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Spatial and circadian regulation of *cry* in *Drosophila*

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Abstract

In *Drosophila*, *cryptochrome* (*cry*) encodes a blue light photoreceptor that mediates light input to circadian oscillators and sustains oscillator function in peripheral tissues. The levels of *cry* mRNA cycle with a peak at ~ZT5, which is similar to the phase of *Clock* (*Clk*) mRNA cycling in *Drosophila*. To understand how *cry* spatial and circadian expression is regulated, a series of *cry*-Gal4 transgenes containing different portions of *cry* upstream and intron 1 sequences were tested for spatial and circadian expression. In fly heads, *cry* upstream sequences drive constitutive expression in brain oscillator neurons, a novel group of non-oscillator cells in the optic lobe, and peripheral oscillator cells in eyes and antennae. In contrast, *cry* intron 1 drives rhythmic expression in eyes and antennae, but not brain oscillator neurons. These results demonstrate that intron 1 is sufficient for high amplitude *cry* mRNA cycling, show that *cry* upstream sequences are sufficient for expression in brain oscillator neurons, and suggest that *cry* spatial and circadian expression are regulated by different elements.

Keywords

cryptochrome; *Drosophila*; gene regulation; circadian clock; pacemaker cells; peripheral tissues

Introduction

In *Drosophila*, *cryptochrome* (*cry*) encodes a blue light photoreceptor that mediates entrainment of circadian oscillators and, in some peripheral tissues, is necessary for circadian oscillator function (Hardin 2005). Unlike extraretinal photoreceptors in Hofbauer-Buchner cells and retinal photoreceptors in compound eyes and ocelli (Helfrich-Forster 2005b), CRY functions in a cell autonomous manner to reset the oscillator by binding TIMELESS (TIM) in response to light (Ceriani et al. 1999). CRY also functions cell autonomously to maintain peripheral oscillator function by regulating circadian transcription (Collins et al. 2006). The close relationship between CRY and the circadian clock implies that *cry* should be expressed in all oscillator cells, but analysis of *cry* spatial expression using *cry* promoter driven Gal4 (*cry*-Gal4) transgenes, *cry* RNA in situ hybridization, and CRY immunolocalization have focused exclusively on the brain and are inconsistent (Emery et al. 2000; Helfrich-Forster et al. 2007; Klarsfeld et al. 2004; Shafer et al. 2006; Zhao et al. 2003).

Circadian oscillators are found in seven sets of neurons in the adult brain (Helfrich-Forster 2005a; Shafer et al. 2006): small ventral lateral neurons (s-LN_vs), large ventral lateral neurons (l-LN_vs), dorsal lateral neurons (LN_ds), lateral posterior neurons (LPNs), dorsal neuron 1s

(DN₁s), dorsal neuron 2s (DN₂s) and dorsal neuron 3s (DN₃s). The pattern of *cry*-Gal4 driven GFP reporter gene expression ranges from l-LN_vs only to all LNs and DN₁s depending on the transgene insertion site and whether the transgene contains the first intron (Emery et al. 2000; Helfrich-Forster et al. 2007; Klarsfeld et al. 2004; Shafer et al. 2006; Zhao et al. 2003). RNA in situ hybridization shows *cry* expression in the LNs, DN₁s and DN₂s, but not DN₃s (Zhao et al. 2003). Immunolocalization of CRY shows expression in LNs and DN₁s, but not in DN₂s or DN₃s (Klarsfeld et al. 2004). Because of the variability within and among these different assays, there is no consensus for *cry* spatial expression in the brain. The loss of circadian oscillator function and/or synchrony in peripheral tissues of *cry*^b flies implies that *cry* is expressed in these tissues (Emery et al. 2000; Ivanchenko et al. 2001; Krishnan et al. 2001; Levine et al. 2002; Stanewsky et al. 1998), yet *cry* expression hasn't been directly assessed in peripheral tissues.

The levels of *cry* mRNA cycle with a peak at ZT5 and a trough at ZT17, which is almost antiphase to *period* (*per*) and *timeless* (*tim*) RNA cycling, but similar to *Clock* (*Clk*) RNA cycling (Egan et al. 1999; Emery et al. 1998; Ishikawa et al. 1999). In addition, *cry* mRNA levels are relatively low in *per*⁰¹ or *tim*⁰¹ mutants and relatively high in *Clk*^{Jrk} and *cyc*⁰¹ (*cyc*⁰¹) single mutants or *per*⁰; *Clk*^{Jrk} and *per*⁰; *cyc*⁰¹ double mutants (Emery et al. 1998; Ishikawa et al. 1999). These results suggest that *cry* transcription is constitutively activated independent of a functional clock, repressed via CLK-CYC, and derepressed via PER-TIM. These results imply that *cry* expression is regulated by a mechanism similar to the one that regulates rhythms in *Clk* transcription, where an uncharacterized activator, possibly assisted by PAR domain protein 1ε (PDP1ε) at dawn, is rhythmically repressed by VRILLE (VRI) during the early to late evening (Benito et al. 2007; Cyran et al. 2003; Glossop et al. 2003). In contrast, CRY protein levels are regulated by light; CRY is low in the light phase, begins to accumulate after lights off and remains at constant high levels during constant dark conditions (Emery et al. 1998).

To determine where *cry* is expressed and understand how *cry* spatial and circadian expression is regulated, a series of *cry*-Gal4 transgenes was generated that contain different portions of *cry* upstream sequences with or without *cry* exon 1 and intron 1, or *cry* intron 1 alone. Transgenes that contain *cry* upstream sequences along with *cry* intron 1 and flanking exon sequences drive Gal4 mRNA cycling with a phase and amplitude similar to that of endogenous *cry* mRNA, and activate *β-galactosidase* reporter gene expression in brain oscillator neurons and eye and antennal peripheral oscillator cells in heads. In contrast, *cry* transgenes lacking intron 1 express low constitutive mRNA levels in brain oscillator neurons and eye and antennal peripheral oscillator cells, whereas *cry* intron 1 drives rhythmic expression only in eye and antennal peripheral oscillator cells. These results demonstrate that *cry* is expressed in essentially all circadian oscillator cells, *cry* intron 1 is necessary and sufficient for high amplitude mRNA cycling, and *cry* spatial and circadian expression are independently regulated.

Materials and Methods

Flies stocks and transgenic flies

The wild-type *D. melanogaster* strain was Canton-S. Construction of the *cry*Gal4 transgene was described previously (Emery et al. 1998). The 4.0*cry*-Gal4, 3.0*cry*Gal4, 2.0*cry*Gal4, 0.7*cry*Gal4 and *cry*In1-*hsbp*-Gal4 transgenes were constructed as described below. Genomic DNA fragments from *cry* were amplified from BAC clone BACR48G05 (BACPAC Resources). The 4.0*cry*-Gal4 transgene was constructed by inserting the MluI-BamHI amplification product generated by the -4.0*crys* (5'-CGACGCGTGGATTGGGCGGCACAAAGG-3') and *cry*ex1a (5'-GGGGATCCCTGCTTTTGGGCTTC-3') primers into the AscI and BamHI sites of the

pCaSpeR4-Gal4:VP16 vector. The *cryex1a* primer ends 11bp upstream from the *cry* translation initiation site. The pCaSpeR4-Gal4:VP16 vector generates a fusion protein containing the GAL4 DNA binding domain (1-147) and the VP16 (411-490) activation domain (Galindo and Smith 2001). The 0.7*cry*-Gal4 transgene was constructed by inserting the SpeI-BamHI amplification product generated by the -0.7*crys* (5'-GGACTAGTCGGCGCAGAGCTAGAAGTGC-3') and *cryex1a* (5'-GGGGATCCCTGCTTTTGGGCTTC-3') primers into the SpeI site and BamHI sites of the pCaSpeR4-Gal4:VP16 vector. The 3.0*cry*-Gal4 transgene was constructed by inserting the KpnI-BamHI amplification product generated by the -2.0*crys* (5'-CTTTGTAAAAATTGGTACCACCTGGCG-3') and *cryEx2a* (5'-CAGGATCCCTTGGTACATGTTAAGG-3') primers into the KpnI and BamHI sites of the pCaSpeR4-Gal4:VP16 vector. Partial sequencing of the 3.0*cry*Gal4 transgene confirmed that *cry* coding sequences from the entire first exon, as well as three amino acids from the second exon, form a fusion protein with GAL4:VP16. The 2.0*cry*Gal4 transgene was constructed in two stages. A KpnI-BamHI amplification product generated by the -2.0*crys* and *cryex1a* primers was inserted into the KpnI and BamHI sites of the pGatB vector just upstream of the Gal4 coding region and the SV40 terminator (Brand and Perrimon 1993). A KpnI-NotI fragment containing *cry* upstream and exon 1 sequences, Gal4 coding sequences and an SV40 terminator were subcloned into the KpnI and NotI sites of pCaSpeR4. The *cryIn1*-hsbp-Gal4 transgene was constructed by inserting the SacII-BamHI amplification product generated by the *cryInt1s* (5'-GAGCCGCGGGAGTGCAGGTAAGAACTGAG-3') and *cryEx2a* primers into the SacII site and BamHI sites of pPTGAL (Sharma). All *cry*-Gal4 transgenes were used to generate transgenic flies via P-element mediated transformation. At least two independent lines were generated for each transgene. Flies with inserts on the second and third chromosome were balanced using In(2LR)CyO or In(3LR)TM6B, respectively. All fly strains were reared on medium containing corn meal, molasses, yeast, agar, and Tegosept (a mold inhibitor) at 25°C.

RNA extraction and qPCR

Heterozygous *cry*-Gal4/+ flies were used for determining Gal4, *cry* and *rp49* mRNA levels. Flies were entrained for three days in 12hr light: 12hr dark (LD) cycles at 25°C and collected at different time points on the fourth day of LD. RNA was isolated and cDNA was synthesized as described previously (Benito et al. 2007). For qPCR, TaqMan assays were performed using the following primers and probes. The *cry* qPCR probe spans the exon2-exon3 junction, and is immediately flanked by the *cry* forward and *cry* reverse amplification primers (*cry* probe: 5' TGTTCTGTCACACGGT 3'; *cry* forward primer: 5' CCACCGCTGACCTACCAA 3'; *cry* reverse primer: 5' GGTGGAAGCCCAATAATTTGC 3'). The Gal4 probe and primers are directed against the GAL4 DNA binding domain (Gal4 probe: 5' CTTTCGGTTTTTCTTTGGAGCACT 3'; Gal4 forward primer: 5' GCATGCGATATTTGCCGACTTAAAA 3'; Gal4 reverse primer: 5' TCCCAGTTGTTCTTCAGACACTTG 3'). *rp49* was used as internal control for mRNA content in each sample (*rp49* probe: 5' CCTCCAGCTCGCGCACGTTG 3'; *rp49* forward primer: 5' CTGCCCACCGGATTCAAG 3'; *rp49* reverse primer: 5' CGATCTCGCCGAGTAAAC 3'). Reactions were run on an ABI Prism 7000. The relative levels of *cry*, Gal4 and *rp49* RNAs were calculated based on standard curves for these transcripts that were run in each assay. *cry* and Gal4 levels were normalized to *rp49* at each time point. Wild-type flies were used as a standard to compare between different transgenes. One-way ANOVA carried out using STATISTICA software (Statsoft) was used to analyze mRNA cycling.

β -Galactosidase staining

Two independent lines of each cry-Gal4 transgene were crossed to UAS-GFP::lacZ.nls flies (BL 6451), which expresses nuclear localized β -galactosidase (Wilson et al. 2008). The resulting flies were cryosectioned and assayed for β -galactosidase activity using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) histochemistry as described (Liu et al. 1988), except flies were fixed in 2% glutaraldehyde for 5 min. and rinsed in phosphate-buffered saline (PBS, pH 6.9) three times before adding X-gal. At least six individuals were sectioned from each independent line.

Immunostaining

For each cry-Gal4 transgene, two to four independent lines were crossed to flies containing a UAS-GFP::lacZ.nls reporter gene (Wilson et al. 2008). The resulting progeny were entrained in LD for three days and collected at ZT21 on the fourth day. Adult fly brains were dissected in cold PBS and fixed in PBS-3.7% formaldehyde for 15 minutes at room temperature. After three 15 minute rinses in PBS at room temperature, the brains were blocked in 5% BSA+0.3% Triton-100+0.3% sodium deoxycholate (PAXD) for 15 minutes. Then the brains were incubated with rabbit anti-PER serum at 1:30,000 (a gift from Dr. J. Hall) and/or Anti- β -galactosidase antibody (Sigma) 1:200 overnight at 4°C. After four 30 minute and four 1hr washes in PAXD at room temperature, the brains were incubated with a 1:200 dilution of Alexa Fluor 488 goat anti-rabbit IgG (H+L) (Molecular Probes) and/or Cy3®-conjugated goat anti-guinea pig IgG (H+L) (Jackson Immuno Research Laboratories) overnight at 4°C. After another four 30 minute and four 1hr washes in PAXD at room temperature, brains were mounted in Vectashield (Vector Laboratories) and examined using an Olympus FV 1000 confocal microscope. Images were processed using Adobe Photoshop. At least five individuals were dissected from each independent line.

Results

cry intron1 is necessary and sufficient for circadian regulation

To determine how rhythms in *cry* transcription are regulated, transgenes containing *cry* upstream, *cry* upstream plus intron 1, and *cry* intron 1 sequences were used to drive a Gal4 reporter gene (Fig. 1). The largest *cry* transgene (crypiGAL4), which contains ~4.0kb of *cry* upstream and ~1kb of *cry* transcribed sequences (including exon 1, intron 1 and the start of exon 2), was generated previously (Emery et al. 2000). Since *cry* translation begins in exon 1, the crypiGAL4 transgene makes a CRY-GAL4 fusion protein containing the first ~50 amino acids of CRY (Fig. 1). The 4.0cry-Gal4 transgene contains ~4.0kb of upstream sequence and ~140bp of the 5' untranslated region fused to Gal4. The 2.0kbcry-Gal4 transgene contains ~2.0kb of *cry* upstream and ~140bp of the 5' untranslated region fused to Gal4. The 3.0cry-Gal4 transgene contains ~2.0kb of *cry* upstream sequence, exon 1, intron 1 and the start of exon 2. Like crypiGAL4, 3.0cry-Gal4 generates a CRY-GAL4 fusion protein. The smallest transgene, 0.7cryGal4, contains 700bp of *cry* upstream sequence and ~140bp of the 5' untranslated region fused to Gal4.

Wild-type flies containing cry-Gal4 transgenes were collected every four hours during a 12hr light: 12hr dark (LD) cycle, and Gal4 mRNA levels were monitored by quantitative real-time RT-PCR (qPCR). In flies bearing the crypiGAL4 transgene, Gal4 mRNA levels are ~ 3-fold higher during the early day than during the early night (Fig. 2A), which are comparable to rhythms of endogenous *cry* mRNA (Egan et al. 1999; Emery et al. 2000; Ishikawa et al. 1999, Fig. 2A). Likewise, the 3.0cryGal4 transgene drives Gal4 mRNA cycling similar to that of crypiGAL4, but with a lower peak level (Fig. 2A). In contrast, 4.0cryGal4, 2.0cryGal4 and 0.7cryGal4 drive constitutive levels of Gal4 mRNA that are much lower than the trough levels of Gal4 mRNA in crypiGAL4 and 3.0cryGal4 flies or *cry* mRNA in wild-type flies (Fig. 2B).

Since the crypiGal4 and 3.0cryGal4 transgenes contain *cry* intron 1 plus flanking exon 1 and exon 2 sequences, but the 4.0cryGal4, 2.0cryGal4 and 0.7cryGal4 transgenes lack these sequences, our results suggest that regulatory elements within intron 1 and/or flanking exon 1 and exon 2 sequences enhance *cry* mRNA levels and mediate *cry* mRNA cycling.

cry intron 1 and/or flanking exon 1 and exon 2 sequences could regulate mRNA cycling and abundance via transcriptional or post-transcriptional mechanisms. To test whether this regulation occurs at the transcriptional level, *cry* intron 1 was fused upstream of heat shock basal promoter and Gal4 coding sequences to form cryIn1-hsbgp-Gal4 (see Materials and Methods). If *cry* intron 1 contains transcriptional enhancers that control mRNA cycling and abundance, it should drive rhythmic Gal4 mRNA at similar levels as wild-type *cry* mRNA. Indeed, Gal4 mRNA is rhythmically expressed, but with a lower cycling amplitude than wild-type *cry* due to a lower peak levels (Fig. 2C). This result demonstrates that *cry* intron 1 is sufficient to drive rhythmic transcription in the same phase as wild-type *cry* mRNA, but with lower peak levels.

***cry* spatial expression in adult brains**

Since cry-Gal4 transgenes containing intron 1 express much higher levels of *cry* mRNA than those that lack intron 1, we wanted to determine whether the low expression levels from transgenes containing only *cry* upstream sequences could be accounted for by a restricted spatial distribution. Previous studies of crypiGal4 expression in the brain show considerable variability between different insertion lines, with expression ranging from l-LN_vs only to expression in the s-LN_vs, l-LN_vs, LN_ds, most DN₁s and several non-oscillator cell types (Emery et al. 2000; Helfrich-Forster et al. 2007; Klarsfeld et al. 2004; Shafer et al. 2006; Zhao et al. 2003). Given that the 3.0cry-Gal4 expresses *cry* mRNA at similar levels and cycling amplitude as the crypiGal4, we initially determined the pattern of 3.0cry-Gal4 expression in the brain and whether this pattern was consistent among strains.

Brains from flies containing 3.0cry-Gal4 driver and UAS-GFP::lacZ.nls responder transgenes were immunostained with β -Galactosidase antibody to reveal 3.0cry-Gal4 expression and PER antiserum to reveal circadian oscillator cells. β -galactosidase is co-expressed with PER in all six groups of canonical clock cells examined including all of the s-LN_vs, l-LN_vs, LN_ds, DN₁s, DN₂s and most of the DN₃s (Fig. 3A-C). In each of the three 3.0cry-Gal4 lines tested, β -galactosidase was detected in all PER expressing cells, though the number of β -galactosidase positive DN₃s varied in different transgenic lines (data not shown). Outside of the oscillator neurons, β -galactosidase was detected in a group of cells in the dorsal portion of the optic lobe (Fig. 3A), which we will refer to as DOL cells. From these results, we conclude that the 3.0cry-Gal4 consistently drives expression in all oscillator neurons and DOL cells.

The 2.0cry-Gal4 transgene contains the same upstream sequence as 3.0cry-Gal4, but produced constant low levels of Gal4 mRNA. When three independent 2.0cry-Gal4 lines were used to drive UAS-GFP::lacZ.nls, β -galactosidase expression was detected in the same groups of oscillator and non-oscillator neuronal groups as the 3.0cry-Gal4 (Fig. 4A-C), except fewer neurons express β -galactosidase in the DN₃ group (Fig. 4B). Similar results were seen with two independent 4.0cry-Gal4 lines (data not shown), suggesting that regulatory elements responsible for expression in brain oscillator neurons and DOL cells are present in the first 2.0kb of *cry* upstream sequence. Thus, despite the low levels of Gal4 mRNA produced by the 2.0cry-Gal4 and 4.0cry-Gal4 lines, their spatial expression pattern in the brain is almost identical to that of 3.0cry-Gal4 lines.

Like the cry-Gal4 strains analyzed above, 0.7cry-Gal4 is expressed in almost all oscillator cells (e.g. s-LN_vs, l-LN_vs, LN_ds, DN₁s, DN₂s and some DN₃s) and a cluster of non-oscillator cells in DOL cells (Fig. 5A-C). Additional non-oscillator cell expression in 0.7cry-Gal4 flies differs

in number and location among the four independent transgenic lines analyzed (Fig. 5, data not shown), suggesting that this expression is due to P-element enhancer trapping at the different insertion sites. These results demonstrate that this short 0.7kb *cry* upstream fragment contains regulatory elements sufficient for expression in essentially all brain oscillator neurons and non-oscillator DOL cells.

Transgenes containing both *cry* upstream sequences and intron 1 drive strong and rhythmic expression in essentially all brain oscillator cells. Since *cry* upstream sequences mediate expression in brain oscillator cells and *cry* intron 1 mediates strong rhythmic expression, we wanted to determine whether *cry* intron 1 also drives expression in brain oscillator cells. When *cryIn1-hsbg-Gal4* was used to drive *UAS-GFP::lacZ.nls*, neither brain oscillator cells nor non-oscillator DOL cells expressed β -galactosidase (Fig. 6A-C). However, each of the three *cryIn1-hsbg-Gal4* lines tested showed expression in different sets of non-oscillator cells in the brain, indicating that these patterns were due to the insertion site rather than *cry* intron 1 sequences. Unlike the other *cry-Gal4* lines, *cryIn1-hsbg-Gal4* didn't drive expression in DOL cells (Fig. 6A). These results demonstrate that *cry* intron 1 lacks regulatory elements for expression in brain oscillator neurons and non-oscillator DOL cells. However, *cry* intron 1 drives strong rhythmic mRNA expression in fly heads, which suggests that expression is being driven primarily in peripheral oscillators.

***cry* expression in peripheral tissues**

The ~150 oscillator neurons in the brain account for a tiny fraction of oscillator cells in fly heads, where most oscillator cells reside in peripheral tissues such as eyes, ocelli, antennae, proboscis, maxillary palps and fat bodies (Bell-Pedersen et al. 2005; Hall 2003; Shafer et al. 2006). The high levels of Gal4 mRNA produced by *cry* intron 1-containing lines compared to those containing only *cry* upstream sequences might suggest that only lines containing *cry* intron 1 are capable of driving expression in peripheral head tissues. To test this possibility *cry-Gal4* lines were used to drive *UAS-GFP::lacZ.nls*, and β -galactosidase activity was measured on sectioned flies via X-Gal staining (see materials and Methods).

All *cry-Gal4* transgenic lines expressed β -galactosidase in eyes and antennae (Fig. 7). Since transgenes containing only *cry* upstream sequences or *cry* intron 1 drove expression in these peripheral oscillator tissues, regulatory elements that control expression in peripheral oscillators from fly heads are present in both *cry* upstream sequences and *cry* intron 1. The intensity of X-gal staining was not uniform among transgenic lines; the *cryiGAL4* and *3.0cryGal4* transgenes showed much lower intensity X-gal staining than other *cry-Gal4* transgenes. This result is surprising since the *cryiGAL4* and *3.0cry-Gal4* transgenes express high levels of Gal4 mRNA and contain both *cry* upstream and intron 1 regulatory elements for expression in eyes and antennae. These results demonstrate that elements that mediate expression in compound eyes and antennae are present in both *cry* upstream sequences and intron 1.

Discussion

Regulation of *cry* mRNA cycling and abundance

In wild-type flies, *cry* mRNA cycles in abundance with a peak around dawn (Egan et al. 1999; Emery et al. 1998; Ishikawa et al. 1999). Here we show that *cry-Gal4* transgenes containing *cry* upstream, intron 1, and exon 1 and exon 2 sequences flanking intron 1 mediate mRNA cycling with a similar phase and amplitude as endogenous *cry* mRNA, but *cry-Gal4* transgenes containing only *cry* upstream sequences are constitutively expressed. Likewise, transgenes containing *cry* intron 1 express much higher overall levels of mRNA than those lacking intron 1. These results demonstrate that *cry* intron 1 and/or flanking exon 1 and exon

2 sequences are necessary for rhythmic mRNA expression at high overall levels. The *cryIn1-hsbg-Gal4* transgene also drives mRNA rhythms at levels similar to endogenous *cry* mRNA, showing that *cry* intron 1 is sufficient for high levels of rhythmically expressed mRNAs. Taken together, these results demonstrate that *cry* intron 1 is both necessary and sufficient to drive high levels of rhythmic mRNA expression. It is possible that *cry*-Gal4 transgenes which drive proper spatial expression in brain oscillator cells also show rhythmic expression in these cells, but the low number of brain oscillator cells and their isolation from peripheral oscillator cells make it difficult to test this possibility. None of our *cry*-Gal4 transgenes contained *cry* intron 2, intron 3 or downstream sequences, so we can't exclude the possibility that additional regulatory sequences capable of driving mRNA cycling or enhancing expression are present. Although sequences that enhance mRNA expression and control mRNA cycling are both present in *cry* intron 1, we don't know if these aspects of expression are mechanistically linked, or whether additional spatial regulatory elements or separate general enhancer elements are responsible for producing high levels of mRNA.

Based on the phase of *cry* mRNA cycling in wild-type flies and levels of *cry* mRNA in different circadian clock mutants, *cry* mRNA cycling may be regulated by the same mechanism that controls *Clk* mRNA cycling (Cyran et al. 2003; Emery et al. 2000; Glossop et al. 2003). A key regulator of *Clk* mRNA cycling is VRI, which rises to high levels from late morning to mid-evening to repress transcription (Cyran et al. 2003; Glossop et al. 2003). VRI binds to VRI/PDP1 regulatory elements (V/P-boxes) that are similar to consensus 5' (T/A/G)T(T/G)(A/C)TGTA(T/C) 3' mammalian E4BP4 binding sites (Glossop et al. 2003). Two sites having at least 8/10 bases identical to the consensus sequence are present in *cry* intron 1 (Fig. 1A), suggesting that one or both of these sequences is involved in mediating rhythmic expression. However, similar consensus VRI binding sites are also found in the *cry* upstream region (Glossop et al. 2003), indicating that VRI binding specificity either does not exactly match that of E4BP4 or that sequences outside of this consensus are important for binding specificity.

Characterization and regulation of *cry* spatial expression

All *cry*-Gal4 transgenes that contain *cry* upstream sequences are expressed in all s-LN_vs, l-LN_vs, LN_ds, DN₁s and DN₂s, most DN₃s, the compound eye, and antennae. The consistent expression of multiple 2.0*cry*-Gal4, 3.0*cry*-Gal4 and 4.0*cry*-Gal4 lines contrasts with the variable expression among independent lines of *crypti*Gal4 and *crypt*Gal4 (Emery et al. 2000; Helfrich-Forster et al. 2007; Klarsfeld et al. 2004; Shafer et al. 2006; Zhao et al. 2003). The expression of both 4.0*cry*-Gal4 lines in brain oscillator cells and the DOL, but few other cells, is surprising given that the only molecular differences are that *crypt*Gal4 produces a CRY-GAL4 fusion protein with ~10 N-terminal CRY amino acids and natural GAL4, whereas 4.0*cry*-Gal4 produces GAL4-VP16 containing no CRY sequences (Zhao et al. 2003). It is not clear how these structural differences could account for differences in non-oscillator cell expression. Expression of each 3.0*cry*Gal4, 4.0*cry*Gal4, 2.0*cry*Gal4 and 0.7*cry*Gal4 line in every cluster of brain oscillator neurons is not a reflection of high expression levels since none of these *cry*-Gal4 lines are expressed at higher levels than *crypti*Gal4, and most (i.e. all lines lacking *cry* intron 1 and flanking exon sequences) are expressed at lower levels. However, we used UAS-GFP::lacZ.nls and β -galactosidase antiserum to monitor *cry*-Gal4 spatial expression, whereas previous studies used UAS-GFP. Perhaps signal amplification via β -galactosidase antibody provided greater sensitivity than direct GFP fluorescence, thereby revealing low level expression in subsets of LNs and DN_s.

All *cry*-Gal4 transgenes containing *cry* upstream sequences are expressed in brain oscillator neurons, eyes, and antennae. In contrast, the *cryIn1-hsbg-Gal4* transgene, which lacks *cry* upstream sequences, is expressed in eyes and antennae only. These results indicate that regulatory elements capable of driving expression in brain oscillator neurons are present in

cry upstream sequences. Although the amount of *cry* upstream sequence tested ranges from ~4.0kb to ~0.7kb, the only difference in brain oscillator cell expression was the number of DN₃s. Thus, regulatory elements for expression in s-LN_vs, l-LN_vs, LN_ds, DN₁s, DN₂s and some DN₃s reside within 0.7kb of the *cry* transcription start site. Given that the *Pigment dispersing factor* (*Pdf*) gene is expressed in s-LN_vs and l-LN_vs only (Helfrich-Forster 1995; Park et al. 2000), expression in different groups of brain oscillator neurons is likely to be controlled by independent regulatory elements.

Two well-characterized peripheral oscillator tissues in fly heads are compound eyes and antennae (Bell-Pedersen et al. 2005; Hall 2003; Helfrich-Forster 1995; Park et al. 2000). The ~13000 photoreceptor cells in compound eyes represent >80% of oscillator cells in fly heads (Glossop and Hardin 2002), thus much of our knowledge about the molecular mechanism that governs oscillator function is based on photoreceptor cell oscillators. Olfactory sensory neurons in the antenna are necessary and sufficient for rhythms in olfaction (Tanoue et al. 2004), which represents the only physiological rhythm that has been characterized in *Drosophila*. We find that both *cry* upstream sequences and *cry* intron 1 drive expression in compound eyes and antennae, demonstrating that regulatory elements for these peripheral oscillators are redundant. The inability of cryIn1-hsbg-Gal4 to drive expression in brain oscillator neurons shows that regulatory elements for compound eyes and antennae are separable from those that mediate expression in brain oscillator cells.

The crypGAL4 and 3.0cry-Gal4 transgenes showed much lower intensity X-gal staining in eyes and antennae than other cry-Gal4 transgenes. One characteristic that is unique to the crypGAL4 and 3.0cryGal4 transgenes is that they both make a CRY-GAL4 fusion protein containing the first ~50 CRY amino acids. Although β -galactosidase staining was done at ZT9, the CRY-GAL4 fusion protein was not sensitive to light because β -galactosidase staining intensity in 3.0cry-Gal4 flies was the same at ZT9 and ZT21 (data not shown). Perhaps the CRY-GAL4 fusion protein is less stable or less active than native GAL4. We tried to assess the levels of CRY-GAL4 fusion protein, but specific staining could not be distinguished from the numerous nonspecific bands using commercially available GAL4 antibodies (data not shown).

None of the cry-Gal4 transgenes was expressed exclusively in brain oscillator cells. The 0.7cry-Gal4 and cryIn1-hsbg-Gal4 transgenes were expressed in different groups of non-oscillator cells in each line, indicating that this expression is due to enhancer trapping. In contrast, all cry-Gal4 transgenes that contained *cry* upstream sequences showed expression in non-oscillator DOL cells. When the crypGAL4-24 transgene was used to generate ectopic oscillators by driving UAS-Clk, *cry* mRNA was detected in the dorsal region of the optic lobe, but no *cry* mRNA was detected in this brain region from wild-type flies (Zhao et al. 2003). Taken together, the cry-Gal4 transgene results argue that regulatory elements for expression in DOL cells are present in *cry* upstream sequences, but not in intron 1. The inability to detect *cry* mRNA in this brain region from wild-type flies suggests that expression is enhanced by transgene expression and/or detection methods. Three different P-element vectors were used to generate transgenes containing *cry* upstream sequences (see Materials and Methods), which argues that expression in DOL cells does not result from vector sequences.

Analysis of the *cry* promoter has revealed expression in each cluster of brain oscillator neurons and a cluster of non-oscillator DOL cells. Immunolocalization studies show a more restricted pattern of CRY expression that includes s-LN_vs, l-LN_vs, and a subset of LN_ds and DN₁s, but not DN₂s, DN₃s or non-oscillator cells (Klarsfeld et al. 2004), Yoshii et al., in press; Benito et al., submitted). The more restricted distribution of CRY suggests that *cry* expression in DN₂s, DN₃s, some DN₁s and LN_ds, and DOL cells is either not high enough to produce detectable amounts of CRY protein, or that *cry* is regulated at the post-translational level in

these cells. Since oscillators are present in cells that do not express detectable amounts of CRY, entrainment of these cells to light must either depend on photoreceptors in compound eyes, ocelli or Hofbauer-Buchner cells (Helfrich-Forster et al. 2001), or other cells that express detectable levels of CRY.

In summary, these results demonstrate that *cry* spatial regulatory elements are separable from cycling elements, that *cry* expression in brain oscillator neurons and peripheral oscillator tissues (in heads) are controlled by different regulatory elements, and that *cry* regulatory elements capable of driving expression in peripheral oscillators in the head are redundant. The expression of *cry* in peripheral oscillators is consistent with local control of light entrainment and/or oscillator function by CRY, whereas the lack of CRY in some brain oscillator neurons suggests that their entrainment is mediated indirectly by other photosensitive cells.

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References

- Bell-Pedersen D, Cassone VM, Earnest DJ, Golden SS, Hardin PE, Thomas TL, Zoran MJ. Circadian rhythms from multiple oscillators: lessons from diverse organisms. *Nat Rev Genet* 2005;6:544–556. [PubMed: 15951747]
- Benito J, Zheng H, Hardin PE. PDP1epsilon functions downstream of the circadian oscillator to mediate behavioral rhythms. *J Neurosci* 2007;27:2539–2547. [PubMed: 17344391]
- Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 1993;118:401–415. [PubMed: 8223268]
- Ceriani MF, Darlington TK, Staknis D, Mas P, Petti AA, Weitz CJ, Kay SA. Light-dependent sequestration of TIMELESS by CRYPTOCHROME. *Science* 1999;285:553–556. [PubMed: 10417378]
- Collins B, Mazzoni EO, Stanewsky R, Blau J. *Drosophila* CRYPTOCHROME is a circadian transcriptional repressor. *Curr Biol* 2006;16:441–449. [PubMed: 16527739]
- Cyran SA, Buchsbaum AM, Reddy KL, Lin MC, Glossop NR, Hardin PE, Young MW, Storti RV, Blau J. *vriille*, *Pdp1*, and *dClock* form a second feedback loop in the *Drosophila* circadian clock. *Cell* 2003;112:329–341. [PubMed: 12581523]
- Egan ES, Franklin TM, Hilderbrand-Chae MJ, McNeil GP, Roberts MA, Schroeder AJ, Zhang X, Jackson FR. An Extraretinally Expressed Insect Cryptochrome with Similarity to the Blue Light Photoreceptors of Mammals and Plants. *J Neurosci* 1999;19:3665–3673. [PubMed: 10233998]
- Emery P, So WV, Kaneko M, Hall JC, Rosbash M. CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* 1998;95:669–679. [PubMed: 9845369]
- Emery P, Stanewsky R, Helfrich-Forster C, Emery-Le M, Hall JC, Rosbash M. *Drosophila* CRY is a deep brain circadian photoreceptor. *Neuron* 2000;26:493–504. [PubMed: 10839367]
- Galindo K, Smith DP. A large family of divergent *Drosophila* odorant-binding proteins expressed in gustatory and olfactory sensilla. *Genetics* 2001;159:1059–1072. [PubMed: 11729153]
- Glossop NR, Hardin PE. Central and peripheral circadian oscillator mechanisms in flies and mammals. *J Cell Sci* 2002;115:3369–3377. [PubMed: 12154068]
- Glossop NR, Houl JH, Zheng H, Ng FS, Dudek SM, Hardin PE. VRILLE feeds back to control circadian transcription of *Clock* in the *Drosophila* circadian oscillator. *Neuron* 2003;37:249–261. [PubMed: 12546820]
- Hall JC. Genetics and molecular biology of rhythms in *Drosophila* and other insects. *Adv Genet* 2003;48:1–280. [PubMed: 12593455]

- Hardin PE. The circadian timekeeping system of *Drosophila*. *Curr Biol* 2005;15:R714–722. [PubMed: 16139204]
- Helfrich-Forster C. The period clock gene is expressed in central nervous system neurons which also produce a neuropeptide that reveals the projections of circadian pacemaker cells within the brain of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* 1995;92:612–616. [PubMed: 7831339]
- Helfrich-Forster C. Neurobiology of the fruit fly's circadian clock. *Genes Brain Behav* 2005a;4:65–76. [PubMed: 15720403]
- Helfrich-Forster C. Organization of endogenous clocks in insects. *Biochem Soc Trans* 2005b;33:957–961. [PubMed: 16246020]
- Helfrich-Forster C, Shafer OT, Wulbeck C, Grieshaber E, Rieger D, Taghert P. Development and morphology of the clock-gene-expressing lateral neurons of *Drosophila melanogaster*. *J Comp Neurol* 2007;500:47–70. [PubMed: 17099895]
- Helfrich-Forster C, Winter C, Hofbauer A, Hall JC, Stanewsky R. The circadian clock of fruit flies is blind after elimination of all known photoreceptors. *Neuron* 2001;30:249–261. [PubMed: 11343659]
- Ishikawa T, Matsumoto A, Kato T Jr, Togashi S, Ryo H, Ikenaga M, Todo T, Ueda R, Tanimura T. DCRY is a *Drosophila* photoreceptor protein implicated in light entrainment of circadian rhythm. *Genes Cells* 1999;4:57–65. [PubMed: 10231393]
- Ivanchenko M, Stanewsky R, Giebultowicz JM. Circadian photoreception in *Drosophila*: functions of *cryptochrome* in peripheral and central clocks. *J Biol Rhythms* 2001;16:205–215. [PubMed: 11407780]
- Klarsfeld A, Malpel S, Michard-Vanhee C, Picot M, Chelot E, Rouyer F. Novel features of cryptochrome-mediated photoreception in the brain circadian clock of *Drosophila*. *J Neurosci* 2004;24:1468–1477. [PubMed: 14960620]
- Krishnan B, Levine JD, Lynch MK, Dowse HB, Funes P, Hall JC, Hardin PE, Dryer SE. A new role for cryptochrome in a *Drosophila* circadian oscillator. *Nature* 2001;411:313–317. [PubMed: 11357134]
- Levine JD, Funes P, Dowse HB, Hall JC. Advanced analysis of a cryptochrome mutation's effects on the robustness and phase of molecular cycles in isolated peripheral tissues of *Drosophila*. *BMC Neurosci* 2002;3:5. [PubMed: 11960556]
- Liu X, Lorenz L, Yu QN, Hall JC, Rosbash M. Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. *Genes Dev* 1988;2:228–238. [PubMed: 3129339]
- Park JH, Helfrich-Forster C, Lee G, Liu L, Rosbash M, Hall JC. Differential regulation of circadian pacemaker output by separate clock genes in *Drosophila*. *Proc Natl Acad Sci U S A* 2000;97:3608–3613. [PubMed: 10725392]
- Shafer OT, Helfrich-Forster C, Renn SC, Taghert PH. Reevaluation of *Drosophila melanogaster*'s neuronal circadian pacemakers reveals new neuronal classes. *J Comp Neurol* 2006;498:180–193. [PubMed: 16856134]
- Stanewsky R, Kaneko M, Emery P, Beretta B, Wager-Smith K, Kay SA, Rosbash M, Hall JC. The *cry^b* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell* 1998;95:681–692. [PubMed: 9845370]
- Tanoue S, Krishnan P, Krishnan B, Dryer SE, Hardin PE. Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in *Drosophila*. *Curr Biol* 2004;14:638–649. [PubMed: 15084278]
- Wilson RJ, Goodman JL, Strelets VB. FlyBase: integration and improvements to query tools. *Nucleic Acids Res* 2008;36:D588–593. [PubMed: 18160408]
- Zhao J, Kilman VL, Keegan KP, Peng Y, Emery P, Rosbash M, Allada R. *Drosophila* Clock Can Generate Ectopic Circadian Clocks. *Cell* 2003;113:755–766. [PubMed: 12809606]

Figure 1.

Structure of *cry*-Gal4 transgenes. (A) The *cry* gene structure is depicted on top. Thin straight line, upstream sequence; +1, transcription start site; white boxes, untranslated sequence; AUG, translation start; bent lines, introns; black boxes, translated sequences; UGA, translation stop; asterisks, consensus E4BP4 binding sites. Double arrows depict fragments of *cry* genomic DNA in the *cry*piGAL4, 4.0*cry*-Gal4, 3.0*cry*-Gal4, 2.0*cry*-Gal4, 0.7*cry*-Gal4 and *cry*In1-hsbp-Gal4 transgenes. Each *cry* genomic fragment is fused to Gal4 at the 3' end except *cry*In1-hsbp-Gal4, which is fused to a heatshock basal promoter driven GAL4 at the 3' end. Note that *cry*piGAL4, 2.0*cry*-Gal4 and *cry*In1-hsbp-Gal4 produce GAL4, whereas 4.0*cry*-Gal4, 3.0*cry*-Gal4, and 0.7*cry*-Gal4 produce GAL4-VP16. (B) Structure of the CRY-GAL4 fusion protein generated by 3.0*cry*-Gal4. The open reading frame encoding the first 60 amino acids of the CRY-GAL4 fusion is shown. Lowercase letters, DNA sequence; uppercase letters, protein sequence; black letters, *cry* sequence; gray letters, vector sequence; outlined letters, Gal4 sequence.

Figure 2.

Gal4 mRNA cycling and abundance in cry-Gal4 lines. qPCR of Gal4 mRNA from the heads of wild-type and heterozygous cry-Gal4 flies collected at the indicated times under LD conditions (ZT). Two independent fly collections were tested for each line, and two or three lines were tested for each cry-Gal4 transgene, thus the average of 4-6 independent collections are shown in each plot. The relative levels of Gal4 and *cry* mRNAs were quantified as described in Materials and Methods. The black and white bars represent times when lights were on or off, respectively. (A) Relative levels of Gal4 mRNA in crypiGAL4 (black squares) and 3.0cryGal4 (white triangles) flies, and *cry* mRNA in wild-type flies (black circles). Gal4 mRNA cycling was significant in both crypiGAL4 (one-way ANOVA; $P < 0.01$) and 3.0cryGal4 (one-

way ANOVA; $P < 0.01$). (B) Relative levels of Gal4 mRNA in 4.0cryGal4 (black diamonds), 2.0cryGal4 (white squares), and 0.7cryGal4 (white circles) flies. Gal4 mRNA did not cycle (one-way ANOVA; $P > 0.1$) in 4.0cryGal4, 2.0cryGal4, or 0.7cryGal4 flies. Gal4 mRNA levels were measured in parallel with *cry* mRNA from wild-type flies shown in A. (C) Relative levels of Gal4 (gray squares) and *cry* (white diamonds) mRNAs in cryIn1-hsbp-Gal4 flies. Gal4 (one-way ANOVA; $P < 0.02$) and *cry* (one-way ANOVA; $P < 0.0001$) mRNA cycling was significant in cryIn1-hsbp-Gal4 flies.

Figure 3.

Co-localization β -galactosidase and PER immunofluorescence in 3.0cryGal4 fly brains. Brains from 3.0cryGal4 flies collected at ZT9 were dissected, prepared for β -galactosidase and PER whole mount immunofluorescence, and imaged via confocal microscopy. (A) A 1 μ m optical section through the left hemisphere of a brain is shown at 20X magnification, where dorsal is at the top. Co-localization of β -galactosidase (red) and PER (green) immunofluorescence is seen as yellow in the superimposed dual laser image (merge). Images on the top focus on dorsal neuron 1 (DN₁), dorsal neuron 2 (DN₂), dorsal neuron 3 (DN₃), and dorsal optic lobe (DOL) cells, and images on the bottom focus on dorsal lateral neuron (LN_D), large ventral lateral neuron (I-LN_V), and small ventral lateral neuron (s-LN_V) cells. (B) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing dorsal neurons in A. (C) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing lateral neurons in A.

Figure 4.

Co-localization β -galactosidase and PER immunofluorescence in 2.0cryGal4 fly brains. Brains from 2.0cryGal4 flies collected at ZT9 were dissected, prepared for β -galactosidase and PER whole mount immunofluorescence, and imaged via confocal microscopy. (A) A 1 μ m optical section through the left hemisphere of a brain is shown at 20X magnification, where dorsal is at the top. Co-localization of β -galactosidase (red) and PER (green) immunofluorescence is seen as yellow in the superimposed dual laser image (merge). Images on the top focus on DN₁, DN₂, DN₃, and DOL cells, and images on the bottom focus on LN_D, I-LN_V, and s-LN_V cells. Oscillator and non-oscillator cell groups are abbreviated as in Fig. 3A. (B) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing dorsal neurons in A. (C) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing lateral neurons in A.

Figure 5.

Co-localization β -galactosidase and PER immunofluorescence in 0.7cryGal4 fly brains. Brains from 0.7cryGal4 flies collected at ZT9 were dissected, prepared for β galactosidase and PER whole mount immunofluorescence, and imaged via confocal microscopy. (A) A 1 μ m optical section through the left hemisphere of a brain is shown at 20X magnification, where dorsal is at the top. Co-localization of β -galactosidase (red) and PER (green) immunofluorescence is seen as yellow in the superimposed dual laser image (merge). Images on the top focus on DN₁, DN₂, DN₃, and DOL cells, and images on the bottom focus on LN_D, l-LN_V, and s-LN_V cells. Oscillator and non-oscillator cell groups are abbreviated as in Fig. 3A. (B) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing dorsal neurons in A. (C) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing lateral neurons in A.

Figure 6.

Co-localization β -galactosidase and PER immunofluorescence in cryIn1-hsbg-Gal4 fly brains. Brains from cryIn1-hsbg-Gal4 flies collected at ZT9 were dissected, prepared for β galactosidase and PER whole mount immunofluorescence, and imaged via confocal microscopy. (A) A 1μm optical section through the right hemisphere of a brain is shown at 20X magnification, where dorsal is at the top. β -galactosidase (red), PER (green), and β -galactosidase + PER (merge) immunofluorescence is shown. Oscillator and non-oscillator cell groups are abbreviated as in Fig. 3A. (B) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing dorsal neurons in A. (C) 40X magnification of β -galactosidase, PER and merged β -galactosidase + PER immunofluorescence in the brain region showing lateral neurons in A.

Figure 7. X-gal staining in cry-Gal4 fly heads. Male flies carrying the crypiGAL4, 3.0cry-Gal4, 3.0cry-Gal4, 3.0cry-Gal4, 3.0cry-Gal4, or 3.0cry-Gal4 transgenes were collected at ZT9, sectioned, and stained with X-gal for 14 hours to detect β -galactosidase activity. A right hemisphere is shown, oriented dorsal on top. Antenna, ANT; Photoreceptor, PR.