetyltransferase activity in chicken retina. *Biol. Signals* **6**, 217–224.

Kasahara T., Okano T., Haga T. and Fukada Y. (2002) Opsin-G11-mediated signaling pathway for photic entrainment of the chicken pineal circadian clock. *J. Neurosci.* **22**, 7321–7325.

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.

Kasal C. A., 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.

Kobayashi Y., Ishikawa T., Hirayama J. *et al.* (2000) Molecular analysis of zebrafish photolyase/cryptochrome family: two types of cryptochromes present in zebrafish. *Genes Cells* **5**, 725–738.

MacKenzie D., Arendt A., Hargrave P., McDowell J. H. and Molday R. S. (1984) Localization of binding sites for carboxyl terminal specific anti-rhodopsin monoclonal antibodies using synthetic peptides. *Biochemistry* **23**, 6544–6549.

McWatters H. G., Bastow R. M., Hall A. and Millar A. J. (2000) The ELF3 zeitnehmer regulates light signaling to the circadian clock. *Nature* **408**, 716–720.

Moutsaki P., Whitmore D., Bellingham J., Sakamoto K., David-Gray Z. K. and Foster R. G. (2003) Teleost multiple tissue (tmt) opsin: a candidate photopigment regulating the peripheral clocks of zebrafish? *Mol. Brain Res.* **112**, 135–145.

Nakamura A., Kojima D., Imai H., Terakita A., Okano T., Shichida Y. and Fukada Y. (1999) Chimeric nature of pinopsin between rod and cone visual pigments. *Biochemistry* **38**, 14 738–14 745.

Okano T. and Fukada Y. (2000) Photoreceptors in pineal gland and brain: cloning, localization, and overexpression. *Meth Enzymol.* **316**, 278–291.

Okano T. and Fukada Y. (2001) Photoreception and circadian clock system of the chicken pineal gland. *Microsc. Res. Techn* **53**, 72–80.

Okano T. and Fukada Y. (2003) Chicktacking pineal clock. *J. Biochem. (Tokyo)* **134**, 791–797.

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.

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.

Okano T., Yoshizawa T. and Fukada Y. (1994) Pinopsin is a chicken pineal photoreceptive molecule. *Nature* **372**, 94–97.

Panda S., Nayak S. K., Campo B., Walker J. R., Hogenesch J. B. and Jegla T. (2005) Illumination of the melanopsin signaling pathway. *Science* **307**, 600–604.

Panda S., Provencio I., Tu D. C. *et al.* (2003) Melanopsin is required for non-image-forming photic responses in blind mice. *Science* **301**, 525–527.

Panda S., Sato T. K., Castrucci A. M., Rollag M. D., DeGrip W. J., Hogenesch J. B., Provencio I. and Kay S. A. (2002) Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science* **298**, 2213–2216.

Qiu X., Kumbalasiri T., Carlson S. M., Wong K. Y., Krishna V., Provencio I. and Berson D. M. (2005) Induction of photosensitivity by heterologous expression of melanopsin. *Nature* **433**, 745–749.

Ruby N. F., Brennan T. J., Xie X., Cao V., Franken P., Heller H. C. and O'Hara B. F. (2002) Role of melanopsin in circadian responses to light. *Science* **298**, 2211–2213.

Saitou N. and Nei M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.

Sancar A. (2003) Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. *Chem. Rev.* **103**, 2203–2237.

Schibler U. (2000) Circadian clocks. Heartfelt enlightenment. *Nature* **404**, 25–28.

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. *Recent. Prog. Horm. Res.* **45**, 279–352.

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.

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.

Tarttelin E. E., Bellingham J., Hankins M. W., Foster R. G. and Lucas R. J. (2003) Neuropsin (Opn5): a novel opsin identified in mammalian neural tissue. *FEBS Lett.* **554**, 410–416.

Tomonari S., Takagi A., Akamatsu S., Noji S. and Ohuchi H. (2005) A non-canonical photopigment, melanopsin, is expressed in the differentiating ganglion, horizontal, and bipolar cells of the chicken retina. *Dev. Dyn.* **234**, 783–790.

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.

Tu D. C., Batten M. L., Palczewski K. and Van Gelder R. N. (2004) Nonvisual photoreception in the chick iris. *Science* **306**, 129–131.

Wang H., Ma L. G., Li J. M., Zhao H. Y. and Deng X. W. (2001) Direct interaction of Arabidopsis cryptochromes with COP1 in light control development. *Science* **294**, 154–158.

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.

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.

Yang H. Q., Wu Y. J., Tang R. H., Liu D., Liu Y. and Cashmore A. R. (2000) The C termini of *Arabidopsis* cryptochromes mediate a constitutive light response. *Cell* **103**, 815–827.

Zatz M. (1994) Photoendocrine transduction in cultured chick pineal cells: IV. What do vitamin A depletion and retinaldehyde addition do to the effects of light on the melatonin rhythm? *J. Neurochem.* **62**, 2001–2011.

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.

