An Extraretinally Expressed Insect Cryptochrome with Similarity to the Blue Light Photoreceptors of Mammals and Plants

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Photic entrainment of insect circadian rhythms can occur through either extraretinal (brain) or retinal photoreceptors, which mediate sensitivity to blue light or longer wavelengths, respectively. Although visual transduction processes are well understood in the insect retina, almost nothing is known about the extraretinal blue light photoreceptor of insects. We now have identified and characterized a candidate blue light photoreceptor gene in Drosophila (DCry) that is homologous to the cryptochrome (Cry) genes of mammals and plants. The DCry gene is located in region 91F of the third chromosome, an interval that does not contain other genes required for circadian rhythmicity. The protein encoded by DCry is ~50% identical to the CRY1 and CRY2 proteins recently discovered in mammalian species. As expected for an extraretinal photoreceptor mediating circadian entrainment, DCry mRNA is expressed within the adult brain and can be detected within body tissues. Indeed, tissue in situ hybridization demonstrates prominent expression in cells of the lateral brain, which are close to or coincident with the Drosophila clock neurons. Interestingly, DCry mRNA abundance oscillates in a circadian manner in Drosophila head RNA extracts, and the temporal phasing of the rhythm is similar to that documented for the mouse Cry1 mRNA, which is expressed in clock tissues. Finally, we show that changes in DCry gene dosage are associated predictably with alterations of the blue light resetting response for the circadian rhythm of adult locomotor activity.

Key words: circadian; cryptochrome; photoreceptor; blue light; Drosophila; extraretinal

Molecular genetic studies in the mold Neurospora, the fruit fly Drosophila, and the mouse have shown that phylogenetically conserved biochemical mechanisms underlie the generation of biological rhythms (Dunlap, 1996; Darlington et al., 1998; Gevakis et al., 1998; Hall, 1998; Young, 1998). The same analysis has culminated in a detailed model describing the circadian timing device. The timing mechanism now can be described in terms of an autoregulatory feedback loop in which circadian changes in the abundance of clock proteins negatively regulate clock gene transcription. Similarly, the clock resetting mechanism can be understood at the molecular level: resetting stimuli such as light or temperature lead to rapid alterations in the abundance of a clock component, effectively shifting the clock to a new time of day (Crosthwaite et al., 1995; Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996; Liu et al., 1998; Sidote et al., 1998). However, the photopigments that function in the circadian system and the phototransduction pathways that serve to reset the circadian clocks of animal species have not yet been subjected to a detailed molecular analysis.

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All authors contributed equally to this work; with the exception of the corresponding author (F.R.J.), they are listed alphabetically.

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Studies in the fruit fly Drosophila and other holometabolous insect species indicate that an extraretinal blue light photoreceptor mediates light input to the circadian clock regulating behavioral rhythmicity (Zimmerman and Ives, 1971; Truman, 1972; Helfrich, 1986; Blaschke et al., 1996; Suri et al., 1998; Yang et al., 1998). In silkmoths, for example, brain transplantation studies demonstrated that both the circadian clock and a photoreceptor for entrainment were located in the CNS (Truman, 1972). Similarly, retinally blind and eyeless mutants of Drosophila retain the capacity to entrain to environmental light/dark cycles (Helfrich, 1986), indicating that an extraretinal photoreceptor mediates circadian resetting. Indeed, recent studies show that degradation of the timeless (TIM) clock protein, which is correlated with light-induced circadian resetting, occurs in visual transduction mutants of Drosophila (Yang et al., 1998). Finally, spectral resetting curves for the Drosophila activity rhythm show that the circadian clock is sensitive to both blue/green light and longer wavelengths (Blaschke et al., 1996; Suri et al., 1998). Sensitivity to the two portions of the spectrum is thought to be mediated by the retinally based opsin system and an extraretinal blue/green photoreceptor.

Although the retinal phototransduction process is well understood in insects (Zuker, 1996), little is known about the molecular basis of extraretinal (blue light) photoreception in these species. In contrast, blue light photoreception has been well characterized in plants (Ahmad and Cashmore, 1996) and depends on photopigments known as cryptochromes (CRY1 and CRY2), which are homologous to DNA repair (photoreactivating) enzymes known as photolyases but which themselves completely lack DNA repair activity (Ahmad and Cashmore, 1993; Malhotra et al., 1995; Todo et al., 1996). Photoreception through CRY1 and CRY2 are required for many different blue light responses, in-
Figure 1. Sequence of a Drosophila cryptochrome-like protein and comparison to cryptochrome and photolyase sequences from several other organisms. A, Comparison of DCry to the human cryptochromes hCRY1 and hCRY2. Gray highlighting indicates residues in cryptochrome and photolyase sequences that are identical to the new Drosophila cryptochrome-like sequence. Circles, squares, and stars indicate similarities to the Escherichia coli PHR protein. Open and filled squares indicate positions that are similar and identical, respectively, to (Figure legend continues)
cluding plant phototropism, the inhibition of hypocotyl elongation, and the early photomorphogenesis of seedlings (Ahmad and Cashmore, 1993; Ahmad et al., 1998b). In addition, CRY1 also has been implicated in signaling pathways necessary for the circadian regulation of plant catalase expression (Zhong et al., 1997), and CRY2 functions in the photic regulation of flowering time (Guo et al., 1998). The photochromes (PhaYa and PhaB), another well studied class of plant photopigments that are sensitive to red/far red light, participate in some of these photically stimulated responses (Zhong et al., 1997; Ahmad et al., 1998a; Guo et al., 1998), and it recently has been suggested that CRY1 physically interacts with PhaYa in vivo (Ahmad et al., 1998a).

Cry-gene homologs encoding related cryptochromes recently have been identified and characterized in mammalian species (Hsu et al., 1996). In the mouse, both Cry1 and Cry2 are expressed in the ganglion cell and inner nuclear layer of the retina, and it has been hypothesized that the encoded cryptochromes mediate light input to the circadian clock in the suprachiasmatic nuclei (SCN; Miyamoto and Sancar, 1998). Interestingly, Cry1 also is expressed in the SCN, and the mRNA oscillates in abundance during the circadian cycle (Miyamoto and Sancar, 1998), although the role of Cry1 in the SCN is not understood. Nonetheless, evidence from both plant and animal species implicates the cryptochromes in circadian photoreception. Therefore, we have characterized a cryochrome homolog in Drosophila to use a molecular genetic approach to evaluate its role in circadian photoreception.

MATERIALS AND METHODS

Culture conditions and behavioral analysis. All fly stocks were obtained from the Indiana University Drosophila Stock Center (Bloomington, IN). Df(3R)Bx12 removes region 91F1-2 to 92D3-6, whereas Df(3R)Cha7 is an overlapping deletion removing region 90F1-4 to 91F5. To generate siblings carrying one or two copies of the DcRy+ gene, we crossed Df(3R)Bx12/TM6B (DcRy+) flies to w118 individuals, which carry two normal copies of the gene. Cultures were reared on Drosophila cornmeal medium according to standard lab procedures (Newby and Jackson, 1993). Adult activity rhythms were monitored and light-induced phase shifts were calculated as previously described (Newby and Jackson, 1993). Samples of adult heads and bodies were prepared by freezing-fracturing adult flies (previously frozen at −80°C) and separating the different body parts by sieving on ice in the cold (4°C).

RNA isolation and northern analysis. The Canton-Special (C-S) strain was used for all Northern, Southern, and RNase protection studies. All RNA in different tissues and to characterize daily changes in abundance.

RESULTS

Identification and sequence of a Drosophila cryptochrome-like gene

On the basis of the reported sequences of mouse and human cryptochromes, we searched the Berkeley Drosophila Genome Project expressed sequence tag (EST) database for similar genes in Drosophila. A number of cDNA clones representing two different Drosophila genes were identified in the initial search. One of these genes is the Drosophila 6-4 photolyase, which previously was reported to be similar to blue light photoreceptors at 42°C to 10 µg of total RNA, and RNA/RNA duplexes then were digested at 37°C for 1 hr with a cocktail of RNase A and T1 according to the manufacturer’s instructions (Ambion, Austin, TX). The sizes of protected fragments were determined by denaturing polyacrylamide electrophoresis, using MspI-digested pBR322 DNAs as size standards. Gels were subjected to autoradiography, and signals were quantitated exactly as previously described (McNeil et al., 1998) by densitometric scanning of films. DCry RNA abundance was quantitated relative to rp49 abundance, which does not change during the diurnal cycle.

Probes representing the HL03779 or GM03047 (Drosophila 6-4 photolyase) cDNAs were [32P]-labeled by random hexamer priming for use in Northern analysis. A labeled rp49 probe (see above) was used as a control for gel loading. Probes were hybridized to RNA blots containing 5 µg poly(A+) RNA or 20 µg of total RNA per lane. Hybridizations contained −0.25–1 × 10−6 cpm probe/ml and were performed in ExpressHyb solution (Clontech, Palo Alto, CA) for 2 hr or overnight at 45°C (DNA probes) or 65°C (rp49 probe). Then the blots were washed three times (15 min each) at room temperature (~23°C) and twice (30 min each) at 50°C before film autoradiography at ~80°C to detect hybridization signals.

We performed in situ hybridization of sense and antisense DCry probes to parafin-embedded adult tissues, using standard methods (Lehmann and Tautz, 1994). Adults were collected at the middle of the subjective day, which corresponds to the high point of DCry RNA abundance (see Results). A 300 bp digoxigenin-labeled RNA probe representing bases 1524–1823 of the HL03779 cDNA was generated for these studies.

Chromosome in situ hybridizations were performed as described by Engels et al. (1986). A cDNA fragment representing the entire HL03779 clone was labeled with Biotin-dATP by nick translation and was used in chromosome localization. Chromosomes were stained lightly with Giemsa (0.4%; Sigma, St. Louis, MO) after the detection reaction. The polytene chromosome maps of Sorsa (1988) were used to localize the position of the DCry gene.

Sequence comparisons. Comparisons of protein sequences were performed with the software of the Genetics Computer Group (Wisconsin Package Version 9.0, Genetics Computer Group, Madison, WI). Direct comparisons among DCry and hCRY1 and hCRY2 were performed by the GAP program with a gap weight of 12 and length weight of 4. The multiple sequence alignment was constructed by using PILEUP with a gap creation penalty of 12 and a gap extension penalty of 4. Sequences were displayed by using PRETTY, and the shading of amino acid residues was accomplished with Microsoft Word. Distance matrices were generated by either DISTANCES or OLDDISTANCES, using the Kimura method of corrected multiple substitutions. A similarity plot for full-length sequences was generated with the DISTANCES matrix for full-length proteins. The phylogram was produced with GROWTREE to generate a UPGMA tree, using a DISTANCES matrix for full-length proteins. Resampling of the data set with the bootstrapping technique yielded trees that were essentially the same as that shown in Figure 1.
A photolyase. This conclusion is supported by the functional idea that DCry functions as a blue light photoreceptor rather than photolyases. The aggregate of our comparisons is consistent with the subfamily that includes mammalian cryptochromes and 6-4 photolyases. The numbers represent the percentage identity for the various comparisons.

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*Threshold of comparison: 2; denominator: length of shorter sequence without gaps; key for column and row indices, 1, hCRY1; 2, hCRY2; 3, D6-4; 4, At6-4; 5, DCry; 6, AtCRY2; 7, SaPHR; 8, AtCRY1; 9, CrPHR; 10, EcPHR; 11, AnPHR; 12, ScPHR. The numbers represent the percentage identity for the various comparisons.

A single *Drosophila* Cry gene maps to chromosome region 91F

Because there are two Cry genes present in the genomes of certain plants and mammals, we wondered whether additional cryptochrome genes existed in *Drosophila*. Southern hybridizations at reduced stringency, however, revealed genomic fragments corresponding to the new *Drosophila* cryptochrome-like gene or to the *Drosophila* 6-4 photolyase gene, but no additional hybridizing fragments were observed (data not shown). Therefore, there is no evidence for additional genes, although we cannot exclude the possibility of more highly divergent fly cryptochrome-like genes. However, both in humans and *Arabidopsis*, the two Cry genes are much more similar to each other than they are to their counterparts in the other species (see Table 1). This also suggests that the Cry gene pairs of these different species evolved from independent duplication events after the divergence of plants and animals.
ization of cDNA sequences to larval salivary gland polytene chromosomes (see Materials and Methods). A single site of hybridization was observed within polytene region 91F of the third chromosome distal to band 91F5 (Fig. 2). No circadian rhythm genes besides DCry have been mapped to this cytogenetic interval.

The DCry mRNA is expressed in the adult brain and other nonretinal tissues

The HL03779 cDNA clone was isolated from a fly cDNA library generated from brain and sensory organ RNA (Berkeley Drosophila Genome Project/HHMI EST Project; unpublished data), and we have confirmed that the DCry mRNA is expressed in head tissues. In head RNA preparations a doublet of ~2 kb is detected in either total RNA or poly(A+) RNA fractions (Fig. 3A; data not shown). As the HL03779 cDNA is 1823 bp in length and most mRNAs contain 100–200 As at their 3’ end, we assume that this clone represents most or all of the DCry mRNA sequence.

In contrast to Dcry expression, a probe representing the Drosophila 6–4 photolyase gene (see Materials and Methods) did not detect a signal when hybridized to a Northern containing the same head RNA preparations (data not shown). This result is not surprising, because our database searches found 6–4 photolyase-homologous cDNAs only in an EST collection representing a Drosophila ovary cDNA library. As previously reported (Todo et al., 1996), the fly 6–4 photolyase gene is expressed at significant levels only in ovary tissues.

We used RNase protection methods to examine various developmental stages and tissues for DCry expression. To determine whether the DCry RNA was expressed in the brain, we prepared RNA samples from hand-dissected adult brains, which were completely devoid of eyes and ocelli. As judged by the control rp49 signal (Fig. 3B, lane Br), DCry mRNA can be detected in a modest amount of total RNA (lane Br), indicating that the message is enriched in the brain. This result also demonstrates that the message is expressed in an extraretinal manner and suggests that it encodes the cryptochrome mediating circadian photoreception.

Consistent with expression in the brain, DCry mRNA was detected readily in head tissues of eyes absent (eya) mutants, which entirely lack compound eyes (data not shown). Interestingly, DCry mRNA can be detected in body tissues (lane B in Fig. 3A,B), which previously have been shown to contain photoreceptive clocks (Plautz et al., 1997), although the relative abundance of the mRNA is apparently lower in the body. Finally, DCry message could not be detected in moderate-to-large amounts of total RNA from 0–24 hr embryos (Fig. 3B, lane E), whole third instar larvae (lane L), or third instar larval brains (lane Br), suggesting that a different photoreceptor might mediate circadian resetting at these developmental stages.

In situ hybridization techniques were used to examine the spatial localization of DCry mRNA within the adult nervous system. As shown in Figure 3C, a low level of expression could be detected throughout the cell body layer of the CNS (see arrowheads in Fig. 3C). A much stronger signal, however, was observed in large cells of the lateral CNS (Fig. 3C, arrow), which are close to or coincident with the ventral group of Drosophila clock neurons (see Discussion). Specific expression also was detected in adult non-neural tissues, including the gut (data not shown). Importantly, sections hybridized with a DCry sense probe did not show any signal within brain or gut cells (data not shown). A small amount of reaction product was observed within the retina (R) with both the sense and antisense DCry probes; thus, we conclude there is no specific signal for DCry mRNA within retinal tissues.

The DCry mRNA oscillates in abundance during the circadian cycle

As the mouse Cry1 mRNA had been reported to oscillate in abundance during the diurnal cycle, we determined whether the same might be true of the DCry mRNA. As shown in Figure 4, the DCry message is more abundant in head RNA samples during the day than at night, relative to rp49 abundance, which does not change during the diurnal cycle (Hardin et al., 1990). In two independent experiments, DCry mRNA was 6- and 11-fold more abundant at peak during the day than it was at the trough of the rhythm during the night (Fig. 4B). Indeed, the amplitude of the DCry rhythm was greater than that observed for the mouse Cry1 mRNA, which oscillates in abundance in the suprachiasmatic nuclei (SCN; Miyamoto and Sancar, 1998). Additional experiments showed that DCry mRNA did not show immediate increases in abundance in response to the lights-on signal (data not shown), indicating that DCry gene expression is not light-inducible. Similar to mouse Cry1 mRNA, the rhythm in DCry abundance persisted in constant conditions (Fig. 4C), demonstrating that it is under circadian regulation.

Changes in DCry gene dosage affect the blue light resetting response

We conducted behavioral genetic experiments to test the notion that the Drosophila cryptochrome mediates blue light resetting of the circadian clock. As a prelude to these experiments, we first examined blue light resetting in normal flies. As shown in Figure 5A, normal individuals exhibited phase shifts of increasing magnitude in response to ~200 lux blue light pulses of increasing duration. Flies receiving 5 min of blue light (5 min b) or 5 min of 2500 lux white light (5 min w) exhibited phase delays of identical magnitude, suggesting that this duration of blue light constituted a saturating light pulse. Importantly, these data indicate that 10 sec and 1 min pulses of blue light cause submaximal phase shifts, and thus such resetting pulses might be appropriate for detecting behavioral alterations that result from changes in DCry gene dosage.

To determine whether changing DCry dosage affected blue light resetting, we characterized the resetting responses of flies carrying one or two doses of the gene. We used two different
third chromosome deletions in these experiments: Df(3R)Dl-BX12 and Df(3R)Cha7. Based on our localization of the DCry gene (in distal 91F), the Dl-BX12 deletion was predicted to remove the gene, whereas Cha7 was expected to delete a region adjacent to the gene (see Materials and Methods). Control flies heterozygous for the Cha7 deletion exhibited phase delays at all three pulse durations that were within the wild-type range and not significantly different from those of normal flies (data not shown), which confirmed that this deletion did not remove the DCry gene. For Df(3R)Dl-BX12, resetting responses were characterized in sibling flies of similar genetic background (see Materials and Methods), which were either heterozygous for the deletion (Df(+)) or homozygous for the normal DCry+ allele (+/+). As shown in Figure 5B, flies heterozygous for Df(3R)Dl-BX12 had significantly smaller phase delays than normal siblings in response to a 10 sec pulse of blue light (p < 0.001). Phase delays for such flies were progressively larger in response to longer pulses (Fig. 5B), but not significantly different from those of siblings, presumably because the system was at or near saturation at the longer pulse durations. These data indicate that flies deficient for DCry product have decrements in blue light resetting.

**DISCUSSION**

**Identification of a Drosophila Cry homolog**

Cryptochrome (Cry) proteins lacking photolyase activity have been identified in mammals and plants (Ahmad and Cashmore, 1993; Malhotra et al., 1995), and these proteins have been demonstrated to mediate blue light photoreception in *Arabidopsis*. We have identified and characterized a new *Drosophila* gene (DCry) encoding a cryptochrome protein (DCry) with significant similarity to the cryptochromes previously described in plant and animal species. Although DCry also has similarity to 6-4 photolyase proteins, it is most similar to the mammalian cryptochromes (hCRY1 and hCRY2) that have been implicated in circadian photoreception (Miyamoto and Sancar, 1998).

Given that the DCry gene is expressed in *Drosophila* brain and body tissues, we postulate that the encoded protein serves as the extraretinal blue light photoreceptor mediating the entrainment of circadian behavioral rhythms. Previous studies have indicated that a circadian photoreceptor is localized in the insect brain (Truman, 1972; Helfrich, 1986), and recent work has demonstrated the existence of photoreceptive circadian clocks in a variety of *Drosophila* tissues (Plautz et al., 1997). Consistent with a role for cryptochrome in light resetting of the circadian clock, *Drosophila* strains that are deficient for DCry product show correlated changes in the resetting response to blue light. In addition, work reported by Stanewsky et al. (1998a) indicates that a mutation in the DCry gene we have characterized leads to altered light resetting of circadian rhythms. The aggregate of these results suggests that the DCry photoreceptor mediates extraretinal light input to the *Drosophila* circadian clock. Because brain-localized photoreceptors mediate clock resetting in other holometabolous insect species (Truman, 1972), it is likely that cryptochromes

**situ** hybridization to a horizontal section of an adult head. The arrow indicates the position of DCry-expressing cells in the lateral CNS. The arrowheads show specific signal in other portions of the brain. The star indicates nonspecific staining, which also was seen with the sense probe. A similar spatial pattern of expression was observed in two independent experiments. R, Retina; L, optic lamina; M, optic medulla; Lo, lobula.
similar to DCry function in circadian phototransduction in such species.

**How might Cry-mediated phototransduction reset the circadian clock?**

Although DCry mRNA is expressed within the CNS, the identities of the cell types relevant for extraretinal photoreception have not been positively determined. However, relative to other parts of the nervous system, DCry mRNA is expressed at high levels in large cells of the lateral CNS. Although we have not yet per-
formed double-labeling experiments with clock cell antibodies, on the basis of the size and position of the hybridizing cells we postulate that they correspond to the ventral group of the so-called “lateral clock neurons.” These cells are known to be critical for circadian function (Frisch et al., 1994; Helfrich-Förster, 1998) and contain the clock proteins Period and Timeless (Young, 1998) as well as a neuropeptide known as pigment-dispersing hormone (PDH; Helfrich-Förster, 1995). We hypothesize that the clock neurons themselves are directly light-sensitive by virtue of containing the DCry cryptochrome. Perhaps less likely, DCry protein might be localized within dedicated circadian photoreceptor cells that are close to the clock neurons and innervate them.

The signaling pathway that transduces light information from Cry proteins to the clock mechanism has not been elucidated in any species. In Drosophila such a signaling mechanism ultimately must result in the degradation of Timeless (TIM) protein, which serves as the sensor for light input to the clock (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Thus, Cry-mediated phototransduction must involve the activation of an appropriate protease, perhaps indirectly via a kinase or phosphatase intermediary. In this regard, it is of interest that Zhao and Sancar (1997) have shown that human CRY proteins can interact with members of the TPR family of proteins, including serine/threonine phosphatase 5 (PP5). Moreover, the activity of PP5 can be inhibited by interaction with hCRY proteins, although the observed inhibition is not light-dependent (Zhao and Sancar, 1997). It also is known that there is a blue light-mediated and CRY-dependent autophosphorylation of an Arabidopsis protein kinase known as NPH1 (Ahmad et al., 1998b). Therefore, it is reasonable to suppose that a light-induced alteration of a specific phosphatase and/or protein kinase might be required for the activation of the protease that mediates Drosophila TIM degradation.

Circadian oscillations in DCry mRNA abundance

DCry mRNA abundance changes in a circadian manner, with peaks of abundance occurring during the photoperiod of a light/dark cycle. Although the daily phasing of the DCry cycle is similar in Drosophila and the mouse (Miyamoto and Sancar, 1998), the significance of the mRNA oscillation is not understood in either species. Indeed, one would expect the abundance of a photoreceptor protein either to be constant during the circadian cycle or to be higher during the night, at the time when the clock is maximally sensitive to resetting stimuli. This could be the case for DCry protein if there is a temporal lag in translation of the DCry message. Alternatively, the DCry rhythm might be required for another aspect of circadian clock function in addition to photoreception.

Although Cry rhythmicity might be relevant to circadian photoreception or another circadian function, it is also possible that the mammalian and Drosophila Cry rhythms reflect the ancestral photolyase activity of the proteins. For photolyases, a higher DNA repair activity might be advantageous during the photoperiod. In this regard, it is known that the expression of certain photolyase genes can be induced by light (Batschauer, 1993; Ahmad et al., 1997), although it is unclear whether the expression of these genes is under clock control. Irrespective of the physiological significance of the Cry mRNA rhythm, the remarkable similarity in the phasing of rhythmicity in different species suggests a conservation of function and/or gene regulatory mechanisms. A more detailed molecular genetic analysis of the DCry RNA rhythm may yield insights about its function.

Note. While this paper was in review, other labs reported the genetic and molecular characterization of the Drosophila DCry gene (Emery et al., 1996; Powney et al., 1998b). Their work reports the behavioral analysis of a DCry mutant and provides additional molecular evidence that DCry protein functions as a circadian photoreceptor.

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