Light-dependent interaction between *Drosophila* CRY and the clock protein PER mediated by the carboxy terminus of CRY

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Background: The biological clock synchronizes the organism with the environment, responding to changes in light and temperature. *Drosophila* CRYPTOCHROME (CRY), a putative circadian photoreceptor, has previously been reported to interact with the clock protein TIMELESS (TIM) in a light-dependent manner. Although TIM dimerizes with PERIOD (PER), no association between CRY and PER has previously been revealed, and aspects of the light dependence of the TIM/CRY interaction are still unclear.

Results: Behavioral analysis of double mutants of *per* and *cry* suggested a genetic interaction between the two loci. To investigate whether this was reflected in a physical interaction, we employed a yeast-two-hybrid system that revealed a dimerization between PER and CRY. This was further supported by a coimmunoprecipitation assay in tissue culture cells. We also show that the light-dependent nuclear interactions of PER and TIM with CRY require the C terminus of CRY and may involve a *trans*-acting repressor.

Conclusions: This study shows that, as in mammals, *Drosophila* CRY interacts with PER, and, as in plants, the C terminus of CRY is involved in mediating light responses. A model for the light dependence of CRY is discussed.

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Background

The ability to synchronize behavior and physiology to predictable cyclical events is of paramount adaptive importance. Rhythmicity is therefore a pervasive feature of life, and most, if not all, higher eukaryotes and some prokaryotes possess circadian 24 hr clocks, which can free-run under constant conditions and be reset or entrained by photic cues. Cryptochromes are pterin/flavin-containing proteins that are presumably derived from photolyases, which mediate circadian photoreception in plants and animals [1, 2]. The *Drosophila melanogaster cry* gene was identified through the cry^b mutation, corresponding to an Asp → Asn substitution at amino acid 410, a highly conserved position on the flavin binding site [3, 4]. Behavioral experiments [4] indicate that CRY function is not fully compromised in these mutants, which have normal 24 hr rhythmicity in constant darkness (DD), entrain normally to light-dark conditions (LD, 12 hr light; 12 hr dark), and also reentrain to a second light regime. However, cry^b mutants do not phase shift in response to 10 min light pulses, and the visually blind double mutants $norpA^{P41}$;cry^b show severely compromised light entrainment, even though, individually, each mutant can entrain to LD cycles [4]. It has been suggested that, in addition to being a photoreceptive molecule, *Drosophila* CRY might be part of the pacemaker mechanism, at least in some tissues [5]. In mammals, the protein products of the two cryptochrome genes, *Cry1* and *Cry2*, are part of the central clock and interact with the major clock components [6, 7, 8]. Therefore, an interaction between CRY and core clock components could also be expected in *Drosophila*. Indeed, a previous report [9] has described CRY/TIM dimerization but has failed to identify an interaction between CRY and PER.

This study analyzes the circadian behavior of double mutant per^S;cry^b flies, revealing a temperature-dependent genetic interaction between the two loci. Furthermore, we provide evidence for the physical association between PER and CRY. This interaction is light dependent in certain conditions and light independent in others, suggesting a possible mode of action for *Drosophila* CRY.

Results and discussion

Temperature-dependent genetic interaction between per^S and cry^b

Stanewsky and coworkers [4] showed that at 25°C, per^S;cry^b flies displayed predominantly 24 hr cycles in a LD 12:12 regime, although ~40% of the flies also have a minor 19 hr per^S component. These two periodic components are not found together in either single mutant.

We monitored locomotor activity for per^S, cry^b, and the per^S;cry^b double mutants at 18°C and 28°C (Table 1). Single

Table 1

Entrained and free-running locomotor rhythmicities for *cry*^{b}, *per*^{S}, and *per*^{S};*cry*^{b} flies.

	18°C				28°C			
Genotype	Period LD (n)	N	Period DD (n)	N	Period LD (n)	N	Period DD (n)	N
cry^b	24.2 ± 0.1 (32)	36	24.3 ± 0.1 (29)	34	23.8 ± 0.1 (33)	33	23.7 ± 0.2 (24)	27
per^S	23.9 ± 0.1 (19)	19	19.8 ± 0.1 (15)	17	23.9 ± 0.1 (18)	18	18.6 ± 0.2 (15)	16
per^S;*cry*^{b}	24.6 ± 0.1 (32)	32	19.3 ± 0.1 (32)	33	24.4 ± 0.6 (5)[b]	31	18.2 ± 0.1 (26)	34
	18.8 ± 0.1 (19)[a]	32			18.3 ± 0.2 (23)	31		

The period of locomotor activity was determined by autocorrelation and Fourier (spectral) analysis, and the results were compared [26]. The period length given by spectral analysis is presented.
N = total number of flies examined.
(n) = number of rhythmic flies.
[a] All flies also showed a rhythm of ~24 hr.
[b] Four flies also showed a rhythm of ~18 hr; one fly showed only the ~24 hr period.

mutant *per*^{S} or *cry*^{b} flies entrain to the LD 12:12 regime at both temperatures, showing a 24 hr period and distribution of activity around the times of light/dark transitions (Figure 1). In DD, they free-run with a period of about 24 hr for *cry*^{b} and a period of about 19 hr for *per*^{S}, with a modest temperature dependence [10, 11]. In DD conditions, *per*^{S};*cry*^{b} flies behave virtually identically to *per*^{S} mutants at both temperatures. However, the behavior of the double mutant changes dramatically in LD, in a temperature-dependent manner. At 18°C, all *per*^{S};*cry*^{b} flies show a periodic component of about 24 hr, but about 60% of them also display a minor 19 hr component, in agreement with previous results [4]. At 28°C, 79% of the rhythmic flies display the endogenous 19 hr period as the main rhythmic component (Figure 2). The breakdown of entrainment at 28°C in double mutant flies could reflect a genuine genetic interaction between the *cry*^{b} and *per*^{S} mutations. Alternatively, perhaps the limits of entrainment at high temperature are reduced in *cry*^{b} mutants so that *per*^{S};*cry*^{b} flies might indeed entrain to a T cycle of 20 hr at this temperature (which is closer to the 19 hr endogenous period of *per*^{S}), whereas *cry*^{b} individuals (whose endogenous period is ~24 hr) might not. To test this hypothesis, we monitored the locomotor activity rhythms of single and double mutant flies at 28°C under an LD 10:10 regime. Both *per*^{S} and *cry*^{b} flies entrain under this condition. However, the double mutants may show some evidence of entrainment during the first two cycles of the new light/dark regime, but any entrainment soon breaks down, and the *per*^{S};*cry*^{b} flies free-run, with their daytime activity advancing by about 90 min on each successive day (Figure 3). Therefore, the entrainment defect

Figure 1

Activity profiles of *cry*^{b} and *per*^{S} mutants in LD 12:12. Average locomotor activity profiles (see Table 1 for the number of flies averaged for each genotype) for each mutant are double plotted (day 1–day 2; day 2–day 3, etc.) for 5 successive days in LD 12:12 cycles at 18°C and 28°C. The mutants show entrainment in both temperature conditions.

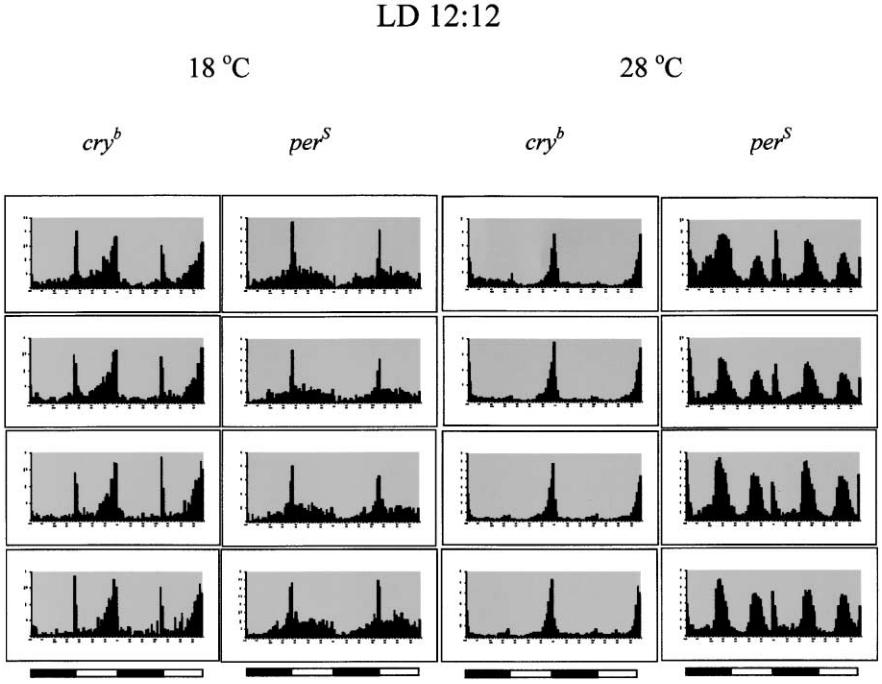
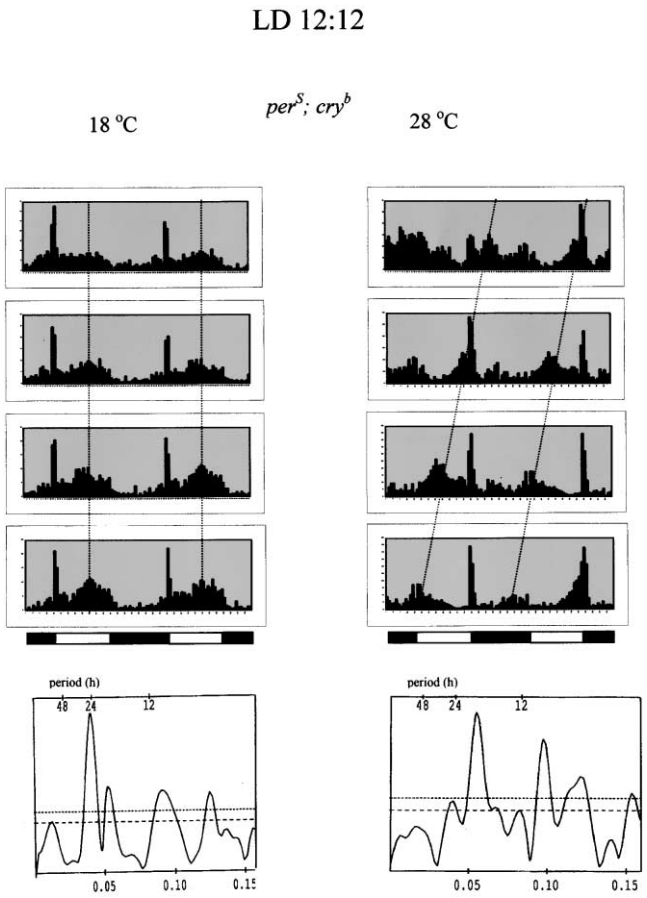


Figure 2



Activity profiles of $per^S;cry^b$ double mutants in LD 12:12. Average locomotor activity profiles (see Table 1 for the number of flies averaged for each genotype) for each group of double mutants is shown double plotted for 5 successive days in LD 12:12 cycles. The dark/light transitions correspond with the startle responses at 18°C, but this shifts to the light/dark transitions at 28°C. This phenomenon is also observed in the wild-type and single mutants. At 18°C, entrained cycles with an ~24 hr period are observed (dashed vertical lines). At 28°C, a free-running locomotor activity cycle with an ~19 hr period is superimposed upon the startle response. Typical spectral analysis for individual flies at each temperature can be seen in the lower panel. The horizontal dashed and dotted lines represent the 95% and 99% confidence limits, respectively, as determined by Monte-Carlo simulations [26].

at high temperature shown by $per^S;cry^b$ flies is the product of a specific interaction between the two mutations rather than a defect in the entrainment of cry^b alone. In *Drosophila*, the visual system is involved in the reception of circadian-relevant light information [4]. This system is perfectly functional in the double mutant [4] and is revealed by the startle response that is evident at the transition points from dark to light (and vice versa) at both temperatures (see Figures 1 and 2). Therefore, $per^S;cry^b$ flies are able to detect light but are deficient in the transmission of light information to the clock mechanism in a temperature-dependent manner.

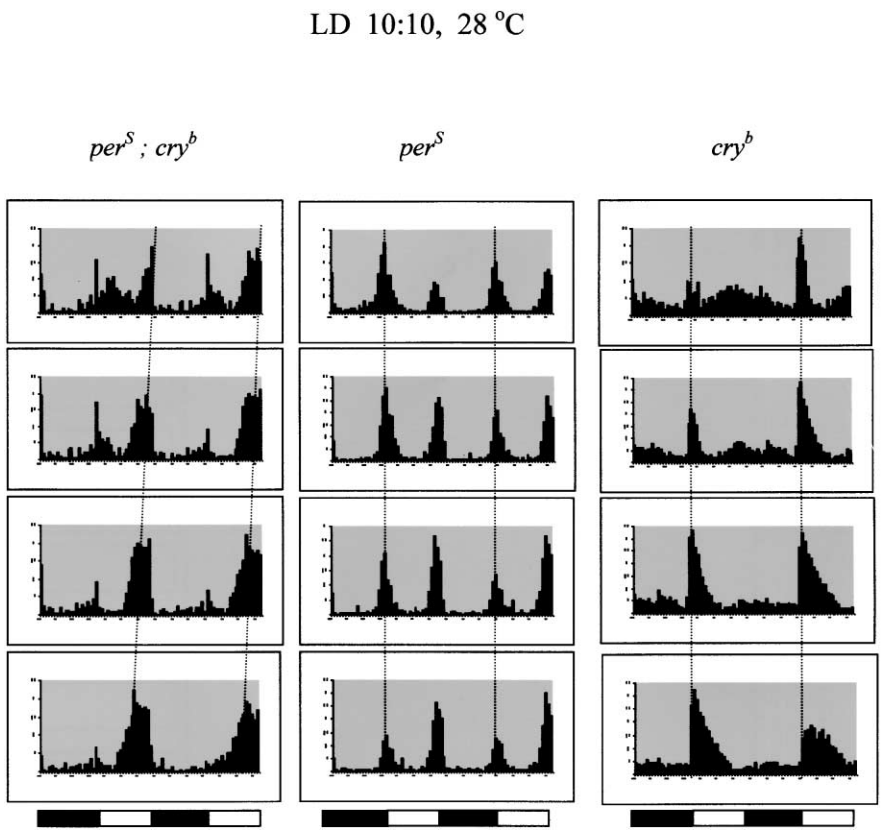
Light-dependent physical association between CRY and PER in a yeast-two-hybrid system

The genetic interaction between per^S and cry^b prompted us to investigate the possibility of a physical interaction between PER and CRY using a yeast-two-hybrid system [12]. A full-length CRY protein, directly fused to LexA (bait), was challenged with PER(233–685) as prey. This fragment includes the major protein/protein interaction domains described for PER. We also tested a fragment of TIM(377–915) that is known to bind to PER [13] and contains the relevant regions for PER/TIM dimerization as prey [14]. No interactions were observed between LexA-CRY and both PER(233–685) and TIM(377–915) fragments in the dark (Figure 4a). It has been reported that CRY interacts with full-length TIM, but not PER, under constant light [9]. We found that in light, LexA-CRY binds strongly to PER(233–685), but not to TIM(377–915) (Figure 4a). We also challenged LexA-CRY with full-length PER and TIM, both in darkness and light. No interactions were observed in the dark. Under constant light, only full-length TIM showed evidence of dimerization with LexA-CRY (Figure 4a), in agreement with the report of Ceriani et al. [9]. We draw three conclusions from these results: PER and TIM interactions with LexA-CRY are light dependent; the N and/or the C terminus of TIM are required for the association with LexA-CRY, and there is an inconsistency between the results obtained from full-length PER and the fragment PER(233–685). In regard to the latter, we retested the well-established PER/TIM interaction using LexA-TIM bait with PER and PER(233–685) preys in darkness and light. No interactions were observed using full-length PER (Figure 4b). Subsequent Western blot analysis revealed that, in our system, full-length PER is poorly expressed, thereby explaining the lack of interactions in yeast with this construct (Figure 4c). Nevertheless, unlike Ceriani et al. [9], we were able to show a strong interaction between LexA-CRY and PER(233–685). This discrepancy between our results must reside in the different yeast-two-hybrid systems employed, plus the fact that we utilized a direct fusion to connect CRY to the LexA moiety, whereas Ceriani et al. used a linker.

Light-independent physical association between CRY and PER in S2 cells

Having showed that CRY and PER can interact in a heterologous system and in the absence of any other *Drosophila* protein, we looked for evidence of whether they can be found in the same protein complex in a more functional context. We therefore employed a coimmunoprecipitation (coIP) assay in S2 cells, a *Drosophila* system in which, unlike in the fly itself, PER is stable in the absence of TIM [14]. This is essential since TIM interacts with both PER and CRY ([9, 14], this report), and complexes other than PER/CRY would mask the coIP result. A stable S2 cell line expressing full-length PER under the *Drosophila actin 5C* promoter was constructed, and cells were trans-

Figure 3



Activity profiles of per^S, cry^b, and double mutant flies in LD 10:10. Average locomotor activity profiles of 18 flies for each genotype are shown double plotted for 5 successive cycles in LD 10:10 at 28°C. per^S and cry^b mutants show entrained cycles with an ~20 hr period (dashed vertical lines). Double mutant per^S;cry^b flies show some evidence of entrainment only during the first two LD 10:10 cycles; afterwards, the entrainment breaks down, and a shorter ~18 hr free-running period is evident (dashed vertical lines).

fected with a construct constitutively expressing full-length CRY with an N-terminal hemagglutinin tag (*ha-cry*). Transfected cells were kept in the dark and analyzed 48 hr later. Immunoprecipitation with anti-HA antibodies detected both HA-CRY and PER proteins, revealing the existence of a TIM-independent, PER/CRY complex (Figure 5). Because of the light labile nature of CRY in S2 cells [9], we did not attempt the coIP experiment in constant light.

Role of the C terminus of CRY in light dependence

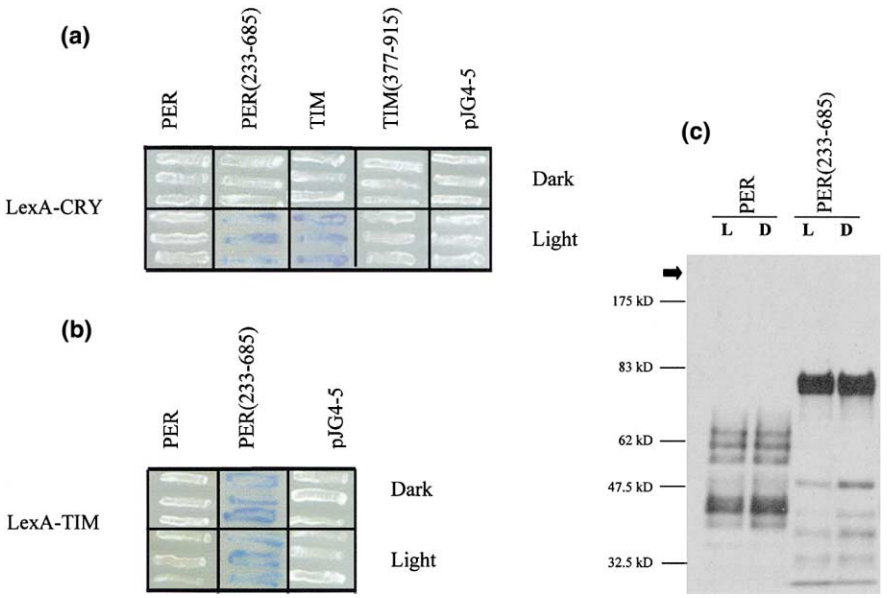
Cryptochromes are believed to interact with a signaling factor after light exposure [1, 15, 16], and evidence of a role of the C-terminal domain in signaling has recently been reported in plants [16]. Since our coimmunoprecipitation result supports the view that the interaction between LexA-CRY and PER(233–685) in yeast reflects a meaningful association between PER and CRY, we exploited the power of yeast genetics to test the regulatory role of the C terminus of *Drosophila* CRY.

We deleted 20 residues from the CRY C terminus to create CRYΔ and challenged it with PER(233–685) and full- length TIM in darkness and light. An interaction was evident in both conditions, with no obvious difference between them (Figure 6a). It has been suggested that LexA-CRY^b is unable to interact with TIM in yeast cells because it may have lost its photoresponsiveness [9]. We observed that both LexA-CRY^b and LexA-CRY^bΔ, which are strongly expressed in yeast ([9], data not shown), are nevertheless unable to interact with PER(233–685) or with TIM (Figure 6b). Given the light independence of CRYΔ, we suggest that the D[410]N substitution in CRY^b probably confers a gross structural defect to LexA-CRY^b, rather than simply affecting its photoreceptor ability.

CRY interacts with the PER C Domain

To further map the interaction between CRY and PER, we then challenged LexA-CRYΔ with several overlapping PER fragments. We confirmed that LexA-TIM (377–915) interacts with the PAS A domain (PER[233–390]) and PER(233–685) ([17], data not shown). We observed that LexA-CRYΔ does not associate with PER(233–390), nor with the PAS A + B region (PER[233–485]), but interacts with the downstream C domain (PER[524–685] [17], which includes the per^S site (Figure 6a). From these results, we speculate that TIM and CRY may interact with different regions of the PER protein and, since CRY associates with region(s) of TIM external to the (377–915) fragment, we hypothesize that PER, TIM, and CRY can be found in the same complex.

Figure 4



LexA-CRY interactions are light dependent. Triplicate yeast patches from independent transformants were grown on plates containing the β-galactosidase substrate X-gal. A blue precipitate represents cumulative β-galactosidase activity produced by activation of the *lacZ* reporter by protein/protein interactions. Baits (rows) and prey (columns) were challenged at 30°C. Assays with pJG4–5 represent the negative control. **(a)** LexA-CRY interacts with PER(233–685) and full-length TIM only under constant light conditions. No interactions were ever observed with either full-length PER or TIM(377–915). **(b)** LexA-TIM interacts with PER(233–685), but fails to do so with full-length PER both in light and dark**. (c)** Full-length PER and PER(233–685) prey fusions were probed with anti-HA antibodies in cells also expressing LexA-CRY. Western blot analysis shows that PER(233–685) is expressed at high levels, whereas the full-length PER fusion (expected at the level of the arrow) cannot be revealed. The same results were obtained under light and dark conditions and also in LexA-TIM-expressing cells. The lower molecular weight bands represent truncation or (most likely) degradation products.

Temperature increase diminishes CRY interactions in yeast

LexA-CRY requires light in order to interact with PER(233–685) and TIM. However, it cannot be ruled out a priori that it is the temperature increase, caused by the continuous light exposure, rather than light per se, that triggers CRY's interactions. LexA-CRY was therefore challenged with PER(233–685) and TIM at 37°C in the dark, but no interactions were observed (data not shown). Furthermore, since LexA-CRYΔ does not require light, we used this variant to investigate the effect of temperature on CRY interactions, adopting an established approach [13, 17].

Yeast patches were grown on X-gal plates at 30°C and 37°C in parallel. We noted that at 37°C, the LexA-CRYΔ interaction with PER(233–685) is considerably weakened (Figure 7a), whereas the control LexA-TIM(377–915)/PER(233–685) dimerization did not show any substantial temperature differences (see legend for Figure 7 and [13]). Liquid assays confirm these results (Figure 7b). The same temperature dependence is also observed when LexA-CRYΔ is challenged with TIM and PER (524–685) (data not shown). This loss of activity is not due to reduced expression of the CRYΔ fusion at higher temperature, since immunoblot analysis of LexA-CRYΔ does not reveal any detectable difference in the abundance of the protein at the two different temperatures (Figure 7c). Similar reductions in the strength of the interactions at high temperature were obtained for LexA-CRY under constant light (data not shown).

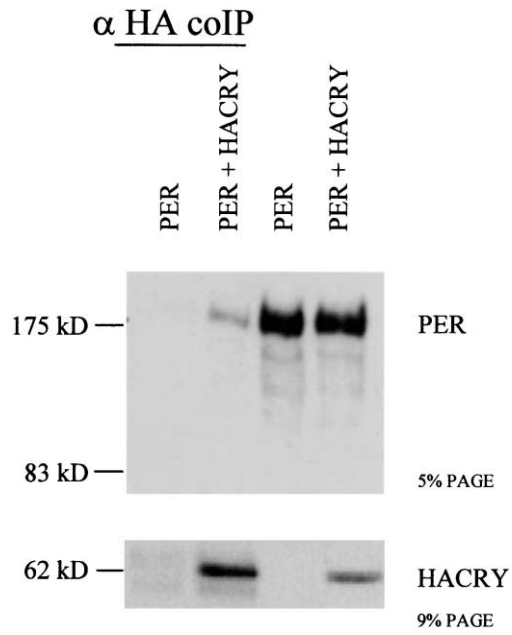
In vitro mutagenesis of *cry*

To further identify those regions of CRY that could suppress the negative effect of darkness, random *Taq*-induced mutations were introduced into full-length *cry* by PCR, and LexA-CRY* mutants were created by in vivo gap repair [18]. The putative LexA-CRYs* were challenged with PER(233–685) in the dark. A total of 14 bona fide light-independent mutations were identified that generated a CRY/PER interaction in darkness. The sequencing of these variants shows that all of these light-independent CRYs* carry either a translational stop or a frame-shift at their C termini (Table 2). Some of the mutants have additional amino acid substitutions scattered across the entire sequence, but because of their sporadic nature, it is very unlikely that these missense mutations are contributing to the light-independent phenotype.

A model for CRY action

The results reported above support the view that the C terminus of CRY is responsible for the light dependence of the interactions with PER and TIM. Perhaps the removal of the C terminus changes CRY conformation to a form that is active in darkness. Alternatively, there could be a carboxy-terminal-bound, light-inhibited nuclear repressor of CRY in yeast (Figure 8). To investigate these possibilities, we mutagenized 5×10^5 yeast cells (carrying

Figure 5



CRY binds to PER in the dark in the cytoplasm of S2 cells. S2 cells expressing PER and HA-CRY (full-length CRY carrying the hemagglutin epitope at its N terminus) were immunoprecitated with anti-HA antibodies (Boheringher) and Protein G-agarose (Sigma). The immunoprecipitates were loaded onto 5% and 9% SDS-polyacrylamide gels and blotted. Filters were probed with a rabbit anti-serum to PER [4] and anti-HA antibodies, respectively, for the 5% and 9% gels. Cells expressing PER, but not HA-CRY, were processed in parallel and used as negative controls. Part of each sample was not subjected to immunoprecipitation and was used as a positive control. A representative blot is shown.

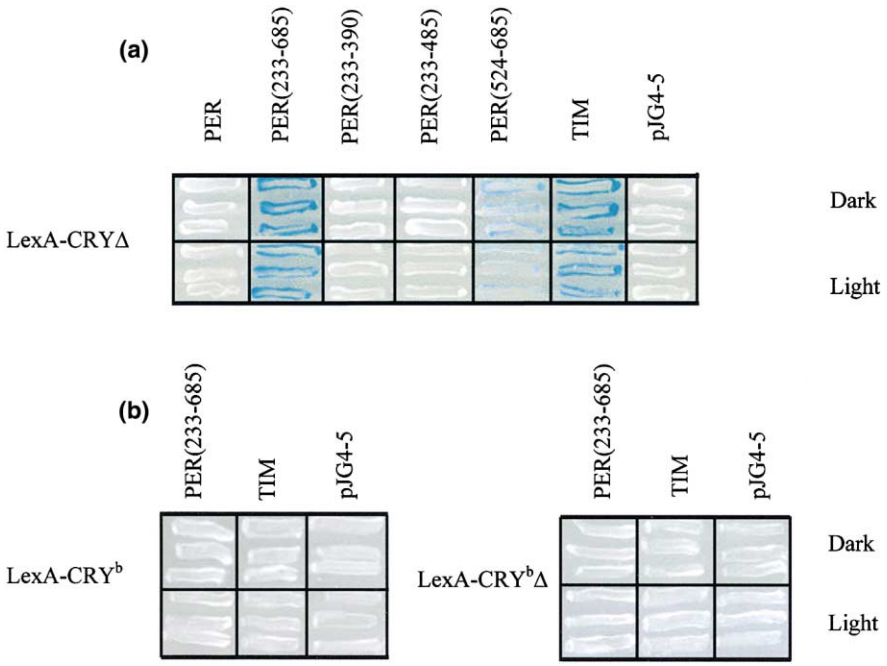
bait, prey, and reporter plasmids) with EMS and tested their ability to support a LexA-CRY/PER(233–685) interaction in the dark. "Blue" colonies (24) were initially isolated. To distinguish between mutations affecting plasmid sequences rather than genomic loci, we replaced the plasmids with nonmutagenized bait, prey, and reporter plasmids and identified two yeast mutants in which the interaction between LexA-CRY and PER(233–685) is light independent and occurs both under dark and light conditions. Thus a *trans*-acting factor in yeast can be mutated to disinhibit nuclear CRY activity in darkness, and we are currently attempting to identify the gene(s) involved.

Conclusions

We have demonstrated that CRY binds PER in yeast and in a *Drosophila* cell culture system. As in yeast, the light-dependent activities of CRY in S2 cells have been reported only in the nucleus, where CRY is suggested to undergo a conformational change after light absorption, allowing it to bind to TIM (and now PER, Figure 8) [9]. However, CRY coimmunoprecipitates with TIM and PER in the cytoplasm of S2 cells under darkness, suggesting that light is not required to change CRY into its active conformation [9]. Consequently, both in yeast and S2 cells, we predict that a nuclear factor may interact with the CRY C terminus in darkness to prevent it from interacting with the two clock proteins (Figure 8). CRY itself is probably not its own repressor, because we tested full-length CRY in a yeast-two-hybrid assay and found that it does not significantly self-associate in light or dark (E.R. and V.C., unpublished data). However, mutagenesis of the yeast genome has identified two variants that can derepress the CRY/PER interaction in darkness. Isolation of this gene(s), irrespective of its function in yeast, will provide candidates for this nuclear repressor(s), which, we predict, will have a clock relevant homolog(s) in *Drosophila*. An analogous situation, in which *Saccharomyces cerevisiae* casein kinase I, HRR25 (without known clock function in yeast) binds and phosphorylates PER with affinities similar to the *Drosophila* casein kinase Iε, DOUBLETIME (DBT) [19], has been recently reported in the literature. The signaling mechanism of cryptochrome is also mediated through the C terminus in *Arabidopsis*. A fusion between β-glucuronidase (GUS) and the C-terminal domain (CCT) of either CRY1 or CRY2 (to create CCT1 and CCT2) mediates a constitutive light response [16]. This means that "isolated" CCTs display properties in the dark that are strikingly similar to those of light-activated CRYs. Within the CRY molecule, the C-terminal domain is repressed under darkness, and light activation might be achieved either by an intramolecular (Figure 8a) or an intermolecular (Figure 8b) redox reaction, but the details of the light-induced activation of CCT are not known [16]. In this study, we show that the intermolecular model is the more appropriate to explain our observations with *Drosophila* CRY. Light-induced activation of CRY removes a regulatory molecule (hatched in Figure 8b), enabling the binding of PER and TIM, although the possibility exists that the regulatory molecule itself, rather than CRY, could act as the primary photopigment. It will be of interest to see if this model also applies to *Arapidopsis* cryptochromes. The C-terminal domain of CRY thus becomes a focal point for further studies, and it is probably not a coincidence that it is this region of the otherwise evolutionary conserved CRY molecule that is the most variable [1].

We cannot unequivocally conclude that the physical interaction revealed between PER and CRY is responsible for the genetic interaction that we have shown in per^{S};cry^{b} mutants at high temperature, even though this was the experiment that led us to test a possible PER/CRY dimerization. However, we have also observed that the PER/CRY interaction is temperature-sensitive in yeast, and it is the PER C domain (which includes the site of per^{S}) that dimerizes with CRY, providing further circumstantial evidence that the genetic interaction between PER and

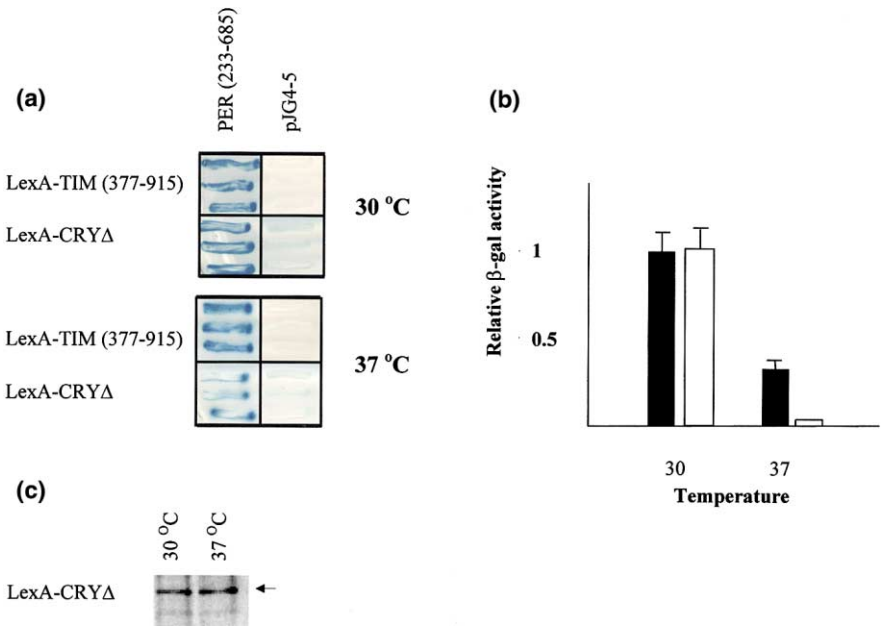
Figure 6



LexA-CRYΔ interacts in a light-independent manner. **(a)** LexA-CRYΔ interacts with PER(233–685) and PER(524–685), but not with PER(233–390) or PER(233–485), both under light and dark conditions. LexA-CRY binds to the same PER fragments, but in light only (data not shown). LexA-CRYΔ also binds to TIM. **(b)** LexA-CRY^b and CRY^{b}Δ are unable to interact with either PER(233–685) or TIM under both light or dark conditions.

CRY may correlate with the physical interaction. Furthermore, it is tempting to speculate that differences in the PER^S/CRY physical interaction may be at the heart of reports that the *per*^{S} mutants are hypersensitive to light [11, 20, 21] and that flies carrying a small deletion (amino acids 515–568) within the PER C domain display short,

Figure 7



Temperature plays a negative role in CRY interactions. **(a)** Yeast patches were grown in parallel on X-gal medium at 30°C and 37°C. Readings were taken at the same time for the two temperatures. This procedure overestimates the interactions occurring at 37°C, since β-galactosidase activity increases with temperature. This assay does not reveal any difference in the interactions between LexA-TIM(377–915) and PER(233–685), whereas the LexA-CRYΔ versus PER(233–685) interaction is greatly reduced at 37°C. **(b)** β-galactosidase activity from yeast liquid cultures expressing PER(233–685) and LexA-TIM(377–915) (solid bars) or LexA-CRYΔ (open bars) grown at 30°C and 37°C. The mean ± SEM of at least six cultures from at least six independent transformants is shown. For LexA-CRYΔ versus PER(233–685) at 37°C, the standard error is too small to be shown on this scale. Consistent with the plate assay, the LexA-CRYΔ interaction decreases dramatically at 37°C, much more than LexA-TIM(377–915). β-galactosidase activities were measured at 30°C and normalized to that of LexA-TIM(377–915) versus PER(233–685) grown at 30°C. **(c)** A protein immunoblot with anti-LexA serum showing that LexA-CRYΔ is present at the same level in cells, also containing PER(233–685), grown at 30°C and 37°C. An arrow marks the expected size of LexA-CRYΔ, the smaller band likely represents a truncation or degradation product (see [9]).

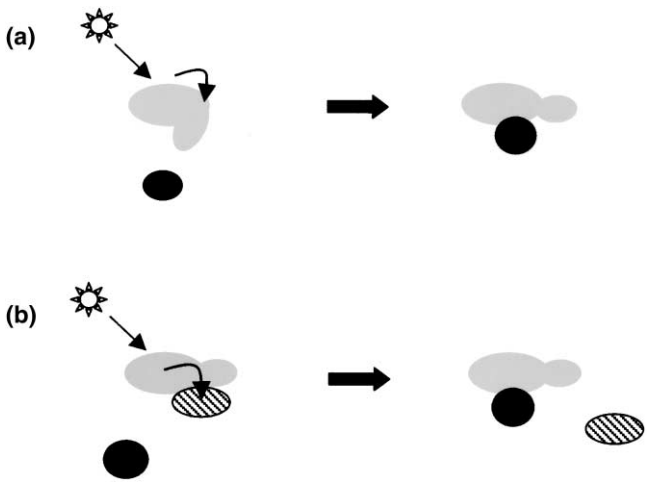
Table 2

Random PCR-induced, light-independent mutations.

C-terminal mutation	Other mutation(s)
V[489]FRAME-SHIFT + STOP	N[253]T
P[519]FRAME-SHIFT	V[297]M
P[520]FRAME-SHIFT	–
P[520]FRAME-SHIFT	T[149]A
P[520]FRAME-SHIFT	R[390]H
C[523]STOP	–
R[524]STOP	–
R[524]STOP	–
R[524]STOP	–
E[529]STOP	–
E[530]STOP	E[200]A, T[303]P
Q[533]STOP	T[438]A, N[514]S
Q[533]STOP	K[445]R, I[167]N
Q[533]STOP	–

poorly temperature-compensated rhythms and an altered behavioral response to light pulses [22]. Perhaps a reduction in the strength of the PER^S/CRY association, further decreased at 28°C below a critical threshold (see Figure 7), might account for the entrainment defect of $per^S;cry^b$ double mutants at high temperature. Finally, genetic interactions between short-period mutations per^S and per^T and the arrhythmic mutation dbt^{ar} implicate the C domain in the dynamics of PER phosphorylation by DBT [23]. Taken together, these results suggest that the PER C domain may provide a convergence point for both CRY and DBT, and we anticipate that future research may disclose a prominent role for CRY in the fly circadian clock.

Figure 8



Models of CRY function. **(a)** In darkness, the C terminus of CRY (gray) blocks access to a binding partner (black). After light absorption, an intramolecular redox reaction relaxes CRY conformation, exposing CRY binding sites. **(b)** In darkness, a nuclear repressor (hatched) binds CRY at the C terminus and other sites, excluding the binding partner. After light absorption, an intermolecular redox reaction releases the repressor, allowing the binding partner to interact with CRY (and perhaps the repressor to become available for further interactions).

Materials and methods

Yeast-two-hybrid assays

The CRY, PER, and TIM fragments are in direct amino-terminal fusion to the LexA and/or "Acid blob" moieties of the bait (pEG202) and prey (pJG4–5) vectors, respectively [12]. CRYΔ, which was generated by deleting residues 521– 540, and full-length CRY, PER, and TIM were generated using a PCR-based strategy and sequenced to control for unwanted mutations. PER(233–685), PER(233–390), PER(233–485), PER(524–685) [17], and TIM(377–915) [13] have been reported previously. Yeast-two-hybrid assays were performed as described in [12] at 30°C or 37°C and were based on the expression of the *lacZ* reporter. Light and dark growth experiments were performed as in [9]. All interactions were assayed at least in triplicate and repeated at least twice with new yeast transformants. LexA-CRYΔ activates some transcription of the *lacZ* reporter, but not at sufficient levels to cloud the interpretation of the results.

Yeast immunoblot analyses

Yeast cells from light- or dark-maintained plates were grown overnight at 30°C or 37°C in liquid selection medium in the appropriate light or dark condition. Culture (500 μl) was then used to inoculate 5 ml of fresh medium, and the new cultures were grown (in light or dark) to OD_{600} = 0.5 at 30°C or 37°C. Samples (1.5 ml) of each culture were spun down and resuspended in 50 μl (30 μl for detection of LexA fusions) of 2× Laemli buffer. Samples (15 μl) were run on either an 8% or 12% SDS-PAGE and blotted onto nitrocellulose. The same samples were also run on an identical gel in parallel and stained with Coomassie to verify that the same amount of total protein was loaded in each lane. At least two independent transformants were assayed for each experiment, which was repeated at least once. Immunodetection against prey fusions were performed with monoclonal anti-HA antibodies (Boehringer, 1:400) and anti-mouse IgG-HRP (Sigma, 1:8000). LexA fusions were detected with goat anti-LexA antiserum (Santa Cruz, 1: 200) and rabbit anti-goat IgG-HRP (Sigma, 1: 40,000). Signals were obtained by enhanced chemiluminescence.

Cell culture and coIP assays

S2 cells were purchased from Invitrogen. A stable S2 cell line expressing full-length *per* under control of the *Drosophila actin 5C* promoter was generated essentially following an Invitrogen protocol but using Effectene (Qiagen) as the transfection reagent; pAct-*per* was a gift from F. Ceriani and S. Kay. For each immunoprecipitation experiment, we transiently transfected ~10^7 cells (of the stable PER-expressing line) with 2 μg pAct-*ha-cry* (generated by PCR and fully sequenced) using Effectene. The same number of PER-expressing cells, but without further transfection, were used as negative controls and processed in parallel. Cells were maintained in the dark for 48 hr before being harvested and extracted under red safe light. The protein extracts were compared using the Bradford reagent (Sigma) and equalized. The coIP assay was performed as in [24], but using 0.1% Triton X-100 in the lysis buffer and agarose-Protein G from Sigma rather than Gammabind (Pharmacia). Protein samples were split into two aliquots and loaded into 5% (detection of PER) and 9% (detection of HA-CRY) polyacrylamide gels and blotted onto nitrocellulose. The coIP experiment was repeated three times. Immunodetection against HA-CRY was performed with monoclonal anti-HA antibodies (Boehringer, 1:400) and anti-mouse IgG-HRP (Sigma, 1:8000). PER was detected with a 1:10000 dilution of rabbit antiserum [4] and goat anti-rabbit IgG-HRP (Sigma, 1: 6000). Signals were obtained by enhanced chemiluminescence.

PCR mutagenesis and in vivo *gap repair*

For each experiment, about 10^8 yeast cells carrying a prey PER(233–685) and a *lacZ* reporter [12] were transformed with 100 ng of a gapped bait plasmid and 2 μg of a PCR product (containing random Taq-induced

mutations) corresponding to *cry* flanked by 69 and 198 nt of the bait 5′ and 3′ end sequences. Tranformant colonies obtained on glucose selection medium were replica-plated on galactose and X-gal selection medium and tested in the dark for blue color. As a result of two independent experiments, ~50 colonies carrying a mutagenized CRY* sequence were identified. The bait plasmid was isolated from each of them and amplified in *E. coli* before retransforming into two sets of yeast cells, one containing both the reporter and the prey plasmids, the other containing only the reporter. This allowed us to discard mutations that did not give consistent results or self-activate the reporter. A total of 14 mutations were finally sequenced.

Yeast mutagenesis

Yeast mutagenesis was conducted as in [25], with modifications. Yeast (EGY48 strain) cells (2×10^8) containing LexA-CRY [pEG202 bait], PER(233–685) [pJG4–5 prey], and reporter plasmids [pSH18–34] [12] were mutagenized with 20 μl EMS in a total volume of 1 ml. Samples (250 μl) were collected after 30, 60, 180, and 240 min and tested for survival. Cells (5×10^5) from the 60 min sample (which gave an ~60% survival rate) were plated on glucose selection medium. Colonies were then replica-plated on galactose and X-gal selection medium and tested in the dark for blue color. Blue colonies were plated again on glucose selection medium and then retested on galactose X-gal selection medium to reduce the number of false-positives. Clones (24) were thus isolated. Each clone was grown in rich liquid medium to stationary phase for several cycles until all plasmids were lost (checked by replica plating on different selection media). The putative mutants were transformed again with new bait, prey, and reporter plasmids and tested twice for their ability to sustain a LexA-CRY/PER(233–685) interaction both in dark and light. Two mutants were finally isolated.

Behavioral analyses

Behavioral analyses were conducted as in [26].

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Note added in proof

Krishnana et al. [*Nature* **411:**313-317] have recently suggested that CRY is part of the pacemaker mechanism in peripheral organs of the fly.