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CRY, a *Drosophila* Clock and Light-Regulated Cryptochrome, Is a Major Contributor to Circadian Rhythm Resetting and Photosensitivity

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Summary

Light is a major environmental signal for circadian rhythms. We have identified and analyzed *cry*, a novel *Drosophila* cryptochrome gene. All characterized family members are directly photosensitive and include plant blue light photoreceptors. We show that *cry* transcription is under circadian regulation, influenced by the *Drosophila* clock genes *period*, *timeless*, *Clock*, and *cycle*. We also show that *cry* protein levels are dramatically affected by light exposure. Importantly, circadian photosensitivity is increased in a *cry*-overexpressing strain. These physiological and genetic data therefore link a specific photoreceptor molecule to circadian rhythmicity. Taken together with the data in the accompanying paper, we propose that CRY is a major *Drosophila* photoreceptor dedicated to the resetting of circadian rhythms.

Introduction

Most living organisms manifest circadian rhythms, which serve to anticipate the daily changes of the external world. Although these approximately 24 hr rhythms persist in constant conditions, fluctuations of the natural environment entrain or reset rhythms to precisely 24 hr periods and to an appropriate phase. Both period and phase are often monitored by assaying behavioral and/or molecular fluctuations (Rosbash et al., 1996).

Genetics has provided a key entrée into identifying oscillating molecules, as most clock genes in cyanobacteria, *Neurospora*, as well as *Drosophila*, undergo circadian oscillations in transcription and transcript levels (Hardin et al., 1990; Aronson et al., 1994; Sehgal et al., 1995; So and Rosbash, 1997; Darlington et al., 1998; Ishiura et al., 1998). For many years, only one *Drosophila* clock gene (*period* [*per*]) was known (Konopka and Benzer, 1971; Bargiello et al., 1984; Reddy et al., 1984). *per* encodes a member of the PAS family, which includes many transcription factors (Crews, 1998). Taken together with other data, a feedback role for *per* in transcriptional regulation was proposed (Hardin et al., 1990, 1992; Marrus et al., 1996; So and Rosbash, 1997). Feedback regulation of transcription was subsequently shown to be a central feature of many other circadian clocks, including those in *Neurospora* and cyanobacteria (Aronson et al., 1994; Ishiura et al., 1998).

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The second identified *Drosophila* clock gene is *timeless* (*tim*) (Myers et al., 1995; Sehgal et al., 1995). *tim* protein (TIM) heterodimerizes with *per* protein (PER) (Gekakis et al., 1995; Zeng et al., 1996), which is likely important for clock function (Rutila et al., 1996). The interaction was suggested to be important for nuclear entry of both proteins (Vosshall et al., 1994; Curtin et al., 1995; Saez and Young, 1996). The direct biochemical functions of both TIM and PER remain unknown.

In contrast, the functions of the three newest *Drosophila* clock genes, *dClock* (*Clk*), *cycle* (*cyc*), and *doubletime* (*dbt*), are apparent from their primary sequence. The first two proteins, CLK and CYC, are bHLH-PAS transcription factors (Allada et al., 1998; Rutila et al., 1998b). CLK/CYC heterodimers are the likely direct regulators of *per* and *tim* by binding to the E boxes present in the promoters of these genes (Hao et al., 1997; Darlington et al., 1998). Consistent with this notion, *per* and *tim* transcript levels are very low and noncycling in both *dClock* and *cycle* mutants (Allada et al., 1998; Rutila et al., 1998b). Furthermore, it has been shown that PER and TIM together are able to inhibit transcriptional activation by the CLK/CYC heterodimer (Darlington et al., 1998), consistent with the negative feedback loop hypothesis (Hardin et al., 1990). *dbt* encodes a casein-kinase I homolog, which has been suggested to regulate *per* phosphorylation and accumulation (Kloss et al., 1998; Price et al., 1998).

In mammals, the molecular mechanisms generating and controlling circadian rhythms are likely to be similar to those of *Drosophila* (Wilsbacher and Takahashi, 1998). But one difference between systems is the response of clock molecules to light. In *Drosophila*, TIM levels decrease in response to illumination (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). Although this biochemical measurement primarily reflects a light response of abundant peripheral clocks, genetic and physiological data indicate that the TIM response is also relevant to lateral neuron circadian photoreception (Suri et al., 1998; Yang et al., 1998). For example, the TIM-SL mutant increases the photosensitivity of behavioral rhythm resetting as well as TIM degradation (Suri et al., 1998). TIM is therefore part of the light input pathway as well as a clock component. In mice, *mper1* and *mper2* mRNA levels in the suprachiasmatic nucleus rapidly increase in response to ocular illumination, and the induction correlates with circadian times of behavioral photosensitivity. Thus, transcription of some clock-relevant genes is likely relevant to circadian photoreception (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). This aspect of clock gene expression is similar to the response of the *frequency* (*frq*) gene to light in *Neurospora* (Crosthwaite et al., 1995). Remarkably, the photoreceptors for these and all other circadian rhythm–relevant light perception are unknown—in *Drosophila*, mammals, and *Neurospora*.

In *Drosophila*, genetic ablation of eyes or mutations in the well-characterized visual phototransduction pathway do not block entrainment of circadian clocks to

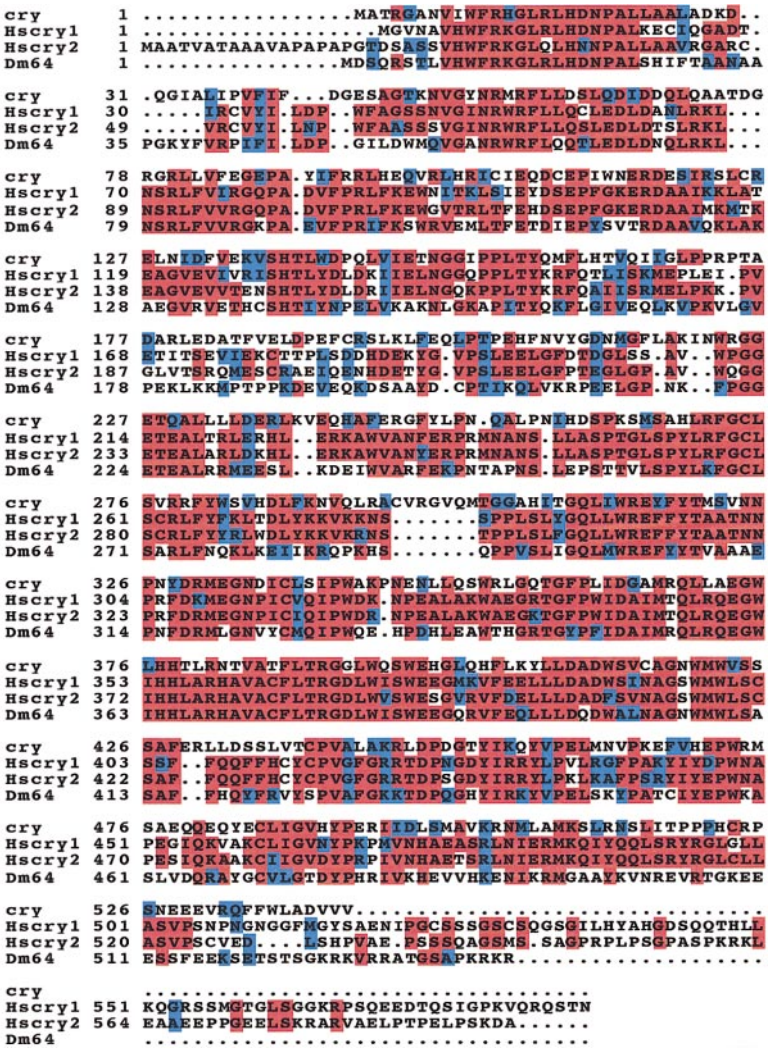

Figure 1. *cry* Is a Novel Member of the 6-4 Photolyase/Cryptochrome Protein Family

Protein sequence of *cry* and sequence alignment with two human cryptochromes (*HsCRY1* and *HsCRY2*) and *Drosophila melanogaster* 6-4 photolyase (*Dm64*). Red, identical amino acids; blue, homologous amino acids.

light. For example, *Drosophila* mutants *norpA* (phospholipase C) and *ninaE* (Rh1 rhodopsin in R1-R6) still have robust and light-sensitive rhythms (Dushay et al., 1989; Zerr et al., 1990; Wheeler et al., 1993; Suri et al., 1998; Yang et al., 1998), indicating that there is at least one additional relevant photoreceptor and signal transduction pathway. Its characteristics can be addressed with action spectra. These had been measured long ago for phase response curves (PRCs) of *Drosophila pseudoobscura* eclosion; they peak at 400–500 nm (Frank and Zimmerman, 1969; Klemm and Ninnemann, 1976). Only recently were similar *Drosophila melanogaster* action spectra derived for adult locomotor activity rhythms and for TIM degradation (Suri et al., 1998). Both curves were significantly different from that of *Drosophila* vision, suggesting a novel blue light circadian photoreceptor that affects behavioral rhythms as well as the response of peripheral clocks, even in the eye. In mammals, the retina is very important for circadian light perception and makes synaptic connections with the suprachiasmatic nucleus via the retino-hypothalamic tract. But the relevant photoreceptor(s) is uncertain, as the well-studied mammalian visual photopigments may be insufficient to account for ocular circadian photoreception (Foster, 1998). As in *Drosophila*, therefore, the mammalian eye may contain another unidentified photoreceptor specialized for circadian light perception (Miyamoto and Sancar, 1998; Soni et al., 1998).

Given the action spectrum, we were intrigued by the possibility that the unknown *Drosophila* photoreceptor might be a member of the photolyase/cryptochrome family, a well-characterized group of blue light–responsive flavoproteins. Photolyases/cryptochromes have five well-characterized subgroups (Kanai et al., 1997). Three groups of cyclobutane pyrimidine dimers (CPD) photolyases are responsible for the repair of thymidine dimers caused by UV irradiation. Another group contains the 6-4 photolyases (found in *Drosophila*, for example; Todo et al., 1996), responsible for the repair of UV-generated 6-4 thymidine dimers and 6-4 photolyase homologs. A fifth group contains plant blue light photoreceptors (Ahmad and Cashmore, 1993; Lin et al., 1998).

We were also directed to the cryptochrome family by evidence from several systems, suggesting that a flavin-based system, in addition to or instead of retinal-containing proteins, contributes to circadian photoreception. Caroteinoid-depleted *Drosophila* show normal photosensitivity (Zimmerman and Goldsmith, 1971). In *Neurospora*, a genetic defect in the riboflavin synthesis pathway inhibits the photoentrainment pathway (Paietta and

Sargent, 1981). In *Arabidopsis*, two cryptochromes have been shown to be involved in three different light-dependent processes: hypocotyl elongation inhibition, phototropism, and photoperiodism of flowering time (Ahmad et al., 1998a; Guo et al., 1998; Lin et al., 1998). Flowering time photoperiodism may be related to circadian rhythms (Guo et al., 1998). In mammals, a recent report described the expression pattern of two novel cryptochromes, mCRY1 and mCRY2 (Miyamoto and Sancar, 1998). The transcripts are expressed in retina and the suprachiasmatic nucleus, tissues highly relevant to circadian rhythms. Moreover, *mcry1* transcript undergoes circadian cycling in the SCN. Although there is no evidence that the SCN is directly light sensitive, the authors proposed that these cryptochromes are involved in mammalian circadian photoreception. Importantly, there are no physiological data that link these or any other specific cryptochromes to circadian light perception in any animal system.

In this communication, we present genetic as well as physiological evidence that a novel clock gene encodes a functionally relevant *Drosophila* cryptochrome/6-4 photolyase family member. Taken together with the results in the accompanying paper (Stanewsky et al., 1998 [this issue of *Cell*]), we propose that *cry* is an input clock gene, a major photoreceptor for behavioral circadian rhythms and the key photoreceptor for peripheral circadian rhythms.

Results

CRY Is a Novel Member of the Cryptochrome/6-4 Photolyase Protein Family

We discovered by BLAST in the *Drosophila* EST sequence database an expressed sequence tag showing high homology with *Drosophila* 6-4 photolyase (Todo et al., 1996; there is also a much more distantly related *Drosophila* CPD photolyase, Yasui et al., 1994; EST HL02979 Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished data). We named the gene *cry*, which is located on the third chromosome at bands 91F8-92A3 (data not shown), and used the coding sequence of the EST clone for the rest of this study.

CRY is more closely related to the two human cryptochromes HsCRY1 and HsCRY2 (10^{-99} and 2×10^{-98}, respectively) than to Dm64 (8×10^{-86}), suggesting that CRY and the human proteins might share functional properties different from those of Dm64 (Figure 1). But an unrooted phylogenetic tree reveals that HsCRY1, HsCRY2, Dm64, and even *Arabidopsis* 6-4 photolyases are more closely related to each other than to CRY (Figure 2A). The blue light photoreceptors of *Arabidopsis*, including the protein responsible for flowering time photoperiodism, are much more distantly related to CRY (4×10^{-23} for AtCRY1 and 3×10^{-21} AtCRY2). Taken together, the sequence relationships do not convincingly indicate a specific biochemical function for *Drosophila* CRY.

The C-terminal half of the protein shows the highest conservation. This region of *E. coli* photolyase contains most of the amino acids involved in binding the two cofactors, MTHF (5,10-methenyltetrahydrofolate) and FAD (flavin-adenine dinucleotide; Figure 2B; Park et al., 1995; Kanai et al., 1997). The MTHF-binding sites are not very well conserved between CRY and its close relative (4/7 homologous amino acids, 2/7 identical to HsCRY1). But these residues are not very strongly conserved, even among family members known to bind MTHF. The FAD-binding site, on the other hand, is highly conserved (12/14 homologous amino acids, 8/14 identical). This is despite the presence of a lysine at conserved position 264, which is either a serine or threonine in all other members of the photolyase/cryptochrome family. Although the crystal structure of *E. coli* photolyase did not contain nucleic acid (Park et al., 1995), other experiments indicated that the same C-terminal half of the protein interacted with DNA (Baer and Sancar, 1993). The putative DNA-binding residues are also well conserved in *Drosophila* CRY (Figure 2B; Park et al., 1995; Kanai et al., 1997).

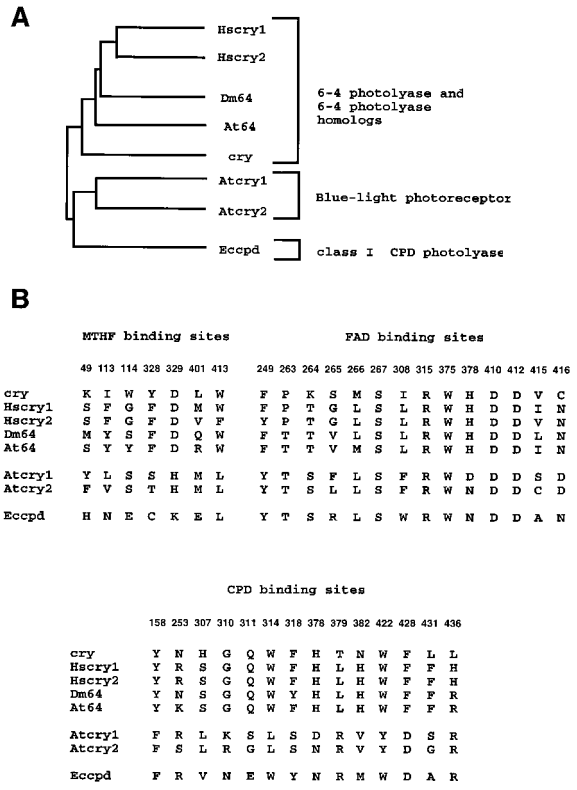

Figure 2. *cry* Phylogeny and Functional Amino Acid Conservation

(A) Unrooted phylogenetic tree based on alignment of several members of the photolyase/cryptochrome family. In addition to the member mentioned above, *Arabidopsis thaliana* 6-4 photolyase (*At64*), cryptochrome 1 and 2 (*AtCRY1* and *AtCRY2*), and *E. Coli* CPD photolyase (*EcCPD*) were included. *AtCRY1*, *AtCRY2*, and *EcCPD* are members of two different classes of photolyase/cryptochrome, the blue light photoreceptors and the class I photolyases.

(B) Alignment of the amino acids putatively involved in MTHF and FAD cofactors and CPD binding.

cry Transcription Undergoes Circadian Cycling

A property shared by many clock gene transcripts is that their abundance undergoes circadian oscillations (Hardin et al., 1990; Aronson et al., 1994; Sehgal et al., 1995; Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997;

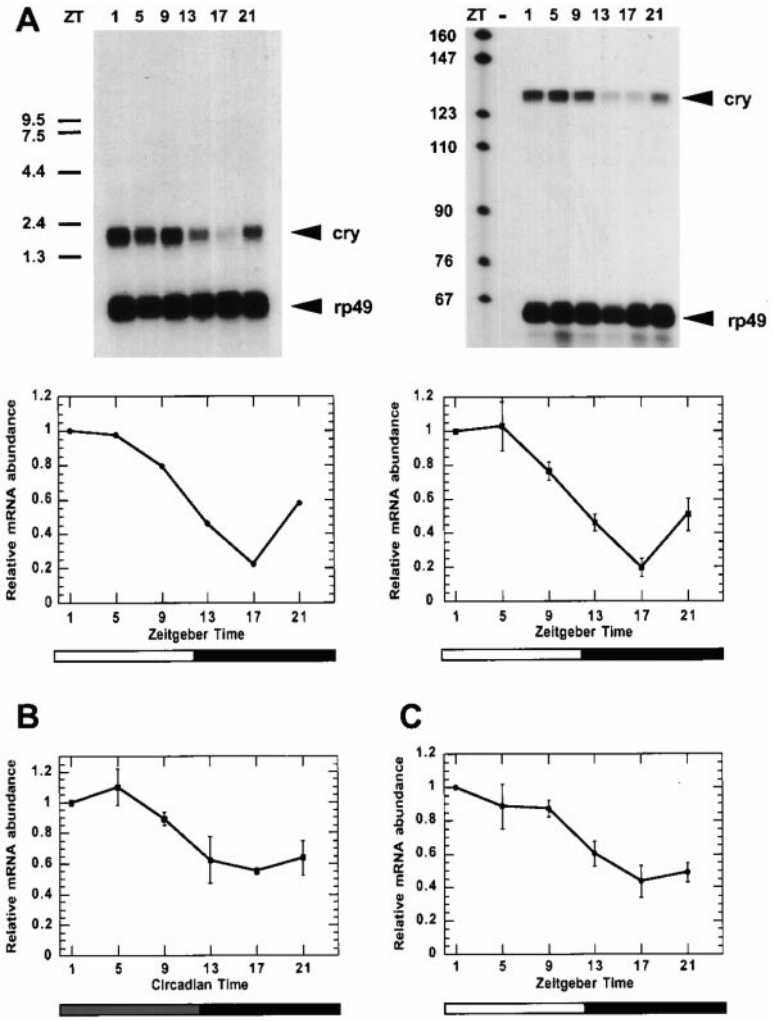

Figure 3. *cry* mRNA Circadian Cycling under LD and DD Conditions

(A) *cry* mRNA cycling in heads was measured both by Northern blot (upper left panel) and by RNase protection (upper right panel). Time of sample collection (Zeitgeber time [ZT]), *cry* and *rp49* (internal control) transcripts, or protected probes are indicated. The (-) lane is a negative control, without head RNA. Bottom panels show the quantitation of one Northern blot (left) and three RNase protections (right, standard deviations are indicated). Open bars correspond to day, and filled bars to night. *cry* mRNA signals were normalized to the *rp49* signal, with the ZT1 *cry* mRNA level set to 1.

(B) *cry* mRNA cycling in heads under DD conditions (two experiments). The gray bar corresponds to the subjective day.

(C) *cry* mRNA cycling in body under LD conditions (two experiments).

Darlington et al., 1998; Takumi et al., 1998; Zylka et al., 1998). To assay *cry* mRNA, we performed Northern and RNase protection analyses on RNA isolated from heads after fly entrainment under standard light/dark conditions (LD). Both assays gave identical results: *cry* mRNA manifests ca. 5-fold amplitude cycling, with a peak at ZT1–5 and a trough at ZT17 (Figure 3A). A higher resolution RNase protection experiment revealed that the peak persists from ZT1 to ZT7, and the trough from ZT17 to ZT19 (data not shown). The Northern analysis indicates that there is only a single *cry* transcript of approximately 2 kb.

We also measured *cry* mRNA cycling under conditions of constant darkness (DD; Figure 3B). Cycling persists under these conditions but with a lower, approximately 2-fold amplitude. Lower amplitude DD cycling was previously described for other clock genes or for clock gene–derived reporter genes (Hardin, 1994; Sehgal et al., 1995; Plautz et al., 1997). Because there is good evidence for peripheral clocks in multiple tissues throughout *Drosophila* (Emery et al., 1997; Giebultowicz and Hege, 1997; Plautz et al., 1997), we also assayed RNA extracted from bodies (Figure 3C). *cry* body mRNA cycling has a similar phase to head mRNA cycling but only a 2.5-fold amplitude. There is precedence for lower amplitude clock mRNA cycling in bodies as compared to heads (Hardin, 1994).

To address transcriptional regulation, we performed nuclear run-on assays on the *cry* gene (So and Rosbash, 1997). There is a 4-fold amplitude of transcriptional cycling under LD conditions, with a peak at ZT1 and a trough at ZT13 (Figure 4A). As this amplitude and phase are similar to the values of mRNA cycling, these oscillations are in large part regulated at the transcriptional level (So and Rosbash, 1997). As with mRNA cycling, the transcriptional cycling persists under DD conditions but with a lower amplitude. This is due in large part to a more shallow trough, which is also observed in the mRNA cycling curve (Figures 4B and 3B, respectively). In both cases (LD and DD), the run-on signal was low and indicates relatively low transcriptional activity (data not shown). This was the cause of the large standard deviations and precluded detecting subtle differences in the phases and shapes of the curves, which could reveal a posttranscriptional contribution to mRNA cycling (So and Rosbash, 1997).

cry Expression Is Altered in Clock Mutants

We next determined how *cry* mRNA cycling is affected by mutations in four clock genes implicated in gene

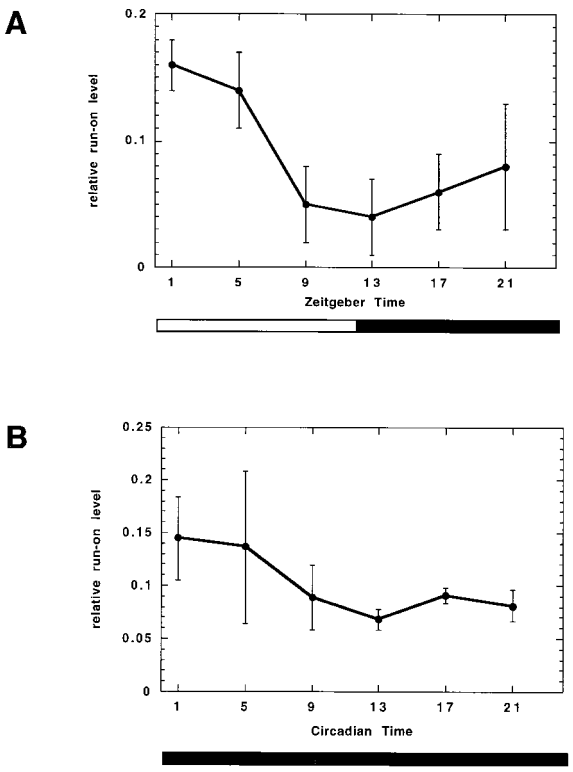

Figure 4. Circadian Transcriptional Control of *cry* mRNA Cycling

Run-on analysis of head RNA in (A) LD (three experiments) and (B) DD conditions (two experiments). Run-on signals were normalized to the *rh*1 signals (So and Rosbash, 1997). Standard deviations are indicated. Open bar corresponds to day, dark bar to night, and gray bar to subjective day.

regulation: *per*, *tim*, *Clock*, and *cycle* (Figure 5). In all single mutants and double mutant combinations, we found little or no mRNA cycling, indicating that it requires a functional pacemaker and is not merely light driven. *cry* mRNA levels were a function of the specific mutant or mutant combination. They were relatively low in the *per* or *tim* null mutants as well as in the *per*;*tim* double mutant combination, whereas they were relatively high

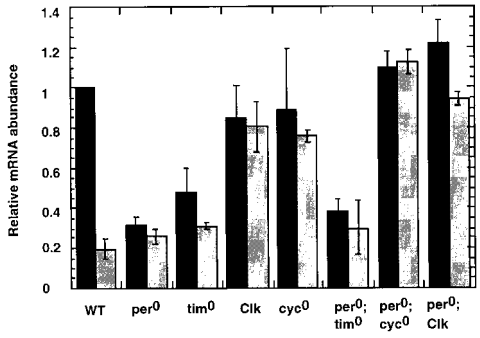

Figure 5. *cry* mRNA Levels in Circadian Mutants

cry mRNA was measured by RNase protection at peak (ZT1, dark bars) and trough (ZT17, gray bars) in wild-type flies (Canton-S) (three experiments), per^0, tim^0, *Clk*, cyc^0 mutants, and per^0;tim^0, per^0;*Clk*, and per^0;cyc^0 double mutant combinations (two experiments). Standard deviations are indicated. Normalization was as described in Figure 3A. Wild-type Canton-S ZT1 level was set to 1.

in the *Clock* and *cycle* mutants. The double mutants *per*;*Clock* and *per*;*cycle* also show high *cry* mRNA levels, indicating an epistatic effect of *Clock* and *cycle* over *per*.

CRY Protein Levels Are Controlled by Light

cry mRNA cycling suggests that the protein abundance also cycles during the day, as shown previously for PER and TIM, and very recently for CLK (Edery et al., 1994a; Zeng et al., 1994, 1996; Lee et al., 1998). To address this question, a rat antibody directed against the N terminus of CRY was used in Western blotting experiments. The antibody shows specific immunoreactivity to in vitro translated CRY (data not shown). The very strong signal obtained with extracts from heat-shocked flies containing the *cry* gene under *hsp70* promoter control shows that the antibody also specifically recognizes CRY in head extracts (Figure 6A). The protein migrates with an apparent molecular weight of approximately 60 kDa, as predicted from the cDNA sequence. Two bands were visible on some gels, whereas a single fuzzy band was detected on others.

We measured the protein abundance in light/dark entrained wild-type (Canton-S) flies, as well as in *y w*;*tim-gal4* flies (see next section and Experimental Procedures). The latter shows a very robust 8-fold amplitude cycle, which is strikingly different from the *cry* mRNA cycle (Figure 6B). Protein is low during the day when the mRNA is high, clearly increases before the mRNA rise, and peaks at ZT23. Two hours later, at ZT1, when the *cry* mRNA peaks, CRY is already strongly reduced (50% of peak value). This indicates that a translational or posttranslational mechanism makes a strong contribution to CRY regulation. In wild-type Canton-S flies, the cycle was less robust (3- to 5-fold), and the trough is reached between ZT11 and ZT13 (Figure 6C, upper panel). Between ZT1 and ZT9, protein levels are approximately 40%–50% of the peak. Lower amplitude CRY cycling in CS versus *y w* flies is probably due to light shielding by eye pigment, as described for the TIM light response (Suri et al., 1998).

To determine whether the CRY cycling is light driven, we measured protein levels under DD conditions. Surprisingly, and in sharp contrast with PER, TIM, and CLK, the CRY DD pattern is completely different from that in LD and increases continuously throughout the subjective day and night (Figure 6C, lower panel), beginning at the levels reached at ZT23 of the previous day. In DD conditions as well as in LD conditions, the protein and mRNA profiles are completely different (see Figure 3). These results strongly suggest that CRY cycling is in great part under light control.

To further address the role of light, we assayed CRY levels in per^0, tim^0, *Clk*, and cyc^0 arrhythmic backgrounds (Figure 6D). As expected, relative protein levels correlate with the relative RNA levels, higher in *Clk* and cyc^0 than in per^0 and tim^0. But there was also robust CRY cycling in all these clock mutants, in contrast to what was observed for *cry* RNA cycling under the same LD conditions (Figure 5). Taken together, the results show that CRY expression is light regulated at the translational or posttranslational level as well as clock regulated at the transcriptional level.

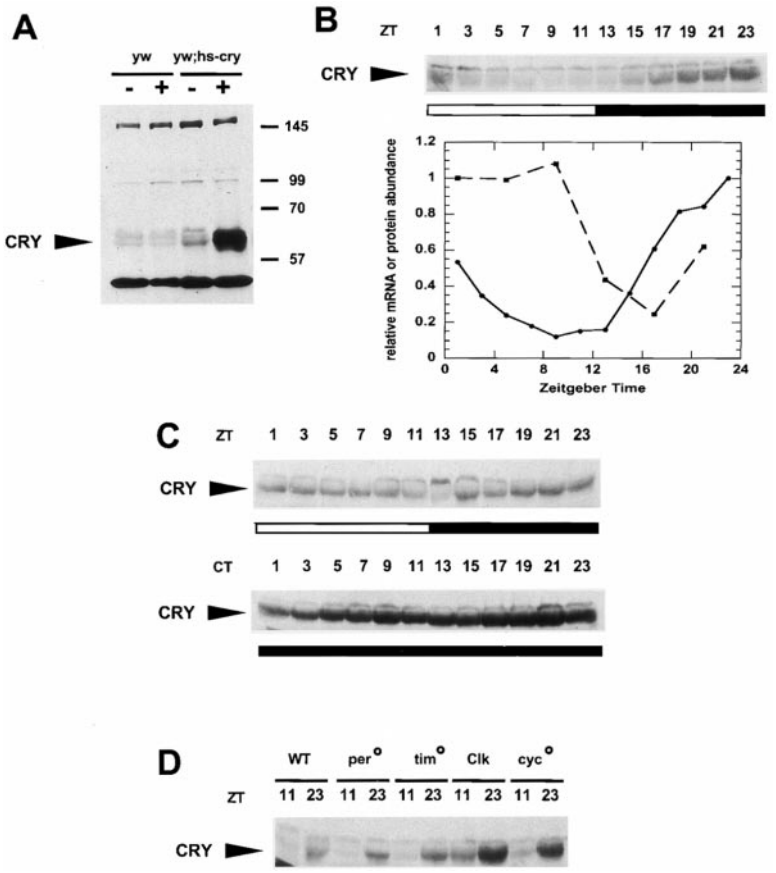

Figure 6. CRY Protein Levels under LD and DD Conditions

(A) The purified anti-CRY antibody specifically recognizes CRY in head extracts. Control (*y w*) and *y w*;*hs-cry* flies were (+) or were not (-) subjected to a 30 min heat shock. After 3 hr in darkness, flies were collected.

(B) CRY protein cycling in *y w*;*tim-GAL4* fly heads under LD conditions. The upper panel shows the Western blot and the lower panel the CRY signal quantitation (solid line); the dashed line corresponds to the mRNA cycling in *y w* flies. Zeitgeber times (ZT) of sample collection are indicated. ZT1 mRNA level and ZT23 protein level were set to 1. This Western blot was repeated with similar results.

(C) (Top) CRY protein cycling in wild-type (Canton-S) fly heads under LD conditions. This Western blot is representative of five experiments. We observed substantial variations of protein levels, but CRY was always clearly more abundant during the late night. (Bottom) CRY protein levels in wild-type fly heads under constant darkness (DD) conditions. A second DD Western blot was performed with very similar results (data not shown). Zeitgeber and Circadian times (ZT and CT) of sample collection are indicated.

(D) CRY protein levels in wild-type (WT, Canton-S), per^0, tim^0, *Clk*, and cyc^0 circadian mutants under LD conditions at Zeitgeber times (ZT) 11 and 23. This experiment was repeated with similar results.

cry-Overexpressing Flies Manifest Hypersensitive Circadian Responses to Light

The RNA oscillations suggest that *cry* is a clock gene, and the primary sequence and light regulation indicate a role in photoreception. To link *cry* to circadian behavior, we used the GAL4 system to overexpress CRY in cells that govern locomotor activity rhythms. A newly generated *tim* promoter–GAL4 strain was crossed with a UAS-*cry* cDNA strain to generate progeny that should overexpress CRY in lateral neurons. Indeed, *cry* mRNA levels were temporally constant and approximately 20-fold higher than the normal ZT1 peak level (data not shown). The protein is also overexpressed (at least 30-fold at each time point) and cycles robustly, as expected from its light sensitivity (see above; Figure 7A).

To measure quantitatively circadian light perception, flies were subjected to an anchored phase response curve (PRC) protocol (Aschoff, 1965; Rutila et al., 1998a; Suri et al., 1998); after entrainment to an LD cycle, flies were exposed to saturating or nonsaturating light pulses and the effect on behavioral phase measured. Control wild-type flies undergo a phase delay when the pulse is administrated during the early night and a phase advance during the late night (Levine et al., 1994; Saunders et al., 1994; Rutila et al., 1998a; Suri et al., 1998). The overexpression had no significant effect on the period or strength of the locomotor activity rhythms (data not shown), and there was no consistent or dramatic effect on the phase shift observed at high light intensities (Figure 7B). However, the CRY overexpression strain was much more sensitive to light at low intensities. Especially in the delay zone, at ZT15, this effect was reproducibly very strong and suggests that CRY levels are normally limiting at low light intensities. In the advance zone, at ZT21, the magnitude of the effect was somewhat more variable from experiment to experiment (Figure 7B). The striking light regulation of protein levels in clock-mutant as well as wild-type flies also indicates that CRY acts upstream of all known central pacemaker components (Figure 8).

Discussion

The properties of the *cry* gene strongly suggest that it encodes a circadian photoreceptor. Indeed, the cry^b mutant strain responds poorly to phase-shifting light pulses (Stanewsky et al., 1998). But this can be an indirect mutant effect (i.e., the cry^b gene only modestly or indirectly affects entrainment and light perception). For example, residual mutant protein could interfere with the function of another photoreceptor. The fact that the mutant flies still entrain to 12 light-dark cycles (Stanewsky et al., 1998) makes this possibility more plausible. But the behavioral phenotype of the overexpression phenotype strongly supports the contention that CRY is a major photoreceptor for *Drosophila* locomotor activity rhythms.

Given the likely cell-autonomous nature and strong light dependence of peripheral clocks in *Drosophila* (Plautz et al., 1997), we presume that *cry* expression

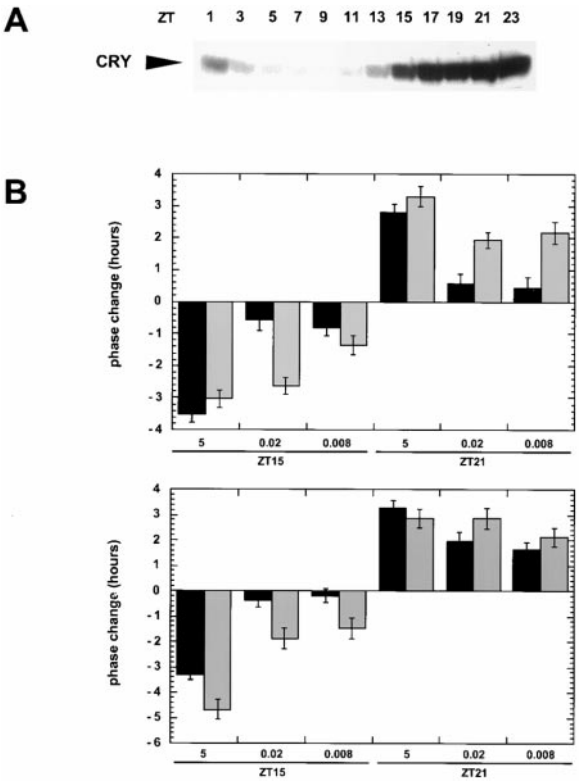

Figure 7. Light Response in CRY-Overexpressing Flies

(A) Western blot showing CRY overexpression and cycling under LD conditions in *tim-GAL4/UAS-cry* flies. Zeitgeber times (ZT) of sample collection are indicated. This experiment was repeated with similar results.

(B) (Top) Change in clock phase at different light intensities with control and *cry*-overexpressing flies at ZT 15 and 21. The Zeitgeber time (ZT) of the light pulse and the intensities in mW/cm^2 (5, 0.02, 0.008) are indicated on the X axis. Phase delays and advances are plotted on the Y axis (± SEM) as negative and positive values, respectively. Solid bars correspond to *tim-GAL4/+* flies (control flies), gray bars to *tim-GAL4/UAS-cry* flies (*cry*-overexpressing flies). Data were pooled from the following numbers of flies (each pair of values referring to control and *cry*-overexpressing flies): control: 14,16; ZT15, 5: 14,15; ZT15, 0.02: 16,16; ZT15, 0.008: 14,13; ZT21, 5: 14,13; ZT21, 0.02: 16,16; ZT21, 0.008: 15,14.

(Bottom) Second experiment, done as in upper panel. Number of flies: control: 13,13; ZT15, 5: 14,12; ZT15, 0.02: 16,12; ZT15, 0.008: 15,14; ZT21, 5: 14,10; ZT21, 0.02: 11,9; ZT21, 0.008: 13,14.

A third experiment was performed at ZT21. Results were very similar to those of the first experiment.

and function are also cell autonomous. Consistent with this notion, *cry* mRNA is abundant and present in fly bodies as well as in most if not all fly head tissues (Figure 3, and L. Sarov-Blat and M. R., unpublished data). A detailed characterization of *cry* expression, probably with antibodies, will be required to determine whether *cry* expression is strictly colocalized with *per* and *tim* expression in all tissues.

We discovered *cry* through its homology to *Drosophila melanogaster* 6-4 photolyase and to HsCRY1, a human cryptochrome proposed to be relevant to mammalian circadian rhythms (Miyamoto and Sancar, 1998). All three proteins are members of the cryptochrome family, which has benefited from detailed biochemical and structural studies of *E. coli* CPD photolyase (Park et al., 1995; Sancar, 1996). These suggest that all family members probably have two chromophores and share common mechanistic features, especially of photoreception. Although we have not shown that *Drosophila* CRY binds FAD and MTHF, this is likely. The FAD-binding amino acids are well conserved, and the mutation in *cry^b* alters a highly conserved FAD-binding residue (Kanai et al., 1997; Stanewsky et al., 1998). *Drosophila* CRY may have DNA repair activity. The relevant residues are well conserved within the family, but some members with comparable conservation, mammalian cryptochromes for example, fail to manifest activity (Hsu et al., 1997).

In addition to sequence conservation between the human and *Drosophila* proteins, we found striking similarities at the level of gene regulation. *cry* mRNA cycles with a 5-fold amplitude in heads, similar to the 4-fold amplitude observed for *mcry1* mRNA in the SCN (Miyamoto and Sancar, 1998). Also, the phases of both cycles are similar. But the *cry* mRNA phase is entirely different, almost antiphase to *per* and *tim* cycling as well as most other cycling curves described so far in *Drosophila* (Hardin et al., 1990; Sehgal et al., 1995; Van Gelder and Krasnow, 1996; Rouyer et al., 1997). Moreover, *cry* mRNA levels are low in *per* and *tim* null mutants, the opposite of what is observed for autoregulation of *per* and *tim* mRNA levels (Hardin et al., 1990; Sehgal et al., 1994, 1995). *cry* mRNA levels are high in *clock* or *cycle* mutants, contrary to the low *per* and *tim* mRNA levels found in these novel clock mutants (Allada et al., 1998; Rutila et al., 1998b). As CLK and CYC are probably direct activators of *per* and *tim* expression (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998b), they may function in an unanticipated manner on *cry*. More likely perhaps, CLK and CYC may regulate *cry* expression only indirectly. All of the cycling mRNA results suggest that *cry* transcription is driven by the canonical circadian circuit but through an unknown and novel set of DNA regulatory elements and factors. The CRY profile is strikingly different from that of *cry* mRNA and increases before the RNA increase during the mid-late night (Figure 6B). The discrepancy is due in large part to light regulation of CRY levels. TIM levels are also light regulated (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996), but the effect is more modest and the TIM LD protein profile more closely follows the *tim* mRNA profile (Zeng et al., 1996). We presume that photon capture by CRY itself increases proteolysis, and the data suggest that CRY is very stable at night and unstable during the day. Perhaps the *cry* mRNA oscillation is important for the proper increase and decrease in CRY levels that take place after lights off and lights on, respectively. These might be more pronounced or more important during the gradual changes in illumination that occur during natural light-dark cycles. Interestingly, *Arabidopsis* CRY2 is also light sensitive and accumulates at low light intensities (Ahmad et al., 1998b; Lin et al., 1998).

Light regulation may explain why the overexpression effect in the advance zone was less striking and more variable. (The least significant of the low-light ZT21 values was $p < 0.059$.) Although this may reflect differences between delay and advance zone signal transduction

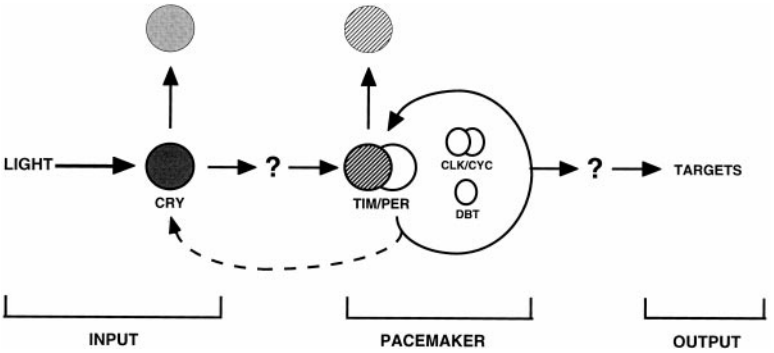

Figure 8. Model of CRY Relationship to the Central Pacemaker

Circadian rhythm system is partitioned into three segments: input, (central) pacemaker, and output. The genetic epistasis experiments place CRY upstream of TIM and TIM downstream of CRY. The question marks represent unknown signal transduction or other pathways. The dashed arrow represents feedback from the pacemaker onto *cry* mRNA cycling. The upper lighter circles represent CRY and TIM, which are subjected to light-mediated regulation. CLK, CYC, and DBT circles represent the product of the *clk*, *cyc*, and *dbt* genes. Overlapping circles (TIM/PER, CLK/CYC) represent heterodimeric proteins.

pathways (e.g., Ding et al., 1998), a more likely explanation focuses on the CRY protein accumulation profile during the night. Late in the night at ZT21, CRY levels are almost maximal. Six hours earlier in the delay zone, CRY levels are low and increasing. Therefore, flies might respond less well to low light intensities and more strongly to CRY overexpression at these earlier times. A more general version of this argument suggests that behavioral photosensitivity or PRC amplitude is sensitive to the prior illumination regime. Although this is valid in some systems (e.g., Shimomura et al., 1998), we have been unable to gather additional evidence that CRY cycling is important for the *Drosophila* PRC amplitude.

Light-mediated changes in CRY levels may be important for signal transduction downstream of CRY, or they may occur subsequent to signal transduction. Signaling may take place by electron transfer, protein–protein contact, or by changes in phosphorylation state. These possibilities are just beginning to be explored in the case of plant and animal cryptochromes (Huala et al., 1997; Zhao and Sancar, 1997; Ahmad et al., 1998a). But the ultimate targets in the case of *Drosophila* CRY are likely to be clock molecules. Indeed, TIM levels are also light sensitive, and there is evidence that this molecular light response is relevant to the behavioral light response (Suri et al., 1998; Yang et al., 1998). As TIM is unresponsive in the cry^b mutant (Stanewsky et al., 1998) and CRY light regulation is independent of other clock molecules including TIM (Figure 6D), this establishes a clear epistatic relationship between CRY and TIM. Yet *cry* mRNA cycling is clock regulated, indicating feedback between the central pacemaker and this clock input component (Figure 8).

Conservation of the putative DNA-binding residues in *Drosophila* suggests another, somewhat audacious possibility: CRY might be a DNA-binding protein and function as a transcriptional regulator of light-induced gene expression. Subcellular localization and mutagenesis studies should aid in determining whether CRY-DNA binding is relevant to the light response in *Drosophila*. In mammals, *mper1* transcription is rapidly induced by light in a rhythm-relevant manner (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997), and there are other data that link immediate early gene expression to phase-shifting light pulses (Aronin et al., 1990; Kornhauser et al., 1990, 1992; Schwartz et al., 1994). mCRY1 and 2 have not been shown to be functionally relevant to rhythms or to rhythm-relevant immediate early gene expression, and no light-regulated genes are known in *Drosophila*. But these differences are probably temporary, indicating that CRY might well connect to transcription in both systems. As CRY autoregulates expression (Stanewsky et al., 1998; data not shown), it is even possible that it participates directly, or once participated directly, in its own transcriptional regulation. Photoreception and transcriptional feedback loops are ubiquitous features of circadian pacemakers. Therefore, circadian rhythms may have begun long ago when a DNA repair protein acquired the ability to autoregulate expression in a light- and ultimately time-dependent manner.

Experimental Procedures

Flies and Generation of *tim-GAL4*, *UAS-cry*, and *hs-cry* Transgenic Flies

Flies were raised and maintained on media consisting of commeal, dextrose, yeast, and agar. Wild-type Canton-S and *y w* flies were used for molecular and behavioral studies.

A 6 kb BamHI/SalI fragment containing the *tim* upstream sequence whose 3′ end is at the *tim* translation start was cloned into a modified version of pPTGAL (provided by D. Eberl) just 5′ to the translation start site of *GAL4* (cloning details will be described elsewhere). The same *tim*-upstream sequence fused to the *tim* cDNA successfully rescued the rhythmic behavior of the tim^{01} mutant (Rutila et al., 1998a). This construct was then used to generate germline transformants by injecting *y w*;*Ki* $p^p[ry^+ \Delta 2\text{–}3]/+$. Twenty-three independent insertion lines were obtained, ten of which were checked for the GAL4-driven expression pattern of *UAS-GFP* (a mutant form of GFPS65T; provided by B. Dickson) in the whole adult fly, and six of which were studied for such patterns in the adult and larval brains. The GFP expression pattern in the whole fly was similar to the pattern previously described for flies carrying *per-GAL4* and *UAS-GFP* (Plautz et al., 1997; data not shown). All the *tim*-expressing cells in the adult and larval brains appear to express GFP in all the *tim-GAL4* lines tested in combination with *UAS-GFP* in addition to a few additional larval CNS cells that have not been detected by TIM immunohistochemistry (Hunter-Ensor et al., 1996; Kaneko et al., 1997; data not shown). Most of this ectopic GAL4 expression is not due to a positional effect of the inserts, since it was observed in five of six *tim-gal4* lines. Locomotor activity rhythms of the adult flies carrying one copy of *tim-GAL4* were tested as described in Hamblen et al. (1986). No significant abnormalities were observed in their rhythmic behavior except that in two of four lines, 16.7% and 25% of the flies tested were arrhythmic in constant darkness (DD). The *tim-GAL4* line (#27) used in this study was normal in terms of both its rhythmic behavior and GAL4 expression pattern (data not shown).

cry cDNA (from the EST clone HL02979) was cloned in the EcoRI and XhoI sites of pUAST (Brand et al., 1994). *y w*;*Ki* $p^p[ry^+ \Delta 2\text{–}3]/+$ flies were transformed with this construct using P element–mediated transformation. Ten lines were obtained. To obtain *cry*-overexpressing flies, *y w*;*tim-GAL4* females were crossed with *y w*;*UAS-CRY24.5*

males. Control flies were obtained by crossing *y w*;*tim-GAL4* females to *y w* males. The progeny of these crosses were then used for molecular and behavioral analyses. No rhythmic abnormalities were observed.

y w;*Ki p*^{P}*[ry*$^{+}$*Δ2–3]/+* flies were also transformed with a pCasper-hs plasmid containing the *cry* cDNA cloned in its EcoRI and StuI sites. Five lines were obtained and analyzed for heat shock–induced *cry* overexpression by RNase protection assay. *hs-cry7* strain showed the strongest induction and was used in this study.

Northern Blotting

Total RNAs were prepared as in Rutila et al. (1996). Poly(A)$^{+}$ RNA was prepared from total RNA using poly(A) Tract kit according to manufacturer's instructions (Promega). Northern analysis used 1 μg of poly(A)$^{+}$ RNA per lane on a 1% agarose formaldehyde gel (Rouyer et al., 1997). Gels were quantified on a Bio-Rad phosphorimager with Molecular Analyst software.

RNase Protection Assays

Total RNA was prepared from 50 heads (50% males and 50% females) per time point as described in Zeng et al. (1994). The assays are described on the Cell web site (http://www.cell.com/cgi/content/full/95/5/669/DC1).

Nuclear Run-Ons

Nuclear run-on analyses were performed as described in So and Rosbash (1997). A mixture of two DNA probes was generated from a genomic cosmid clone (P. E. and M. R., unpublished data) using primers located at cDNA position −13/+549 and +209/+1457 (+1 is the A of the initial ATG). Rhodopsin 1 was used as an internal control.

Anti-CRY Antibodies and Western Blotting

A PCR fragment corresponding to CRY amino acids 1–183 was cloned in the EcoRI and XhoI restriction sites of pGEX6p-1 (Pharmacia). The GST-CRYNt fusion protein was overexpressed in bacteria and purified from an SDS-polyacrylamide gel. Eluted protein was then injected into rats. Anti-CRY antibodies were affinity purified from serum and diluted 1:10 in TBST/5% milk for Western blot.

Head protein extracts were obtained as follows. Twenty heads from frozen flies were homogenized in 20 μl extraction buffer (20 mM HEPES [pH 7.5], 100 mM KCl, 5% glycerol, 10 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 0.5 mM PMSF, 20 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A). SDS loading buffer was then added, and the extract was incubated 10 min at 95°C. After 10 min of full-speed microcentrifugation, half of the supernatant was loaded on a 9% SDS-polyacrylamide gel (29/0.8 acryl/bisacrylamid ratio). Western blots were performed as described in Edery et al. (1994b), except that TBST/5% milk was used for blocking the membrane after transfer and for the incubation with purified anti-CRY serum, and TBST/1% milk was used for the incubation with the secondary antibody. Autoradiographs were quantified with Molecular Analyst software.

Behavioral Light Response Experiments

Anchored PRC experiments were performed as in Suri et al. (1998) and Rutila et al. (1998). *y w*;*tim-GAL4* and *y w*;*tim-GAL4/UAS-cry24.5* PRCs were produced by first entraining flies during 4 days in 12 hr light:12 hr dark (LD), then subjecting them to a 5 min light pulse (white light, 5, 0.02, and 0.008 mW/cm^{2}) during the dark phase of the final LD cycle, at ZT15 and 21. Flies were then kept in DD for 5 days, and activity rhythm shifts were determined by formal analysis of the new versus old phases, using locomotor data from the second day.

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GenBank Accession Number

The sequence of *cry* has been submitted to GenBank with accession number AF099734.