The two CRYs of the butterfly

Haisun Zhu[1], Quan Yuan[1], Oren Froy, Amy Casselman and Steven M. Reppert

Animal flavoproteins called cryptochromes (CRYs) are generally believed to have distinct circadian clock functions in insects and mammals: *Drosophila* has one CRY (dCRY) which functions primarily as a blue-light photoreceptor [1], whereas mouse has two CRYs, mCRY1 and mCRY2, which while not directly photoreceptive, are potent transcriptional repressors acting within the clockwork itself [2]. We have discovered that the monarch butterfly, *Danaus plexippus*, has two *cry* genes: one encodes a fly-like protein with photosensitive properties, while the other encodes a mouse-like protein with potent transcriptional repressive activity. Database searches show that other non-drosophilid insects also have two *cry* genes. These findings change our view of how some insect clocks may work and redefine the evolution of animal CRYs.

As part of research on navigational clock mechanisms in the monarch butterfly, a brain EST library was made for the species. Sequence analysis of 21,212 clones revealed two distinct *cry* cDNA fragments. The predicted protein encoded by one cDNA fragment matched a *Drosophila*-like CRY cloned previously from monarch brain [3], designated dpCRY1. Phylogenetic analysis showed that the other cDNA encoded a predicted CRY-like protein, designated dpCRY2, that aligned more closely with mammalian CRYs than with dpCRY1 (Figure 1).

To analyze the functions of the two monarch butterfly CRYs, we expressed the full-length coding region of each in *Drosophila* Schneider 2 (S2) cells to assay for photosensitive and transcriptional activities. As dCRY undergoes a light-dependent reduction in protein levels in S2 cells because of proteasome-mediated degradation [4], we assessed the ability of a 6 hour light pulse to promote dpCRY1 and dpCRY2 degradation, and compared the responses to those of dCRY (the positive control) and mCRY1 (the negative control). The levels of dCRY and dpCRY1 decreased substantially (95% and 53%, respectively) after the 6 hour light exposure, while mCRY1 and dpCRY2 levels were unaltered (Figure 2A). So in S2 cells dpCRY1, like dCRY, is degraded in response to light, while dpCRY2 and mCRY1 are not.

To assess transcriptional activity, we used a luciferase reporter construct with an E-box enhancer from the monarch *period (per)* gene promoter; the butterfly *per* gene is under circadian control *in vivo*, likely through transcription via the enhancer element [5]. Co-transfection of the reporter with monarch CLOCK and CYCLE, two clock-relevant transcription factors [6], elicited a 26-fold increase in transcriptional activity (Figure 2B). Transcription was not inhibited by co-transfection of dpCRY1 or dCRY, but it was abolished by co-transfection with dpCRY2, an inhibition similar to that elicited by mCRY1 (Figure 2B). So in S2 cells dpCRY2, like mCRY1, can act as a potent transcriptional repressor, while dpCRY1 and dCRY [7] cannot.

The occurrence of two *cry* genes is not unique to the monarch butterfly. Rather, they provide a window into a more global view of CRY evolution in insects: analysis of other insect genomic and EST databases have revealed two distinct *cry* genes in the genomes of the Chinese oak silkmoth (*Antheraea pernyi*), the commercial silkworm (*Bombyx mori*) and the mosquito (*Anopheles gambiae*); only the mammalian-like *cry* has been identified so far in the genomes of the honeybee (*Apis mellifera*) and the red flour beetle (*Tribolium castaneum*) (Figure 1). Importantly, the mosquito proteins agCRY1 and agCRY2 also have distinct functions in S2 cells, identical to those of the monarch butterfly CRYs (Figure 2A,C). These findings extend the distinct functions of insect CRY1 and CRY2 to two orders, *Lepidoptera* and *Diptera*.

In contrast to the other insects examined, only the previously characterized, photoreceptive dCRY is found in the annotated *Drosophila* genome. Studies have shown that dCRY is involved in circadian clock function in peripheral tissues through a photoreceptor-independent mechanism [8–10], so it is conceivable that dCRY has transcriptional acitvity in peripheral clocks. The only bona fide function of dCRY in the central clock, however, is as a blue-light photoreceptor [1,6].

In the butterfly and mosquito, CRY1 and CRY2 are functionally distinct: the CRY1s are closer in sequence to dCRY and are photosensitive, while the CRY2s are closer in sequence to mCRY1 and mCRY2 and are repressors of E-box-mediated transcription. In *Drosophila*, PERIOD is the major transcriptional repressor in the

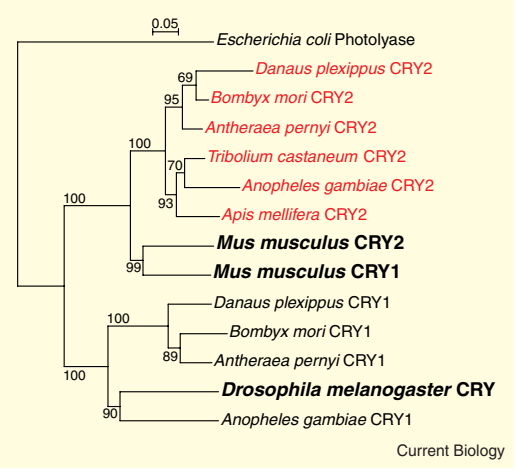

Figure 1. Insect CRY phylogeny.

Phylogeny of insect CRYs relative to *Drosophila* CRY, and CRY1 and CRY2 from the mouse (*Mus musculus*). The insect CRY2 clade is highlighted in red. Bootstrap values (percent of branching in 100 replicate searches) are indicated on the horizontal branches. See Figure S1 in the Supplemental data for a more extensive tree.

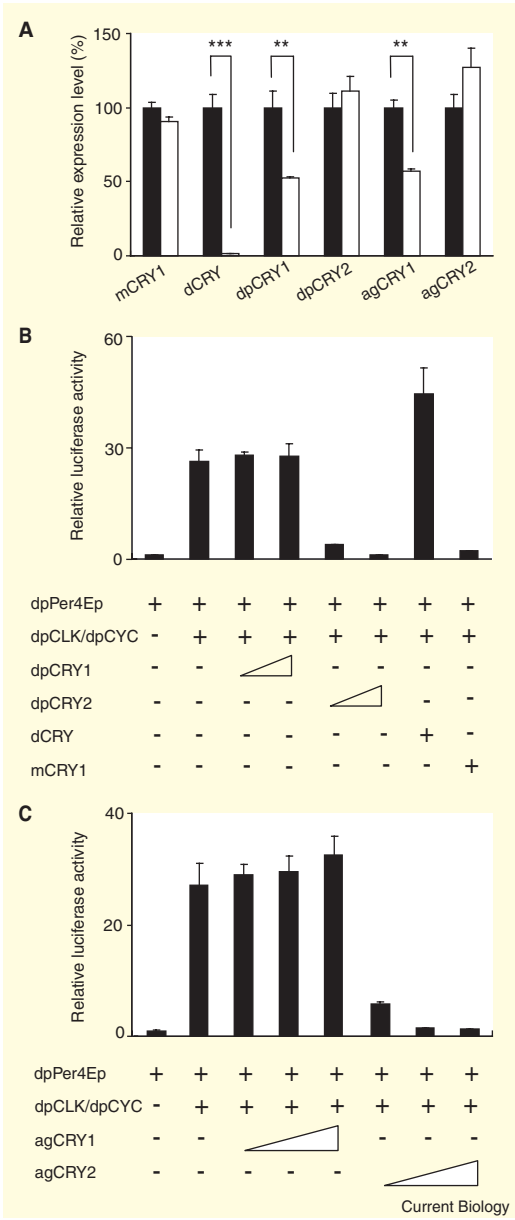

Figure 2. Functional analysis of monarch butterfly and mosquito CRY proteins.

(A) Light suppresses monarch butterfly (dp)CRY1 and mosquito (ag)CRY1 levels in S2 cells. V5 epitope tagged mCRY1, dCRY, dpCRY1, dpCRY2, agCRY1, or agCRY2 was co-expressed with V5 tagged β-galactosidase. After either exposure to a 6 hour light pulse (open bars) or constant darkness (dark bars), cell extracts were collected, western blotted, and probed with anti-V5 antibody. CRY levels were quantified by densitometry of antibody staining after normalization with β-galactosidase. The dark value for each CRY was plotted as 100%. The results are the mean ± SEM of three separate transfections. **, $p<0.01$; ***, $p<0.001$. (B) Monarch butterfly (dp)CRY2 inhibits CLOCK/CYCLE-activated transcription. The monarch *per* E box luciferase reporter (dpPerEp; 10 ng) was used in presence (+) or absence (–) of dpCLK/dpCYC expression plasmids (5 ng each). dpCRY1 (1 and 5 ng), dpCry2 (1 and 5 ng), dCRY (200 ng) or mCRY1 (200 ng) were used. Luciferase activity was computed relative to β-galactosidase activity. Each value is mean ± SEM of three replicates. (C) Mosquito (ag)CRY2 inhibits CLOCK/CYCLE-mediated transcription. The dpPerEp reporter (10 ng) was tested in presence (+) or absence (–) of dpCLK/dpCYC expression plasmids (5 ng each); agCRY1 (1, 5, and 10 ng) or agCry2 (1, 5 and 10 ng) was used. Luciferase activity relative to β-galactosidase activity was computed. Each value is mean ± SEM of three replicates.

circadian clock [6], while in several other insects, including the monarch butterfly [3], PERIOD is not detected in the nucleus, suggesting that another clock protein may fulfill this function [11]. Therefore, it is significant that CRY2 exists in other insects and can potently repress transcription in cell culture, as it may be a major transcriptional repressor for the central clockwork of some non-drosophilid insects, acting like CRY in mammals [2]. From an evolutionary vantage point, the transcriptionally active insect CRY2s share a common ancestor with the two mammalian CRY*s* (Figure 1 and Figure S1 in the online Supplemental Data).

Acknowledgments

We thank the W.M. Keck Center for Comparative and Functional Genomics for the EST library, Larry Zwiebel for *A. gambiae* RNA, Danielle Metterville for technical assistance, Adriana Briscoe for suggesting that two *cry* genes exist in *A. gambiae*, and Dave Weaver and Patrick Emery for discussions. This work was supported in part by NIH grant R01NS047141.

Supplemental data

Supplemental data and experimental procedures are available at http://www.current-biology.com/cgi/content/full/15/23/R953/DC1/

References

1. Emery, P., Stanewsky, R., Helfrich-Forster, C., Emery-Le, M., Hall, J.C., and Rosbash, M. (2000). *Drosophila* CRY is a deep brain circadian photoreceptor. Neuron *26*, 493–504.
2. Reppert, S.M., and Weaver, D.R. (2002). Coordination of circadian clocks in mammals. Nature *418*, 935–941.
3. Sauman, I., Briscoe, A.D., Zhu, H., Shi, D., Froy, O., Stalleicken, J., Yuan, Q., Casselman, A., and Reppert, S.M. (2005). Connecting the navigational clock to sun compass input in monarch butterfly brain. Neuron *46*, 457–467.
4. Lin, F.-J., Song, W., Meyer-Bernstein, E., Naidoo, N., and Sehgal, A. (2001). Photic signaling by cryptochrome in the *Drosophila* circadian system. Mol. Cell. Biol. *21*, 7287–7294.
5. Froy, O., Gotter, A.L., Casselman, A.L., and Reppert, S.M. (2003). Illuminating the circadian clock in monarch butterfly migration. Science *300*, 1303–1305.
6. Stanewsky, R. (20032). Genetic analysis of the circadian system in *Drosophila melanogaster* and mammals. J. Neurobiol. *54*, 111–147.
7. Ceriani, M.F., Darlington, T.K., Staknis, D., Mas, P., Petti, A.A., Weitz, C.J., and Kay, S.A. (1999). Light-dependent sequestration of TIMELESS by CRYPTOCHROME. Science *285*, 553–556.
8. Vanchenko, M., Stanewsky, R., and Giebultoxicz, J.M. (2001). Circadian photoreception in *Drosophila*: functions of cryptochrome in peripheral and central oscillators. J. Biol. Rhythms *16*, 205–215.
9. Krishnan, B., Levine, J.D., Lynch, M.K., Dowse, H.B., Funes, P., Hall, J.C., Hardin, P.E., and Dryer, S.E. (2001). A new role for cryptochrome in a *Drosophila* circadian oscillator. Nature *411*, 313–317.
10. Levine, J.D., Funes, P., Dowse, H.B., and Hall, J.C. (2002). Advanced analysis of a cryptochrome mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues in *Drosophila*. BMC Neurosci. *3*, 5.
11. Rosato, E., and Kyriacou, C.P. (2001). Flies, clocks, and evolution. Philos. Trans. R. Soc. Lond. B Biol. Sci. *356*, 1769–1778.

Department of Neurobiology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, Massachusetts 01605, USA. [1]Equal contributions.
E-mail: Steven.Reppert@umassmed.edu