

Chickens' Cry2: molecular analysis of an avian cryptochrome in retinal and pineal photoreceptors

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Abstract We have identified and characterized an ortholog of the putative mammalian clock gene cryptochrome 2 (*Cry2*) in the chicken, *Gallus domesticus*. Northern blot analysis of *gCry2* mRNA indicates widespread distribution in central nervous and peripheral tissues, with very high expression in pineal and retina. In situ hybridization of chick brain and retina reveals expression in photoreceptors and in visual and circadian system structures. Expression is rhythmic; mRNA levels predominate in late subjective night. The present data suggests that *gCry2* is a candidate avian clock gene and/or photopigment and set the stage for functional studies of *gCry2*. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Circadian rhythm; Cryptochrome; Pineal gland; Retina; Chicken; Clock gene

1. Introduction

The biological clock(s) that control the wide variety of behavioral, physiological and biochemical circadian rhythms in vertebrates are now believed to reside in multiple photoreceptive and oscillatory tissues [1–3]. Nowhere has this multiplicity of circadian function been more apparent than in birds [4–7]. Circadian oscillators are located in the ocular retinae, pineal gland and in the avian homolog of the mammalian suprachiasmatic nucleus (SCN). Photoreceptors capable of entraining these oscillators have been localized in the retinae, pineal gland and several brain structures, in the septum and tuberal hypothalamus [4,5,7].

The molecular components that comprise these clocks have been identified in diverse animal species ranging from *Drosophila melanogaster*, where the molecular mechanisms of clock function are best understood, to several species of mammals,

including humans, with an apparently extraordinary degree of evolutionary conservation [8]. In *Drosophila*, pacemaker cells in the brain, retinae, and perhaps other tissues express rhythmic patterns of transcription and translation of 'positive elements' comprised of the gene products of *clock* (*clk*) and *brain muscle ARNT-like protein 1* (*bmal1*), which dimerize to activate the transcription of 'negative elements' *period* (*per*) and *timeless* (*tim*), which in turn are translated, dimerize themselves and feedback to inhibit their own transcription by interfering with the *clk/bmal1* activation [1–3,9]. This autoregulatory loop is believed to be entrained to light:dark cycles (LD) via the action of both opsin-based photopigments and the flavin-based blue-light photopigment *cryptochrome* (*cry*) [10–12].

Based on cross-species comparisons of gene sequence, mutation analysis and in vitro data, a homologous autoregulatory transcriptional/translational feedback loop comprised of gene products with remarkable similarity to those demonstrated in *Drosophila* has been postulated as the underlying mechanism in mammals [1–3,13,14,18]. According to the current mammalian model, the positive elements are *clk* and *bmal1*, as it is in flies, while the negative components are a quartet of genes comprising *period 1* (*per1*), *period 2* (*per2*) and the two cryptochromes (*cry1*) and (*cry2*). In the mouse, *Mus musculus*, *mCrys* are expressed in retina, brain and peripheral tissues [10,11,15,16]. Mice lacking both *mCry1* and *mCry2* are behaviorally arrhythmic [16,17]. It is interesting to note that, in mammals, the cryptochromes play a central role in the oscillation itself, co-opting the function of *timeless*, while in *Drosophila*, cryptochrome acts both as a photopigment [1] and in oscillator functions, at least in some tissues [2–5]. These data indicate that *Crys* are key components of the circadian system in both *Drosophila* and mammals. However, their function as circadian photoreceptors in mammals is still under debate [11,12].

Recent studies have reported the cloning and initial characterization of several avian clock factors including *Clk*, *bmal1* and *per* genes [18–21]. However, very little is known about their contribution to avian physiology, although in vitro evidence has strongly suggested that chicken clock gene heterodimers can directly activate the gene for chicken arylalkylamine *N*-acetyltransferase (AANAT), a crucial enzyme in the biosynthetic pathway for the hormone melatonin [22]. In addition, the sequences of all the known genes involved in melatonin biosynthesis in the chick pineal gland are well characterized [23–26]. In order to examine further the molecular clock and photoreceptor components of the avian circadian

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Abbreviations: cry, cryptochrome; SCN, suprachiasmatic nucleus; LD, light:dark; AANAT, arylalkylamine *N*-acetyltransferase; per, period; clk, clock; bmal1, brain muscle ARNT-like protein 1; ZT, Zeitgeber time; TeO, optic tectum; GCL, ganglion cell layer; RBP, retinol-binding protein

clock, the chicken pineal gland has been studied because both photoentrainment and generation of circadian rhythms can be analyzed in vitro [23,24]. We report here the cloning of a mammalian ortholog of *Cry2* from the chicken pineal gland, designated *gCry2* (GenBank accession number AY046568), and have characterized its expression. The data are consistent with the notion that *gCry2* is an evolutionarily conserved member of the animal cryptochrome family and plays a crucial role in avian circadian organization. The question whether *gCry2* serves as a photopigment and/or clock component will be discussed.

2. Materials and methods

2.1. Animals

White leghorn cockerels were obtained from Hy-Line International (Bryan, TX, USA) and maintained for 2 weeks in a LD cycle of 12:12 h (lights on Zeitgeber time (ZT) 0–12) with food (Purina Startena) and water ad libitum. Thereafter, the lighting cycle was altered as described in the figure legends.

2.2. Isolation of *gCry2*

A fragment of *mCry2* corresponding to bases ~700–1200 of the

coding region was used to screen a chick pineal cDNA library. The cDNA library was constructed from pineal mRNA collected at ZT-18 using a Lambda Zap II cDNA Synthesis Kit (Stratagene). A positive clone, ~1.5 kb, was isolated and sequenced to confirm identity. The cDNA fragment shared high sequence similarity to *mCry2*, and was therefore screened against a chicken bacterial artificial chromosome (BAC) library (HGMP Human Resource Centre, UK). Positive clone 64m7 was obtained from the Medical Research Centre HGMP Human Resource Centre (UK). BAC DNA isolation was performed using a Large Construct Isolation Kit (Qiagen). Direct BAC clone sequencing in the presence of Thermofidase (Fidelity Systems) was performed using an ABI 377 sequencer under the following cycling conditions: 95°C for 5 min, followed by 100 cycles of: 95°C for 30 s, proper annealing temperature for 20 s, and extension at 60°C for 4 min.

2.3. Bioinformatic analysis of *gCry2* sequence

Cladistic analysis was performed using the neighbor joining (NJ) method in the Vector Nti Molecular Biology analysis software (Informax). The NJ method works on a matrix of distances among all pairs of sequence to be analyzed. These distances are related to the degree of divergence among the sequences. The phylogenetic tree is then calculated after the sequences are aligned.

Further, homology modeling of chicken cryptochrome *Cry2* was conducted, based on the high sequence similarity between *gCry2* and DNA photolyase from two bacterial sources, *Escherichia coli*

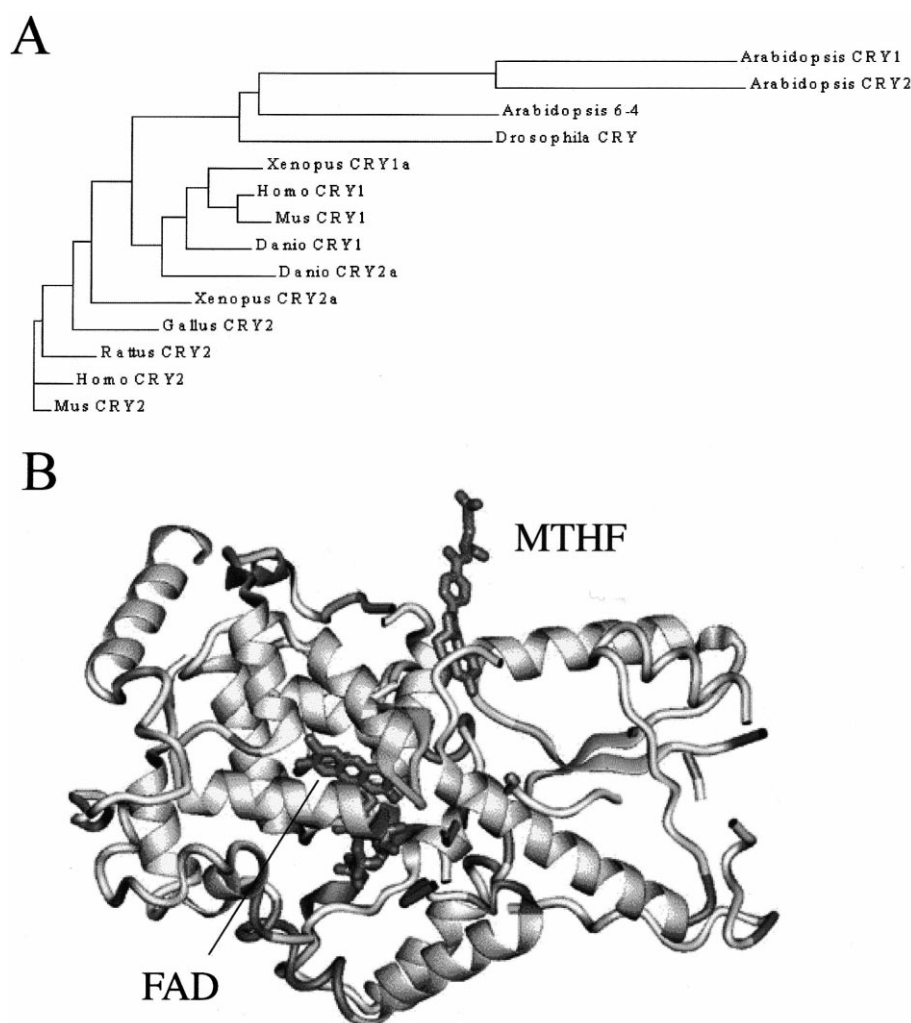


Fig. 1. Cryptochrome expression and putative structure. A: Cladogram indicating that *gCry2* is a phylogenetically conserved member of the animal cryptochromes and is more closely related to other vertebrate *Cry2* than to either *Cry1* or plant 6-4 photolyases or cryptochromes. B: Ribbon diagram based upon homology modeling of *gCry2* showing the likely positions of the flavin chromophore (FAD) in the center of the molecule and the pterin co-factor (MTHF) on the surface.

and *Synechococcus elongatus* (nee *Anacystis nidulans*) for which high resolution crystal structures are available [25,26]. Three-dimensional protein structural modeling for *gCry2* was performed in QUANTA/CHARMM (version 2000, Accelrys) molecular modeling environment using a UNIX Silicon Graphics O2 workstation. The structural coordinates of the bacterial photolyase proteins (1DNP, 1QNF) were extracted from Protein Data Bank (<http://www.rcsb.org/pdb/>), and modeling was performed using the primary structure alignment between *gCry2* and the bacterial photolyases using Clustal W [25]. The raw alignment result was manually refined using iterative alignment tools in the Protein Design module of QUANTA. Statistical significance of the pair-wise sequence similarities was evaluated by an alignment-independent program PRSS, which calculates the probability of similarities of randomly shuffled and unshuffled sequences using the distance matrix Monte Carlo procedure [27]. The analysis was carried out by setting the gap-opening penalty as 12 and gap-extending penalty as 2, and by performing 1000 global shuffling iterations using the BLOSUM62 scoring matrix.

After alignment, the template proteins were matched and superimposed. The coordinates of the aligned amino acid residues were averaged and copied to the modeled sequences. The newly defined coordinates were refined with a structural regularization tool. The connecting loop sequences were not modeled at this time. Cryptochromes are known to share the same chromophores, pterin (5,10-methyl-6,7,8-trihydrofolic acid, MTHF) and flavin (flavin adenine dinucleotide, FAD), as does the photolyase from *E. coli*. The coordinates of the chromophores from *E. coli* were thus transferred directly to the cryptochrome protein model. The overall raw structure was energy minimized using the CHARMM procedure [29]. The hydrogen-bonding pattern of the constructed PSII model was calculated on the Protein Design module and the secondary structure of the cryptochrome protein was derived.

2.4. RNA analysis

Total RNA was isolated from tissues using RNA Aqueous Midi-Kit (Ambion) as described by the manufacturer. Poly(A)+ RNA was isolated from total RNA using a MicroPure PolyA Kit (Ambion). Northern blots were performed as previously described [28,29]. Unless otherwise, total (10 µg each lane) or Poly(A)+ (2 µg) RNA was fractionated on 1.5% agarose/0.66 M formaldehyde gel, and probed for *gCry2*. Probes were labeled with [α - 32 P]dATP by random priming (DECA Prime II kit, Ambion). Typically, blots were first hybridized with the *gCry2* probes (1 kb 3'-UTR) and subsequently stripped (2×15 min in boiling water) before hybridization with actin probe. The final wash was at 55°C in 0.1×SSC containing 0.1% sodium dodecyl sulfate for 30 min. Blots were exposed to X-ray film (Biomax MS, Kodak) for 2 to 3 days and their images scanned and analyzed using the Image software (Scion Image). Transcript sizes were estimated by comparison with standard RNA markers (Roche). Data were normalized for variation in RNA loading and transfer efficiency by probing the Northern blots with β -actin cDNA.

2.5. In situ hybridization (ISH)

Animals were sacrificed by decapitation; brains and eyes were removed and rapidly frozen in isopentane at -40°C. ISH techniques were carried out as previously described [28,29]. Following fixation, deproteinization, and acetylation, slides were hybridized with sense and antisense cRNA probes for *gCry2*. Probes encoding the 3'-UTR of *gCry2* were generated in the presence of [α - 33 P]dUTP, in vitro with T3 and T7 RNA polymerases for sense and antisense probes, respectively. Sections were incubated overnight at 50°C and then subsequently washed in SSC and then dehydrated in 100% ethanol. Sections were exposed to BioMax MS film (Kodak) for 36 h.

Digoxigenin-labeled probes were synthesized encoding the antisense of the 3'-end of *gCry2* and for the corresponding sense sequences using a DIG RNA Transcription Kit (Roche). Following prehybridization, sections were incubated with the RNA probe (200 pmol/ml) in hybridization buffer at 50°C for 16 h. To visualize the hybridization a color reaction was then performed overnight.

3. Results

3.1. Bioinformatic analysis of *gCry2*

The *Cry2* gene isolated from the chicken BAC library cor-

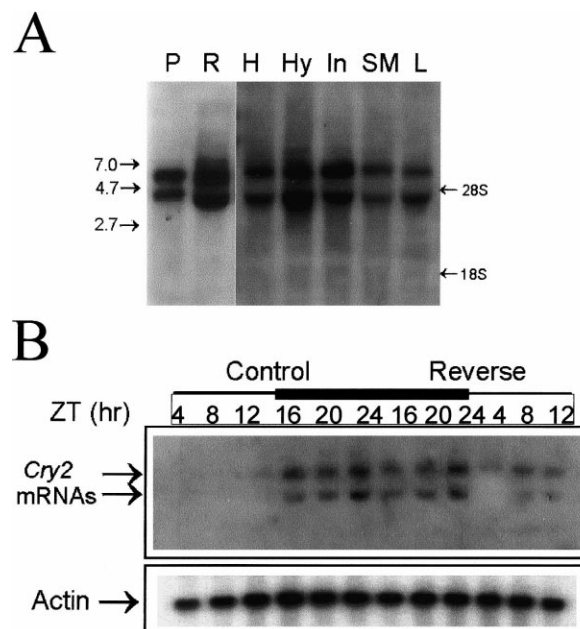


Fig. 2. Northern blot analysis of *gCry2* mRNA expression. A: Two transcripts (~4.2 and 5.2 kb) were present in all tissues examined. Poly(A)+ RNA (2 µg) from pineal gland and retina and total RNA (20 µg) from other tissues were loaded. All RNA samples were isolated from the indicated tissues dissected at ZT-20. The blots were repeated with similar results on independently obtained samples. P = pineal, R = retina, H = heart, Hy = hypothalamus, In = intestine, L = liver, SM = skeletal muscle. B: Rhythm in *gCry2* mRNA persists in LD in chicken pineal gland. Levels are high during late night and are entrainable to LD cycles, since reversal of the LD cycle in the birds reverses the phase of *gCry2* levels.

responds very closely to the mammalian *Cry2* (human and mouse). Cladistic analysis of the *Cry* genes indicates that the *gCry2* sequence belongs within the general animal *Cry* family of genes (Fig. 1A). It is important to note that *gCry2* is closer to *Cry2* sequences of other taxonomic groups than it is to the *Cry1* of other species, or to preliminary sequence we have obtained from chicken *gCry1* (Bailey et al., unpublished), indicating that this set of genes represents separate and very ancient lineages, certainly preceding the divergence of amniotes from anamniote species. Genomic sequence indicates that the open reading frame (ORF) of the *gCry2* gene is spread across at least 8 kb of genomic DNA, consisting of at least five exons and six introns (data not shown).

The predicted amino acid sequence from the ORF of the cDNA sequence indicated that *gCry2* is 86% identical to human and mouse *Cry2*. Remarkably, the sequence is 29.5% identical and 59.6% similar to the 6-4 DNA photolyase in *S. elongatus* ($P = 7.3 \times 10^{-53}$) and 21.7% identical and 58.0% similar to the homologous *E. coli* enzyme ($P = 2.9 \times 10^{-41}$). The predicted amino acid sequence of *gCry2* contains a probable FAD-binding site, a MTHF (pterin)-binding domain and a DNA photolyase domain. The residues that form the FAD-binding pocket are located in the middle of the predicted protein and are significantly positively charged, including residues 233, 243, 257–261, 264–265, 296, 299, 301–302, 305, 361–365, 367–368, 371, 390, 394, 396, 401–403, and 405–406. The pterin-binding pocket is much smaller, since this cofactor is partially bound at the surface. The putative binding residues are 112–114, 326 and 399.

Because of the close similarity of *gCry2* to the prokaryote photolyases, it was possible to model *gCry2* in homologous regions and to construct a putative structure for the predicted protein, such as the ribbon diagram of the overall modeled chicken cryptochrome structure including MTHF and FAD cofactors (Fig. 1B). The FAD-binding domain contains residues within 3 Å of the FAD chromophore, buried in the center of the protein. The MTHF (pterin)-binding domain contains residues within 5 Å of the flavin molecule. This cofactor is partially bound at the surface.

3.2. Tissue distribution of *gCry2* mRNA

Northern blot analysis at high stringency revealed that *gCry2* mRNA is expressed at high levels in the pineal gland and retina (Fig. 2A): *gCry2* probes hybridized to two transcripts (approximately 4.2 and 5.2 kb). Multiple tissue Northern analysis revealed that *gCry2* mRNA is widely expressed in the chicken, including the heart, liver, skeletal muscle, intestine and brain (Fig. 2A). The existence of daily rhythms in *gCry2* mRNA was examined using Northern blot analysis in RNA prepared from pineal tissues (ZT 4 to ZT 24). The expression of *gCry2* mRNA oscillated on a 24 h basis in a LD cycle such that *gCry2* exhibited high levels at late night (Fig. 2B). ISH of the chick brain, using radioactive-labeled probes, revealed an expression of *gCry2* mRNA in areas associated with phototransduction and the visual system, including the visual SCN (vSCN), optic tectum (TeO), and lateral septum (LS) (Fig. 3). Non-radioactive digoxigenin-labeled

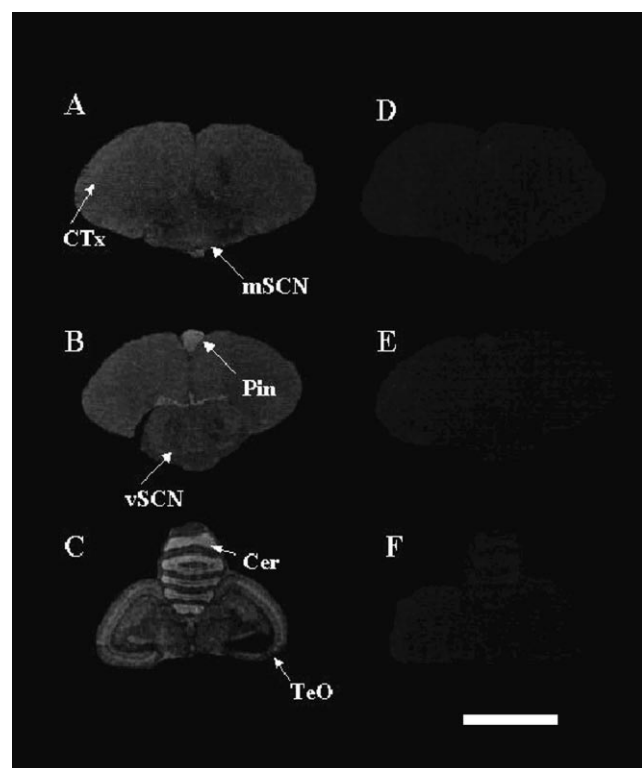


Fig. 3. ISH analysis of *gCry2* mRNA expression in the chicken brain. These coronal sections are displayed in rostral (A), intermediate (B), and caudal (C) aspects of the brain. Note high expression in the TeO, Pin, and cerebellum (Cer). Relatively low levels of expression are found in the mSCN, vSCN and cortex (CTx). Corresponding sense controls (D, E, F) exhibit very little, if any, hybridization. Bar = 1 cm.

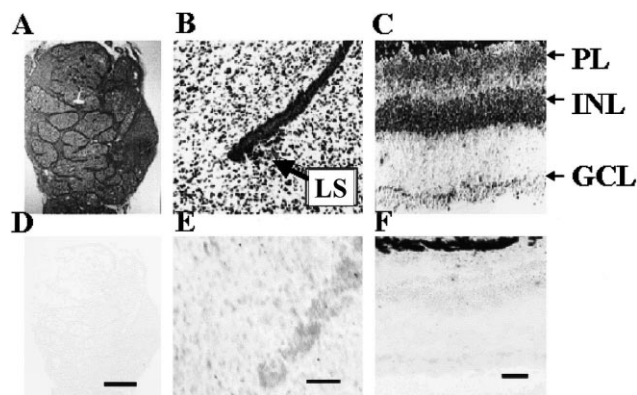


Fig. 4. Digoxigenin ISH for *gCry2* mRNA in the pineal gland (A), LS (B), retina (C), and corresponding sense controls (D, E, F). These data show broadly distributed, but specific, expression in most of the pineal gland (A, D). Bar in D corresponds to 200 µm for both A and D. In the septum (B, E), a concentration of *gCry2* cells were observed in ependymal regions, which have been shown to contain opsins. Bar in E corresponds to 100 µm for both B, and E. Finally, the retina (C, F) expresses *gCry2* in PLs, the INL and GCL. Bar in F corresponds to 100 µm for C and F. In all cases, no expression is seen with sense control probes (D–F).

ISH confirmed *gCry2* mRNA expression in the pineal gland, LS, and also revealed expression in the chick retina. Retinal *gCry2* mRNA expression is observed primarily in the inner nuclear layer (INL), photoreceptor layer (PL), and to a lesser extent, in the ganglion cell layer (GCL) (Fig. 4). *gCry2* mRNA is expressed in both photoreceptive pinealocytes (Pin) and interstitial cells of the pineal gland (Int), vSCN, and ventrolateral geniculate nucleus (GLv), stratum opticum (Sop), stratum griseum et fibrosum (SGF), and stratum griseum centrale (SGC) layers of the TeO (Fig. 5A–C). However, it is important to point out that the level of expression in either the vSCN or medial SCN (mSCN), albeit present, is not particularly strong, when compared to either pineal or retinal expression (Figs. 3–5).

4. Discussion

We report here the isolation and initial characterization of *gCry2*. Analysis of the predicted amino acid sequence indicates that *gCry2* is a phylogenetically conserved ortholog of mammalian *Crys* (Fig. 1A), complete with a flavin-binding site, a pterin-binding site and a DNA photolyase domain (Fig. 1B). Northern blot analysis of *gCry2* detected two transcripts in all tissues examined, which is similar to human *Cry2* mRNAs [30] but not the mouse, where there appears to be only one transcript [15]. It is conceivable that the two *Cry2*

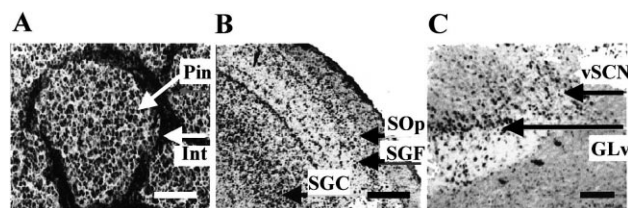


Fig. 5. Digoxigenin ISH for *gCry2* mRNA in the pineal gland indicating expression in both Pin and Inter (A), vSCN, and GLv (B), and Sop, SGF, and SGC layers of the TeO (C). Bar corresponds to 100 µm.

transcripts in chicken and human were due to alternative polyadenylation site usage as sequence analysis revealed a consensus polyadenylated tail at a premature location in the 3'-UTR, approximately 1 kb from the polyA+ tail for *gCry2*. The wide distribution is similar to the profile seen in mammals [11,15,30].

There are, however, several important differences in the expression patterns among the mammalian cryptochromes and *gCry2*. First and foremost, *gCry2* is expressed by known photoreceptive cells in the retinae, the pineal gland and in the putative deep-brain photoreceptor region of the LS (Figs. 3 and 4), whereas, in mammals, the cryptochromes are not expressed by canonical photoreceptor cells [11]. This expression pattern coincides with opsin and opsin-like immunohistochemical staining in these structures in a variety of non-mammalian vertebrate species [12,31–35] and resembles the cryptochrome expression pattern in the zebrafish and *Xenopus* [30,35]. In addition, ISH revealed *gCry2* mRNA in the retinal ganglion cell and INLs of the retina, also similar to the situation in *Xenopus* [35] and in the mouse [15]. Further, we find broad *gCry2* expression in retinorecipient and integrative structures of the visual system (Fig. 3), which is not the case in mammals [11]. It is interesting to note that, while we observe strong hybridization in the photoreceptive elements of the circadian clock in retinal, pineal and brain photoreceptors, we see only moderate expression in the two candidates for the avian SCN, which is also the case in mammals [36]. This observation stands in sharp contrast to the situation for the *per* genes, which are expressed abundantly in the mSCN [20,21].

Light is a major environmental time cue in the entrainment of circadian rhythms [37]. Visual phototransduction has been extensively characterized at the molecular level, although the identity of the photoreceptors mediating circadian photoentrainment in vertebrates is uncertain [12]. Conceivably, molecules that mediate circadian photoreception may include both opsin and non-opsin-based pigments [11,12,36]. In addition to the better-known visual pigments, several novel non-visual opsins have been identified in vertebrates, including pinopsin [39,40] melanopsin [41] and parainopsin in the pineal [41], among many others [11,12].

In non-mammalian vertebrates, the pineal gland is a directly photoreceptive structure on which light has three major effects: (1) the acute suppression of melatonin production, (2) resetting the phase of the endogenous circadian oscillator and (3) the prevention of damping of the output rhythm [24,42,45]. It is possible that some or all of these effects are mediated by opsin-based photopigments, which mediate phototransduction via a vitamin A-dependent retinaldehyde chromophore [12]. Certainly many of these photopigments are present in the avian pineal gland [12]. However, it is important to point out that, although the acute effects of light on chick pineal melatonin are reduced with vitamin A deprivation, the phase-shifting effects of light in cultured chick Pin are unaffected by >95% depletion of total and of protein bound retinaldehyde [38]. This observation raises the possibility that a non-opsin-based photopigment may underlie circadian phase-shifting and entrainment in the chick pineal. There is a growing body of evidence in favor of this scenario in mammals. Selby et al. [43], using triple-mutant mice lacking rods and most cones (rd/rd) as well as both mCRY proteins, have recently reported that classical opsins and CRYs serve

functionally redundant roles in circadian phototransduction. Further, Thompson et al. [44] examined the circadian photoresponse in vitamin A-depleted retinol-binding protein (RBP)-/- mice as measured by acute *mper* gene induction in the SCN in response to light. These authors reported that ocular retinal is not required for light signaling to the murine circadian pacemaker.

In spite of recent molecular breakthroughs and high sequence similarities to the mammalian clock genes, these genes role in the avian circadian system is undetermined. However, co-expression of the putative positive elements in COS-7 cells activates a chicken AANAT E-box luciferase reporter construct [22], suggesting elements of the proposed transcription/translation feedback model interact with a known circadian output. It is not clear at this point whether *gCry2* is involved in the phototransduction associated with entrainment and/or is a clock component itself. However, the presence of this molecule in the cell-types associated with photoreception and clock function provides strong circumstantial evidence that *gCry2* is one more cog in the avian biological clock.

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