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[38] Cryptochromes and Circadian Photoreception in Animals

By CARRIE L. PARTCH and AZIZ SANCAR

Abstract

Cryptochromes are flavin- and folate-containing blue-light photoreceptors with a high degree of similarity to DNA photolyase, which repairs ultraviolet-induced DNA damage using blue light to initiate the repair reaction. Cryptochromes play essential roles in the maintenance of circadian rhythms in mice and *Drosophila*, and genetic data indicate that cryptochromes function as circadian photoreceptors in these and other animals. However, the photochemical reactions carried out by cryptochromes are not known at present.

Introduction

In animals, synchronization of the circadian clock with the environmental light/dark cycle requires contribution from multiple photoreceptor systems. Genetic studies in mice have revealed functional redundancy between retinaldehyde-based opsins and flavin-based cryptochromes in circadian photoreception. These studies have revealed the role of three

0076-6879/05 $35.00

photoreceptor systems in this process: (1) visual opsins, (2) the nonvisual opsin melanopsin, and (3) cryptochromes (Sancar, 2003; Van Gelder and Sancar, 2003). This article reviews the experiments used thus far to elucidate the role of cryptochromes in circadian photoreception in animals. In addition to their putative photoreceptor function, cryptochromes also constitute an integral component of the transcriptional feedback loop that generates the circadian clock (Thresher *et al.*, 1998; van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999). However, this light-independent function of cryptochromes is not covered in any detail.

Cryptochromes were initially identified as putative photoreceptors by their high degree of homology to the light-activated DNA repair enzyme photolyase (Ahmad and Cashmore, 1993; Hsu *et al.*, 1996). Animal cryptochromes are 60- to 70-kDa proteins that share 30–50% sequence identity with photolyase along the first 500 amino acids and contain the same two chromophore/cofactors: methenyltetrahydrofolate (MTHF) and a flavin in the form of FAD. A small number of photolyases contain 8-hydroxy-5-deazariboflavin instead of folate as the second chromophore. Photolyase family members have no apparent sequence homology to other classes of flavoproteins, perhaps because photolyase utilizes flavin in its two electron-reduced and photochemically excited state $1(FADH^-)^*$ as opposed to most other flavoproteins, which operate from the oxidized, ground state of flavin (FAD_{ox}). Both cryptochromes and photolyases have a positively charged groove along one face of the protein that binds the phosphodiester backbone of DNA, with a hole in the middle that, in the case of photolyase, allows entry of an ultraviolet (UV)-induced cyclobutane pyrimidine dimer or pyrimidine-pyridimidone (6–4) photoproduct into the active site cavity close to the flavin for repair. The significance of the conservation of this groove and the hole in cryptochromes is not yet understood.

Despite strong genetic evidence in plants and animals for its role as a photoreceptor, the mechanism of action, or photocycle, of cryptochrome is currently not known. However, detailed mechanistic studies have been carried out on photolyase and its mechanism of action is well understood (Sancar, 2003). The enzyme binds its substrate independently of light, and catalysis is initiated by light (Fig. 1). The photoantenna chromophore MTHF ($\lambda_{max} = 380–420$ nm) absorbs a photon of blue light (350–450 nm) and transfers the excitation energy to the catalytic cofactor $FADH^-$ by Förster resonance energy transfer. Alternatively, the $FADH^-$ ($\lambda_{max} = 360$ nm) may become excited by absorbing a photon directly. The excited $1(FADH^-)^*$ singlet state transfers an electron to the pyrimidine dimer, generating an $FADH^\circ$ blue neutral radical and a pyrimidine dimer radical. The latter undergoes bond rearrangements to generate two canonical pyrimidines and restores $FADH^\circ$ to its catalytically competent form ($FADH^-$)

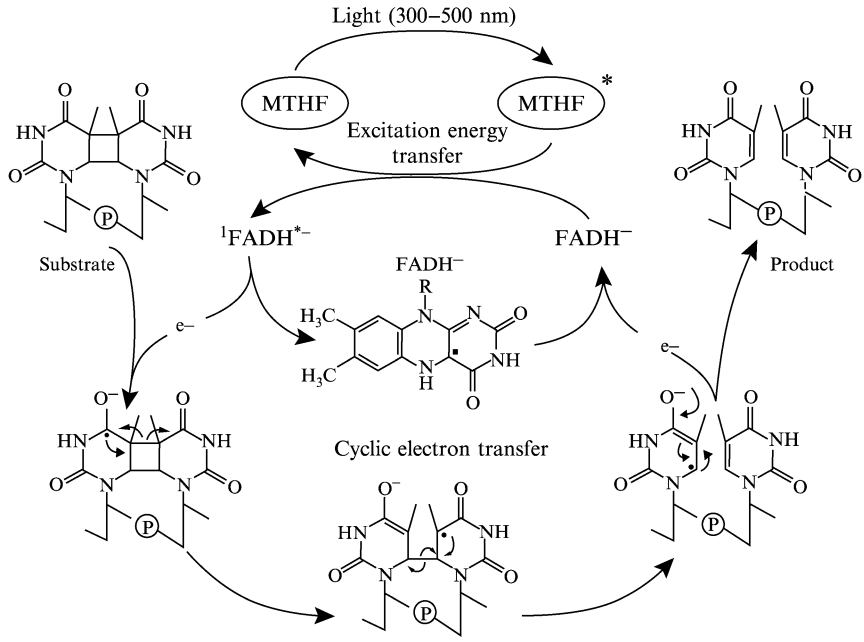

FIG. 1. Reaction mechanism of *Escherichia coli* cyclobutane pyrimidine dimer photolyase. MTHF absorbs a 300- to 500-nm photon and transfers the excitation energy to $FADH^-$ by resonance energy transfer. The $1(FADH^-)^*$ transfers an electron to the pyrimidine dimer to generate a pyrimidine and pyrimidine$^{\circ -}$; back electron transfer to $FADH^\circ$ restores the catalytic cofactor to the active reduced form and the dimer is converted to canonical bases (Sancar, 2003).

by back electron transfer to complete the photocycle; the repaired DNA subsequently dissociates from the enzyme. Although cryptochromes by definition lack DNA repair activity, it is hypothesized that they utilize a similar photocycle to regulate light-dependent signaling. Structurally, cryptochromes are also defined by the presence of extended C-terminal domains ranging from 40 to 220 amino acids that are not homologous to any known protein. Studies of cryptochromes from two different organisms (*Arabidopsis thaliana* and *Drosophila melanogaster*) indicate that these unique C-terminal domains are involved in regulating light-dependent signaling by cryptochromes (Rosato *et al*., 2001; Yang *et al*., 2000). All mammalian and bird species analyzed so far, including humans and mice, have two cryptochrome isoforms (Cry1, Cry2), and some amphibians possess up to seven cryptochromes. The variable C-terminal domain sequences are the only predominant difference between most cryptochrome isoforms.

Mammalian Cryptochromes

Biochemical Characterization

Structural Aspects. Although cryptochrome/photolyase family members contain two noncovalently bound chromophores, only the FAD is absolutely required for activity. The crystal structure of *Escherichia coli* photolyase, the prototype of this family of proteins, is shown in Fig. 2A. The enzyme consists of an N-terminal α/β domain and a C-terminal α-helical domain connected by a long interdomain loop (Park *et al*., 1995). The photolyase-like domain of human cryptochrome 2 (hCRY2) was homology modeled on the *E. coli* crystal structure and is predicted to have a similar tertiary structure (Fig. 2B) (Ozgur and Sancar, 2003). The FAD is deeply buried within the C-terminal α-helical domain, held tightly in place by contact with 14 amino acids in photolyase, most of which are conserved in cryptochromes (Park *et al*., 1995). The second chromophore, MTHF, is loosely bound in a shallow cleft between the two domains and is easily lost

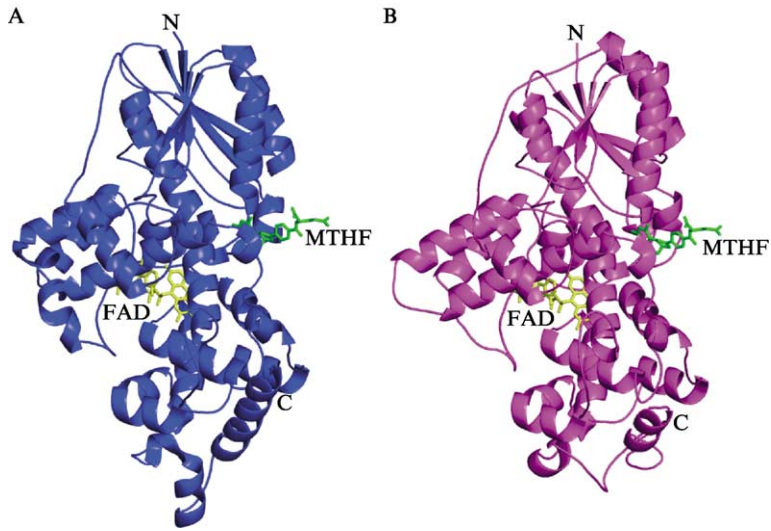

FIG. 2. Crystal structure of *E. coli* photolyase and homology-modeled human cryptochrome 2. (A) Ribbon diagram representation of *E. coli* photolyase showing the N-terminal α/β domain, the C-terminal α-helical domain, and the positions of the two cofactors (Park *et al*. 1995). (B) The model of the hCRY2 tertiary structure was generated using the experimentally determined structures of *E. coli* and *A. nidulans* photolyases as templates, excluding the N-terminal 22 and C-terminal 80 amino acids of hCRY2 that have no homology to photolyase (Ozgur and Sancar, 2003).

during purification. It acts as a photoantenna, increasing the efficiency of DNA repair by photolyase five- to 10-fold and dominates the absorption spectrum with a peak ranging from 377 to 410 nm depending on the source of the enzyme. In the absence of folate, the absorption spectrum of purified cryptochrome/photolyase family members is characteristic of the FAD and its oxidation state. During purification, the $FADH^-$ cofactor of photolyases becomes oxidized in the majority of photolyases to yield either the flavin neutral radical ($FADH^\circ$) or FAD_{ox}. Enzyme preparations that are blue in color contain the neutral radical form of flavin, due to its strong absorbance at long wavelengths, from 380 to 625 nm, and preparations with oxidized flavin are yellow, due its absorbance at 370 and 430 nm. The catalytically inactive $FADH^\circ$ can be reduced *in vitro* to $FADH^-$ either chemically or by photoreduction, in which a tryptophan residue in the apoenzyme transfers an electron to the excited state $FADH^\circ$ (Payne *et al*., 1987). The oxidation state of the catalytic flavin in cryptochromes is not known at present, although an action spectrum of hypocotyl elongation performed in *Arabidopsis*, a cryptochrome-dependent response, suggests that the flavin may be active in the one ($FADH^\circ$)- or two-electron (FAD_{ox}) oxidized form in plants, which would suggest a radically different photochemistry from photolyase (Ahmad *et al*., 2002). In contrast, a *Vibrio cholerae* cryptochrome purified from *E. coli* contains the flavin in the FADH-form, suggesting a photolyase-like reaction mechanism (Worthington *et al*., 2003).

Purification and Spectroscopic Properties. With current protocols, purification of most animal cryptochromes from heterologous sources does not yield protein with stoichiometric amounts of chromophores in sufficient quantities for biochemical studies. In contrast to *Arabidopsis* cryptochrome 1 (AtCry1), which can be purified as a recombinant protein from *E. coli* with stoichiometric amounts of FAD, expression and purification of human CRY1 and CRY2 as MBP fusion proteins in *E. coli* yielded moderate quantities of protein with grossly substoichiometric amounts (1–5%) of FAD and even less folate; efforts to supplement the apoprotein with FAD and folate were unsuccessful (Hsu *et al*., 1996). Absorption spectra of recombinant hCRY1 and hCRY2 expressed in *E. coli* (Fig. 3A) show the characteristic absorbance of oxidized flavin at 420 nm, with residual absorbance extending all the way to 700 nm. However, it is doubtful that this represents the active form of cryptochrome, as many photolyases known to be active only when the flavin is in the $FADH^-$ form exhibit similar spectra when overexpressed and purified from heterologous sources (Sancar, 2003).

Attempts to purify animal cryptochromes from native sources have been difficult because of the lack of a biochemical assay for cryptochrome

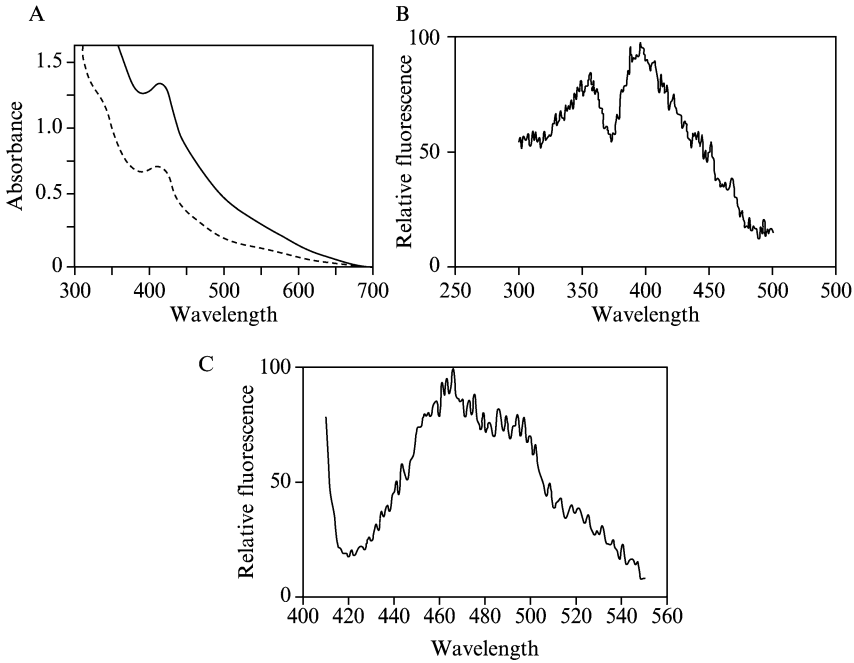

FIG. 3. Spectroscopic properties of mammalian cryptochromes. (A) Dashed and solid lines represent the absorbance spectra of hCRY1 and hCRY2, respectively, purified from *E. coli* (Hsu *et al.*, 1996). (B) Uncorrected fluorescence emission spectrum of hCRY2 purified from HeLa cells with λ emission at 520 nm, indicative of FAD. (C) Uncorrected fluorescence emission spectrum of hCRY2 purified from HeLa cells with λ excitation set at 400 nm reveals a peak at 460 nm and a shoulder at 510 nm, indicative of the presence of both FAD and MTHF, respectively (Ozgur and Sancar, 2003).

function. However, affinity purification of recombinant, FLAG-tagged hCRY2 from a stably transfected HeLa cell line yielded small quantities of protein (5–15 μg hCRY2 from 10-liter HeLa suspension cultures) with an estimated chromophore stoichiometry of 30% (Ozgur and Sancar, 2003). Chromophore stoichiometry was estimated by fluorescence spectroscopy; the fluorescence excitation spectrum of purified hCRY2 with emission set at 520 nm (Fig. 3B) is characteristic of FAD with maxima at 370 and 430 nm, and the fluorescence emission spectrum with excitation set at 400 nm (Fig. 3C) is indicative of the presence of both MTHF (major peak, 460 nm) and FAD (shoulder, 505 nm) (Sancar *et al.*, 1984). Finally, expressing hCRY2 in insect cells using the baculovirus system yielded abundant protein with no detectable chromophore.

Enzymatic Activities. Several *in vitro* activities associated with animal cryptochromes, such as DNA binding and autophosphorylation, have been described (Bouly *et al*., 2003; Ozgur and Sancar, 2003; Shalitin *et al*., 2003). Because mammalian cryptochromes have dual roles as light-independent regulators of the molecular clock and as circadian photoreceptors in the eye, it is unclear whether these *in vitro* activities are physiologically relevant for cryptochrome in the photocycle, the molecular clock, or both.

Purified hCRY2 binds to single-stranded DNA with high affinity ($K_D \sim 5 \times 10^{-9}$ *M*) and double-stranded DNA weakly ($K_D \sim 10^{-7}$ *M*), as measured by electrophoretic mobility shift assay (Ozgur and Sancar, 2003). This is in contrast to photolyase, which binds to damage in single- and double-stranded DNA with comparable affinities ($K_D \sim 10^{-9}$ *M*) (Sancar *et al*., 1985). hCRY2 also bound with slightly higher affinity to UV-damaged DNA, although the magnitude of increase in affinity for damaged over undamaged DNA is significantly less than that of photolyase. Unlike photolyase, DNA binding by hCRY2 was not affected by light, and no repair by cryptochrome has been detected *in vivo* or *in vitro*.

It has been reported that plant and human cryptochrome 1 have autophosphorylating kinase activities (Bouly *et al*., 2003; Shalitin *et al*., 2003). The kinase activity of purified AtCry1 was also tested on a variety of classic kinase substrates such as histones, casein, and myelin-binding protein and it appears that the kinase activity is limited to autophosphorylation. *In vitro* autophosphorylation of AtCry1 occurred only on serine residues, depended on the presence of flavin in a reducing environment, and was stimulated by blue light. Because both AtCry1 and AtCry2 have previously been shown to be phosphorylated rapidly *in vivo* in response to blue light, this autophosphorylation may be involved in regulating signal transduction *in vivo* (Shalitin *et al*., 2002, 2003). hCRY1 purified from insect cells was shown to bind to ATP cellulose and autophosphorylate in solution (Bouly *et al*., 2003).

Expression of Cryptochrome in the Retina

The retina is the exclusive site of circadian photoreception in mammals (Wright and Czeisler, 2002). While visual pigments in rods and cones unquestionably contribute to circadian photoreception, they are not essential for circadian phototransduction. Mice and humans with certain retinal degeneration diseases lose complete function of the visual photoreceptors in the outer retina and retain circadian photoreception. Therefore, the inner retina must contain photoreceptors capable of sensing and transmitting light information in the absence of the visual photoreceptors. Currently, two candidate photoreceptive pigments are known to be expressed

in the inner retina: melanopsin and the two mammalian cryptochromes (Miyamoto and Sancar, 1998; Provencio *et al*., 2000).

Cryptochrome Expression in Mouse Retina. To examine the expression of cryptochromes in the retina by bright-field microscopy, polymerase chain reaction fragments of *mCry1* (nucleotides 1074–1793) and *mCry2* (nucleotides 1040–1649) are subcloned into the pBluescipt SK+ plasmid, and ^{35}S-UTP-labeled sense and antisense RNA probes are generated *in vitro* with T3 and T7 RNA polymerase. Frozen sections of retina (20 μm thick) are fixed for 20 min in 4% formaldehyde in phosphate buffer, treated with proteinase K (10 μg/ml) for 10 minutes, acetylated with acetic anhydride in 0.1 *M* triethanolamine, and dehydrated with sequential ethanol dehydration. ^{35}S-labeled sense and antisense probes diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 20 m*M* Tris–HCl, pH 8.0, 0.3 *M* NaCl, 0.2% Sarcosyl, 0.02% salmon sperm DNA, and 1X Denhardt's solution) are placed on the sections and incubated at 55° overnight. The sections are washed at 65° (50% formamide, 2X SSC, 0.1 *M* dithiothreitol) for 30 min and then treated with RNase A (1 μg/ml) for 30 min at 37°. Sections are washed again for 30 min at 65°, dipped in nuclear emulsion (Kodak NTB-2), and exposed to X-ray film for 2 weeks at 4°. Slides are stained after the emulsion autoradiography for 1 min with hematoxylin, washed with dH_2O, dehydrated with ethanol, and then treated with xylene and mounted. Examination of *Cry1* and *Cry2* mRNA levels in the mouse retina by *in situ* hybridization reveals moderate expression of *mCry1* and a high level of *mCry2* mRNA in both the inner nuclear layer and the ganglion cell layer of the retina (Fig. 4A) (Miyamoto and Sancar, 1998). The arrows in Fig. 4 indicate clusters of ganglion cells that express *Cry1* and *Cry2*.

Cryptochrome Expression in Human Retina. CRY2 protein levels are measured in the human retina by immunohistochemistry (Fig. 4B) as follows: 5-mm trephine punches of preserved human donor eyes are cryosectioned (10 μm), pretreated in 0.15% H_2O_2, and washed thoroughly in phosphate-buffered saline (PBS) before incubation in 0.02 mg/ml affinity-purified CRY2 antibody (Alpha Diagnostics, Inc.) on 0.1 *M* PBS, 0.5% Triton X-100, and 10% normal goat serum for 12–26 h at 4° (Thompson *et al*., 2003). Sections are then washed in PBS three times and incubated in a goat antirabbit biotinylated secondary antibody (1:50; Jackson ImmunoResearch) for 2 h. After washing with PBS, sections are incubated in an avidin–biotin–peroxidase mixture (Vectastain ABC Kit; Vector Laboratories) for 1 h and in 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) for 10 min followed by a brief treatment with DAB and 0.03% H_2O_2. Slides are washed in PBS, mounted in a glycerin–PBS mixture, and analyzed with a microscope equipped with either epifluorescence or differential interference contrast optics. Antibody specificity is

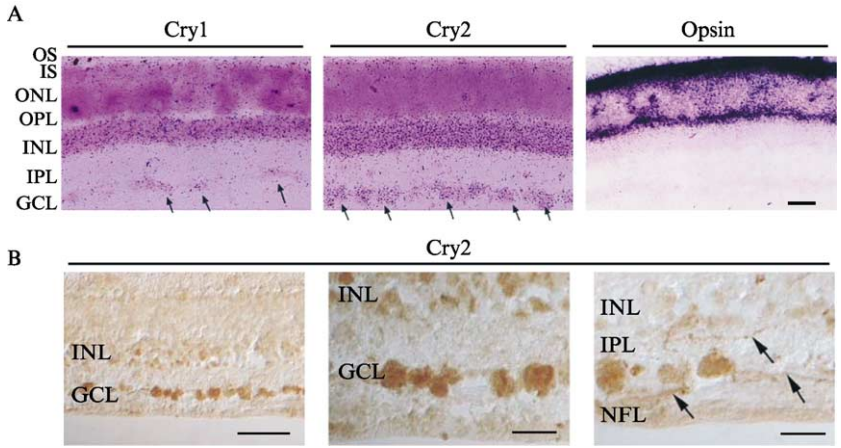

FIG. 4. Expression of cryptochrome in the mammalian retina. (A) Bright-field micrograms of *in situ* hybridizations comparing the expression of *mCry1*, *mCry2*, and opsins in the mouse retina. Bar: 30 μm (Miyamoto and Sancar, 1998). Arrows indicate clusters of ganglion cells expressing cryptochromes. (B) hCRY2 immunoreactivity in the human retina. Arrows indicate cryptochrome expression in ganglion cell extensions. Bar: 50 μm in the lower left box and 25 μm in the lower right two boxes (Thompson *et al*., 2003). Retinal histology: OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

determined either by eliminating primary antibody or by preadsorption of the primary antibody with 0.1 mg/ml CRY2 peptide (Alpha Diagnostics, Inc.) overnight at 4° before incubation with tissue. Expression of hCRY2 is detected in approximately 70% of retinal ganglion cells in both the macula and the peripheral retina with some staining also present in the inner nuclear layer. Interestingly, hCRY2 in the retina was found to be mostly cytoplasmic using 4′,6-Diamidino-2-phenylindole (DAPI) labeling of nuclei and anti-CRY2 immunofluorescence. This was confirmed by subcellular fractionation and Western analysis of retinal extracts. Intriguingly, hCRY2-reactive immunostaining was visible in some axonal processes extending into the inner plexiform layer and nerve fiber layer, as indicated by the arrows in Fig. 4B.

Retinal ganglion cells that directly innervate the site of the molecular clock in the brain (the suprachiasmatic nucleus, SCN) represent approximately 1% of total ganglion cells in the mouse. The majority of these cells are directly photosensitive by whole cell current clamp recordings, depolarizing in response to blue/green light with a maximum response at 480 nm (Berson *et al*., 2002; Hattar *et al*., 2002). This response was

attributed to melanopsin; however, reconstituted melanopsin has an absorption peak at 420 nm (Newman *et al.*, 2003).

Genetic Analysis

Genetic studies have been carried out on mice with mutations inactivating each of the various candidate circadian photopigments to quantitatively assess the contribution of each candidate gene to circadian photoreception. These studies have highlighted the contributions of three classes of photopigments in this process: the visual opsins, melanopsin, and cryptochromes. There are two common assay end points used to quantify photoreceptor input to the suprachiasmatic nucleus (SCN): behavioral analysis, which measures the synchronization of circadian behavior with a given light/dark cycle, and quantification of gene induction in the SCN in response to light. The use of behavioral analysis to analyze the effects of the loss of cryptochromes on photoreception is complicated by the essential, light-independent role of cryptochromes in the molecular clock mechanism (Griffin *et al.*, 1999; Kume *et al.*, 1999; Thresher *et al.*, 1998; van der Horst *et al.*, 1999; Vitaterna *et al.*, 1999). Both $Cry1^{-/-}$ and $Cry2^{-/-}$ mice exhibit abnormalities in the lengths of their intrinsic circadian rhythms, and $Cry1^{-/-}$ $Cry2^{-/-}$ mice are arrhythmic in constant darkness, indicative of total loss of the molecular clock. The apparently normal behavioral response of $Cry1^{-/-}$ $Cry2^{-/-}$ mice in light/dark cycles can be attributed to masking, which is the acute behavioral response to light with no lasting effect on the phase and period of the rhythm. Given that several processes, circadian and noncircadian, govern an animal's behavioral response to light, molecular analysis of phototransduction by gene induction in the SCN in response to light is the most quantitative and reliable assessment of the contribution of a photoreceptor to circadian phototransduction.

A molecular readout of light signaling to the SCN is typically measured by irradiating mice in the middle of the dark period of their circadian cycle (ZT18-20) with a range of white light doses to generate a dose–response curve. Light given at this point in the circadian cycle rapidly induces robust expression of mRNA of the immediate early gene c-*fos* and the clock genes *Per1* and *Per2*. The level of gene induction is measured quantitatively by *in situ* hybridization of 20-μm slices of the SCN using ^{35}S-labeled probes against a specific gene. The c-*fos* gene is used as the molecular target in assays involving cryptochrome knockout mice, as the disruption of both cryptochrome genes causes constitutively high expression of *Per1* and *Per2*, making direct comparisons of *Per* levels between cryptochrome knockout mice and other genotypes impractical (Selby *et al.*, 2000; Vitaterna *et al.*, 1999). Although Fos protein is not necessary for light-induced phase

shifting, induction of c-*fos* transcription in the SCN serves as a robust marker of photic input to the circadian clock (Honrado *et al.*, 1996). Examination of c-*fos* induction by a variety of chemical agents in immortalized fibroblast lines generated from wild-type and *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice indicates that there are no gross alterations of the well-established signal transduction pathways involved in c-*fos* induction in *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice and that c-*fos* is a suitable target for use in comparing photoresponses in wild-type and *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice (Thompson *et al.*, 2004).

To deconvolute the contributions of various pigments in the retina, genetic approaches were used to eliminate one or more of the candidate pigments (rod and cone opsins, cryptochromes, all opsins, or cryptochromes plus all opsins) and then c-*fos* induction was tested in these animals. *rd/rd* mice were used to eliminate pigments from the outer retina, as the *rd* mutation causes retinal degeneration, resulting in complete histological destruction of the outer retina and a near complete loss of visual pigments by 12 weeks of age and has been used in many studies investigating the role of nonvisual pigments in circadian photoreception. A second approach has been to utilize *rbp*$^{-/-}$ mice whose retinas are histologically normal but when placed on a vitamin A-free diet lack all opsin photoreception due to depletion of the opsin chromophore retinaldehyde (Quadro *et al.*, 1999). These mice lack plasma retinol-binding protein (RBP), the only known serum transport protein for mobilizing hepatic retinol stores to other tissues, including the retina where retinol is converted to retinaldehyde for use as the opsin chromophore. In *rbp*$^{-/-}$ animals maintained on a vitamin A-free diet, the animals become progressively blind; after 6–10 months on a vitamin A-free diet, no electroretinogram signal can be detected and HPLC measurements show that retinal is below sensitive detection limits (0.5 ng per pair of eyecups), reduced 500-fold from wild-type levels (Thompson *et al.*, 2001).

rd/rd *and* rd/rd Cry1$^{-/-}$ Cry2$^{-/-}$ *Mice.* To assess the role of cryptochromes in circadian phototransduction, *rd/rd* and *rd/rd Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice on a 12-h light/dark cycle are irradiated at ZT18 with various doses of white light for a total of 30 min, sacrificed immediately, and the brains frozen under yellow light. Coronal sections of frozen brain (18 μm) are fixed and hybridized with a ^{35}S-labeled c-*fos* antisense RNA probe (nucleolides 855–1577) using the *in situ* hybridization protocol described earlier with standard autoradiography and quantified using a density-calibrated Leica M420 macroscope. Representative SCN slices and quantification of gene induction in wild-type, *rd*, *Cry1*$^{-/-}$ *Cry2*$^{-/-}$, and *rd/rd Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice are shown in Fig. 5 (Selby *et al.*, 2000). Under low irradiance (10^4 μmol/m^2 or less photons), c-*fos* induction was severely attenuated in *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ and *rd/rd Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice, virtually indistinguishable from

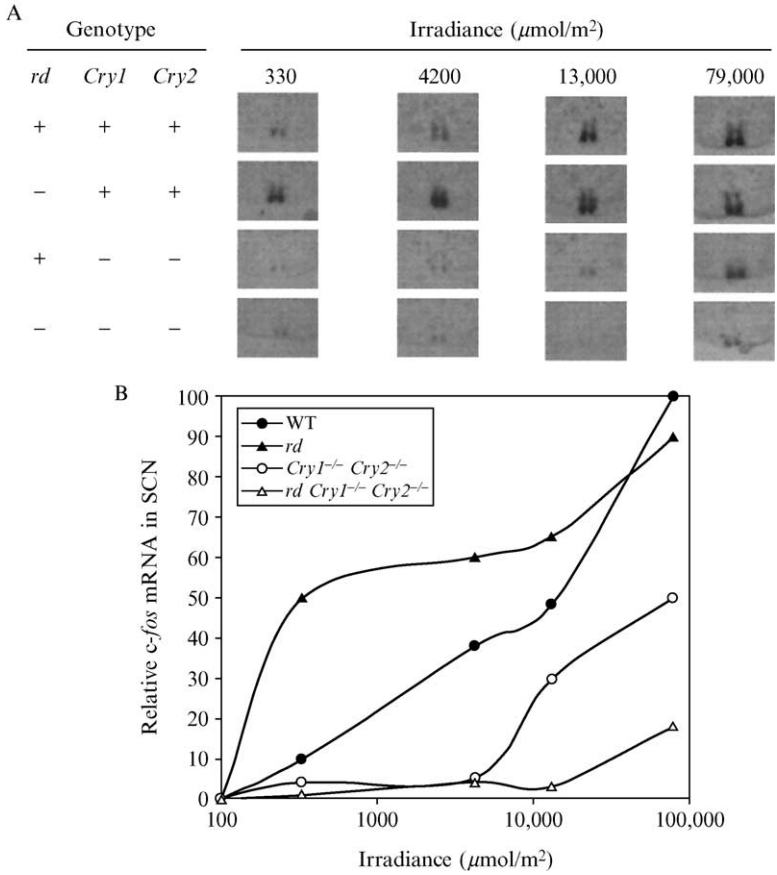

FIG. 5. Analysis of the role of cryptochromes and visual opsins in the photoinduction of c-*fos* in the SCN by *in situ* hybridization. (A) Representative slices exhibiting the strongest signal at each light dose in the SCN are shown for each of the four genotypes. (B) Dose–response plot of c-*fos* induction in the SCN of wild-type and mutant mice. Levels of c-*fos* are plotted relative to the wild type at the highest dose used (79,000 μmol/m^2 photons), which is taken as 100% (Selby *et al.*, 2000).

the uninduced, background level. From these induction curves, it is estimated that the loss of cryptochromes reduces photosensitivity approximately 10- to 20-fold in animals with intact rods and cones and 3000-fold in *rd/rd* animals. Thus, it appears that even in the presence of the visual opsins, the lack of cryptochromes seriously compromises photoinduction of c-*fos*, which is then reduced drastically in their absence. The residual gene induction measured in the *rd/rd* *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ is attributed to

melanopsin (*Opn4*). Data from *Opn4*$^{-/-}$ mice indicate that there are only minor effects of the loss of melanopsin on circadian phototransduction in the presence of the visual opsins; however, all photoresponses are lost in the *rd/rd Opn4*$^{-/-}$ mice (Hattar *et al*., 2003; Panda *et al*., 2002, 2003; Ruby *et al*., 2002). These data considered in their entirety suggest that phototransduction to the SCN by cryptochrome requires melanopsin or the outer retina—how this is accomplished in mechanistic terms is not known at present.

rbp$^{-/-}$ *and* rbp$^{-/-}$ Cry1$^{-/-}$ Cry2$^{-/-}$ *Mice.* Studies with mice of the *rbp*$^{-/-}$ genotype were conducted to assess the relative contributions of opsins and cryptochromes to circadian photoreception. Mice of this background raised on a vitamin A-free diet for 6–10 months have less than 0.2% of the ocular retinal of wild-type mice and yet induction of both *Per1* and *Per2* mRNA in the SCN of *rbp*$^{-/-}$ mice is normal (Thompson *et al*., 2001). In order to address the role of cryptochromes in the remaining photoresponsiveness in *rbp*$^{-/-}$ mice, *rbp*$^{-/-}$ *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice are generated and depleted of ocular retinal on a vitamin A-free diet (Thompson *et al*., 2004). Gene induction in response to light is performed essentially as before; mice are exposed to 80,000 μmol/m^2 photons of white light at ZT18-20 and killed 30–45 min after initiation of the light pulse. As seen in Fig. 6A, triple mutant mice raised on a vitamin A-free diet have virtually no c-*fos* induction compared with *rbp*$^{-/-}$ controls, indicating that cryptochromes are required for photoreception in animals depleted of ocular retinal. Accordingly, the majority of triple mutant mice have lost all behavioral responses to light/dark cycles, as shown in Fig. 6B. Moreover, the sensitivity of pupillary photoresponse in these animals was reduced three logs relative to wild-type mice and one log relative to *rd/rd Cry1*$^{-/-}$ *Cry2*$^{-/-}$ animals, indicating that retinal in both the outer and the inner retina had indeed been depleted. These data strongly indicate a photoreceptive role for mouse cryptochromes, although their light-dependent mechanism of signaling to the SCN remains to be determined.

Zebrafish Cryptochromes

Unlike mammals, which rely strictly on their eyes for all photoreception, some animal species receive extraocular photoreceptive input into the circadian clock. Among the most well studied are avian species such as the Japanese quail and chicken, where the pineal gland in the brain has demonstrated activity as a photoreceptive organ for the circadian clock, and zebrafish (*Danio rerio*), where peripheral clocks in internal organs such as heart and liver are locally entrained by light (Whitmore *et al*., 2000). Several cell lines (PAC1, Z3) have been derived from zebrafish embryos and retain

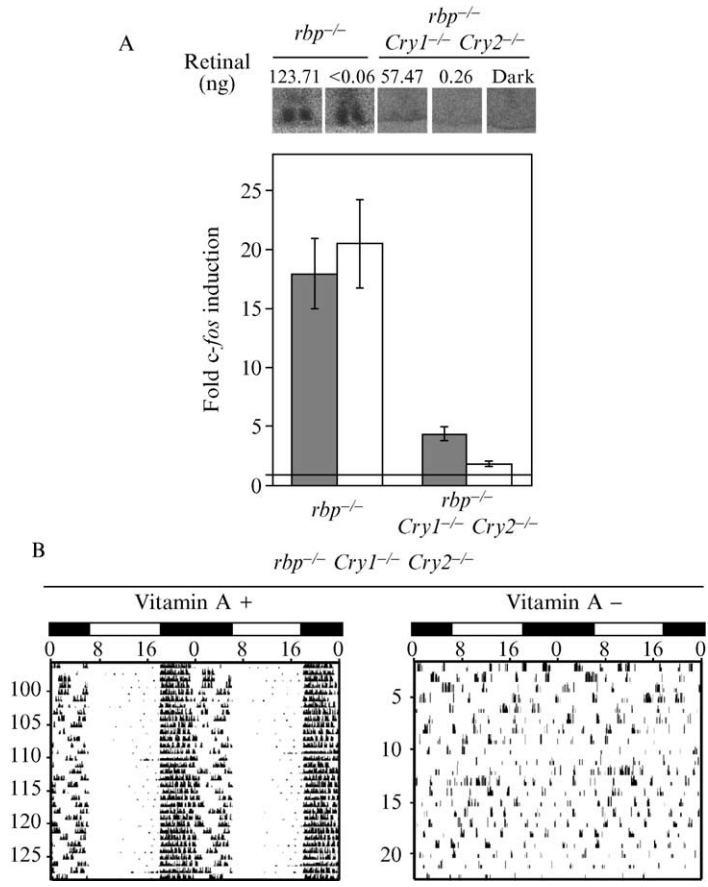

FIG. 6. Elimination of photoresponses in the absence of functional opsins and cryptochromes. (A) Photoinduction of c-*fos* in the SCN analyzed by *in situ* hybridization. Representative slices of the SCN are shown for each genotype. The bar graph represents fold-induction over unirradiated controls. Genotypes are indicated along the *x* axis. Gray bars represent mice on a vitamin A-supplemented diet, and open bars indicate mice on a vitamin A-deficient diet (ocular retinal <10 ng). Error bars represent SEM. (B) Behavioral analyses of $rbp^{-/-}$ $Cry1^{-/-}$ $Cry2^{-/-}$ mice on control and vitamin A-free diets. Actograms of mice from each diet regimen are shown. Ocular retinaldehyde levels in these two mice were 98.7 and 0.5 ng, respectively (Thompson *et al.*, 2004).

photoreceptive input into the clock. These are attractive model systems for studying light input into circadian clocks (Pando *et al.*, 2001; Whitmore *et al.*, 2000). In particular, the Z3 cell line has been rather useful in studying the circadian clock and photoentrainment mechanism in zebrafish.

The zebrafish Z3 cell line expresses all the animal core circadian clock components: Period, Clock, Bmal, and Cryptochrome. Most notably, the cell line undergoes circadian rhythms of clock gene expression that conform to the given light/dark cycle, indicating that the cell line contains the requisite photoreceptors for entraining the clock to light (Pando *et al.*, 2001). The molecular readout of circadian photoreception in the Z3 cell line is the light-dependent gene induction of *zPer2* mRNA. Induction of *zPer2* mRNA is rapid and robust, reaching levels 10- to 15-fold over dark controls within 2 h and is easily measured quantitatively by the RNase protection assay (Cermakian *et al.*, 2002). Unlike the mammalian clock, in which *Per2* expression is rhythmically regulated as a key component of the molecular clock, *zPer2* expression is strictly regulated by light and is therefore thought to be the mechanism by which the zebrafish clock synchronizes to changes in light cycles.

Zebrafish express seven cryptochrome isoforms; four of the zebrafish cryptochromes (zCry1a,b and zCry2a,b) share significant homology with mammalian cryptochromes and can act as inhibitors of the Clock/Bmal heterodimer that acts as the core transcriptional regulator of the clock, similar to the light-independent function of cryptochromes in the mammalian clock (Kobayashi *et al.*, 2000). Two cryptochromes (zCry3, zCry4) that have lost the ability to act as transcriptional repressors in reporter gene assays *in vitro* and an additional gene related to bacterial cryptochromes all have unknown function. Expression of six zebrafish cryptochromes (zCry1a,b-4) has been measured in the Z3 cell line; each cryptochrome displays a distinct expression profile with several of the cryptochrome mRNAs expressed abundantly in naïve, dark-grown Z3 cells and nearly all are strongly induced by exposure to a light/dark cycle (Cermakian *et al.*, 2002).

Action Spectrum of zPer2 *Induction by Light*

The isolation of the Z3 cell line as a model system for studying circadian photoreception has, for the first time, facilitated the identification of dedicated circadian photoreceptors in a simple, well-defined system. An action spectrum is a measurement of the efficiency of the output response (*zPer2* mRNA induction) as a function of the wavelength of light used. Various doses of monochromatic light at wavelengths ranging from 320 to 580 nm were used to induce *zPer2* expression, and the slope of the dose response of each wavelength was calculated as the relative efficiency of that wavelength to elicit the response (Fig. 7A) (Cermakian *et al.*, 2002). The action spectrum of *zPer2* induction in Z3 cells, shown in Fig. 7B, reveals a peak located at 380–400 nm and minimal induction over 450 nm. These data are

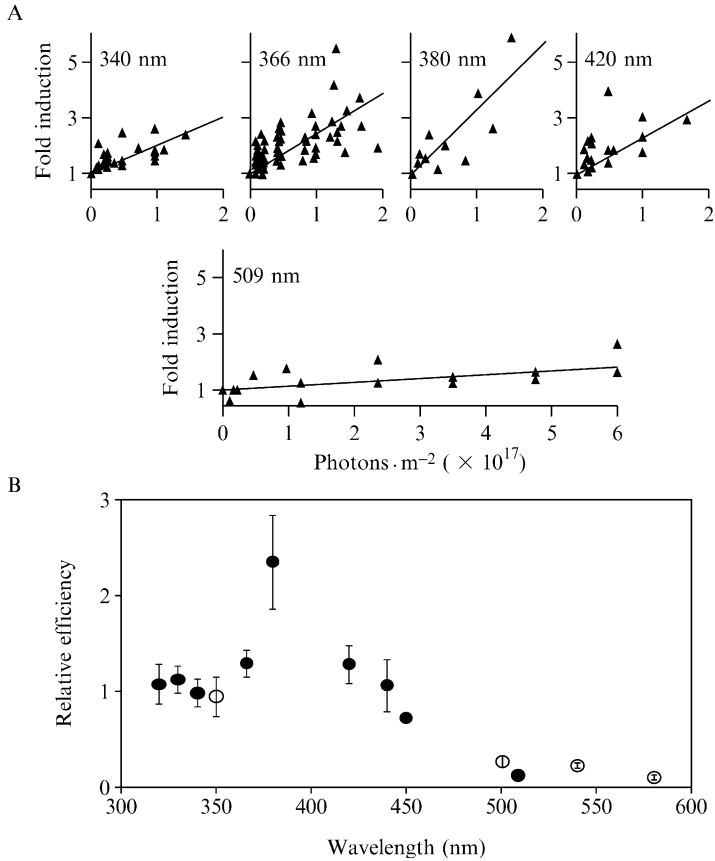

FIG. 7. Action spectrum of *zPer2* induction in the Z3 cell line. (A) Dose-dependent induction of *zPer2* by five monochromatic wavelengths of light. (B) Action spectrum showing the relative efficiency of *zPer2* induction by each wavelength of light used. Relative efficiency is the slope of the linear regression of data in A ($\times 10^{-17}$). Open circles represent single experiments. Error bars represent standard errors for the slope of the regressions (Cermakian *et al.*, 2002).

consistent with either a cryptochrome or UV/blue opsin absorbance spectrum, and the shape of the action spectrum is remarkably similar to the absorption spectrum of the *V. cholerae* cryptochrome VcCry1 (Worthington *et al.*, 2003). The zebrafish Z3 cell line provides an attractive model system for the investigation of the signal transduction of circadian photoreception and cryptochrome function *in vivo*.

Drosophila Cryptochrome

As in mammals, circadian photoreception in *Drosophila* consists of multiple photoreceptive input pathways utilizing both compound eyes and extraocular photoreception in the Hofbauer–Buchner eyelet and pacemaker cells. *Drosophila* has one cryptochrome (dCRY) that acts as a cell autonomous photoreceptor and is sufficient for most aspects of circadian light sensitivity and entrainment to light/dark cycles (Hall, 2000). There are currently no null mutations in *dCry*; the sole mutant available for study of cryptochrome function (cry^b) has a single amino acid substitution in the highly conserved flavin-binding domain (D542N), which presumably cannot interact stably with the catalytic flavin chromophore (Stanewsky *et al.*, 1998). cry^b mutant flies retain behavioral rhythmicity in light/dark conditions but are unable to shift the phase of their behavior in response to pulses of white light, indicating functional redundancy with rhodopsin and other opsins. Interestingly, wild-type flies exhibit two peaks in the action spectrum for phase shifting: a 420- and a 480-nm peak (Helfrich-Forster *et al.*, 2002). $glass^{60j}$; so^1 double mutants, which lack all known external and internal eye structures, lost only the 480-nm peak, suggesting that the remaining 420-nm peak was contributed by cryptochrome. Combination of the cry^b mutation with the $glass^{60j}$ mutation generated flies that lacked all known external and internal eye structures in addition to cryptochromes and resulted in flies that were visually and circadian blind (Helfrich-Forster *et al.*, 2001).

Functionally, dCRY is thought to signal light information to the clock through light-dependent interactions with the integral clock proteins dTIM and dPER, regulating the ability of the dPER-dTIM complex to inhibit CLOCK-mediated transcription (Ceriani *et al.*, 1999; Rosato *et al.*, 2001). Interestingly, in yeast two-hybrid assays, the C-terminal extension of dCRY was required to modulate the light dependence of these interactions; in its absence, all interactions became light independent, suggesting a light-dependent conformational change in dCRY involving the C-terminal domain. In addition, dTIM and dCRY protein levels are sensitive to blue light, undergoing rapid proteolytic digestion in response to light, whereas protein levels in mutant cry^b flies appear to lack light sensitivity (Lin *et al.*, 2001; Naidoo *et al.*, 1999). The signal transduction mechanisms utilized by dCRY, involving light-dependent protein–protein interactions and light-mediated protein degradation, are in agreement with its proposed role as a cell autonomous circadian photoreceptor.

Conclusions

Cryptochromes are flavin and folate-containing blue-light photoreceptors. Their role in regulating the circadian clock in mice and *Drosophila* has been shown unambiguously. Genetic data strongly indicate that

cryptochromes function as circadian photoreceptors in these and other animals. However, direct photochemical evidence for their photoreceptive function remains to be determined.

Acknowledgments

This work was supported by NIH Grant GM31082 to A.S. C.L.P. is supported by NIMH predoctoral National Research Service Award MH70151–01.

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