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Insect Cryptochromes: Gene Duplication and Loss Define Diverse Ways to Construct Insect Circadian Clocks

Quan Yuan, Danielle Metterville,* Adriana D. Briscoe,† and Steven M. Reppert**

*Department of Neurobiology, University of Massachusetts Medical School; and †Comparative and Evolutionary Physiology Group, Department of Ecology and Evolutionary Biology, University of California, Irvine

Cryptochrome (CRY) proteins are components of the central circadian clockwork of metazoans. Phylogenetic analyses show at least 2 rounds of gene duplication at the base of the metazoan radiation, as well as several losses, gave rise to 2 cryptochrome (*cry*) gene families in insects, a *Drosophila*-like *cry1* gene family and a vertebrate-like *cry2* family. Previous studies have shown that insect CRY1 is photosensitive, whereas photo-insensitive CRY2 functions to potently inhibit clock-relevant CLOCK:CYCLE-mediated transcription. Here, we extended the transcriptional repressive function of insect CRY2 to 2 orders—Hymenoptera (the honeybee *Apis mellifera* and the bumblebee *Bombus impatiens*) and Coleoptera (the red flour beetle *Tribolium castaneum*). Importantly, the bee and beetle CRY2 proteins are not light sensitive in culture, in either degradation of protein levels or inhibitory transcriptional response, suggesting novel light input pathways into their circadian clocks as *Apis* and *Tribolium* do not have CRY1. By mapping the functional data onto a cryptochrome/6-4 photolyase gene tree, we find that the transcriptional repressive function of insect CRY2 descended from a light-sensitive photolyase-like ancestral gene, probably lacking the ability to repress CLOCK:CYCLE-mediated transcription. These data provide an evolutionary context for proposing novel circadian clock mechanisms in insects.

Introduction

Organisms from prokaryotes to humans exhibit circadian (circa 24 h) rhythms. The rhythms drive daily and seasonal photoperiodic changes associated with observed changes in organismal physiology and behavior (Reppert and Weaver 2002). Circadian rhythms are particularly important for timing or regulating key biological events in insects (Saunder 2002). Some examples of critical circadian rhythm outputs in holometabolous insects include the time of day of egg hatching, the time of day of adult eclosion, the seasonal timing of adult development, and time-compensated sun compass navigation.

At the molecular level, the central circadian clock in *Drosophila melanogaster*, the most well studied of animal clocks, is driven mainly by a negative transcriptional feedback loop that involves the products of the *period* (*per*) and *timeless* (*tim*) genes, and the transcription factors *Clock* (*Clk*) and *cycle* (*cyc*)(Stanewsky 2003). CLK and CYC heterodimers drive *per* and *tim* transcription through E-box promoter elements. The resultant PER and TIM proteins form dimers that translocate back into the nucleus to repress their own transcription via inhibitory effects on CLK and CYC. *Drosophila* cryptochrome (CRY) protein is colocalized in clock cells with PER and TIM and functions as a blue-light photoreceptor involved in photic entrainment (Emery et al. 1998, 2000; Stanewsky et al. 1998). CRY inhibits the formation of PER and TIM heterodimers by directly interacting with TIM in a light-dependent process, and it also participates in its own light-dependent proteasomal degradation (Lin et al. 2001).

Based on studies of the 2 mouse CRY proteins (mCRY1 and mCRY2), the mammalian CRYs on the other hand, work within the circadian clockwork itself (van der Horst et al. 1999; Vitaterna et al. 1999) as potent repressors of CLK:BMAL1 (the mammalian ortholog of CYC)-mediated transcription in a light-independent manner (Griffin et al. 1999; Kume et al. 1999). The repressive function of mammalian CRY proteins on CLK:BMAL1-activated transcription has been extended to homologous CRY proteins from other vertebrates, including those from zebra fish *Danio rerio* (zCRY1A, 1B, 2A and 2B) (Kobayashi et al. 2000), *Xenopus laevis* (xCRY1 and xCRY2b) (Zhu and Green 2001), and the domestic chicken *Gallus gallus* (cCRY1 and cCRY2) (Yamamoto et al. 2001).

A second *cry* gene was recently discovered in insects, which is present in all nondrosophilid species so far examined (Zhu et al. 2005). This second *cry* encodes a vertebrate-like protein designated insect CRY2. Functional studies in *Drosophila* Schneider 2 (S2) cells show that the insect CRY2 proteins are potent transcriptional repressors of CLK:CYC-mediated transcription, but are not light sensitive. In contrast, *Drosophila*-like CRY, designated CRY1, is light sensitive in S2 cells, but does not show transcriptional repressive activity.

Drosophila expresses CRY1 only, whereas several insects, like mosquitos and butterflies, express both CRY1 and CRY2 (Zhu et al. 2005). Surprisingly, the honeybee *Apis mellifera* and the beetle *Tribolium castaneum* were identified through Blast searches of whole genomes to contain only CRY2 (Zhu et al. 2005; Rubin et al. 2006). This suggests 2 remarkable possibilities. First, the core oscillator in insects has itself evolved such that at least 3 kinds of clocks exist, those containing only CRY1 as in *Drosophila*, those containing CRY1 and CRY2 as in monarch and mosquito, and those containing CRY2 alone as in beetle and honeybee. Second, in insects containing only CRY2, the cryptochrome may serve dual functions, as both a transcriptional repressor and a photoreceptor.

Here we contribute functional data for bee and beetle CRY2, as well as for silk moth CRY1 and CRY2, and for *Drosophila* and monarch 6-4 photolyase. We then map the ability to repress CLK:CYC(BMAL1)-mediated transcription onto a gene tree of cryptochromes and DNA photolyases to examine the evolutionary origins of transcriptional repressive activity. Finally, we expand the current model of the circadian clockwork in insects to include new models based on the observation that some insects

Key words: cryptochromes, circadian clocks, CLOCK, CYCLE, photoreceptor, transcriptional activity, monarch butterfly.

E-mail: abriscoe@uci.edu; steven.reppert@umassmed.edu.

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have both CRY1 and CRY2, whereas other insects have only CRY2, and based on our findings that all insect CRY2 proteins so far examined are light-insensitive transcriptional repressors.

Materials and Methods

Polymerase Chain Reaction, Cloning, and Sequencing

cDNA fragments were cloned by either primer-specific or degenerate polymerase chain reaction (PCR). cDNA templates for PCR were prepared from RNA purified from the bodies of the red flour beetle *T. castaneum*; the heads of the fly *Drosophila melanogaster*, mosquito *Anopheles gambiae*, and worker honeybee *A. mellifera*; and the brains of the worker bumblebee *Bombus impatiens* and monarch butterfly *Danaus plexippus*. The ends of the coding regions were obtained by rapid amplification of cDNA ends (RACE, Marathon cDNA Amplification Kit). Complete open reading frames were obtained by *Pfu* Turbo (Stratagene, La Jolla, CA) PCR from cDNA. Clones were sequenced at core facilities at University of Massachusetts Medical School and the University of California, Irvine.

Insect Cell Culture, Transfections, and Transcription Assays

S2 cells were maintained at 25 °C, in Schneider's *Drosophila* media (Gibco/Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco/Invitrogen). The reporter was generated by subcloning a tandem repeat of an E-box element from the monarch *per* gene promoter into a luciferase reporter vector containing the *heat shock promoter 70* (Chang and Reppert 2003). S2 cell expression constructs were generated by subcloning cDNAs into the pAc5.1V5/HisA vector (Gibco/Invitrogen, Carlsbad, CA). Transient transfections and transcriptional assays were performed as previously described (Chang and Reppert 2003).

Photosensitivity Assays

Light treatments involved placing S2 cell culture plates under fluorescent lighting (0.2–0.3 mW/cm^2) at 24 °C in a temperature controlled incubator (Percival Scientific Perry, IA). The duration of light exposure was set according to the experiment. Dark control plates were wrapped in aluminum foil and incubated beside the light-treated plates.

Western Blots

Western blotting was performed as described previously (Lee et al. 2001). The V5 antibody used for western blots was a monoclonal anti-V5 IgG (Gibco/Invitrogen).

Phylogenetic Analysis and Character Mapping of Transcriptional Repression

To determine the evolutionary relationships between the newly cloned insect cryptochromes and DNA photolyases and other known gene family members, we conducted a GenBank search. We focused primarily on species for which whole-genome sequences are available. We also examined the literature for sequences assayed for transcriptional repressive activity in cell culture. Among those sequences included in our analysis for which no functional data are available were 3 sequences from the mosquito *Aedes aegypti* genome (*cry1*, *cry2*, and 6-4 photolyase), 1 sequence from *X. laevis* (*xcry4*), and 3 from the sea urchin *Strongylocentrotus purpuratus* (*cry1a*, *cry5*, and *cry1*). Altogether, we included 36 sequences from 13 species in our phylogenetic analysis (for species names and GenBank accession numbers, see supplementary table S1, Supplementary Material online). Amino acid sequences were aligned using ClustalW (Thompson et al. 1994) in the Alignment Explorer in MEGA version 3.1 (Kumar et al. 2004).

Both Neighbor-Joining (NJ) and maximum likelihood (ML) analyses were conducted in MEGA 3.1 (Kumar et al. 2004) and PAUP* (Swofford 2000), respectively. A total of 1,056 first + second nucleotide sites were included. For the NJ analysis, complete deletion of gaps and the Tamura–Nei model of nucleotide evolution were used. For the ML analysis, the GTR + Γ + I model was used with parameters estimated from the data. The reliability of the ML trees was tested by bootstrap analysis using PhyML (Guindon and Gascuel 2003; Guindon et al. 2005) and 500 bootstrap replicates.

Because we were interested in examining the evolution of transcriptional repression, and when this property of vertebrate cryptochromes evolved, we examined the literature for cell culture studies performed in comparable ways to the assays described above. We therefore included in our character mapping those studies which used an E-box promoter, coexpressed with CLK and CYC- ("or" vertebrate BMAL1-) containing plasmids in the absence of PER. Cryptochromes assayed in this way were scored as either being transcriptional repressors (+) or not (−) depending upon the results of the surveyed studies, and these binary data were mapped onto the multi-gene family phylogeny.

Results and Discussion

The Transcriptional Inhibitory Activity of Insect CRY2 Extends to Bees and Beetles

We expanded our functional analyses of insect cryptochromes by cloning the full-length coding regions of CRY2 from species in 2 additional insect orders—Hymenoptera (which included *Apis* and the bumblebee, *B. impatiens*) and Coleoptera (which included *Tribolium*)—increasing our evolutionary survey of cryptochrome function in holometabolous insects (fig. 1*A*; supplementary fig. 1, Supplementary Material online). As previously mentioned, analysis of the completed genomes of *Apis* and *Tribolium* showed no *cry1* homologs, suggesting that the *cry1* gene has been lost and that only *cry2* is present in those species (Zhu et al. 2005; Rubin et al. 2006). We also cloned the full-length cDNAs of CRY1 and CRY2 from another lepidopteran, the Chinese oak silk moth *Antheraea pernyi* for inclusion in our functional analysis (supplementary fig. 1, Supplementary Material online).

Collectively, we analyzed CRY protein function in 7 insect species, representing 4 insect orders: 2 dipterans, *Drosophila* and *Anopheles*; 2 lepidopterans, the monarch butterfly and silk moth *A. pernyi*; 2 hymenopterans, the honeybee and bumblebee; and the coleopteran *Tribolium*.

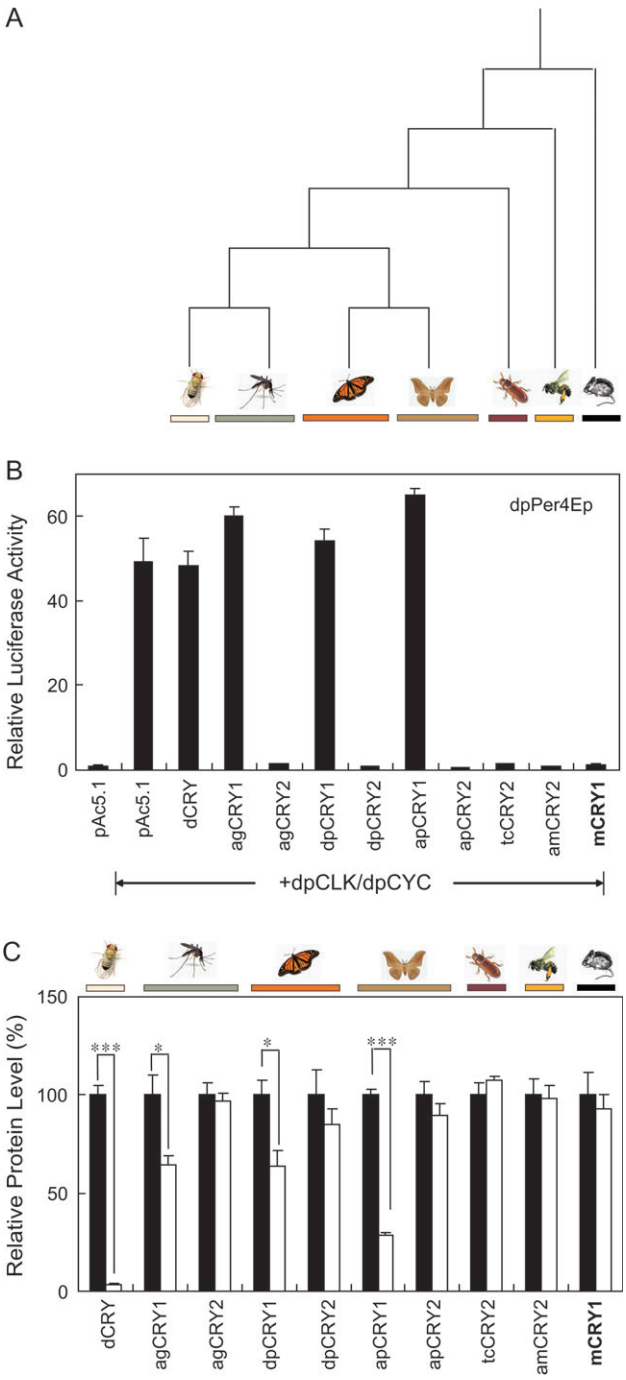

FIG. 1.—Functional analyses of CRY1 and CRY2 proteins from 4 insect orders, Diptera, Lepidoptera, Coleoptera, and Hymenoptera. (*A*) Cladogram (from left to right) of phylogenetic relationships between *Drosophila melanogaster*, *Anopheles gambiae*, *Danaus plexippus*, *Antheraea pernyi*, *Tribolium castaneum*, and *Apis mellifera*, redrawn from (Savard et al. 2006) with *Mus musculus* as the outgroup. (*B*) Insect CRY2 proteins inhibit CLK/CYC-activated transcription. The monarch per E-box luciferase reporter (dpPer4Ep; 10 ng) was used in the presence (lanes 2–12) or absence (lane 1) of dpCLK/dpCYC expression plasmids (5 ng each). The dose of the pAc5.1 expression vector or of each CRY expression construct was 200 ng. Luciferase activity was computed relative to β-galactosidase activity. Each value is the mean ± standard error of the mean of 3 replicates. (*C*) Light suppresses insect CRY1 levels in S2 cells. V5 epitope tagged dCRY (100 ng), agCRY1 (30 ng), agCRY2 (30 ng), dpCRY1 (30 ng), dpCRY2 (50 ng), apCRY1 (100 ng), apCRY2 (100 ng); amCRY2 (200 ng), tcCRY2 (50 ng), or mCRY1 (50 ng) was expressed with V5 tagged β-galactosidase (20 ng). After either exposure to a 6-h 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 was plotted as 100%. The results are the mean ± SEM of 3 separate transfections. $*P < 0.05$; $***P < 0.001$.

For comparison, we also analyzed mouse mCRY1 function. To analyze CRY function, we expressed the full-length coding region of each in S2 cells to assess transcriptional activity and light sensitivity.

To evaluate transcriptional activity, we used a luciferase reporter construct with an E-box enhancer from the monarch butterfly *period (per)* gene promoter, as the butterfly *per* gene is under circadian control in vivo, likely through transcription via the enhancer element (Froy et al. 2003). Cotransfection of the reporter (dpPer4Ep) with monarch CLOCK and CYCLE elicited a large increase in transcriptional activity. Transcription was potently inhibited by all 6 of the insect CRY2 proteins, similar to that found for mouse mCRY1 (fig. 1*B*; supplementary fig. 2, Supplementary Material online). For *Apis* and *Tribolium*, further analysis revealed that transcriptional inhibition was dose dependent (fig. 2*A*). In addition, western blot analysis of the expressed CRY proteins showed that dpCRY2, amCRY2, and tcCRY2 are equally potent transcriptional repressors (fig. 2*A*). *Drosophila* CRY and the other 3 insect CRY1 proteins, including *A. pernyi* CRY1, did not inhibit CLK:CYC-mediated transcription, similar to what has been previously reported (Zhu et al. 2005).

To assess photosensitivity, we took advantage of the fact that dCRY undergoes a light-dependent reduction in protein levels in S2 cells, likely through proteasome-mediated degradation (Lin et al. 2001). For each CRY protein, we therefore examined the ability of a 6-h light pulse to cause a reduction in CRY levels (Froy et al. 2002). Although there was a variation in the degree of the light-induced decrease, all 4 insect CRY1 proteins exhibited a significant reduction in levels with light exposure, compared with nonlight exposed proteins (fig. 1*C*). As previously shown, mCRY1 was not degraded by light in S2 cells (Zhu et al. 2005). Importantly, amCRY2, biCRY2, and tcCRY2 did not show any light sensitivity (fig. 1*C*; supplementary fig. 2, Supplementary Material online). Because these insects appear to only express CRY2, it was possible that their CRY2 proteins served dual functions—not only functioning as transcriptional repressors but also serving as light sensors. However, we found that this is probably not the case, based on their lack of degradation in response to light in S2 cells (fig. 1*C*).

The possibility of insect CRY2 light sensitivity was further evaluated by determining whether light alters the ability of dpCRY2, amCRY2, or tcCRY2 to inhibit CLK:CYC-mediated transcription in S2 cells. Parallel sets of cells were transfected with each of the insect CRY2s, with 1 group cultured under constant light and the other group under constant darkness. Compared with constant darkness exposure, the 48 h of constant light during culture caused a modest increase in basal luciferase activity when only the reporter (dpPer4Ep) was expressed (fig. 2*B*, first

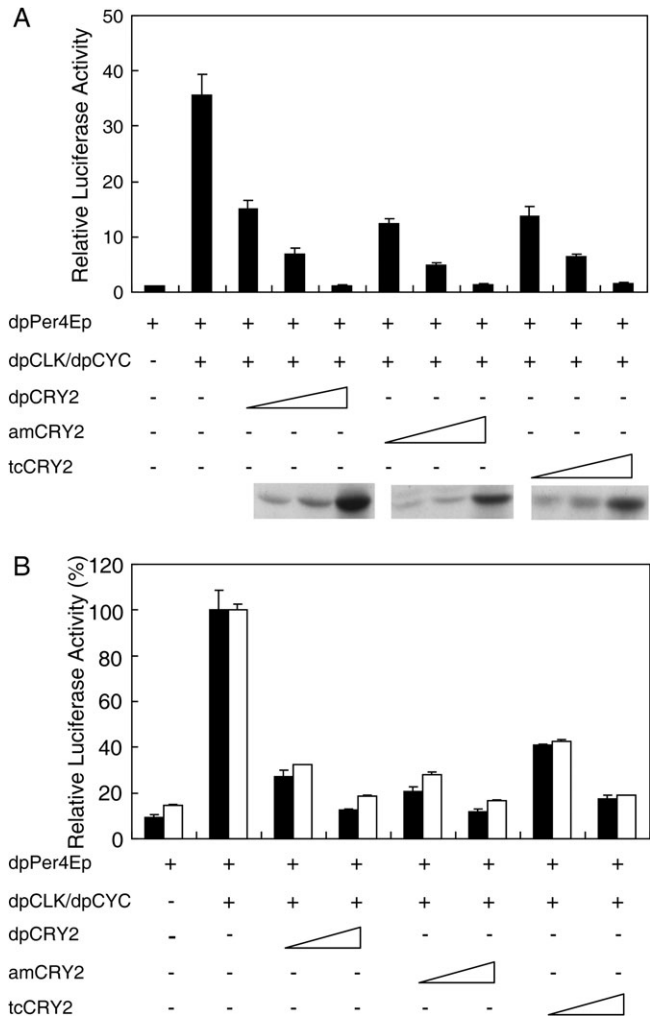

FIG. 2.—Dose-dependent transcriptional responses. (*A*) Dose-dependent transcriptional inhibition of CLK/CYC-activated transcription by *D. plexippus* dpCRY2, *A. mellifera* amCRY2, and *T. castaneum* tcCRY2. The monarch butterfly *per* E-box enhancer luciferase reporter (dpPer4Ep; 10 ng) was used in the presence (+) or absence (−) of dpCLK/dpCYC expression plasmids (5 ng each). dpCRY2 (1, 2, and 10 ng), amCRY2 (10, 20, and 100 ng), or tcCRY2 (1, 2, and 10 ng) was used. Luciferase activity was computed relative to β-galactosidase activity. Each value is the mean ± SEM of 3 replicates. Western blot of V5 epitope–tagged CRY2 protein expression levels for each concentration is depicted below the graph. (*B*) Effect of constant light on dose-dependent transcriptional inhibition. The monarch butterfly *per* E-box enhancer luciferase reporter (dpPer4Ep; 10 ng) was used in the presence (+) or absence (−) of dpCLK/dpCYC expression plasmids (5 ng each). dpCRY2 (2 and 10 ng), amCRY2 (20 and 100 ng), or tcCRY2 (2 and 10 ng) was used. For each construct and dose, cells were cultured for 48 h after transfection in either constant darkness (black bars) or constant light (open bars) and then harvested. Luciferase activity was computed relative to β-galactosidase activity and normalized such that the value in the presence of dpCLK/dpCYC alone was 100%. Each value is the mean ± SEM of 3 independent transfections.

2 lanes). Even when cultured in constant light, each of the 3 insect CRY2 proteins was still able to robustly inhibit CLK:CYC-mediated transcription (fig 2*B*). The small decrease in CRY2-mediated inhibition for both dpCRY2 and amCRY2 could be accounted for by the light-induced increase in basal luciferase activity. We therefore conclude that light has no significant effect on the ability of the insect CRY2s to inhibit CLK:CYC-mediated transcription in cell culture.

We also assayed *Drosophila* and monarch 6-4 photolyases for transcriptional repressive function because, phylogenetically, the 6-4 photolyases and nonmammalian vertebrate CRY4 proteins are most closely related to mCRY1 and mCRY2 and insect CRY2. The 6-4 photolyases use blue light to repair UV induced DNA damage, specifically the pyrimidine–pyrimidone (6-4) photoproduct (Pyr[6-4]Pyr) (Sancar 2003), whereas nonmammalian CRY4 is a protein of unknown function found in pineal gland and retina (Kubo et al. 2006).

Neither *Drosophila* nor monarch photolyase (epitope tagged or untagged proteins) inhibited CLK:CYC-mediated transcription at expressed levels that were greater than those of dpCRY2 that maximally inhibited transcription (fig. 3). Importantly, the V-5 tagged proteins were located primarily in the nucleus (>90% of cell examined, data not shown), showing that the lack of transcriptional inhibition was not due to inaccessibility to transcriptional machinery in the nucleus (fig. 3).

Differential Gene Loss Contributes to the Evolution of Insect Cryptochromes

Both NJ and ML methods were used to reconstruct the gene family tree. For the ML tree reconstruction, hierarchical likelihood ratio tests and the Akaike information criterion were used to select the best fitting model of nucleotide evolution (Posada and Crandall 1998). Both selected the GTR + Γ + I model as the best fit to the data. Similar trees were recovered with both NJ and ML analyses of all sequences with some differences in tree topology. We note, however, that when we removed the shortest sequence from the alignment, *S. purpuratus cry1*, and reran the analysis, the structure of the NJ tree was identical to that of the ML tree with respect to the basal nodes (data not shown).

Our ML tree of cryptochrome/DNA photolyase genes (fig. 4) indicated that both insect *cry1* and *cry2* homologues existed at the base of the metazoan radiation, and at least 2 gene duplication events occurred leading to the evolution of the *cry2* cluster. The first gene duplication led to the insect *cry1* cluster and a second duplication led to the evolution of the vertebrate *cry* plus insect *cry2* cluster. The 6-4 photolyase and vertebrate *cry4* gene clusters evolved from a third duplication. Interestingly, the *cry*/photolyase gene family phylogeny is marked by gene losses in several lineages. Homologues of the vertebrate *cry4* gene cluster, for instance, have not been reported in insects even though this cluster arose before the radiation of metazoans. The homolog of insect *cry1* appears to have been lost, probably more than once, in the lineages leading to ray-finned fish, tetrapods, *Tribolium* and *Apis*. The 6-4 photolyase is missing from the *Tribolium* and *Apis* genomes. *cry2* in *Drosophila* was lost sometime after the split between that lineage and mosquitos, 223–240 MYA (Wiegmann et al. 2003). These results show that the 3 combinations of *cry* gene expression in insects (those expressing *cry1* and *cry2*, *cry1* only, and *cry 2* only) are due to differential gene loss of *cry1* and *cry2*.

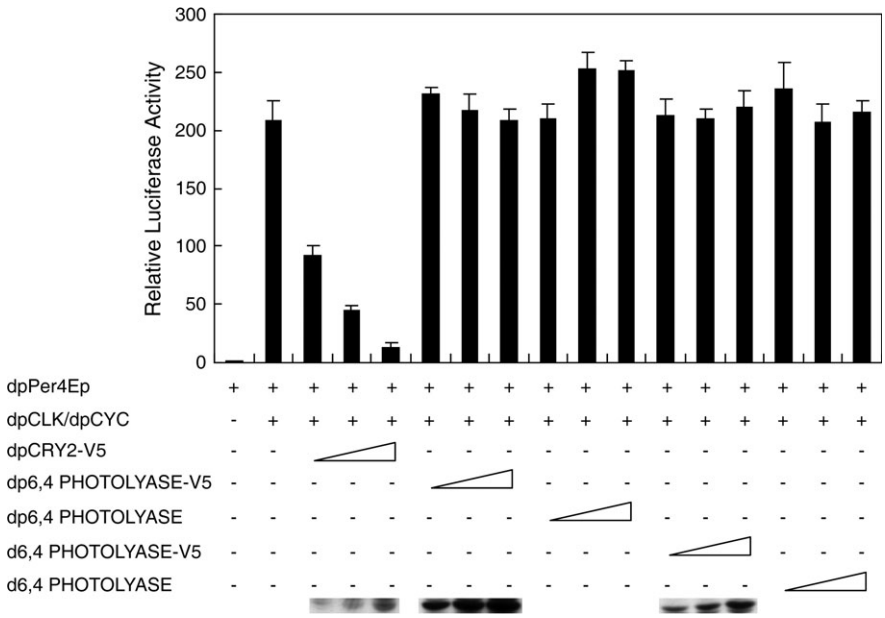

FIG. 3.—Neither *Drosophila* d6-4 photolyase nor *D. plexippus* dp6-4 photolyase repress CLK/CYC-mediated transcription. The monarch butterfly *per* E-box enhancer luciferase reporter (dpPer4Ep; 10 ng) was used in the presence (+) or absence (−) of dpCLK/dpCYC expression plasmids (5 ng each). dpCRY2-V5 (1, 2, and 10 ng), dp6-4 photolyase-V5/or untagged (1, 2, and 10 ng), or d6-4 photolyase-V5/or untagged (1, 2, and 10 ng) was used. Luciferase activity was computed relative to β-galactosidase activity. Each value is the mean ± SEM of 3 replicates. Western blot of V5epitope–tagged protein expression levels for each concentration is depicted below the graph.

CRY Repression of CLK:CYC-Mediated Transcription Is a Recent Evolutionary Innovation

We were interested in using this phylogeny to examine the functional evolution of DNA photolyase/cryptochrome gene family members' ability to repress CLK:CYC (BMAL1)-mediated transcription. We thus mapped this functional character onto the ML tree (fig. 4). The resultant character map revealed that all members of the vertebrate CRY plus insect CRY2 cluster possess this transcriptional repressor activity, with the exception of zCRY3 (Kobayashi et al. 2000). These results indicate that the ability to repress CLK:CYC (BMAL1)-mediated transcription, at least in the context of expression in cell culture and in the absence of PER, evolved from a photolyase-like ancestral gene that lacked this function (branch leading from Node A to Node B, fig. 4). These data provide a strong framework for further studies examining the functional evolution of transcriptional repressive activity by ancestral state reconstruction and expression of key nodes along the cryptochrome/photolyase gene tree.

Novel Insect Clock Models

The discovery of insect CRY2 and its potent transcriptional inhibitory function challenges our *Drosophila*-centric view of how circadian clocks work in different insects. *Drosophila*, which has the most well-studied circadian system in animals, lacks CRY2. Nonetheless, in the fly, CRY1 has more than 1 circadian function. The primary role of *Drosophila* CRY1 is to function as a blue-light photoreceptor that entrains the central clock in lateral neurons in brain (fig. 5, panel 1a) (Emery et al. 2000). But studies have also shown in peripheral tissues that *Drosophila* CRY1 functions both as a circadian photoreceptor and as a core clock component via a photoreceptor-independent mechanism (Krishnan et al. 2001; Vanchenko et al. 2001; Levine et al. 2002) (fig. 5, panel 1b). Overexpression of CRY1 and PER in fly eyes represses CLK:CYC-activated transcription, suggesting that CRY1 can actually function as a transcriptional repressor of the circadian oscillator in peripheral clocks (Collins et al. 2006). This transcriptional repressive function of *Drosophila* CRY1 is dependent on coexpression of PER and does not occur in the lateral neuron clocks driving locomotor behavior. Thus, in flies, based on CRY1 alone, at least 2 different circadian clock mechanisms occur.

A novel type of circadian clock that could exist in nondrosophilid insects, based on the existence of 2 *cry* genes, is an ancestral clock in which both CRY1 and CRY2 are expressed in clock cells of the same species, revealing clock mechanisms characteristic of both flies and mice. The circadian clock found in the monarch butterfly exemplifies this type of clock mechanism (fig. 5, panel 2). In the butterfly, CRY1 functions primarily as a circadian photoreceptor, whereas CRY2 appears to function as a major transcriptional repressor of the core clock feedback loop (Zhu H, Yuan Q, Casselman A, Sauman I, Emery P, Reppert SM, unpublished data). It is also possible that peripheral clocks exist in the butterfly in which only CRY1 is expressed, where the protein could function as a photoreceptor and/or perhaps as parts of a core clock mechanism (as in *Drosophila*), or in which only CRY2 is expressed so those clock cells would receive light input through CRY1-independent pathways (e.g., adult stemmata) (Briscoe and White 2005).

Perhaps the most curious types of insect clock mechanisms are ones in which only CRY2 is expressed, as in *Apis* and *Tribolium*, in which our functional data suggest

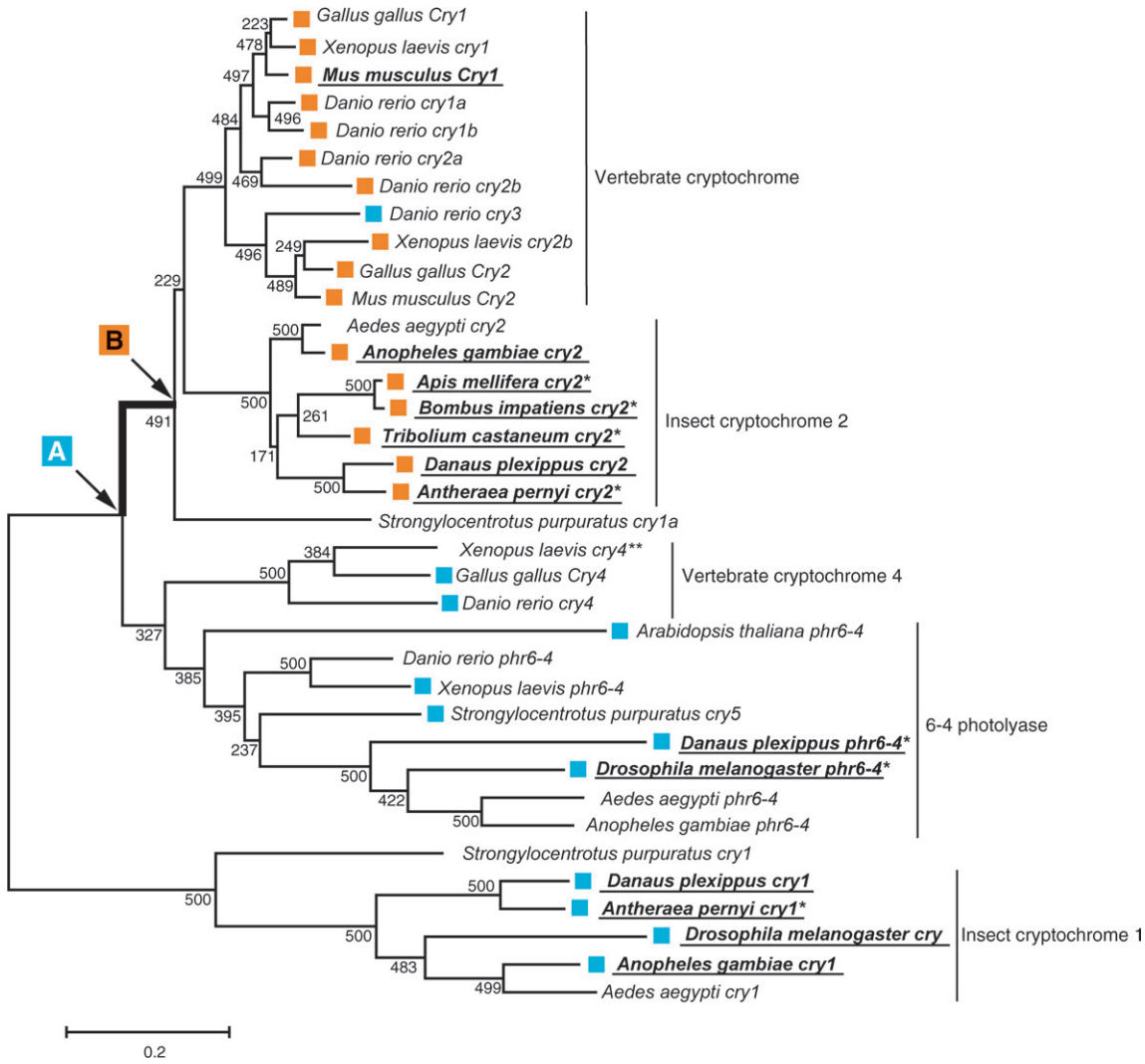

FIG. 4.—DNA photolyase/cryptochrome gene family tree. Tree shown is the result of a ML analysis using first + second nucleotide positions. Bootstrap values shown are based upon 500 ML bootstrap replicates determined using the GTR + Γ + I model with estimated gamma shape parameter = 0.889 and proportion of invariant sites = 0.1062. GenBank accession numbers for sequences are provided in supplementary table S1 (Supplementary Material online). Orange squares indicate the ability to repress CLK:CYC- or CLK:BMAL1-mediated transcription in cell culture. Blue squares indicate proteins, which have been shown to lack this ability in cell culture. The branch along which a repressor type (node B) cryptochrome evolved from a non-repressor 6-4 photolyase-like ancestral gene (node A) is indicated by a thick black line. The *xcry4* sequence shown was originally named *cry1* by Klein et al. (2002). We have renamed it here based upon its homology to other vertebrate cryptochrome 4 family members and previous use of the *cry1* name for a different *Xenopus* homolog.

that the protein could function as a core clock element; in further support of this contention in *Apis* is the finding that *cry2* mRNA levels are rhythmically expressed over the circadian cycle in bee heads (Rubin et al. 2006). In both the bee and beetle, the loss of CRY1 strongly suggests that other modes of light input are used to entrain the circadian clock (fig. 5, panels 3a and b). For *Tribolium*, light input might act through opsin-based retinal and extraretinal photoreceptors (Gilbert 1994) to synchronize the clock via CRY-independent TIM degradation, as occurs, in addition to the CRY1-dependent pathway, in *Drosophila* (Stanewsky et al. 1998; Helfrich-Forster et al. 2002).

The circadian light-sensing situation in *Apis* is even more interesting as analysis of the honeybee genome has not revealed a TIM homolog (fig. 5, panel 3b) (Rubin et al. 2006). This suggests a completely novel light input mechanism to the bee central clock. As bees possess both insect-like and vertebrate-like opsins (Velarde et al. 2005), whereas *Drosophila* possess only insect-like opsins, it is possible that bees rely exclusively on an opsin-based photoreceptor system (both retinal and extraretinal) to entrain their circadian clocks. Importantly, a novel putative light-sensitive organ was recently discovered in the third layer of the optic lamina of bumblebees, the "lamina organ" which expresses a UV and potentially other opsins (Spaethe and Briscoe 2005). The lamina organ may thus be a key extra-retinal light input channel to the bee circadian clock.

It is possible that insect CRY2 may have a light-sensing function not detected by our S2 assay system. And, the central clock function of CRY2 remains to be determined in vivo. *Tribolium*, in which knocking down genes with RNA interference can be readily accomplished and which has a

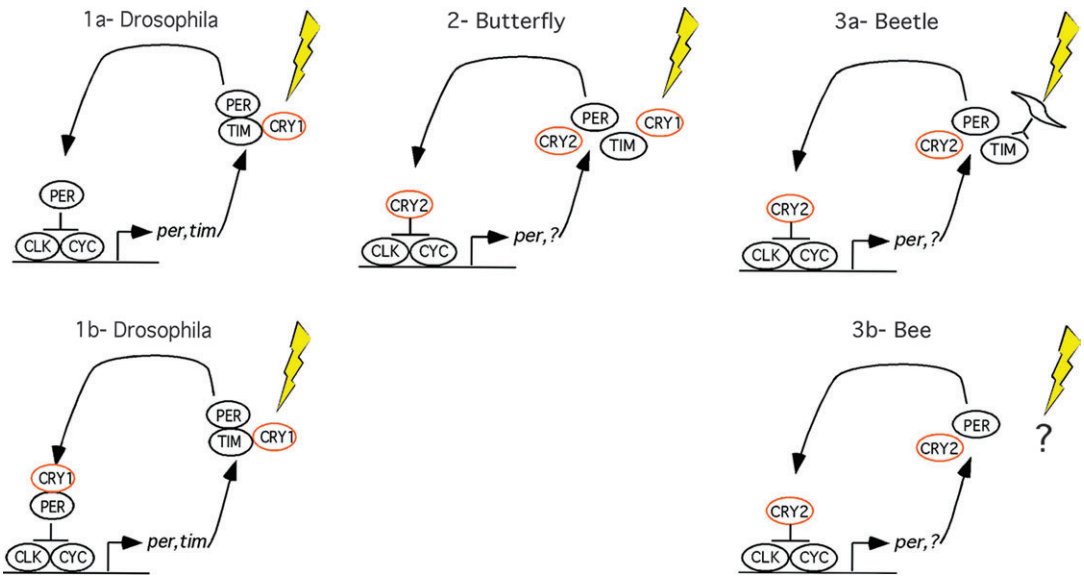

FIG. 5.—Insect clockwork models. With the existence of 2 functionally distinct CRYs in insects, 3 major types of clockwork models can be proposed. Type 1 (the *Drosophila* form) in which CRY1 only functions in the central brain clock as a circadian photoreceptor (panel 1a) or in peripheral clocks as both a photoreceptor and central clock component (panel 1b). Type 2 (the ancestral form apparent in the monarch butterfly) in which both CRY1 and CRY2 exist and function differentially within the clockwork. Type 3 in which only CRY2 exists and functions within the clockwork. In beetles, CRY2 acts as a transcriptional repressor of the clockwork and light input may be mediated through the degradation of TIM (panel 3a). In bees, which lack TIM, CRY2 acts as a transcriptional repressor and novel light input pathways (?) are used to entrain the clock (panel 3b).

genetically accessible genome (Wang et al. 2007), would seem to be the model organism for further clarifying in vivo a clockwork and/or photoreceptive function for insect CRY2.

Supplementary Material

Supplementary table S1 and figures 1 and 2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

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