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CRYPTOCHROME: The Second Photoactive Pigment in the Eye and Its Role in Circadian Photoreception

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■ **Abstract** Circadian rhythms are oscillations in the biochemical, physiological, and behavioral functions of organisms that occur with a periodicity of approximately 24 h. They are generated by a molecular clock that is synchronized with the solar day by environmental photic input. The cryptochromes are the mammalian circadian photoreceptors. They absorb light and transmit the electromagnetic signal to the molecular clock using a pterin and flavin adenine dinucleotide (FAD) as chromophore/cofactors, and are evolutionarily conserved and structurally related to the DNA repair enzyme photolyase. Humans and mice have two cryptochrome genes, *CRY1* and *CRY2*, that are differentially expressed in the retina relative to the opsin-based visual photoreceptors. *CRY1* is highly expressed with circadian periodicity in the mammalian circadian pacemaker, the suprachiasmatic nucleus (SCN). Mutant mice lacking either *Cry1* or *Cry2* have impaired light induction of the clock gene *mPer1* and have abnormally short or long intrinsic periods, respectively. The double mutant has normal vision but is defective in *mPer1* induction by light and lacks molecular and behavioral rhythmicity in constant darkness. Thus, cryptochromes are photoreceptors and central components of the molecular clock. Genetic evidence also shows that cryptochromes are circadian photoreceptors in *Drosophila* and *Arabidopsis*, raising the possibility that they may be universal circadian photoreceptors. Research on cryptochromes may provide new understanding of human diseases such as seasonal affective disorder and delayed sleep phase syndrome.

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HISTORICAL PERSPECTIVE

Rhodopsin, the photoreceptor for vision, was discovered in 1877 (1). Its mechanism of action was elucidated by the work of many researchers over a period of more than a century (2–4). In fact, the last landmark discovery in visual photoreception was the cloning of human genes encoding the blue, green, and red opsins in 1986 (5). Because of the rich history of opsin research, the notion that all photosensory responses mediated by the eye are initiated by opsins became widely accepted. Thus, the recent discovery that in addition to the vitamin A–based opsins the eye contains a second, vitamin B_2–based pigment, cryptochrome, which is unrelated to opsin and which regulates the circadian clock, was unexpected (6, 7), and initially the idea was widely rejected (8).

The existence of a second class of photoreceptors in the eye might have been predicted from recent research in circadian rhythms. Animals use light for vision as well as to sense time of day and adjust their daily behavior (circadian rhythm) accordingly. Data that have been accumulating from circadian research over the past 30 years have revealed that the two photosensory systems differ from one another with regard to the manner of integrating the light stimuli, the type of retinal cells used for absorbing light, and the central nervous system location where the information is processed. Visual information is processed in the cerebral cortex and used to construct a three-dimensional representation of the outside world; in contrast,

photic input into the circadian system is processed in the circadian pacemaker in the hypothalamus to tell the time of day. The issues and views on circadian photoreception have been reviewed recently (9–11) and the molecular aspects of the circadian clock including the important developments of the past three years are covered in two detailed reviews (12, 13). A recent review on cryptochromes (14) provides a historical perspective on the discovery of these pigments as the photoreceptor involved in morphogenesis in plants and circadian photoreception in animals.

CIRCADIAN RHYTHMS

Circadian rhythms are oscillations in the biochemical, physiological, and behavioral functions of organisms with a periodicity of approximately 24 h (9, 13, 15). The circadian (from Latin *circa* = about and *dies* = day) rhythm is perhaps the most widely observed biological rhythm in nature, conceivably because the majority of organisms are exposed to daily cyclic variation of light (day) and dark (night) and it is advantageous to them to synchronize their physical and behavioral activities with these cycles. Circadian rhythms are observed in organisms ranging from cyanobacteria to humans, and their conservation during evolution suggests that they confer a selective advantage. Indeed, it has been experimentally shown that mutant cyanobacteria with an altered rhythm (16) and ground squirrels with no rhythm (17) were overtaken by their wild-type counterparts either in the test tube or in a simulated field condition. However, the circadian rhythm is not universal: the *Archaea* and most of the eubacteria display no circadian rhythm, and several model organisms, including *Escherichia coli*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*, lack circadian rhythms (13).

Circadian rhythms have three basic features. First, the rhythm is an innate property of the organism and is maintained under constant environmental conditions. In fact, the circadian rhythm was discovered in 1729 by the Frenchman Jean-Jacques d'Ortous de Mairan, who found that the daily leaf movements of a heliotrope plant persisted even when the plant was kept in the dark (18). The length of the innate circadian period varies among species but is quite precise for each species and ranges from 22 to 25 h (e.g. *Drosophila*, 23.6 h; mice, 23.7 h; hamsters, 24.0 h; humans, 25.1 h). [A recent study of the period length in humans reports it as 24.2 h (19).] Second, the period length is temperature compensated, so that it is maintained at a constant value throughout the physiological range of external temperature. Third, circadian rhythms are synchronized with the outside world by light. Although heat (20, 21) and other environmental cues can synchronize the rhythm with the environment under specific conditions, light is the predominant and perhaps the only physiologically relevant environmental cue (or *zeitgeber*, from German *zeit* = time and *geber* = giver) for synchronizing the circadian rhythm with the solar day. Figure 1 shows the role of light and dark cycles in regulating activity cycles (photoentrainment), and the changes that occur in activity when light is removed from the cycle.

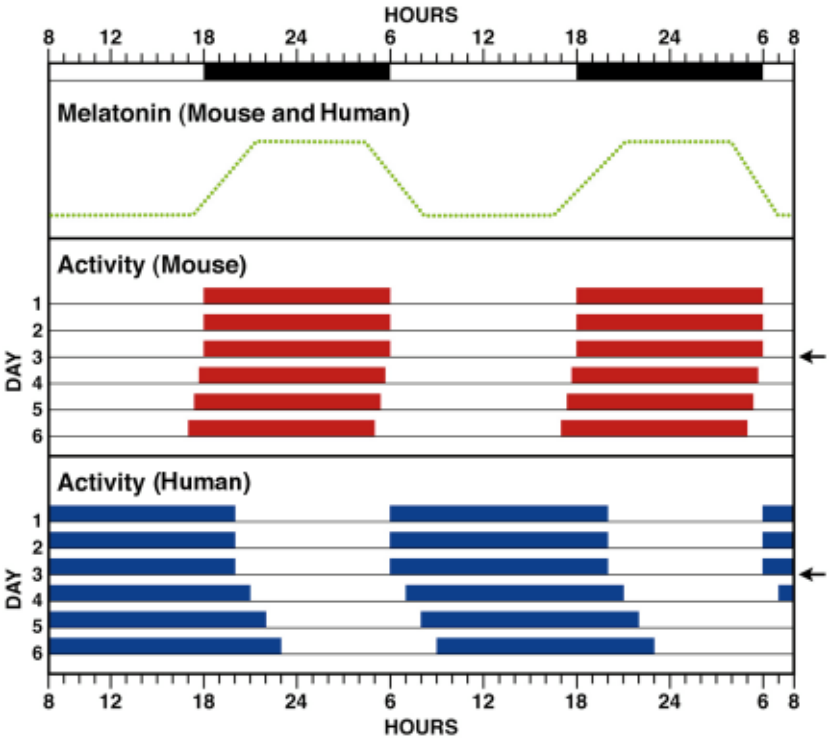


Figure 1 Circadian rhythms in mouse and human. This idealized figure shows the daily oscillation of a physiological variable (melatonin secretion) and a behavioral variable (physical activity) as a function of a cycle of 12 h of light and 12 h of darkness (LD12:12). The bar at the top shows the light and dark phases where light is turned on at 0600 and turned off at 1800. (*Top*) Circadian rhythm of plasma melatonin concentration. Note that in both nocturnal (mouse) and diurnal (human) animals, melatonin levels increase during the night and fall during the day. (*Middle*) Activity record for mouse. Traditionally the activity records are double plotted such that the *first line* shows activity for the first day on the *left side* and for the second day on the *right side*; the *second line* shows activity for the second day on the *left* and the third day on the *right*, and so on. Plotting the data in this manner facilitates comparison of successive days both horizontally and vertically. *Black bars* indicate locomotor activity. At the end of day 3 the light was turned off for the remainder of the experiment (DD, indicated by *arrow*). Under DD, mouse locomotor activity "free-runs" with an intrinsic period of 23.7 h, so the activity phase shifts forward (advances) by about 0.3 h each day. (*Bottom*) Wakefulness record for human. *Black bars* indicate wakefulness. At the end of the third day the subject was switched to a DD condition. Under DD, human circadian rhythm free-runs with a period of 25.1 h. As a consequence, upon transition from LD12:12 to DD the wakefulness phase exhibits a 1-h delay on successive days.

The mechanism by which light is sensed has been a source of great interest in the field of circadian research. Chronobiologists have searched for the circadian photopigment using systems of varying complexities. This review includes a brief survey of naturally occurring photoreceptors and a detailed analysis of past and current research on circadian photoreceptors in mammals.

PHOTORECEPTORS IN NATURE

The eye is required for circadian photoreception in mammals (9), and until recently the only known pigments in the eye were the opsin/retinal-based rhodopsin and color opsins. Thus the circadian photoreceptor was assumed to be either an opsin utilized for both vision and circadian entrainment or a special opsin used for circadian entrainment only. However, other naturally occurring photoactive pigments could function as circadian photoreceptors, especially in plants and protozoa. Although there are many naturally occurring light-absorbing compounds, the number of molecules that convert light energy into either chemical energy (ATP), or information via signal transduction is limited (22). The terms pigment, photoactive pigment, and photoreceptor have been used interchangeably in the literature and we have followed this common practice in the current review where the context makes the meaning clear. Strictly speaking, however, these terms have different meanings, detailed next.

Photoactive Pigments

A photoactive pigment is an organic molecule that absorbs in the near UV–visible light range and upon absorption of a photon initiates a chemical reaction. It has been argued that a photoactive pigment must fulfill three criteria in order to be physiologically relevant (22, 23). First, the absorption spectrum of the pigment should overlap with the wavelengths that are abundantly represented in sunlight. Second, the pigment must have a high extinction coefficient so that it absorbs light with high efficiency. Finally, the excited state of the photopigment (or the photoreceptor) must have a long lifetime so that it initiates a photochemical reaction before it returns to the ground state by radiationless decay. A list of the currently known photopigments that satisfy one or more of these criteria follows; their structures are in Figure 2.

Carotenoids The carotenoids are photoantenna pigments in the photosynthetic system and the catalytic pigments in animal and bacterial rhodopsins. Retinal is the chromophore for the opsin-based visual pigments in animals, and for bacteriorhodopsin in *Halobacteria*, which use light energy for phototaxis and to create a proton gradient across the cell membrane and convert light energy into ATP by chemiosmotic coupling. Carotenoids are also found as photochemically inert pigments in carrots, oranges, and pink flamingos.

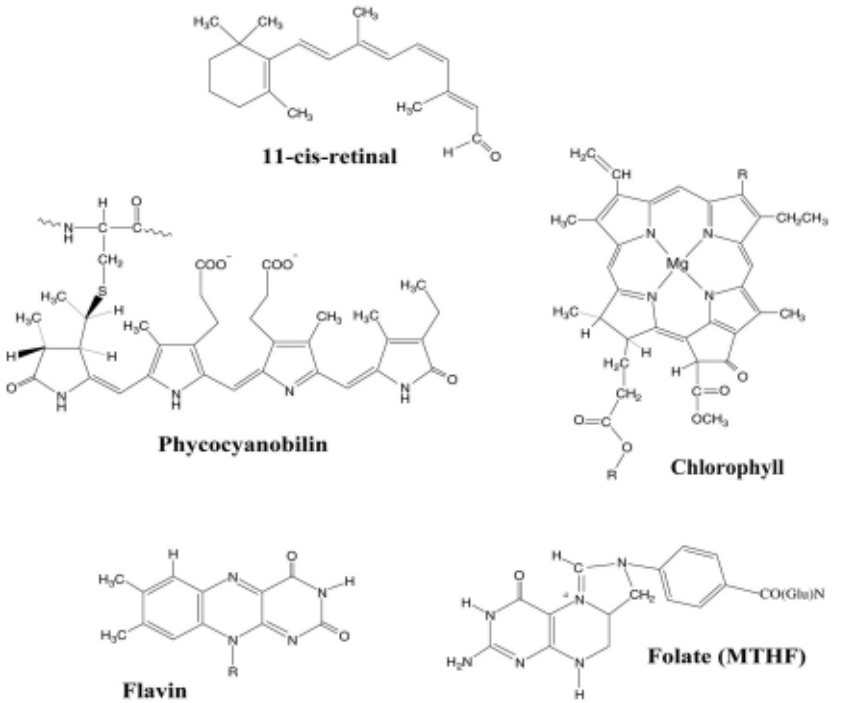


Figure 2 Photoactive pigments (chromophores). The structures of the chromophores found in most photosystems in nature are shown. Retinal-containing photoreceptors absorb in the 350- to 550-nm region. Bilins absorb both in the blue (400–500 nm) and red (600–700 nm) regions. Chlorophylls absorb in the near UV (350–450 nm) and red (600–700 nm) regions. The flavin has an absorption peak at 360 nm in two-electron reduced form; two peaks at 370 and 440 nm in two-electron oxidized form; and peaks at 380, 480, 580, and 625 nm in one-electron reduced (blue neutral radical) form. The unique form of pterin (MTHF) found in the photolyase-cryptochrome family absorbs in the 360- to 420-nm range.

Bilins The bilins are linear tetrapyrroles that function as photoantennas in the light harvesting complex (LHC) of photosynthetic systems and as the chromophore of the plant photoreceptor, phytochrome.

Chlorophylls Chlorophylls are cyclic tetrapyrroles and are utilized both as photoantennas in the LHC and as the primary photoinduced electron donors in the reaction center (RC) of the photosynthetic systems.

Flavins The flavins are redox-active compounds that are cofactors in many light-independent enzymatic reactions. Flavin adenine dinucleotide (FAD) is the photoactive cofactor for the photolyase/blue-light photoreceptor family of proteins (24–30), and flavin mononucleotide (FMN) is the chromophore of the phototropin plant blue-light photoreceptor encoded by the *NPH1* (nonphototropic hypocotyl)

gene in *Arabidopsis* (31, 32). Deazaflavin, despite its name, is chemically more similar to NAD, which is an obligate one-electron donor/acceptor, than to flavin, which can function as both a one- and a two-electron donor/acceptor (33). 5-Deazariboflavin is found in photolyases from a few species including *Cyanobacteria* (34); it functions as a photoantenna in these enzymes (35, 36).

Pterins A special form of pterin, 5,10-methenyltetrahydrofolate (MTHF), is the photoantenna in the majority of the photolyase/cryptochrome blue-light photoreceptor family of proteins (6, 29, 37).

Other Potential Photoactive Pigments This list of photoactive pigments shows that few molecules from the vast repertoire of naturally occurring compounds with conjugated bonds and light-absorbing properties are used in photobiological reactions. However, this is not necessarily a final list. A few other pigments may also be photoactive, even within the narrow range of criteria applied in this article. For example, parahydroxycinnamic acid may act as a photoactive pigment in association with green fluorescent protein (GFP). The parahydroxycinnamic acid cofactor of GFP is excited by either intermolecular energy transfer from aequorin or by direct absorption, and it fluoresces in the 500-nm range concomitant with *cis-trans* isomerization of the polypeptide backbone of GFP at the junction with the chromophore. Because there is no evidence that this photocycle engenders a chemical reaction (38), parahydroxycinnamic acid is not included in the list of photoactive pigments. However, a recent report indicates that parahydroxycinnamic acid is the photoactive pigment of the phytochrome of a photosynthetic bacterium (39).

Photoreceptors

A photoreceptor is an apoprotein containing one or more photoactive pigments that convert light energy into chemical energy or information (i.e. an intracellular signal). Although *photoreceptor* and *photoactive pigment* are often used synonymously, photoactive pigment is actually the chromophore of the photoreceptor. The currently known photoreceptors are listed below.

Rhodopsin Opsin/retinal photoreceptors, along with the photosynthetic system, are the most completely characterized photosystems. Opsins are 30- to 40-kDa transmembrane proteins attached to the chromophore via a Schiff base. In animals, opsins are attached to retinal to form rhodopsin and color opsins, which are the photoreceptors for vision. The absorption maxima are affected by the apoprotein sequence and range from 380 to 560 nm. Humans have four types of opsin/retinal pigments in the eye: rhodopsin ($\lambda_{max} = 500$ nm), blue opsin ($\lambda_{max} = 426$ nm), green opsin ($\lambda_{max} = 530$ nm) and red opsin ($\lambda_{max} = 560$ nm). In *Halobacteria* bacteriorhodopsin is attached to retininyl aldehyde, and the photoreceptor converts light energy into an electrochemical gradient and ultimately into chemical energy in the form of ATP. The primary photochemical reaction in this group of photoreceptors

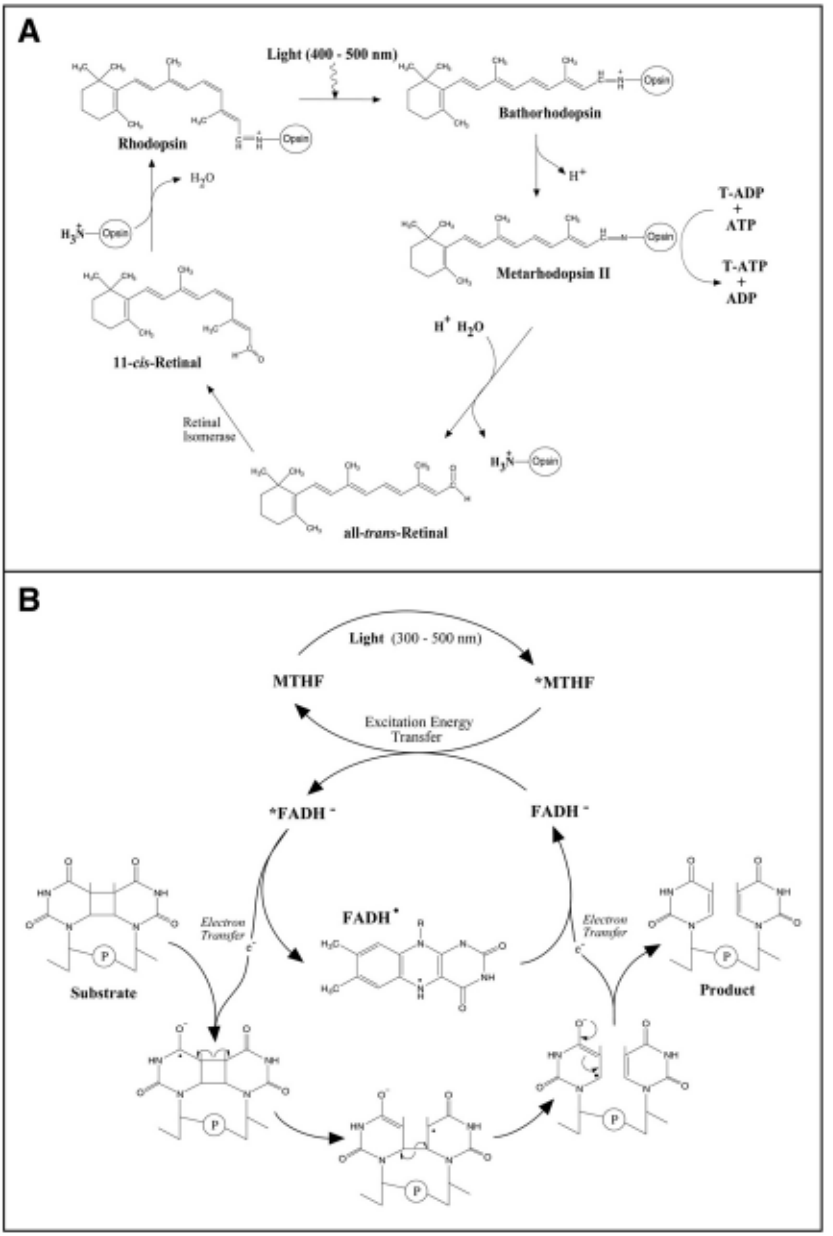
is *cis* to *trans* isomerization of retinal, which in the visual system initiates a signal transduction cascade through a G protein called transducin. The visual photocycle (Figure 3*A*) is among the best-understood signal transduction systems.

Photosynthetic System The photosynthetic system consists of the light harvesting complex (LHC) plus the photosynthetic reaction center (RC), which contains the redox-active special chlorophyll pair attached to the RC polypeptides (RC complex). The LHC contains hundreds of molecules of antenna pigments including chlorophylls, carotenoids, and bilins. Because of the multiple pigments involved, photosynthesis employs photons of nearly the entire sunlight spectrum to harvest energy. The reaction mechanism of the photosynthetic system is known in exquisite detail. A photon absorbed by one of the antenna pigments is transmitted to the RC through the other pigments in the LHC via a series of dipole-dipole interactions and eventually excites the special pair, causing photoinduced electron transfer down one arm of the virtually symmetrical RC complex. This pathway ultimately leads to the splitting of water and the generation of ATP (40).

Phytochrome Phytochromes are cytosolic proteins made up of a homodimer of a 125-kDa polypeptide covalently linked to a linear tetrapyrrole (41). They regulate many plant photoresponses including photomorphogenesis. Phytochromes absorb in the red and far red as well as in the blue region. Light absorption causes *cis* to *trans* isomerization and converts the photoreceptor from the red-light–absorbing phytochrome (Pr) to the far-red-absorbing phytochrome (Pfr). The plant phytochrome is a serine/threonine kinase (42), and the cyanobacterial phytochrome is a histidine kinase (43, 44). Light absorption causes autophosphorylation as well as phosphorylation of phytochrome kinase substrate 1 (PKS1), suppressor of phy A (SPA1), and phyA-phyB-interacting protein (PIF3) in *Arabidopsis* (45–47). Recently it was reported that red light stimulated the binding of phytochrome B to PIF3 (47a) and that red light-activated phytochrome A bound specifically to nucleoside diphosphate kinase2 (47b). However, the precise mechanism of signal transduction by phytochromes is not known.

Phototropin The *NPH1* (nonphototropic hypocotyl) gene encodes the apoprotein for the photoreceptor for phototropism in *Arabidopsis*. It is a 120-kDa protein kinase containing FMN and associated with the membrane. It regulates

Figure 3 Two types of photocycles in nature. (*A*) Visual photocycle. The primary photochemical reaction is the *cis-trans* isomerization of retinal by light, which initiates the signal transduction cascade through the G protein transducin (T). (*B*) Photolyase photocycle. MTHF functions as a photoantenna, absorbing light and transferring the excitation energy to flavin. The primary photochemical reaction is photoinduced electron transfer from $FADH^-$ to the cyclobutane pyrimidine dimer (substrate), which initiates bond rearrangement in the dimer and results in generation of two canonical pyrimidines (product) concomitant with restoration of $FADH^o$ to $FADH^-$ by back electron transfer.


A
Light (400 - 500 nm)
Rhodopsin
Bathorhodopsin
Metarhodopsin II
T-ADP + ATP
T-ATP + ADP
11-cis-Retinal
Retinal Isomerase
all-trans-Retinal
Opsin
B
Light (300 - 500 nm)
MTHF
*MTHF
Excitation Energy Transfer
*FADH -
FADH -
FADH •
Electron Transfer
Substrate
Product

phototropism in response to blue light and contains two light, oxygen, voltage (LOV) motifs. The LOV repeats in other proteins mediate redox-status-dependent responses to light, oxygen, and voltage (31, 48). Interestingly, a phytochrome from the fern *Adiantum capillus-veneris* has sequence features of both phytochrome and NPH1 (49); thus, it may function as a super-photoreceptor that regulates responses to red and blue light. The LOV domains of both *Arabidopsis* and *Adiantum* phototropins bind FMN stoichiometrically (32) and apparently function as blue-light sensors.

Photolyase Photolyase is a 55- to 65-kDa protein that repairs UV-induced DNA damage in a reaction dependent on near UV to blue light (350–450 nm) (50). There are two types of photolyases: one (called photolyase) repairs cyclobutane dipyrimidines and the other [(6-4) photolyase] repairs pyrimidine-pyrimidone (6-4) photoproducts (51). The two types are found in various organisms and exhibit 20–30% sequence identity (52–54). The cyclobutane pyrimidine dimer photolyase is found in many bacteria, some *Archaea*, and some eukaryotes and a grasshopper virus (50, 54a). The (6-4) photolyase has not as yet been found in bacteria or *Archaea* but has been found in *Drosophila*, *Xenopus*, rattlesnake, fish and *Arabidopsis* (54). Humans do not have either enzyme (6, 55). Both types of photolyases contain two photoactive pigments (chromophores). One is invariably FAD in the form of $FADH^-$ (24, 27, 28, 50, 54), and the other so-called second chromophore is a pterin, methenyltetrahydrofolate (28, 37) in most species but 5-deazariboflavin in certain rare species that synthesize this compound (25, 56).

Photolyase repairs DNA as follows (Figure 3*B*). The damage is recognized in a light-independent manner by the enzyme, which forms a Michaelis complex with the substrate. Upon exposure of this complex to light, the second chromophore absorbs a photon and transfers the excitation energy to the flavin, which in turn transfers an electron to the DNA photoproduct; the cyclobutane ring of the pyrimidine dimer or the oxytane ring of the (6-4) photoproduct is broken to generate two pyrimidines (28, 50). Back electron transfer restores the $FADH^0$ neutral radical to the catalytically competent $FADH^-$ form, and the enzyme dissociates from DNA to enter new cycles of catalysis (50, 57–59).

Photolyase has certain functional similarities to the photosynthetic system. First, it contains a photoantenna whose sole function is to gather energy and thus increase catalytic efficiency as measured by the extent of reaction per incident photon. Second, the catalytic cofactor can be excited by nonradiative energy transfer from the photoantenna or by direct absorption of a photon. Third, catalysis is initiated by photoinduced electron transfer. Finally, both processes are very efficient with a quantum yield (number of reaction products per absorbed photon) in the range of 0.7 to 1.0. However, the two systems have significant differences as well. First, the photosynthetic system is membrane-bound whereas photolyases are soluble proteins. Second, the photosynthetic system contains hundreds of antenna molecules per reaction center whereas photolyases have a single second chromophore (photoantenna) and a single FAD (catalytic center) per monomeric

polypeptide. Third, photosynthesis involves a photoinduced electron transfer that results in a net oxidation-reduction reaction. In photolyase, repair occurs by cyclic electron transfer such that at the end of the photocycle the redox states of the enzyme and the substrate/product have not changed (Figure 3*B*). Finally, photosynthetic systems use the energy of the entire solar spectrum, whereas photolyases are UV–blue-light photoreceptors with activity maxima at 370–420 nm for the folate class and 420–440 nm for the deazaflavin class of enzymes.

Blue-Light Photoreceptors/Cryptochromes Studies of the effect of light on organisms have revealed that, remarkably, blue-light responses are universal to nearly all species tested from bacteria to protists, and from plants to animals. Among the responses amenable to phenomenological and photophysical studies are photoreactivation in bacteria, phototropism and photomorphogenesis in plants, phototaxis in protists, and entrainment of circadian rhythms in fungi and *Drosophila* (22, 60–63a). Carotene-, flavin-, and pterin-based photoreceptors have been proposed to mediate these photoresponses, but until recently supporting biochemical or genetic data for most of these claims were not available. A major problem in identifying the blue-light photoreceptors has been that the endpoints of blue-light responses are cellular in nature and not readily amenable to biochemical analysis. A phenomenon called light-induced absorbance change (LIAC) in cell-free extracts, which results from the light-induced photobleaching of *b*-type cytochrome absorption, has been used as an in vitro assay for identifying the blue-light photoreceptors. However, the physiological significance of LIAC has been controversial (62, 63) and this assay has not led to identification of any blue-light photoreceptor. The term cryptochrome was coined as a shorthand for blue-light photoreceptors with the following explanation: "The pigment system(s) responsible for many of the photoprocesses (as ascertained by action spectra) has been nicknamed 'cryptochrome' because of [its] importance in cryptogamic plants and its cryptic nature. This term, despised by many, will suffice us here just because it is shorter than other terms used, such as 'blue (UV) light photoreceptor,' and it will be a useful term until the pigments are identified" (60).

By this definition, photolyase, which mediates near UV–blue-light-dependent reversal of far UV effects, was the first cryptochrome to be cloned (64), sequenced (65), purified to homogeneity (24), and characterized (37, 57, 65, 66). An important reason for the early success in characterization of this blue-light photoreceptor is that in contrast to other blue-light responses, photoreactivation has simple and quantitative in vitro assays (67). Characterization of photolyase revealed an important fact: many photoreceptors may contain more than one chromophore; hence, any attempt to identify a photoactive pigment by action spectrum measurements may be of limited value. Indeed, because the action spectrum maxima of all photolyases are in the 370- to 440-nm range (the two wavelengths where flavin has absorption maxima) it was speculated early that the chromophore was a flavin (68). In fact the chromophores of photolyases are flavin and pterin (or deazaflavin) and because the flavin is in two-electron reduced (bleached) form ($\lambda_{max} = 360$ nm),

it contributes only 10%–20% to the absorption and action spectrum maxima, which are dominated by the pterin or deazaflavin (50). In recent years, other "cryptochromes" have been cloned and characterized. One is the protein encoded by the *HY4* gene of *Arabidopsis thaliana* (69) and another is the protein encoded by the *NPH1* gene of the same organism (31, 46). Both are flavoproteins; NPH1 was discussed briefly above and the FMN-containing photoreceptor encoded by *NPH1* is now called phototropin (32).

Thus, there are currently three blue-light photoreceptors that have been genetically and, to varying degrees, biochemically characterized: photolyase, HY4, and phototropin. They all qualify for the term cryptochrome as originally defined. However, HY4, which has a high sequence homology to photolyase, was called the cryptochrome (30). Two human genes with a high degree of sequence homology to photolyase and HY4 were discovered (6, 52, 70) and shown to encode proteins that have the two photolyase chromophores, FAD and pterin, but no photolyase activity (6). These proteins were presumed to mediate a blue-light response such as entrainment of the circadian clock in humans and thus were called cryptochromes as well (6). Hence, cryptochrome has now assumed a precise definition: a photoreceptor with sequence homology to photolyase but that has no photolyase activity and mediates other blue-light responses. It will be used as such throughout this review. A brief summary of plant cryptochromes is presented below. The rest of this article will discuss the structure and function of mammalian cryptochromes, which is the main focus of this review.

The first cryptochrome gene was isolated from mustard (*Sinapis alba*) as a sequence homolog of photolyase and was thought to be the gene for the mustard photolyase (71). Shortly afterward the *HY4* gene, which encodes one of the two cryptochromes in *Arabidopsis*, was isolated and assigned a function in regulating hypocotyl elongation in response to blue light (69). This gene was later called *CRY1* for cryptochrome (30), and when a second gene homologous to photolyase was found in the *Arabidopsis* genome (72, 73) it was named *CRY2* (73). Mutations in *CRY2* confer a late-flowering phenotype (74). A *CRY* gene has been isolated from the alga *Chlamydomonas reinhardtii* (75) and five *CRY* genes were found in the fern *Adiantum capillus-veneris* (76). However, there are no genetic data on the functions of *CRY*s in these organisms.

Plant cryptochromes exhibit about 30% sequence identity with cyclobutane pyrimidine dimer photolyases (69) of microbial origin and 50% sequence identity with (6-4) photolyase (51). The CRY1 of *Arabidopsis thaliana* and the *Sinapis alba* cryptochrome, which is homologous to CRY2 of *A. thaliana*, have both FAD and pterin cofactors but lack photolyase activity (29). Some CRYs, including CRY1 and CRY2 of *Arabidopsis*, have C-terminal extensions of 80–240 amino acids with no homology with photolyases and very little homology among themselves. This region is thought to be involved in effector function, but it is not necessary for CRY activity.

The reaction mechanism of plant cryptochromes is not known. In *Arabidopsis*, both CRY1 and CRY2 appear to be nuclear proteins (14, 74, 76a, 76b) and both bind

to and are phosphorylated by phytochrome A (a cytosolic protein) in a red-light–dependent manner (77). Significantly, CRY2, but not CRY1, is rapidly degraded by light (blue or red) in a phytochrome-dependent manner (76a, 78). These are important clues to the mechanism of photoreception and phototransduction in plants, but at present the pathway from photoreception by cryptochromes to gene regulation is not known (14).

Following the discovery that the mammalian cryptochromes are circadian photoreceptors (7, 79), it was found that in *Arabidopsis* maintained under dim blue light, the period of gene expression in a *CRY1* mutant was increased by about 4 h. This result implicated cryptochromes in circadian photoreception for *Arabidopsis* (80). However, the interpretation of this result is complicated by the contribution of phytochromes to most photoresponses to both blue and red light, including circadian response. Thus, the roles of cryptochromes and phytochromes in plant circadian responses remain to be more precisely defined. A detailed discussion of mammalian circadian photoreceptors and the methods used to identify them is presented below.

CIRCADIAN PHOTORECEPTORS

Photolyase homologs have been discovered in humans that have a high homology to *Arabidopsis* cryptochromes (6, 52, 81) and no photolyase activity (6), which suggests that the human cryptochromes might also act as blue-light photoreceptors (6, 7). In contrast with plants, in which essentially every aspect of development and behavior is light regulated, photoregulated physiological responses in humans are limited to vision and photoentrainment of the circadian clock. Hence it was proposed that hCRY1 and hCRY2 may be circadian photoreceptors (6). Traditionally, three approaches—action spectra, genetic analysis, and the search for novel opsins—have been used to identify the circadian photopigments. These are discussed below with particular reference to whether or not existing data are consistent with the cryptochromes being circadian photoreceptors.

Action Spectra

An action spectrum is a plot of the rate of a photochemical or photobiological reaction as a function of the wavelength of light eliciting the reaction. In a simple system the action spectrum matches the absorption spectrum of the photoactive pigment. Hence, action spectra have been derived in a number of model systems to identify the circadian photoreceptor. The circadian response action spectrum was measured in *Drosophila* (82, 83) using eclosion (pupae emergence) as an endpoint. The results indicated a peak in the 420- to 480-nm region and thus were considered "consistent with the possibility that a carotenoid is the photoreceptor, but they do not rule out pterins, flavins and other compounds" (82). Recently, the action spectrum for phase shifting in *Drosophila* locomotor activity was determined and essentially the same results were obtained (84).

The first action spectrum for entrainment of the circadian clock in mammals was conducted on golden hamsters (85). This study revealed three important features of the animal circadian photosystem. First, the threshold of light for shifting circadian phase (locomotor activity) is higher than that required for eliciting a visual response. Second, the time-dose reciprocity relationship holds for only about 3 s for vision; that is, a light dose delivered at high intensity within a millisecond has the same effect as the same dose delivered at lower intensity over a period of up to 3 s. In contrast, for circadian photoresponse, as measured by phase shifting with light pulses, the time-dose reciprocity relationship held for at least 45 min. Finally, in hamsters, in which the retina contains almost exclusively rods and very few cones, the action spectrum maximum was at 500 nm, which is consistent with a rhodopsin-like photopigment as the circadian photoreceptor. However, because the visual and the circadian photoresponses showed such a dramatic difference in their time-dose reciprocity relationship, it was suggested that the retinal cells that mediate the circadian photoresponse may be different from those involved in image formation, even though both types of cells might employ the same photoreceptor molecule, rhodopsin (85). Action spectra measurements for phase shifting in mice also revealed a peak near 510 nm, which was ascribed to an opsin-retinaldehyde type of photopigment (86, 87).

Thus, even though the action spectrum in *Drosophila* raised the possibility of a non-opsin photopigment, action spectra in mammals were consistent with opsin-based photopigments and not with "cryptochromes," which absorb in the 370- to 440-nm range. However, action spectra on entire organisms are not highly reliable for identifying photoreceptors for a variety of reasons, including shielding of certain wavelengths by other pigments and light scattering (22, 88). Indeed, genetic and biochemical genetic experiments described below raised different possibilities from the action spectra regarding the nature of the circadian photoreceptor.

Genetic Analysis

A *Neurospora* strain defective in flavin biosynthesis has a severely reduced sensitivity to photoentrainment (89), suggesting that the photoreceptor in this organism may be a flavoprotein, although it is not known whether this organism possesses a cryptochrome. In an interesting experiment, *Drosophila* raised on an aseptic diet without β-carotene (the precursor of retinal) had essentially no visual responsiveness yet maintained normal circadian photosensitivity (90), leading to the conclusion that the circadian photoreceptor in *Drosophila* is not opsin-based. Indeed, *Drosophila* that are visually blind because of mutations in either rhodopsin biosynthetic genes or in the phototransduction pathway have nearly normal circadian photoentrainment (84, 91), again consistent with a non–opsin-based pigment being the circadian photoreceptor in flies.

From the perspective of mammalian circadian photoreception, the studies on *rd* mice are especially illuminating. These mice, which include the common laboratory strain C3H/HeJ, have a mutation in the β subunit of rod cGMP

phosphodiesterase (92). As a consequence of the *rd/rd* genotype, the rods start to degenerate shortly after birth and by 9 weeks of age all rods are lost. The rod loss causes secondary degeneration of cones and at 1 year of age more than 99% of the cones have disappeared as well (see Figure 6). When these rodless and coneless mice were tested for circadian functions, their photoentrainment to a cycle of 12 h of light and 12 h of darkness (LD12:12) and their phase shift of locomotor activity by light pulses were indistinguishable from those in wild-type mice (93). When the action spectrum of *rd* mice was determined for phase shifting, a maximum was found near 510 nm, which was ascribed to the middle-wavelength-sensitive cone photoreceptors (M-cones). However, it was also found that light at 357 nm was more effective than at 510 nm and hence it was concluded that in the *rd* mice, photoentrainment was mediated by a few surviving cones with green- and ultraviolet-sensitive opsins (86). Because the number of surviving cones and the amount of detectable retinal in these animals was so low as to be insignificant, it was also suggested that the mouse retina may contain a novel class of photoreceptor cells with green and UV opsins that are dedicated to circadian photoreception (86). However, in a different strain of *rd* mouse (CBA/J), the action spectrum maximum for phase shifting was 500 nm for the wild type and 480 nm for the *rd* mice, which led to the conclusion that subsets of both rods and cones contain a unique photopigment that works for circadian photoreception but has no role in vision (87, 94).

Finally, the possibility of any role for rods and cones in circadian photoreception was virtually eliminated by the use of transgenic mice in which the rods and cones were selectively destroyed by joining the opsin promoters to the diphtheria toxin gene (95, 96). Tissue-specific expression during development ablates both cell types and there are no detectable rod or M-opsin photopigments in these animals (95, 96). Yet circadian responses in these animals measured by a phase shift in locomotor activity or suppression of melatonin production by 509-nm light were indistinguishable from those of wild-type animals. Hence, novel opsins located in a different part of the retina were proposed as circadian photoreceptors (95, 97). Apparently, certain blind people with no conscious visual perception of light but normal circadian photoresponse have retinal lesions analogous to the rodless and coneless mice (98).

Novel Opsins

The normal circadian behavior of *rd* mice and the well-established presence of extraocular circadian photoreceptors in the pineal gland and deep brain of birds (99, 100) and in the pineal gland, deep brain, and parietal eye of reptiles (101) led to the search for new opsins that might be specialized for the circadian photosystem. These searches have identified several new opsins: pinopsin in the chicken pineal gland (102, 103); melanopsin in the dermal melanophores, the hypothalamus, and the retina of *Xenopus* (104); vertebrate ancient opsin (VA-opsin) in some blind rodents and in the amacrine cells of salmon retina (105, 106); and peropsin and encephalopsin in the mammalian brain (107). However, no genetic or biochemical

data link these opsins to circadian photoentrainment. In particular, with the possible exception of melanopsin no opsin has been found in mammalian retinal cells other than rod and cone photoreceptors, and the genetic data from mice discussed above show that circadian photoreceptors are not located in these cells.

ANATOMY OF THE MAMMALIAN CIRCADIAN SYSTEM

As stated above, the circadian system has three fundamental properties: an intrinsically generated oscillator with a periodicity of approximately 24 h, insensitivity of period length to temperature, and the ability to be adjusted and regulated by light. Thus, the system is conceived as having three components (108): photoreceptor (afferent), oscillator (clock), and output (efferent). The synchronization of the clock with the solar day is so important that a number of organisms have multiple photosensory systems for the circadian input pathway (9, 109, 110). Plants, the dinoflagellate *Gonylaux polyhedra*, and *Cyanobacteria* appear to have both a red and a blue photoreceptor for setting the circadian clock (80, 110–112). Reptiles and birds have three or four photoreceptor organs (109): the eyes, the parietal eye (reptiles), the pineal gland, and the deep-brain photoreceptors. In mammals, in contrast, all existing evidence indicates that the photoreceptors for both vision and the circadian clock are located in the eye (10). A recent report on circadian photoreception through the skin in humans (113) awaits confirmation. In blind hamsters exposure of shaved skin to light does not elicit a circadian response (114).

In mammals total retinal degeneration, enucleation, or severing the optic nerve causes both visual and circadian blindness (96, 98). However, the photoreceptors for the two systems have different histological locations within the retina (7, 93, 96), and the centers for processing the imaging and circadian photic input have different anatomical locations within the brain (115), as shown in Figure 4. Light for vision is absorbed by rhodopsin and color opsins in rods and cones, respectively, which are located in the outer retina near the pigmented epithelium. In contrast, light for synchronizing the circadian clock is absorbed by cryptochromes in ganglion cells and cells in the inner nuclear layer (amacrine cells, Müller cells, interneurons), which are in the front part of the retina (7) (see Figure 6). Signal phototransduction for both systems is through the optic nerve. However, whereas the axons for vision continue their path in the optic nerve to the visual centers in the cortex, the axons of the circadian system part from the optic nerve at the optic chiasma and go upward to a pair of neuron clusters in the anterior hypothalamus called the suprachiasmatic nucleus (SCN). The SCN is the master circadian pacemaker organ (116).

Although recent research has shown that the circadian oscillator is cell-autonomous in organisms ranging from *Drosophila* (117, 118) to mammals (119), the SCN is the master pacemaker that apparently overrides all local oscillators and coordinates the peripheral oscillators with the pacemaker clock. An independent clock exists in the retina, but in the absence of the SCN it cannot act as a pacemaker

Figure 4 The retinohypothalamic tract for circadian phototransduction in mammals. Light absorbed by the rods and cones generates a signal that is transmitted through the optic nerves to the visual cortex (*green*). Light absorbed by the neurons in the inner nuclear layer and ganglion cell layers of the retina produces a signal that travels through the optic nerve to the suprachiasmatic nucleus (*red*), the circadian pacemaker above the optic chiasma. (Adapted from Reference 115.)

to regulate the organism's circadian rhythm (120). Retrograde labeling experiments have shown that a small subset of the retinal ganglion cells form the basis of the retinohypothalamic tract that transmits photic information from the eye to the SCN. Remarkably, cell lines derived from the rat SCN maintained a robust circadian rhythm as measured by the uptake of the metabolic marker 2-deoxyglucose. Most significant, the transplantation of SCN cell lines, but not mesencephalic or fibroblast lines, restored the circadian activity of SCN-lesioned rats (121). These properties clearly explain the unique pacemaker function of the SCN as opposed to other tissues that also have circadian oscillator systems. Similarly, even though transcription of the rat period 2 gene (*rPER2*) oscillates with circadian periodicity in both the SCN and peripheral tissues, destroying the SCN abolished the peripheral oscillation of the *rPER2* transcription, leading to the conclusion that SCN-released humoral factors control the peripheral oscillators (122). The mechanism by which the SCN itself is synchronized with the outside world through cryptochromes is discussed below.

STRUCTURE AND FUNCTION OF MAMMALIAN CRYPTOCHROMES

Physical and Biochemical Properties

Cryptochromes have 20%–25% sequence identity with microbial photolyases and 40–60% sequence identity with (6-4) photolyase; and the sequence identity between CRYs from various sources is remarkably high: human, *Drosophila*, and *Arabidopsis* cryptochromes have about 60% sequence identity (54). However, CRYs from different sources have nonhomologous C-terminal extensions ranging from very short ones in *Drosophila* to as many as 240 amino acids in plants. The two human CRYs are 73% homologous to each other but exhibit no sequence homology within the C-terminal 75 amino acids. It is thought that this domain may bind to effector molecules (14, 54). The hCRY1 protein is 586 amino acids in length and encodes a protein of 66 kDa. The hCRY2 protein is 593 amino acids in length and has a mass of 67 kDa (6). The mouse CRY proteins are nearly identical to the human proteins with the exception of a short region in the C-terminal domain (7, 123).

At present there is no crystal structure of a cryptochrome. However, the crystal structures of two photolyases (*E. coli* and *A. nidulans*) have been solved (124, 125). Even though these two enzymes share only 30% sequence identity and use different second chromophores (a pterin versus a deazaflavin), the two structures are virtually superimposable. Hence it is reasonable to assume that the human cryptochrome structure would be very similar to that of *E. coli* photolyase (Figure 5). The enzyme has an overall dimension of $80 \times 60 \times 30$ Å and consists of two well-defined domains interconnected by a loop of 62 amino acids. The N-terminal α/β domain adopts a fold (the Rossman fold) typical for dinucleotide binding domains although the primary sequence has no homology to other flavoproteins with the Rossman fold and this domain does not actually bind the FAD dinucleotide cofactor of the enzyme. The pterin cofactor is located in the interdomain cleft near the surface and makes intimate contact with residues in the α/β domain. The C-terminal helical domain is made almost entirely of α helices and resembles a slab of $60 \times 40 \times 20$ Å.

The most prominent feature of the α/β domain is a hole in the center of the flat face that leads to FAD in the bottom of the hole. The dimensions of the hole match those of a pyrimidine dimer. It has been proposed that the enzyme flips out the pyrimidine dimer from the DNA helix into the hole in close proximity with the catalytic flavin cofactor so that photoinduced electron transfer and repair can occur with high efficiency (124). The FAD in photolyase has a U-shaped (or *cis*) conformation with the isoalloxazine ring and adenine in close proximity. This conformation is not found in flavoproteins, which carry out redox reactions from the ground state, and it is thought to be unique to proteins of the photolyase/blue-light photoreceptor family, which carry out catalysis from an excited state (124).

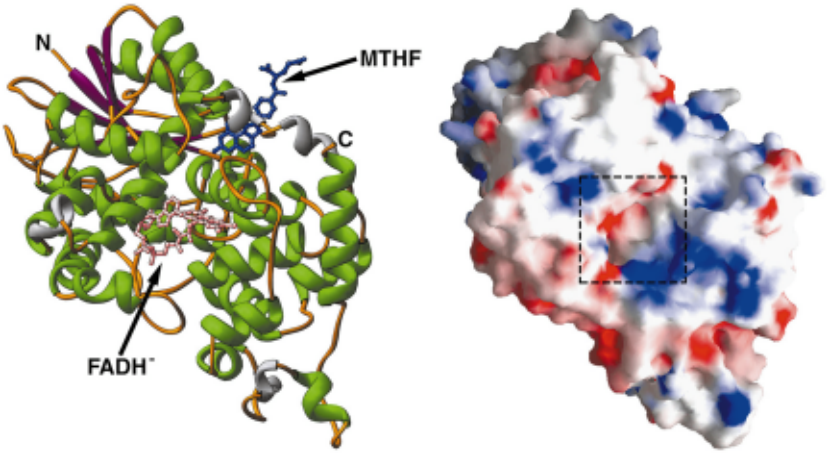


Figure 5 Structure of photolyase. The crystal structure of *E. coli* photolyase is shown in two forms. (*Left*) Ribbon diagram. Note the location of the MTHF photoantenna in the crevice between the α/β domain and the α-helical domain and exposed to the solvent. The $FADH^-$ catalytic cofactor is in *cis* conformation (the adenine ring stacked on the flavin ring) and is deeply buried within the α-helical domain. The center-to-center distance between the two chromophores is 17 Å. (*Right*) Electrostatic surface potential and solvent accessible surface of the enzyme. Note the positively charged groove running the length of the molecule and the hole (*boxed*) in the center of the groove; the hole leads to the $FADH^-$ in the core of the protein.

Remarkably, molecular modeling of hCRY1 and hCRY2 onto the α backbone of *E. coli* photolyase indicate that the overall geometry of *E. coli* photolyase, including the hole in the center, is retained in these proteins. These observations raise the interesting possibility that cryptochromes have retained the unique features of photolyase reaction mechanism: dinucleotide flipping and photoinduced electron transfer.

The human cryptochromes contain both chromophore/cofactors of the photolyase/cryptochrome family of proteins. When expressed as recombinant proteins, they have identical near UV–blue absorption spectra with a 420-nm peak (6). However, cryptochromes purified from their natural sources are not currently available, and hence it is not known if the absorption spectra of the recombinant proteins are identical to those of the native proteins. The cofactors of this class of proteins are readily lost or oxidized during purification from the natural sources (37, 66). Thus, the active form of photolyase contains the flavin in the two-electron reduced $FADH^-$ (or $FADH_2$) form, but during purification the flavin in all but the *S. cerevisiae* photolyase (58) becomes oxidized to the catalytically inert flavin neutral radical ($FADH^o$) and fully oxidized FAD (57, 66). Thus, caution must be exercised in comparing the absorption spectra of purified cryptochromes to the circadian action spectra.

Expression of Cryptochromes in the Retinohypothalamic Axis

In humans, cryptochromes are expressed in all organs and hence the expression pattern is not particularly revealing of function. It is noteworthy that the expression of other genes thought to be strictly clock genes is not confined to the two organs known to be critical for the circadian mechanism, the retina and the SCN. Thus, *CLOCK*, *BMAL1*, *PER1*, *PER2*, *PER3*, and *TIM* are expressed throughout the body (126–132). However, histological examination of the expression of *mCry1* and *mCry2* in the mouse retina and the brain is quite informative (7). The visual photoreceptors, rhodopsin and the color opsins, are expressed in the inner segment and to a lesser degree the outer segment of the retina. In contrast, *mCry1* and *mCry2* are expressed almost exclusively in the ganglion cell layer (GCL) and inner nuclear layer (INL) and are evenly distributed in the central and peripheral retina (Figure 6). These are the layers that remain intact in *rd* mice, which are visually blind but have normal circadian photoresponse (93). Both genes are also expressed throughout the brain. In general, *mCry2* transcript is more abundant than that of *mCry1* in the brain. In the pyramidal cell layer of the hippocampus, the granular cell layer

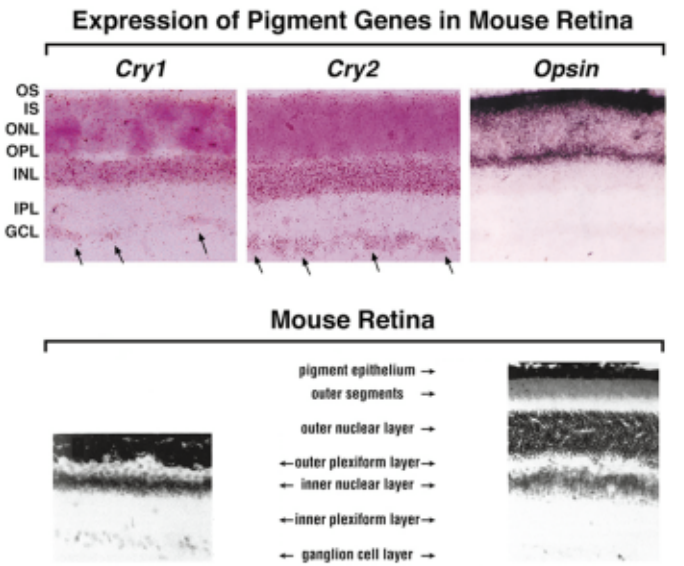


Figure 6 Expression of *mCry1* and *mCry2* in mouse retina. In situ hybridization was performed with probes for cryptochromes and for the mouse opsin gene. Both *Cry* genes are expressed in the ganglion cell layer (GCL) in a subset of ganglion cells indicated by *arrows* and in the inner nuclear layer (INL), whereas the opsin is expressed in the inner segment (IS) and the outer nuclear layer (ONL). The last two frames show the retinas of a control mouse (*right*) and an aged *rd* mouse (*left*) in which only the regions expressing cryptochromes remain intact. (From References 7, 93.)

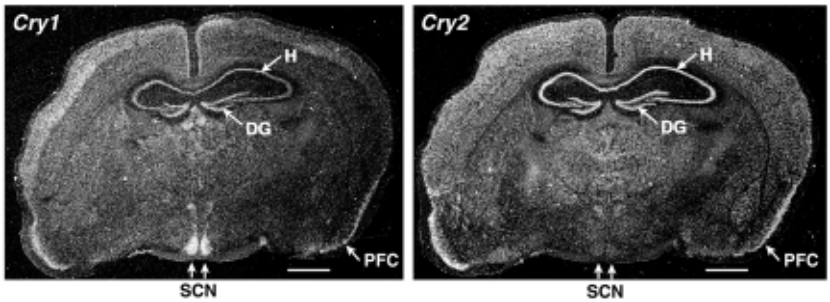


Figure 7 Expression of *mCry1* and *mCry2* in the mouse brain. Coronal brain sections made at the *zeitgeber* time of ZT = 6 (see Figure 8 for a *zeitgeber* scale) were probed with appropriate antisense RNAs. Both genes are highly expressed in all cerebral cortical layers but particularly in the pyramidal cell layer of the hippocampus (H), the granular cell layer of the dentate gyrus (DG), and the pyramidal cell layer of the piriform cortex (PFC). Strikingly, the strongest signal of *mCry1* is in the SCN, where *mCry2* expression is modest. (From Reference 7.)

of the dentate gyrus, and the pyramidal cell layer of the piriform cortex, the ratio of *mCry2* to *mCry1* transcript is about 2 to 1. However, *mCry1* is expressed at a high level in the SCN, whereas *mCry2* expression in this region is almost negligible (Figure 7). These expression patterns suggest that CRY1 and CRY2 perform partly redundant and partly complementary functions in running the circadian clock.

Circadian Oscillation of Cryptochrome Expression

The transcription of the *mCry1* gene in the SCN exhibits a circadian oscillation (7) comparable to those of other circadian genes including *mPer1* (127, 128), *mPer2* (133, 134), *mPer3* (129, 135), and *mTim* (130, 136). The *mCry1* mRNA level reaches a maximum at ZT 6–8 (ZT = 0 by convention, the time at which the light is turned on) and declines to a minimum at ZT 24 (Figure 8). The oscillation of *mCry1* transcript persists when the mice are kept in constant darkness (137), as is observed for the *mPer* genes and as is expected of a true circadian regulator. *mPer1* and *mPer2*, but not *mPer3*, can be induced from a low at nighttime to a high level of expression with light pulses of 5- to 60-min duration (133, 134). *mCry1* behaves like *mPer3* in that its expression is not inducible with acute light pulses (137). Because light pulses that induce *mPer1* and *mPer2* cause a proportional shift in the phase of locomotor activity, it appears that acute phase shifting can occur without changing the circadian expression pattern of *mCry1* in the SCN.

mCry1 expression also shows circadian oscillation in internal organs, most notably in the liver (Figure 8); however, the phase of expression in internal organs is delayed relative to the phase of SCN expression by about 8 h (7, 137, 144). Similar observations were made for *mPer1*, *mPer2*, and *mPer3* (129). Taken together

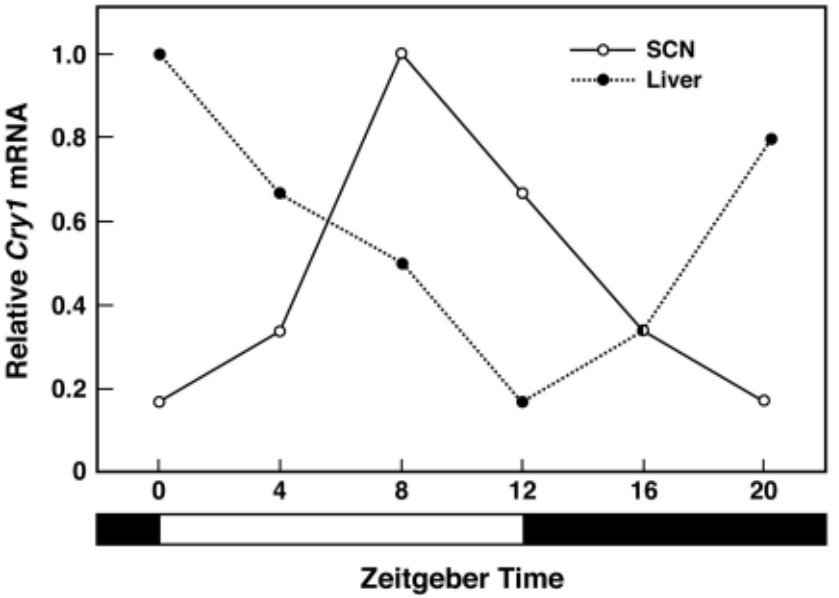


Figure 8 Circadian oscillation of *mCry1* expression in the SCN and liver. The *mCry1* RNA levels were quantified by in situ hybridization in the SCN and RNase protection in the liver. The values are expressed relative to the maximum within each set. (Adapted from References 7, 137.)

these data constitute a strong case for the presence of peripheral clocks that are presumably subordinate to the master clock in the SCN. Interestingly, even though *mCry1* is expressed at a high level in the testis, there is no obvious transcriptional oscillation of *mCry1* mRNA in this organ (137). Furthermore, the *mCry1* expression in testis seems to be restricted to spermatogonia with little or no expression in Leydig cells (137). It has been reported that during meiosis, which occurs in the development of spermatocytes, many genes are turned on but the transcripts from these genes are not translated (138). This is not the case for *mCry1*. In fact, testicular tissue contains the highest relative amount of mCRY1 protein of any tissue tested. However, as mice lacking *Cry1* have apparently normal reproductive functions (139, 140), the significance of such high levels of mCRY1 in testis has yet to be determined.

Cellular Localization of Cryptochromes

All known animal clock proteins are located either in the nucleus or shuttle between the cytoplasm and nucleus to exert their feedback into the circadian transcription loop. Thus, the location of a potential photoreceptor molecule is important from the standpoint of the phototransduction mechanism. A membrane-bound

photoreceptor such as rhodopsin must transduce the light signal through intermediate molecules to the effector clock proteins or genes located in the nucleus (12, 13). In contrast, a nuclear photoreceptor can interact directly with the clock genes and proteins and thus function both as a photoreceptor and phototransducer. Cryptochromes appear to function by the latter mechanism because the following lines of evidence show that they are located in the nucleus. First, yeast two-hybrid analysis revealed that CRY1 and CRY2 interact with the nuclear serine/threonine phosphatase 5 (141, 142). Second, transient transfection of human cells with full-length *hCRY1-GFP* and *hCRY2-GFP* fusion genes lead to nuclear accumulation of the fusion proteins as revealed by fluorescence microscopy (79, 143). A report of mitochondrial localization of mCRY1 protein (123) has not been confirmed (143, 144). Third, both mCRY1 and mCRY2 bind to and affect the activities of the PER1 and PER2 proteins, which are known to shuttle from the cytoplasm to the nucleus (144). Finally, mCRY2 has the bipartite nuclear localization signal PKRK-X13-KRAR in its C-terminal domain (79, 123). The fact that CRY1 and CRY2 are essential components of the molecular clock, which resides in the nucleus, is further evidence for their nuclear localization.

Interactions of Cryptochromes with Other Clock Proteins

A yeast two-hybrid screen with the C-terminal domain of 381 amino acids, which includes the flavin binding site of hCRY2, detected the protein serine/threonine phosphatase 5 (PP5) as a hCRY2-interacting protein (141). PP5 is the only known nuclear serine/threonine phosphatase in mammalian cells (142). It contains three tetratricopeptide repeats (TPR) and it binds to both hCRY1 and hCRY2 through this motif, which is found in many proteins as a protein-protein interaction motif. CRY proteins do not have the TPR motif. Binding of hCRY2 to PP5 reduces the phosphatase activity by 80% in a light-independent manner (141). However, these experiments were conducted with recombinant hCRY2, which is severely depleted of FAD, and this may explain why the effect of hCRY2 on PP5 was not affected by light. Hence the physiological significance of CRY-PP5 interaction remains to be determined. However, it is known that in *Drosophila* phosphorylation/dephosphorylation of TIM and PER is an integral part of the photoentrainment mechanism (12, 145, 146).

An in vitro assay with immobilized hCRY2 revealed specific interaction with PER1 and TIM1 but not CLOCK (139). A detailed study using a cotransfection/coimmunoprecipitation assay coupled with reporter gene assay in transient transfection experiments in NIH-3T3 cells showed the following (144): (*a*) Both mCRY1 and mCRY2 bind to mPER1, mPER2, mPER3, and mTIM; (*b*) CRY-PER interaction results either in nuclear transport or nuclear retention of PERs; (*c*) mCRY1 and mCRY2 inhibit transcription of a clock gene (*mPer1*) and an output gene (vasopressin) mediated by CLOCK•BMAL1 and MOP4•BMAL1, respectively. However, another study using both the yeast two-hybrid assay, and a CLOCK•BMAL1-driven luciferase assay with the *mPer1* promoter as the target,

identified PER2, BMAL1, and CLOCK as the main targets of CRY1 and CRY2 (146a). It was found that both CRY1 and CRY2 bound to PER2 strongly and that CRY1 bound to BMAL1 whereas CRY2 bound to CLOCK with high affinity. Both CRY1 and CRY2 inhibited the transactivation of *mPer1* promoter by the CLOCK•BMAL1 complex. There were some quantitative differences between the effects of hCRY1 and hCRY2 on CLOCK•BMAL1 activity, which may explain the differential effects of *mCRY1* and *mCRY2* knockouts on circadian and molecular behavior of the mutant mice addressed below. Significantly, in this study neither the interactions of CRY1 and CRY2 with the clock components, nor their negative regulatory effects were affectd by light (146a). Clearly, the light-dependent function of mammalian cryptochromes needs to be addressed by more direct assays with native proteins and under physiological conditions.

Of potential relevance to the CRY photoreception/phototransduction mechanism is the finding that in *Arabidopsis* all morphogenetic phototransduction reactions depend on COP9 signalosome, which is related to the 26S proteasome complex (147, 148). Interestingly, the subunits of the COP9 signalosome are conserved between plants and animals, and the complex is located in the nucleus where it can directly interact with the molecular clockwork. However, at present no evidence links the COP9 signalosome to the mammalian clockwork.

GENETICS OF MAMMALIAN CRYPTOCHROMES

No human or mouse genetic diseases are known to be caused by mutations in cryptochrome genes. However, the biochemical and photochemical properties of mammalian cryptochromes, the photoreceptor function of all other members of this family of proteins [photolyase, (6-4) photolyase, plant cryptochromes], and the circadian oscillation of *mCry1* in the SCN of mice constitute compelling evidence for the involvement of cryptochromes in circadian photoreception and regulation. Direct evidence for the role of *Cry* genes in circadian regulation has been obtained from the characteristics of *Cry* knockout mice lacking *Cry1*, *Cry2*, or both.

Phenotype of Cry Mutant Mice

Cry1 knockout mice have seemingly normal circadian behavior under a regimen of 12 h of light and 12 h of darkness (LD12:12) (Figure 9), as tested by locomotor (wheel-running) activity (139, 140). In constant darkness, however, the animals exhibited a free-running clock with 22.7-h periodicity, which is about 1 h shorter than normal (Figure 9*B, E*). These data are consistent with an integral role of mCRY1 protein in the transcription loop that generates the molecular clock.

Cry2 knockout mice show near-normal locomotor activity under the LD12:12 condition but exhibit a free-running period 1 h longer than wild-type animals (79) when kept in darkness (Figure 9*C, E*). Thus, both mCRY1 and mCRY2 are important for maintaining a circadian clock of normal periodicity independent of their

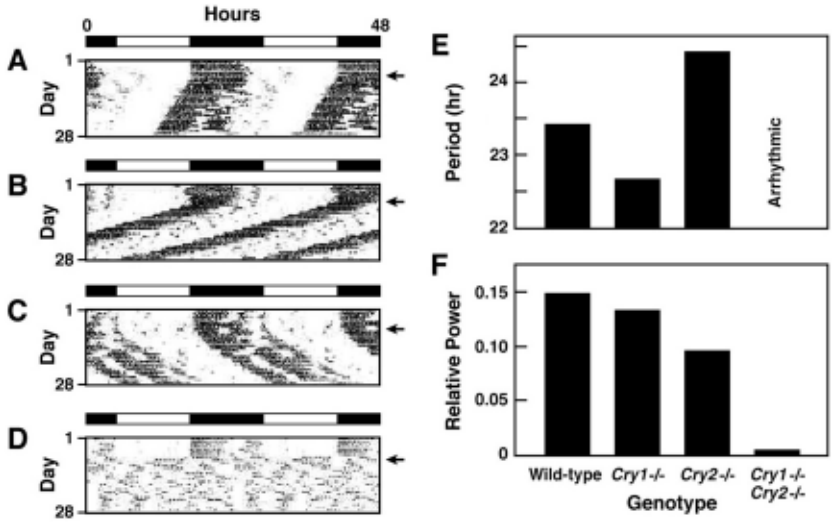


Figure 9 Effect of mutations in *mCry* genes on locomotor (wheel-running) activity rhythms. (*A–D*) Wheel-running activity records of individual mice in the conventional double-plotted format. *Black* represents times of activity. The animals were kept under an LD12:12 cycle and were transferred to constant darkness (DD) on the day indicated by an *arrow*. (*A*) Wild-type mouse. (*B*) $Cry1^{-/-}$ mouse. (*C*) $Cry2^{-/-}$ mouse. (*D*) $Cry1^{-/-}Cry2^{-/-}$ mouse. (*E*) Effect on circadian period of disrupting the *Cry* genes. The free-running period is about 0.8 h shorter and 1.0 h longer than normal in $Cry1^{-/-}$ and $Cry2^{-/-}$ animals, respectively. The double knockout is arrythmic. (*F*) Fourier analyses of activity records show no periodicity, circadian or otherwise, in the double knockout and unstable circadian periodicity in the $Cry2^{-/-}$ mutant. (From Reference 139.)

photoreceptor function (79, 139, 140). Interestingly, the response of $Cry2^{-/-}$ mice to acute light pulses was somewhat paradoxical. In wild-type mice, acute light pulses given in the early hours of the morning cause an approximately 2-h phase delay on the following day as measured by the onset of locomotor activity. At face value one would expect that if the level of the circadian photoreceptor in the retina is reduced because of the *Cry2* knockout, the mutant mice would be less sensitive to phase shifting. In fact, a light pulse delivered at CT17 (CT12 being defined as the onset of activity in mice under a DD [constant darkness] regimen) caused a 7-h phase delay in $Cry2^{-/-}$ mice compared to 2 h in wild-type mice (79). This suggests that the *Cry2* gene dampens the effect of the photic input on the amplitude of the circadian oscillator (79). Indeed, as measured by the amplitude of *mPer1* and *mPer2* mRNA oscillation and the length of the free-running period of locomotor activity, *m*CRY1 and *m*CRY2 seem to pull the circadian oscillator in opposite directions (139). However, the precise molecular mechanism of this unexpected effect of *Cry2* on the mouse circadian behavior remains to be elucidated.

The *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice appear to have normal circadian locomotor activity under an LD12:12 regimen; however, they instantly become arrhythmic upon switching to DD (Figure 9*D, E, F*), indicating a complete collapse of the molecular oscillator under these conditions (139, 140). In fact, even under the LD12:12 regimen, the seemingly entrained behavior might be caused by the "masking" activity of light (140), which is due to a decrease in activity in a nocturnal animal in a direct (visual) response to light (149, 150). These behavioral studies, then, show that mCRY1 and mCRY2 proteins are core components of the circadian clock but do not affect the visual (imaging) pathway of light perception. Interestingly, the complete collapse of the clock mechanism in the double mutant makes it impossible to assess by behavioral tests whether cryptochromes act as photoreceptors in synchronizing the circadian clock. This question was addressed at the molecular level by examining the effect of light on *mPer1* and *mPer2* transcription in mutant mice.

Status of the Molecular Clock in Cryptochrome Mutant Mice

Currently, the known components of the molecular oscillator in mice are CLOCK (126, 151), BMAL1 (131, 132), PER1, PER2, PER3 (127–129, 132–135), and TIM (129, 130, 136). The expression patterns of *Per1, 2,* and *3* show robust circadian oscillation in the SCN and peripheral tissues. In animals under an LD12:12 cycle, the expression of all three *Per* genes in the SCN increases during the light period (ZT 4–8) and declines at night, reaching a minimum at ZT 18–20. If the animals are exposed to acute light pulses during the time of minimal expression (night or subjective night), the transcriptions of *mPer1* and *mPer2* are induced to daytime levels (133, 134). Hence, the oscillation of *mPer1* and *mPer2* transcription and their inducibility with acute light pulses were analyzed in cryptochrome mutant mice to evaluate the role of cryptochromes in photoreception (79, 139).

In *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice, the inducibility of *mPer1* is severely blunted. In *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, there is no induction by acute light pulses (139), although in the double knockout *mPer1* is expressed at a high basal level at all times (Figure 10*A*). These data are consistent with mCRY1 and mCRY2 being the sole photoreceptors for *mPer1* photoregulation as well as light-independent regulators of *mPer1* transcription. This interpretation is based on the fact that in the absence of cryptochromes *mPer1* is expressed at near-maximal levels at all times, including ZT 18 when *mPer1* transcription is normally at a minimum (139).

Surprisingly, the transcriptional behavior of *mPer2* in the SCN of the *Cry* mutant mice (139) is radically different from that of *mPer1* (Figure 10*B*). First, under LD12:12 conditions *mPer2* transcription oscillates with higher than wild-type amplitude in both *Cry1*$^{-/-}$ and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mutants. Second, an acute light pulse at ZT 18 induces *mPer2* transcription to higher than wild-type levels in both *Cry1*$^{-/-}$ and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. Finally, under all conditions the levels of *mPer2* transcript in the SCN of the mutant is higher than that of the wild type. These results are consistent with *mPer2* transcription being negatively regulated by cryptochromes

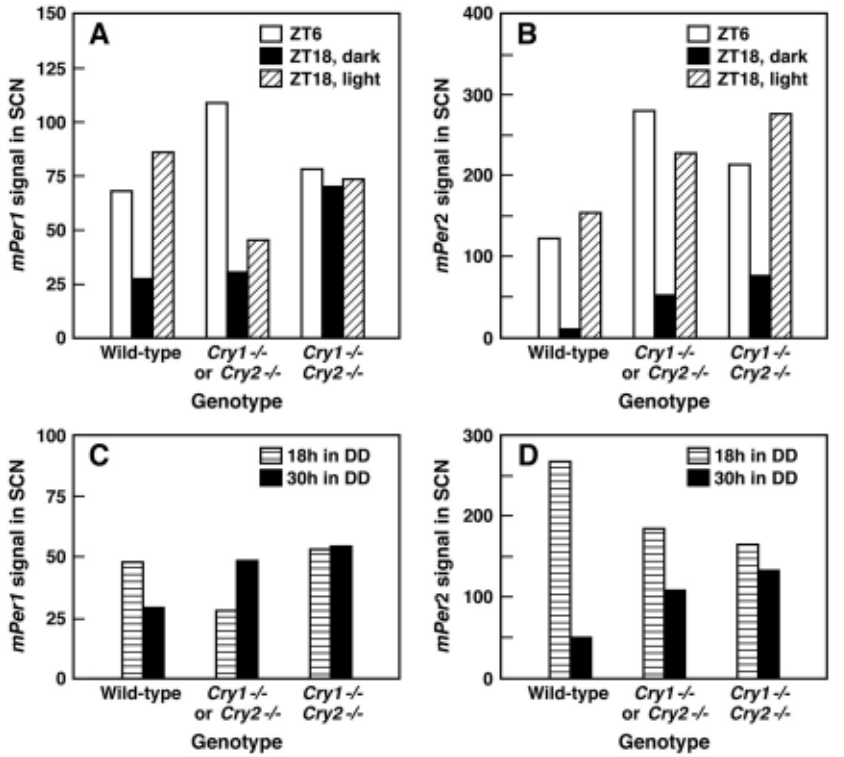


Figure 10 Effects of disruption of *Cry* genes on diurnal and circadian expression of *mPer1* and *mPer2* in the SCN. The expression level was determined by in situ hybridization. (*A*, *B*) Diurnal expression patterns of *mPer1* and *mPer2* and acute light induction of *mPer* genes by a light pulse in the dark phase (*hatched*). The *mPer2* oscillation was not tested in $Cry2^{-/-}$ animals. (*C*, *D*) Circadian expression patterns of *mPer* genes under free-running conditions. Both $Cry1^{-/-}$ and $Cry2^{-/-}$ (not shown) mice exhibit circadian oscillation of *mPer1* and *mPer2* expression although the phases of peak activities are different because of different period lengths in the mutants. The $Cry1^{-/-}Cry2^{-/-}$ animals express constitutively high levels of *mPer1* and intermediate levels of *mPer2* with no circadian oscillation of either, consistent with the arrythmic behavior of these mice. (From References 79, 139.)

in a light-independent manner and, more significantly, indicate that there is a cryptochrome-independent photic input pathway for induction of *mPer2* transcription (139). At this point it is unclear whether the *mPer2* oscillation under LD and its induction by light pulses are mediated by another circadian photoreceptor or by the visual system through a masking mechanism. Interestingly, under constant darkness neither *mPer1* nor *mPer2* oscillate in the SCN of the $Cry1^{-/-}Cry2^{-/-}$ mice (Figure 10*C*, *D*), consistent with the arrhythmic behavior of these animals under a DD condition (Figure 9*D*).

Cryptochrome Genetics in Other Animals

Cryptochrome genes have been found in zebrafish, *Xenopus laevis*, and *Drosophila* (152–157) but, significantly, not in *C. elegans*. The *Drosophila* cryptochrome has been characterized in some detail. There is only one cryptochrome gene in *Drosophila* and it is clearly involved in circadian photoreception (153, 156, 157). However, it is unclear at present whether photic input into the circadian clock comes from the visual photoreceptor system (i.e. rhodopsin) in addition to cryptochrome. A *Drosophila* mutant was identified by its failure to express *Per* in a cyclic manner under LD conditions and it was found to have a mutation in the *dCry* gene (153). Furthermore, based on the crystal structure of DNA photolyase (124), this CRY mutant (CRY^b) has an amino acid alteration in one of the residues involved in binding to FAD (D410N). In the *Drosophila Cry* mutants, PER and TIM are expressed at constitutive levels in the eye and the rest of the body with no detectable oscillation under either LD or DD conditions; in contrast, wild-type animals show robust circadian oscillation of both PER and TIM proteins. Surprisingly, however, the mutants were photoentrainable. To explain this paradox the expression of PER and TIM was examined in the lateral neuron (LN) cluster, which is the circadian pacemaker in *Drosophila*. PER and TIM oscillated weakly in these neurons, which might explain the normal circadian behavior of the mutant flies.

The nearly normal photoentrainment of the Cry^b mutant was ascribed to photic input from the visual (rhodopsin) photoreceptor. Surprisingly, a double mutant (*norp*A Cry^b) that is totally deficient in the visual phototransduction system and presumably in cryptochrome function was still photoentrainable at high light intensities but became unresponsive with low-intensity light (153). As no third photoreceptor is known in *Drosophila*, there is no satisfactory explanation of the data. One possibility is that since the mutation in CRY is conservative (Asp to Asn) Cry^b does not have a null phenotype and the residual photoresponse in the double mutant is mediated by the mutant CRY photoreceptor. This may also explain why cryptochromeless mice are arrhythmic but the Cry^b homozygotes retain normal circadian rhythm under DD: Even though CRY^b is severely defective as a photoreceptor, it may be capable of carrying out nearly normal clock function, which requires only protein-protein interactions. However, it is also possible that there are fundamental differences between the insect and mammalian circadian photoregulatory systems.

The *Drosophila* CRY, like its mammalian counterparts, binds to TIM. Importantly, this binding appears to be enhanced by light when tested by the yeast two-hybrid system, and light stimulated binding was reported to sequester TIM and thus induce CLOCK•BMAL1-controlled transcription of the *Per* gene in a cotransfection experiment (158). This effect is opposite to the light-independent inhibition of gene transcription in mammalian cells (144, 146a) but parallels the light-dependent and *mCry1*- and *mCry2*-dependent induction of *mPer1* induction in mice (79, 139).

MOLECULAR MODEL FOR THE MAMMALIAN CIRCADIAN CLOCK

Evidence from mice to cyanobacteria indicate that circadian rhythms at the organism level are engendered by a cell-autonomous and autoregulatory transcription loop (12, 13, 15, 111, 159). The main feature that distinguishes the circadian negative feedback loop from other biochemical feedback loops is that the circadian system has a time delay between the transcription of the "clock gene" and the production or availability of the negative feedback inhibitory protein. This is accomplished either by a delay of the translation of the transcript, a delay in activation by posttranslational modification, or a delay in the entry of the feedback inhibitor into the nucleus (12, 13, 15). Many components of the circadian clock have been identified and characterized, so a reasonable model for the circadian clock and the circadian system can now be constructed (Figure 11).

The cryptochromes are the photoreceptors and essential components of the circadian oscillator, performing partly redundant and partly complementary functions. Currently it is not known how the cryptochromes transmit the light signal to the molecular clock and how they engender a nerve impulse transmitted to the brain. In contrast, there is a reasonably detailed understanding of the molecular clock: CLOCK and BMAL1 are positive transcription factors, which dimerize, bind to the E-box of and turn on the *Per* and *Tim* genes (131, 132). There is a delay between the transcription and translation of these genes such that significant accumulation of PER1 and TIM in the nucleus is delayed by about 6 h from the time of maximum transcription (160). When PER1, PER2, and PER3 enter the nucleus, they compete with BMAL1 for heterodimerization with CLOCK using the PAS dimerization domain. The CLOCK•PER, CLOCK•TIM or BMAL1•PER, BMAL1•TIM heterodimers are inactive as transcription factors. Alternatively, as has been shown in *Drosophila*, PER and TIM may bind to the CLOCK•BMAL1 heterodimer and interfere with its activity (161). As a consequence, the transcription of *Per* and *Tim* is turned off and the corresponding proteins are degraded, allowing the formation of the CLOCK•BMAL1 complex and initiation of a new cycle. CRY1 and CRY2 proteins play a central role in this cycle, independent of their photoreceptor function, by directly interacting with CLOCK, BMAL1, PER, and TIM proteins, transporting or retaining the PER proteins in the nucleus. In doing so they interfere with the CLOCK•BMAL1 transcriptional activator and thus inhibit the transcription of *Per* genes and other clock genes. This model is by necessity general and qualitative because all the components of the molecular clock have not yet been identified, and the precise roles and mechanisms of action of the known components have not yet been elucidated. In particular, the mechanisms of photoreception and signal phototransduction (most likely by electron transfer) by cryptochromes must be clarified in order to construct a more specific model. Nevertheless, the working model is likely to be correct in outline and should serve as a guide for testing and developing more accurate models.

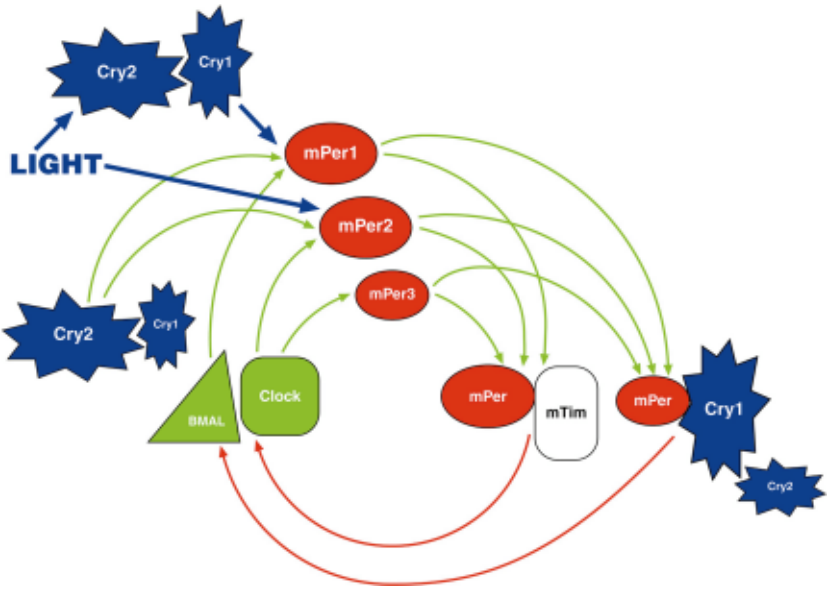


Figure 11 Molecular model for the mammalian circadian clock. The clock is located within the nucleus. The positive and negative regulatory effects of the circadian loop are indicated by *green* and *red arrows*, respectively. The CLOCK•BMAL1 heterodimer is a transcriptional activator that turns on the *mPer* genes. The PER proteins enter the nucleus either as PER heterodimers or as PER•CRY heterodimers and bind to CLOCK•BMAL1 to interfere with its activator functions either as such or as PER•TIM heterodimers and thus complete the circadian autoregulatory loop. Cryptochromes mediate light induction of *mPer1* but not *mPer2*, but they may positively affect the steady-state cycling of both *Per* genes (*left*). In addition, the cryptochromes dimerize with PERs, and perhaps directly interact with CLOCK•BMAL1 and thus function in the negative feedback loop as well (*right*). Finally, differences in the relative contributions of *CRY1* and *CRY2* in the positive drive and negative feedback components of the loop must account for the differences in their influence on circadian period. (Adapted from Reference 139.)

CRYPTOCHROMES AND HUMAN HEALTH

The following diseases and syndromes are known or thought to be caused by abnormal clock function. To what degree, if any, abnormal functioning of cryptochromes contribute to pathogenesis of these conditions is not known at present.

Seasonal Affective Disorder

Seasonal affective disorder (SAD) may affect up to 5% of the population, who manifest classic symptoms of depression including withdrawal, morbid thoughts, sadness, decreased activity, and loss of libido and some atypical features such as

overeating, weight gain, and hypersomnia (18). It occurs during the winter months in the Northern Hemisphere. Its pathogenesis is not understood. However, up to 75% of the patients go into remission with daily exposure to high-intensity white light for 30 min (162). This is consistent with an abnormality in the circadian system and conceivably with mutations in the clock components including cryptochromes, or even with vitamin B_2 and folic acid deficiency, which results in a lower than normal level of active cryptochromes.

Delayed Sleep Phase Syndrome

Patients with delayed sleep phase syndrome typically go to sleep around 4:00 AM and wake up around noon. A substantial number of these patients benefit from phase shifting with intense light exposure of short duration. Interestingly, some patients benefit from vitamin B_{12} given at high doses (163). It is conceivable that some of these patients carry a missense mutation in one of the cryptochrome genes.

Jet Lag (Syndrome of Rapid Change in Time Zone)

Changing time zones in a short period imposes a new phase-shifted dark-light cycle on the traveler. Until the individual's internal clock synchronizes to the new environment, the social demands of the new time zone and the person's capability for fulfilling them are out of phase. As a consequence, the person suffers from insomnia, fatigue, and irritability. As a rule it takes about one day for each time zone change to synchronize the biological rhythm with the new environment (18). In mammals including humans, the pineal hormone melatonin reaches its maximum during the night, and its secretion can be suppressed by exposure to acute light pulses. The SCN contains high-affinity melatonin receptors (164, 165), and treatment with melatonin has been used to remedy the effects of jet lag. However, even though the drug can entrain locomotor activity in hamsters (164), its usefulness in humans for treating jet lag and a host of other ailments is controversial (165).

Rotating Shift Work

Shift work can cause sleep disturbances and fatigue, and impair mental and physical performance. The syndrome is caused by the discrepancy between social demand on the individual to perform certain tasks and the individual's ability to perform these tasks optimally as dictated by the circadian clock. A person working the night shift cannot perform at peak mental and physical levels. It has been suggested that the disasters at Three Mile Island, Chernobyl, and Bhopal occurred between midnight and dawn at least in part because of errors committed by workers who had not been synchronized to work the night shift (166). The symptoms can be ameliorated by exposure to high-intensity light during the night to shift the phase of the circadian clock (167).

Circadian Clock and Breast Cancer

In industrialized countries the incidence of breast cancer has steadily increased during this century. It has been suggested that one cause is exposure to light for longer periods than that afforded by the natural daily light-dark cycle. This so-called melatonin hypothesis proposes that the suppression of melatonin secretion at night by artificial light increases breast cancer risk by increasing exposure to estrogen (168, 169). Since the isolation of melatonin receptors and the finding of widespread expression of these receptors in neural and nonneural tissues (170, 171), the scope of the melatonin hypothesis has expanded to include other mechanisms by which reduced melatonin concentrations may affect hormone homeostasis and induce breast and perhaps other cancers (172). A retrospective survey found that severely blind women had about half the incidence of breast cancer of women with normal vision (173). A recent study that compared the breast cancer incidence in women with various degrees of visual impairment to that in women with normal vision found that women with no conscious perception of light had a 60% lower incidence of breast cancer than women with normal vision. Women with less severe visual impairment had intermediate incidence of breast cancer (174). Further studies are needed to understand the contributions of the circadian and visual phototransduction systems to these phenomena and to provide a mechanistic basis for the epidemiological data.

CONCLUDING REMARKS

Since the discovery of the first mammalian circadian mutant in 1988 (175) and the isolation of the first mouse circadian mutant in 1994 (151), there has been considerable progress in the genetics and molecular biology of the circadian clock. At present eight human and mouse genes that qualify for the definition of "clock gene" have been isolated and characterized: *Clock*, *BMal1*, *Per1*, *Per2*, *Per3*, *Tim*, *Cry1*, and *Cry2*. From the initial characterization of these genes and their proteins a reasonably detailed molecular model for the mammalian circadian clock has been developed that appears to be based, as in other species, on an autoregulatory transcriptional loop.

Recent research has forced a conceptual change in the models for circadian rhythms. Whereas the classical circadian rhythm models consisted of three components—input, clock, and output—it is now clear that at the molecular level the input molecules (cryptochromes) are also basic components of the molecular clockwork. The same may also be true for some of the output molecules. It is expected that in the near future the entire set of the mammalian clock genes will be identified. This should make it possible to develop a cell-free system that oscillates with circadian periodicity and that can be reset by light through cryptochrome blue-light photoreceptors. The study of clock-setting by cryptochromes will undoubtedly reveal a novel signal transduction mechanism whose significance may well transcend the field of circadian research. Finally, understanding of circadian

photoreception and of the circadian clock at the molecular level should help in rational drug design for clock-related illnesses, and in development of rational behavioral approaches to enhance and optimize health, well-being, and physical and intellectual performance.

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