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Circadian Rhythms in Drosophila Can Be Driven by *period* Expression in a Restricted Group of Central Brain Cells

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Summary

Neural tissues controlling circadian rhythmicity have been identified in a variety of organisms and are often closely associated with the visual system. In Drosophila, the clock gene *period* (*per*), which is required for circadian rhythms, is expressed in many neurons and glia throughout the eye and brain. We asked whether biological rhythms could be generated if *per* expression were restricted to a subset of these cells that is involved in photoreception. Here we demonstrate that expression of *per* under the control of the *glass* promoter confers both behavioral and molecular rhythmicity. *glass* is required for development of Drosophila photoreceptors, and this promoter is active in eyes, ocelli, and certain cells of the central brain. When we genetically removed all external photoreceptor cells, rhythms persisted in these transgenic animals. This suggests that a few central brain cells producing *glass* and *per* are capable of generating biological rhythms.

Introduction

A biological clock present in most eukaryotes imposes daily rhythms on multiple biochemical processes and behaviors (reviewed in Takahashi and Zatz, 1982; Edmunds, 1988; Dunlap, 1993; Hall and Rosbash, 1993; Young, 1993). Although these rhythms can be entrained to environmental cycles, primarily cycles of light and dark, they persist in the absence of external cues. The cellular and molecular bases of such light-sensitive biological clocks are being explored in certain model organisms. Mutations have identified clock genes in Neurospora (Feldman and Hoyle, 1973; Dunlap et al., 1993), Drosophila (Konopka and Benzer, 1971; Jackson, 1993; Sehgal et al., 1994), hamster (Ralph and Menaker, 1988), and mouse (Vitaterna et al., 1994). These mutations speed up, slow down, or eliminate rhythms. Molecular analyses of the *period* (*per*) gene of Drosophila and the *frequency* (*frq*) gene of Neurospora point to possible roles in transcriptional regulation. Both genes are transcribed with a circadian rhythm, and the rhythms are altered by *per* and *frq* mutations, respectively (Hardin et al., 1992, 1993; Huang et al., 1993; Aronson et al., 1994; Page, 1994). In Drosophila, transcriptional rhythms are further regulated by the recently discovered clock gene *timeless* (*tim*; Sehgal et al., 1994). A null mutation of *tim* abolishes circadian cycling of *per* transcription, behavioral rhythmicity, and nuclear localization of certain PER reporter proteins (Sehgal et al., 1994; Vosshall et al., 1994; M. Myers and M. Y., unpublished data). The mutation also leads to reduced levels of PER that do not cycle (J. Price, M. Dembinska, M. Y., and M. Rosbash, submitted). How the action of any such clock genes might transduce environmental signals into whole animal rhythms is only beginning to be explored.

The nervous systems of several species contain discrete pacemaker tissues. These tissues receive input from the visual system or are themselves directly light sensitive, and they possess intrinsic oscillatory function. In many instances, removing these tissues renders the animal arrhythmic, and transplanting them into an arrhythmic host restores rhythmicity. In rodents (Moore and Eichler, 1972; Rusak, 1977) and birds (Klein, 1978; Takahashi and Menaker, 1979), pacemaker tissues are found in the suprachiasmatic nucleus and pineal gland, respectively. In the mollusks Bulla and Aplysia, the retina has pacemaker activity (Lickey et al., 1976; Eskin, 1979; Roberts and Block, 1983), and for Bulla this activity is expressed by single cells in culture (Michel et al., 1993). Xenopus also produces a retinal pacemaker (Cahill and Besharse, 1993). In the beetle and cockroach, circadian tissues have been described in the eyes and optic lobes (Fleissner, 1982; Page, 1982). In contrast, the central brain probably contains pacemaker tissues in the housefly, as behavioral rhythms persist in the absence of the eyes and optic ganglia (Truman, 1976; Helfrich et al., 1985).

Although the first molecular cloning of a clock gene occurred in Drosophila (Bargiello and Young, 1984; Reddy et al., 1984), only a few studies have implicated defined regions of the brain in pacemaker function. Transplantation studies suggested that a circadian pacemaker is located in the head (Handler and Konopka, 1979). Later studies ruled out an essential pacemaker contribution for the eyes, ocelli, and optic lobes, because mutants that lacked these tissues had normal circadian rhythms (Engelmann and Honegger, 1966; Helfrich and Engelmann, 1983; Helfrich, 1986; Dushay et al., 1989; Wheeler et al., 1993). Since all of these mutants continued to entrain to light–dark cycles, the data indicated that pacemaker cells must be found in the brain, and that extraocular photoreceptors can modulate the activity of pacemaker tissues.

Since *per* is essential for normal rhythms, further clues about the location of Drosophila pacemaker tissues should come from an examination of patterns of *per* RNA and protein synthesis, through manipulation of *per* expression in the head. For example, PER protein is widely distributed in the adult head, throughout the eye, optic lobe, and brain, in both neurons and glia (Liu et al., 1988, 1992; Saez and Young, 1988; Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992). A subset of these cells has been further implicated in circadian rhythmicity by investigation of the mutation *disconnected* (*disco*). *disco* mutants show behavioral

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arrhythmia and disrupt connections between the eye and brain. The mutants also appear to delete certain PER-expressing neurons found in the central brain bordering the optic lobes. These cells are often referred to as lateral brain neurons (LNs; Dushay et al., 1989; Zerr et al., 1990; Helfrich-Forster and Homberg, 1993; Helfrich-Forster, 1995). The behavioral arrhythmia is probably not due to the disruption in eye–brain connectivity, since rare flies with patently normal optic lobes on one or both sides of the brain are still arrhythmic. The missing LNs in *disco* mutants include neurosecretory cells that express both pigment-dispersing hormone (PDH) and PER (Helfrich-Forster, 1995). Evidence linking PDH-expressing neurons to regulation of circadian rhythms has now been obtained in several insects (reviewed in Helfrich-Forster and Homberg, 1993; Stengl and Homberg, 1994). Although *disco* flies are behaviorally arrhythmic, molecular rhythms of *per* transcription persist in the mutants owing to the retention of many *per*-expressing cells in other regions of the brain and in the eyes. Thus, the affected brain neurons may contribute to circadian rhythmicity but cannot be the only cells with pacemaker activity.

More evidence bearing on the identity of pacemaker cells has come from analysis of flies genetically mosaic for *per* in the head (Ewer et al., 1992) and from investigation of a promoterless *per* transgene (Frisch et al., 1994). These studies have indicated a role for certain central brain neurons and glia in pacemaker activity.

The *glass* gene encodes a transcription factor essential for the development of all known photoreceptor cells in Drosophila (Moses et al., 1989; Moses and Rubin, 1991; Ellis et al., 1993). Since circadian pacemakers have been mapped to cells with photoreceptor function in a variety of organisms (Deguchi, 1979; Eskin, 1979; Robertson and Takahashi, 1988; Zatz et al., 1988; Cahill and Besharse, 1993), we sought to limit patterns of *per* expression by replacing the *per* promoter with the photoreceptor-active promoter *glass*. In this paper we show that circadian molecular and behavioral rhythms can be generated by such transgenes. The results indicate that cells with photoreceptor properties might be directly involved in the generation of circadian rhythms in Drosophila.

We further show that expression of PER protein in the eyes and ocelli, as directed by the *glass*-promoted transgene, is dispensable for generation of these circadian rhythms, since mutations that delete all visual system structures fail to diminish behavioral rhythmicity. Thus, cells in the brain must be responsible for the observed behavioral rhythms. *glass*-expressing brain cells are identified. Some of these appear to correspond to a small subset of the previously implicated LNs. However, most *glass*-expressing cells appear to belong to a novel cell group occupying a ventrolateral location in the central brain.

Results

Genetically Ablating Eye, Optic Lobe, and Brain Structures Does Not Eliminate Circadian Rhythms

We screened existing mutations that disrupt the visual system and the brain for circadian phenotypes. Flies were raised under standard conditions for several generations until tested and entrained for at least 5 days to a 12 hr light–12 hr dark cycle (LD12:12). Their locomotor behavior was assayed after transfer to constant darkness. The mutants tested can be divided into three broad phenotypic categories (Table 1): roughened eye mutants, visual signal transduction mutants, and eye–brain morphology mutants. All of these mutations displayed locomotor activity rhythms with circadian periods despite varied and dramatic effects on the structure and/or function of cells in the adult head. It should be noted that for some of the mutants, although sample sizes were small, low penetrance of rhythmicity was observed (e.g., *Drop-Miopthalmia* [Dr^{Mio}] and certain *lozenge* [*lz*] alleles). We also point out that for some strains the average period lengths of the locomotor activity rhythms were rather short (e.g., *ocelliless* [*oc*] and Dr^{Mio}). We have not yet determined whether these effects are in fact genetically linked to the indicated eye–optic lobe–brain mutations. Most of the mutations were tested with homozygotes; however, some of the mutations are homozygous lethal (described in Lindsley and Zimm, 1992). For the latter, except as described Table 1, effects on visual system organization are dominant, and viable heterozygotes were tested for behavioral rhythms.

Expressing *per* under the Control of the *glass* Promoter Rescues Behavioral Rhythms

We used the *glass* promoter to direct *per* expression because this promoter is active in a subset of *per*-expressing cells throughout development. *glass* is expressed in the larval photoreceptor, in the developing eye disc, and in a few cell bodies of the larva central brain (Moses et al., 1989; Moses and Rubin, 1991; Ellis et al., 1993). In the adult, *glass* is expressed in the R1–R8 photoreceptors of the eye and is active in the ocelli (Moses et al., 1989; Moses and Rubin, 1991; Ellis et al., 1993). Expression in the adult brain was not examined in these earlier studies.

A 5.1 kb DNA fragment containing the *glass* promoter (see Experimental Procedures) was cloned upstream of a *per* genomic DNA fragment to form a transgene designated *glass*–PERc. This *per* DNA lacks the *per* promoter and sequences corresponding to most of intervening sequence 1 (Baylies et al., 1993), but is sufficient to encode a wild-type PER protein (see Experimental Procedures). Seven independent autosomal transformants were obtained in a per^{o} background for behavioral testing (see Experimental Procedures). Behavioral tests were performed with flies heterozygous for each transgene and are summarized in Table 2. All seven lines produced rhythmic flies, with penetrance ranging from 25% to 69%. The rhythms produced by the flies showed strain-specific periods ranging from 27.7 to 34.4 hr. Transgenic lines expressing *per* under the control of its own promoter (designated PER^{+}) also showed incomplete penetrance and some variation in period length (Table 2). As described previously, such effects on penetrance and period length are likely to reflect chromosomal position effects (Baylies et al., 1987, 1992). The results obtained in this study with *glass*-promoted *per* expression also can be compared with re-

Table 1. Existing Mutations That Disrupt Eye, Optic Lobe, and Brain Morphology and Function Continue to Produce Circadian Locomotor Activity Rhythms

Mutant Name	Mean Period Length ± SD (hr)	Penetrance (% of Rhythmic Flies)
Roughened eye mutants		
almondex (*amx*)	23.9 ± 0.9	4/5 (80%)
chaoptic (*chp*)		
chp^{1}	23.6 ± 0.5	4.4 (100%)
chp^{3}	23.8 ± 0.3	3/4 (75%)
chp^{5}	24.2 ± 0.3	3/3 (100%)
chp^{6}	23.7 ± 0.4	5/6 (83%)
chp^{8}	23.8 ± 0.4	2/2 (100%)
Irregular facets (*If*)	24.1 ± 0.7	10/11 (91%)
pebbled (*peb*)	23.7 ± 0.2	3/4 (75%)
rough (*ro*)	23.8 ± 0.6	8/10 (80%)
roughex (*rux*)	23.7 ± 0.4	6/6 (100%)
scabrous (*sca*)	23.5 ± 0.5	3/3 (100%)
sevenless (*sev*)	23.3 ± 0.6	3/3 (100%)
Visual transduction mutants		
ninaA^{P228}	23.7 ± 0.8	6/6 (100%)
ninaE^{P318}	23.9 ± 0.5	10/12 (83%)
Eye and brain mutants		
drop dead (*drd*)	23.9 ± 0.6	19/22 (86%)
Drop-Miopthalmia (*Dr*^{Mio})	22.5 ± 0.5	5/10 (50%)
Ellipse (*Elp*)	23.6 ± 0.5	4/4 (100%)
eyeless (*ey*)		
ey^{1}	23.3 ± 1.5	3/4 (75%)
ey^{2}	22.8 ± 1.2	12/14 (86%)
ey^{4}	23.9 ± 0.7	5/6 (83%)
eyes absent (*eya*)		
eya^{1}	24.1 ± 0.6	4/4 (100%)
eya^{ph}	23.5 ± 0.7	7/8 (88%)
glass (*gl*)		
gl^{1}	24.3 ± 0.3	6/8 (75%)
gl^{2}	24.0 ± 0.7	9/9 (100%)
gl^{3}	23.7 ± 0.8	7/8 (88%)
gl^{60j}	23.9 ± 0.6	26/32 (81%)
Glued (*Gl*)	23.4 ± 1.2	7/8 (88%)
lozenge (*lz*)		
lz^{1}	24.1 ± 1.3	4/6 (66%)
lz^{2}	22.9 ± 1.7	3/5 (60%)
lz^{34}	23.8 ± 0.4	2/2 (100%)
lz^{36}	23.0 ± 1.0	3/5 (60%)
lz^{50e}	23.2 ± 1.3	5/5 (100%)
lz^{9}	24.7 ± 1.7	3/3 (100%)
lz^{rv4}	23.7 ± 0.3	3/6 (50%)
Microcephalus (*Mc*)	23.7 ± 0.9	11/15 (73%)
mushroom body defect (*mud*)	23.9 ± 0.6	11/14 (79%)
no ocelli, narrow eyes (*none*)	23.8 ± 0.4	5/5 (100%)
ocelliless (*oc*)	22.7 ± 0.8	7/8 (88%)
reduced ocelli (*rdo*)	23.7 ± 1.2	9/13 (69%)

Flies were entrained to LD12:12, and their locomotor activity was measured as described (Sehgal et al., 1994) in constant darkness, *amx, chp, If, peb,* and *rux* result in a roughened surface of the eye. Analysis of *sev, sca,* and *ro* mutants has shown that they lack a subset of photoreceptor cells (Harris et al., 1976; Tomlinson and Ready, 1987; Tomlinson et al., 1988; Ready, 1989). Most of these roughened eye mutants have a range of effects on the optic lobe axon array and the morphology of optic lobe structures (Johannsen, 1924; Meyerowitz and Kankel, 1978; Garen and Kankel, 1983). The two *nina* genes are involved in visual signal transduction. *ninaE* is the structural gene for the Rh1 opsin in photoreceptor cells R1-R6 (O'Tousa et al., 1985; Zuker et al., 1985). A Drosophila homolog of prolyl cis-trans isomerase specifically required for the processing of *ninaE* is encoded by the *ninaA* gene (Shieh et al., 1989; Colley et al., 1991). Both *eya* (Bonini et al., 1993) and *ey* (Quiring et al., 1994) suppress eye development and have a morphological phenotype similar to that of *sine oculis* (Fischbach and Technau, 1984). The number of ommatidia in the compound eye is reduced in *Elp, Gl,* and *lz,* resulting in an oval- or crescent-shaped eye (Lindsley and Zimm, 1992). *glass* mutants lack photoreceptor cells in the retina and ocelli and have small and disorganized optic lobes (Johannsen, 1924; Meyerowitz and Kankel, 1978; Garen and Kankel, 1983; Moses et al., 1989). A preliminary study (Sehgal et al., 1991) suggested that the *gl*^{1} mutation might cause behavioral arrhythmicity. However, subsequent tests have shown that this behavioral phenotype does not map to the *glass* locus (L. B. V., unpublished data). Earlier studies of a second null allele (*gl*^{60j}) also failed to indicate an effect of *glass* mutation on circadian rhythms (Sehgal et al., 1991). *mud* affects the mushroom bodies in the central brain, and *none* and *rdo* modify the ocelli (Lindsley and Zimm, 1992). *Mc* and *Dr*^{Mio} affect the size of the eye and the brain (Lindsley and Zimm, 1992). *drd* results in progressive brain degeneration, leading to death within a week of eclosion (Hotta and Benzer, 1972; Lindsley and Zimm, 1992). Homozygous *drd* males were entrained as pupae and placed into the locomotor assay on the first day of adult life. Thus, they were found to have circadian rhythms with a wild-type period throughout their abbreviated life span.

Table 2. Behavioral Analysis of Transgenic Flies Carrying PER$^+$ and *glass*–PERc Transgenes

Genotype (listed by line number)	Mean Period Length ± SD (hr)	Penetrance (% of rhythmic flies)
per^o; PER$^+$		
2	26.4 ± 1.2	45/59 (76%)
3	26.7 ± 1.3	55/60 (92%)
4	25.6 ± 0.8	39/50 (78%)
per^o; *glass*–PERc		
1	27.7 ± 1.2	123/177 (69%)
2	33.3 ± 3.4	33/68 (49%)
3	31.4 ± 4.1	16/44 (36%)
4	33.5 ± 3.4	42/97 (43%)
6	34.3 ± 3.2	25/81 (31%)
8	32.3 ± 3.6	16/63 (25%)
9	34.4 ± 2.6	12/26 (46%)

Animals heterozygous for each transgene were entrained and tested as described in Table 1. Three independent *per^o*; PER$^+$ lines carried the *per* gene under the control of the *per* promoter (see Baylies et al., 1992). Seven independent lines carried the *glass*–PERc transgene in a *per^o* background.

sults derived from expression of a related transgene in which *per* genomic DNA was expressed in the absence of any promoter (Frisch et al., 1994). In the latter study, only two of ten tranformed strains produced rhythmic flies. This indicates that inclusion of the *glass* promoter has a significant effect on the pattern or strength of *per* expression (or both), and this promoter activity is conducive to expression of behavioral rhythmicity with high penetrance. Figure 1 shows a plot of locomotor activity and a periodogram analysis for a representative fly from one of the transformed lines, *glass*–PERc1. *glass*–PERc1 flies produced rhythms 1–2 hr longer than PER$^+$ control transformants (Table 2; also see Baylies et al., 1992).

Expressing *per* under the Control of the *glass* Promoter Rescues Molecular Rhythms in the Adult Eye

In wild-type flies, the presence of PER protein in nuclei is under circadian control (Siwicki et al., 1988; Zerr et al., 1990; Vosshall et al., 1994): high levels of nuclear PER are found late at night and early in the day. Little or no PER is detectable near dusk. We asked whether PER produced from the *glass* promoter also exhibited time-dependent nuclear staining. Flies from *per^o* stocks, *per^o*; *glass*–PERc1 transformants, and control *per^o*; PER$^+$ transformants were entrained to LD12:12 for at least 5 days and collected at two time points, 12 hr apart (ZT2 and ZT14; ZT [Zeitgeber Time] is a convention for designating time in reference to an imposed light cycle, with ZT0 corresponding to lights on and ZT12 corresponding to lights off). Frozen sections were prepared and stained with an anti-PER antibody (Saez and Young, 1988; Vosshall et al., 1994). Representative sections are shown in Figure 2. Figure 2A shows that, as for *per$^+$* flies, in *per^o*; PER$^+$ transformants PER immunoreactivity is evident in photoreceptor nuclei R1–R8 at ZT2 but not at ZT14. Similarly, *per^o*; *glass*–PERc1 sections show accumulation of nuclear PER at ZT2 only (Figure 2B). PER immunoreactivity was examined in a third line of transformants, *glass*–PERc2, and was also found to be present at ZT2, but not at ZT14 (data not shown). Figure 2C shows that no immunoreactivity is observed at either time point in sections from *per^o* flies.

Expression of *glass* RNA Is Not under Circadian Control

In adult heads, *per* mRNA displays a circadian rhythm of expression, with peak levels occurring about 2 hr after lights off and lower levels found near lights on (Hardin

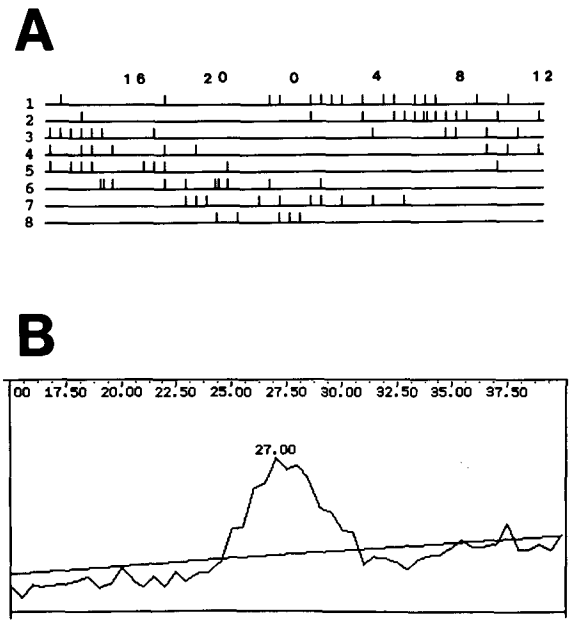

Figure 1. Locomotor Activity Profile in a *glass*–PERc1 Fly

(A) Locomotor activity of a representative *glass*–PERc1 fly recorded for 8 days in constant darkness. Horizontal lines represent successive 24 hr days. Vertical deflections from the time line indicate activity. Time of day is shown at the top of the record in Zeitgeber time (ZT0, lights on; ZT12, lights off during prior entrainment).

(B) Periodogram analysis of the locomotor behavior of the fly represented in (A). Records were analyzed for evidence of periodicity in the 15–40 hr range. The sloping line represents the lower limit of statistical significance ($p < .05$).

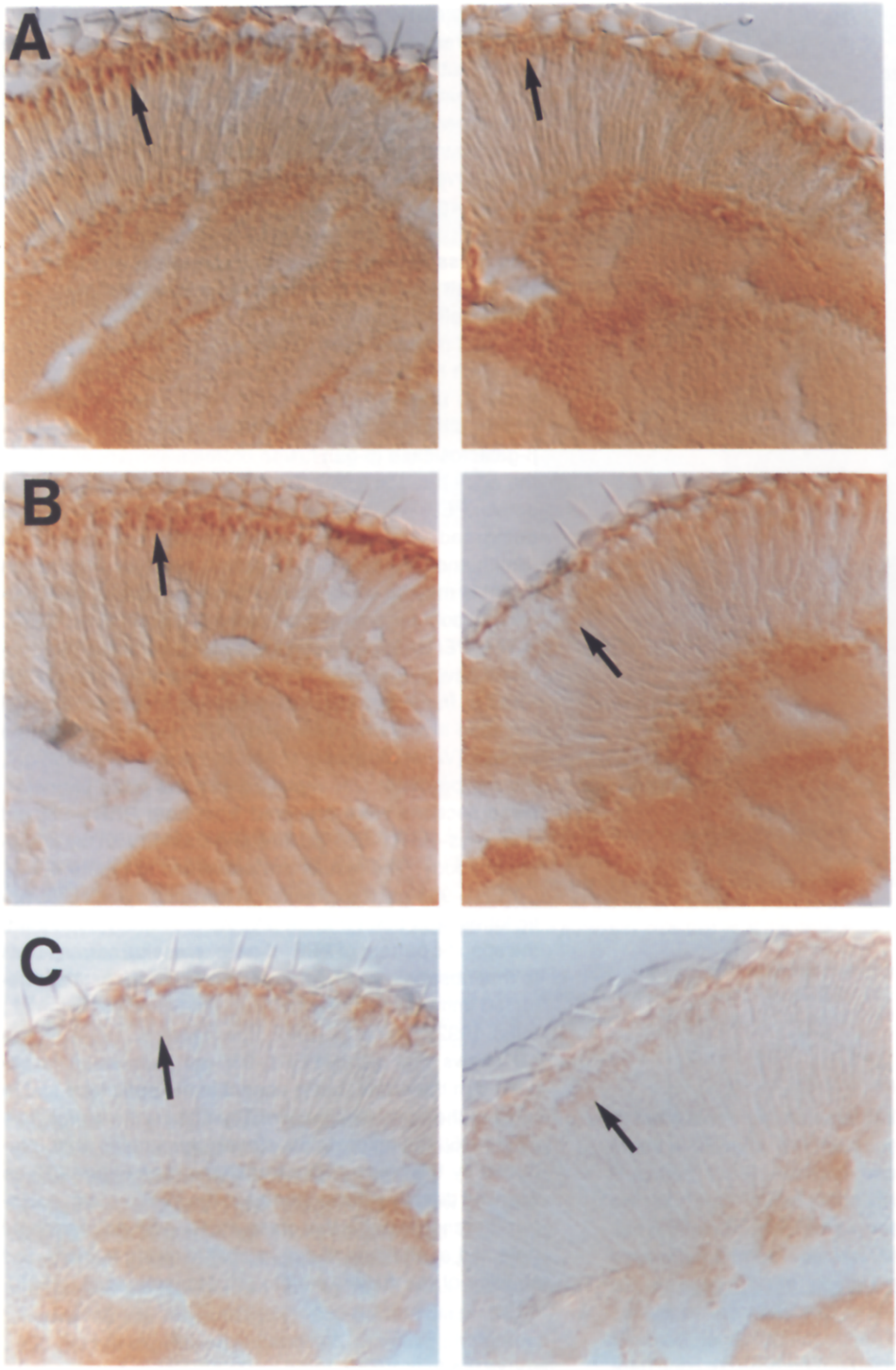

Figure 2. Molecular Rhythm of PER Protein in PER$^+$ and *glass*–PERc1 Transformants

Sections (14 μm) from adult head were prepared at ZT2 (left) and ZT14 (right) and incubated with anti-PER antibody. Immunoreactivity of photoreceptor nuclei in *per*^{o}; PER$^+$ (A) and *per*^{o}; *glass*–PERc1 (B) transformants is visible at the ZT2 time point. No nuclear staining is visible in these two genotypes at ZT14 (right). Control *per*^{o} sections (C) are unstained at both time points. The nuclear layer of photoreceptor cells R1–R6 and R7 is marked in each section (closed arrows).

et al., 1990; Sehgal et al., 1994). We asked whether the endogenous *glass* gene produces an oscillating mRNA. Wild-type (Canton-S) flies were raised in LD12:12 and collected at four 6 hr intervals, two during the light portion of the cycle (ZT2 and ZT8) and two during the dark (ZT14 and ZT20). RNA was prepared from mass-isolated head tissue, and levels of *glass* and endogenous *per* RNA were assayed by RNase protection. In Figure 3A, protected frag-

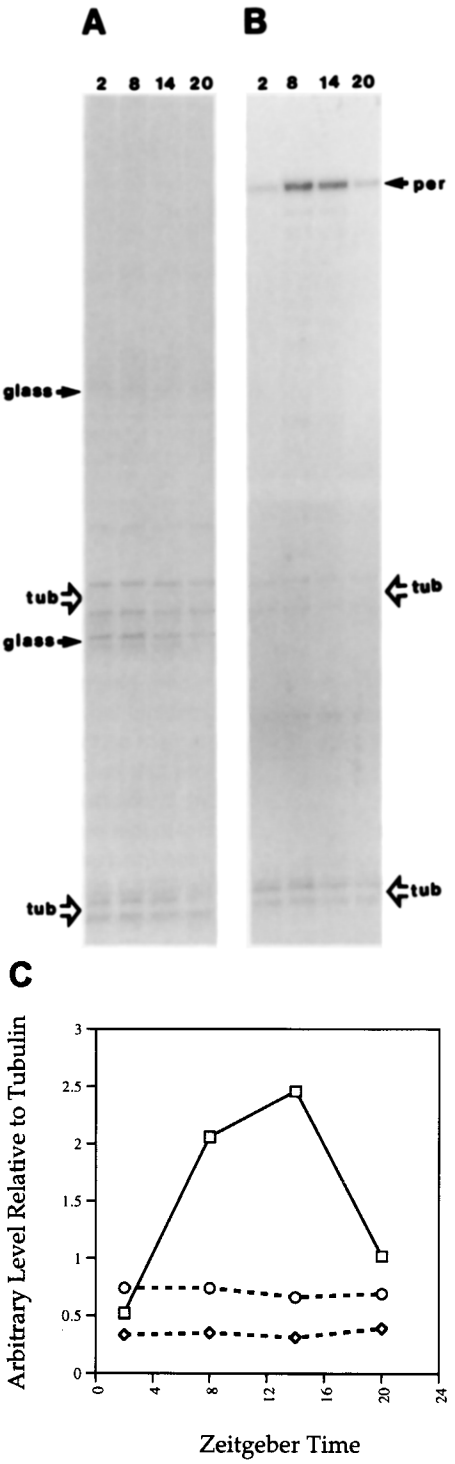

Figure 3. Levels of *glass* and *per* Expression in Wild-Type Flies in a Light–Dark Cycle

(A) Total wild-type adult head RNA collected from the indicated time points was incubated with *glass* and control tubulin probes and assessed by RNase protection as described in Experimental Procedures. Animals were entrained to a 12 hr light–12 hr dark cycle (LD12:12) and were collected in this light cycle, with ZT2 and ZT8 collected in the light, and ZT14 and ZT20 in the dark. The bands of protected *glass* RNA are indicated with closed arrows. The upper band corresponds to exon 1 and the smaller band to exon 2. Doublet bands corresponding to tubulin are marked with open arrows.

(B) The same preparation of head RNA used in (A) was protected with *per* and tubulin probes. The fragment corresponding to *per* exons 1 and 2 is indicated with a closed arrow, and the two tubulin doublet bands are marked by open arrows.

(C) Protected bands were quantitated by densitometric analysis of scans made by a phosphorimager. Control tubulin levels were assessed in each lane from the lower doublet. Levels of *per* exons 1 and 2 (open squares), *glass* exon 1 (open diamonds), and *glass* exon 2 (open circles) were normalized to tubulin. *per* RNA shows robust oscillation, with peak accumulation at ZT14. In contrast, *glass* RNA does not oscillate and is present at constitutive levels at all time points.

ments corresponding to *glass* exons 1 and 2 show constitutive expression at all four time points tested, relative to a control tubulin probe. In contrast, in the same animals, a probe that protects portions of *per* exons 1 and 2 shows robust *per* RNA oscillation (Figure 3B). Protected bands were quantitated by densitometry using a phosphorimager. Levels of *glass* and *per* RNA normalized to tubulin are shown in Figure 3C.

The *glass* Promoter Directs Expression of PER in the Adult Eye and Ocelli and in a Few Central Brain Cells

We next mapped sites of expression of *per* and *glass* in the adult head. Since the appearance of PER protein in nuclei is under circadian control and since cytoplasmic PER is difficult to detect (Vosshall, 1993), we used PER–β-galactosidase (β-gal) fusion proteins to mark all cells in the adult head in which the *per* and *glass* promoters are active. PER–β-gal fusion proteins have the advantage of being readily detectable in both the nucleus and cytoplasm (Smith and O'Kane, 1991; Vosshall, 1993), and the fusion proteins may be more stable than endogenous PER proteins at both intracellular locations (Vosshall et al., 1994).

The PER1–95β-gal construct contained the *per* promoter driving expression of the first 95 amino acids of PER fused in frame to β-gal. PER1–95β-gal localizes to nuclei because it contains N-terminal PER nuclear localization signals (Vosshall et al., 1994). In *glass*–PER1–95β-gal, the *per* promoter was replaced with the *glass* promoter. Head sections were prepared from both PER1–95β-gal and *glass*–PER1–95β-gal flies and stained with the anti-β-gal antibody. The results are presented in Figure 4.

PER1–95β-gal is expressed in the adult eyes, ocelli, optic lobes, and brain (Figure 4A). As previously described, the specific pattern of PER–β-gal expression corresponds to that observed when endogenous PER proteins are detected immunocytochemically in wild-type flies (Liu et al., 1988, 1992; Ewer et al., 1992; Vosshall et al., 1994). Figure 4B shows that *glass*–PER1–95β-gal expression is observed in the retina, but is absent in the optic lobe and in most of the brain (see below). Thus, the pattern of *glass*–PER1–95β-gal expression closely resembles that produced by the *glass*–PERc transgene (see Figure 2B) as assayed directly with antibodies to the PER protein. A useful difference is that is that *glass*–PER1–95β-gal is strongly detected at all times of day, probably owing to increased stability of the fusion protein (cf. Monsma et al., 1988).

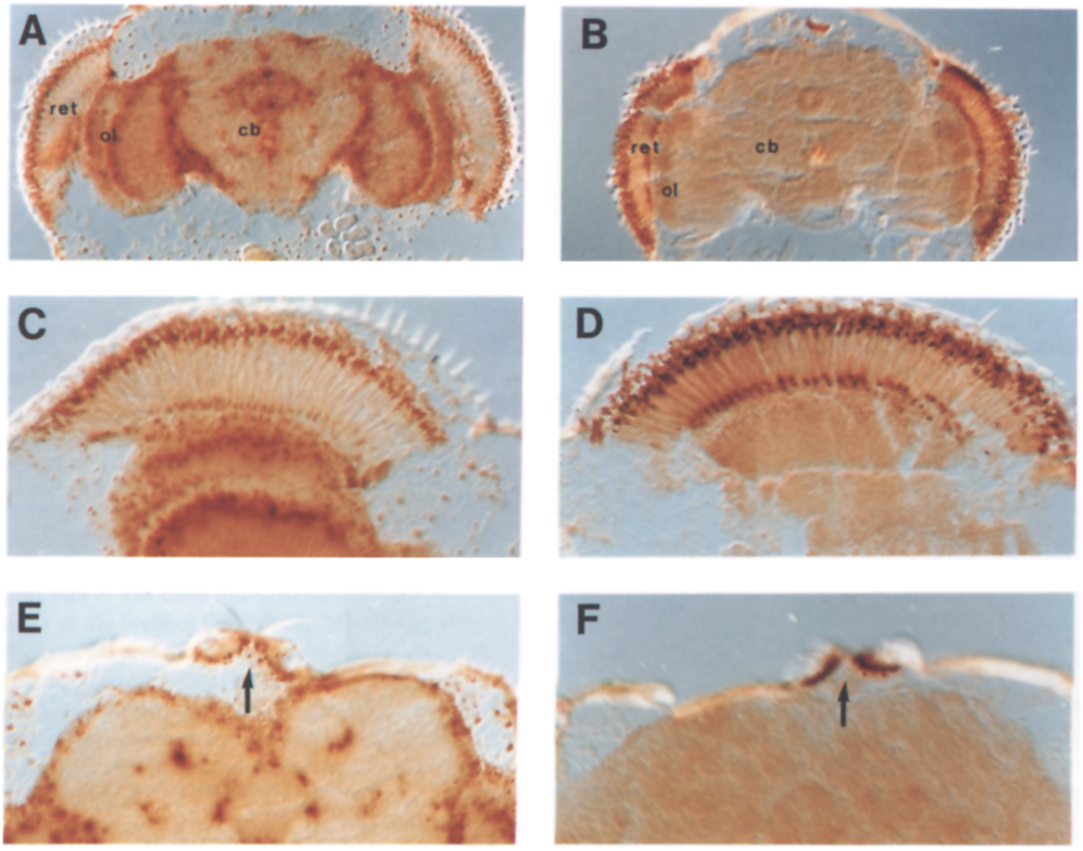

Figure 4. Patterns of *per* and *glass* Expression in the Adult Head Determined by β-gal Reporter Proteins

Expression of PER1–95β-gal (A, C, and E) and *glass*–PER1–95β-gal (B, D, and F) in various tissues of the adult head.

(A) A low power view of a frontal section shows PER1–95β-gal expression throughout the retina (ret), optic lobe (ol), and central brain (cb).

(B) *glass*–PER1–95β-gal expression is observed in the visual system. At the top of the frame, positive ocellar photoreceptor cells are visible.

(C) PER1–95β-gal is expressed in the retinal photoreceptor cells, the accessory cells that lie at the distal edge of the eye, and cells in the lamina and medulla of the optic lobe.

(D) *glass*–PER1–95β-gal is found in a subset of the tissues marked by PER1–95β-gal: the retinal photoreceptors and the accessory cells. There are no positive cells in the lamina and medulla of the optic lobe apposed to the eye. (E and F) Both PER1–95β-gal (E) and *glass*–PER1–95β-gal (F) are expressed in the ocellar photoreceptors. These are primitive photoreceptors located at the top of the head. Both frames show sections through two of the three clusters of ocellar photoreceptors.

The pattern of *glass*–PER1–95β-gal transgene expression also closely resembles that previously described for *glass* (Moses and Rubin, 1991; Ellis et al., 1993).

Figure 4C shows a higher magnification view of PER1–95β-gal expression in all photoreceptor and accessory cells of the adult eye. The same cells expressing *glass*–PER1–95β-gal are shown in Figure 4D. As shown in Figures 4E and 4F, both PER1–95β-gal and *glass*–PER1–95β-gal are expressed in the ocelli, primitive photoreceptors located on the top of the head. PER1–95β-gal expression was visible in many cells in the lamina and medulla of the optic lobe and in a subset of neurons and glia in the central brain (Figures 4A and 4C). In contrast, *glass*–PER1–95β-gal staining was never observed in the lamina and medulla (Figures 4B and 4D). Hofbauer and Buchner (1989) identified cells between the retina and lamina of the optic lobes that had some properties of photoreceptor cells. Although we do not see *glass*–PER1–95β-gal staining in the lamina, we cannot rule out that this transgene is expressed in Hofbauer–Buchner cells closely apposed to the retina. It is notable that these cells persist in flies made eyeless by the mutation *sine oculis* (Hofbauer and Buchner, 1989). Although most *glass*–PER1–95β-gal expression was limited to the visual system, close examination revealed consistent expression in three groups of cells between the optic lobe and the central brain (Figures 5A–5E, arrows). These labeled cells appear to represent a subset of the cells expressing PER1–95β-gal (see also Figure 5, legend). Inspection of two independently generated lines expressing the *glass*–PER1–95β-gal transgene indicated bilateral staining of a ventral-most group of 3–5 cells. Frontal sections of the head placed these cell groups in the central brain close to the medulla and ventral to the esophagus (Figures 5A–5E). The second location for *glass*–PER1–95β-gal transgene expression also involved lateral regions of the central brain adjoining the optic lobes, but in this case at the level of the esophagus. Labeling in this region was bilateral and involved 1–2 cells (Figure 5D, open arrow). Staining of the third group of cells, also restricted to 1–2 cells on each side of the brain, was

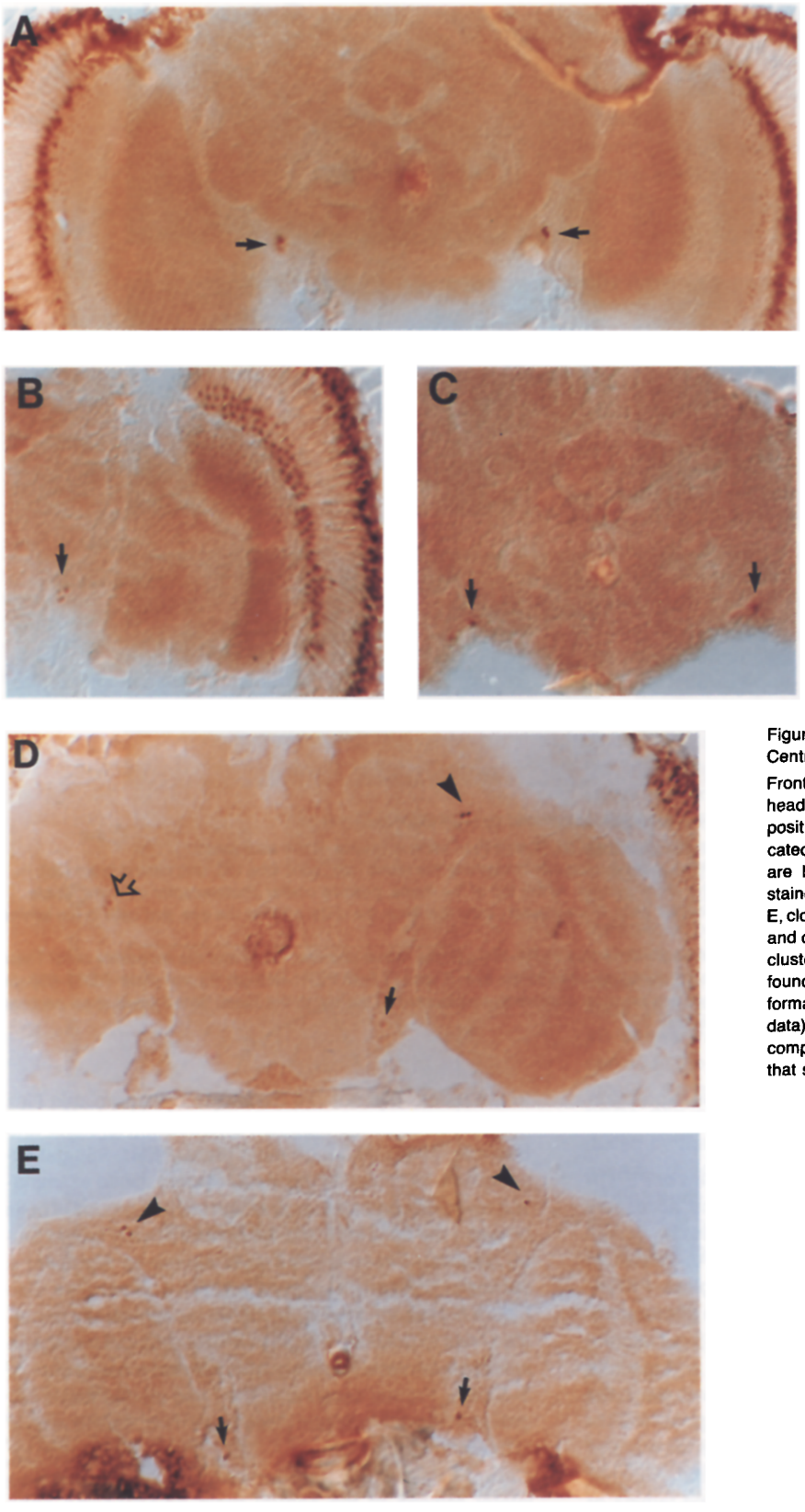

Figure 5. Expression of *glass*–PER1–95 in the Central Brain

Frontal sections through *glass*–PER1–95β-gal heads stained with anti-β-gal antibody reveal positive central brain cells. The cells are located between the optic lobe and the brain and are bilaterally symmetric. Three clusters of stained cells have been observed: ventral (A–E, closed arrows), dorsolateral (D, open arrow), and dorsal (D and E, closed arrowheads). The clusters correspond to a subset of positive cells found in the brains of PER1–95β-gal transformants (see Figure 4A) (L. B. V., unpublished data). Dorsal and dorsolateral cell clusters may compose a subset of previously described LNs that show PDH immunoreactivity (see text).

Table 3. Behavior Rescue of the *glass*–PERc1 Transgene in Mutants Lacking Retinal and Ocellar Photoreceptors

Genotype	Mean Period Length ± SD (hr)	Penetrance (% of rhythmic flies)
Wild type	23.4 ± 0.6	49/49 (100%)
per°	Arrhythmic	0/39 (0%)
per°; PER$^+$	26.2 ± 0.4*	139/169 (82%)
per°; *glass*–PERc1	27.7 ± 1.2	123/177 (69%)
eyes absent (*eya^{ph}*)	23.5 ± 0.7	7/8 (88%)
per°; *eya^{ph}*	Arrhythmic	0/33 (0%)
per°; *eya^{ph}*; *glass*–PERc1	28.2 ± 2.0	41/61 (67%)
ocelliless (*oc*)	22.7 ± 0.8	7/8 (88%)
per° oc	Arrhythmic	0/4 (0%)
per° oc; *glass*–PERc1	28.5 ± 1.8	3/3 (100%)
per° oc; *eya^{ph}*	Arrhythmic	0/19 (0%)
per° oc; *eya^{ph}* *glass*–PERc1	28.9 ± 1.7	14/20 (70%)

All mutants were entrained and tested as described in Figure 1. The *glass*–PERc1 transgene produces behavior rescue with a period of ~28 hr in both *eya^{ph}* and *oc* mutants, which lack eyes and ocelli, respectively. The *glass*–PERc1 transgene also rescues circadian rhythms, with periods of ~29 hr in flies doubly mutant for *eya^{ph}* and *oc*, and thus lacking both eyes and ocelli.
* Values are mean ± SEM of data in Table 2.

still more dorsal. *glass*–PER1–95β-gal transgene expression was in this case found in lateral areas of the central brain at the level of the calyces of the mushroom bodies (Figures 5D and 5E, arrowheads). For each of these three central brain locations, an estimate of the total number of *glass*–PER1–95β-gal cells is given above. These estimates were derived by comparison of serial frontal sections encompassing the entire head (see Experimental Procedures).

As discussed further below, the two smaller (and more dorsal) *glass*-expressing cell clusters may correspond to a subset of the PER-expressing LNs previously implicated in some aspects of circadian rhythmicity (3–7 and 4–7 LNs were previously counted in each of the two clusters on either side of the head; Siwicki et al., 1988; Zerr et al., 1990; Ewer et al., 1992; Frisch et al., 1994). Most of the *glass*-expressing cells are, however, located in central brain regions ventral to the LNs. To summarize, our immunocytochemical studies of the adult head show *glass* promoter activity in all cells composing the external photoreceptors as previously described (Moses et al., 1989; Moses and Rubin, 1991; Ellis et al., 1993). *glass* promoter activity was newly detected in small groups of central brain cells.

Behavior Rescue by the *glass*-PERc Transgene Is Independent of External Photoreceptors

To determine whether the rescuing activity of the *glass*–PERc transgene derived from expression of PER protein in the external visual system or in central brain cells, we genetically removed the eyes and ocelli and tested for behavior rescue. We used *eyes absent^{pinhead}* (*eya^{ph}*), a highly penetrant, viable allele of *eyes absent* (N. Bonini, personal communication), to remove both compound eyes completely (Bonini et al., 1993). *eya^{ph}* leaves the ocellar photoreceptors intact (Bonini et al., 1993). To remove ocelli specifically, we used the *oc* mutation, which deletes ocellar photoreceptors without affecting the compound eye (Lindsley and Zimm, 1992).

The rhythm phenotypes of various combinations of mutants with the *glass*–PERc1 transgene are listed in Table 3. Wild-type flies displayed robust 23.4 hr rhythms. The null *per°* mutation caused arrhythmic behavior. As shown earlier in this work, *per°* mutants carrying a wild-type *per* transgene (PER$^+$) were restored to rhythmic behavior with an average period of 26.2 hr, while *per°*; *glass*–PERc1 flies produce rhythms with a period of 27.7 hr. Both *eya^{ph}* and *oc* have wild-type rhythms in a *per$^+$* background and are arrhythmic in a *per°* background. Again, these data confirm that Drosophila pacemaker cells likely lie outside of conventional photoreceptors.

We moved the *glass*–PERc1 transgene onto an *eya^{ph}* chromosome by genetic recombination and tested circadian behavior in a *per°* background. These animals were rhythmic, with periods indistinguishable from those found with normal eyes. Therefore, the *glass*–PERc1 transgene rescue must derive from expression in ocelli or in the central brain. To determine whether ocellar expression of *glass*–PERc1 was required for behavior rescue, we analyzed the behavior of a *per° oc*; *glass*–PERc1 strain. These also displayed rhythmicity, with the same period as animals with functional ocelli.

Since removal of eyes or ocelli did not affect behavioral rhythmicity, expression of *per* in a few brain neurons seemed the likely source of rescue activity. To test this, we constructed *per° oc*; *eya^{ph}* triple mutants with and without *glass*–PERc1. Crosses were designed to produce equal numbers of *per° oc*; *eya^{ph}* and *per° oc*; *eya^{ph}* *glass*–PERc1 progeny. Transgenic flies could not be identified directly since eye color was used to mark the construct. We therefore used PCR to genotype single flies at the conclusion of behavioral testing (see Experimental Procedures). Of 39 flies tested, 20 carried the transgene. Of these, 14 showed robust rhythms with an average period of 28.9 hr (Table 3). All 19 *per° oc*; *eya^{ph}* flies not carrying the transgene were arrhythmic. Since *per° oc*; *eya^{ph}* mutants lack all external photoreceptors, *per* expression in a few central brain cells appears to be sufficient to rescue the arrhythmia of *per°* mutants.

We also analyzed locomotor activity records from three of the transgenic lines listed in Table 3 for evidence that *glass*–PERc transformants might have been entrained to

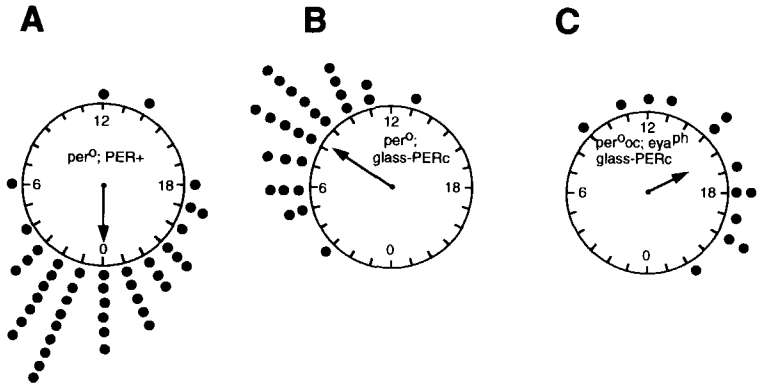

Figure 6. Distribution of Phases for Flies Carrying the PER^{+} and *glass*–PERc Transgenes in Various Mutant Backgrounds

All data were taken from flies whose locomotor activity rhythms were measured for 7 days after release into free run (constant darkness) from LD12:12. Exposure to the indicated light–dark cycle was for 5 days prior to the free run. The three large circles represent 24 hr clock faces. For each clock face, lights on occurred at 0 and lights off at 12 during entrainment. Closed circles show locomotor activity of individual flies. The position of each closed circle on the clock face indicates the time of activity offset for a single fly at the end of 7 days of free run. Clustering of activity offsets should occur only if the flies have similar periods and were entrained to the same Zeitgeber prior to initiation of the free run (see Sehgal et al., 1992). The phase of the population of flies is indicated for each strain by the orientation of the arrow at the center of the clock face. The length of the arrow reflects the degree of synchrony in the population and corresponds to the r value as reviewed in Sehgal et al., 1992. The r values were calculated as 0.73 (A), 0.87 (B), and 0.58 (C). For (A) and (B), the significance of the phase was calculated from the r values by the Rayleigh test as $p < .001$; (C) has a significance value of $p < .01$ (see Sehgal et al., 1992).

the light–dark cycle supplied prior to the free run. Since locomotor activity rhythms were assessed in constant darkness (free run) following exposure to LD12:12 for several days, in Figure 6 the mean phase of locomotor activity at the end of the free run for *per^{0}*; PER^{+} flies (A) is compared with those of *per^{0}*; *glass*–PERc1 (B) and *per^{0} oc*; *eya^{ph}*; *glass*–PERc1 (C) transformants. The average phase of locomotor activity for each strain is indicated by the direction of the arrow on the clock face. All three genotypes showed strong phasing consistent with the original entrainment regime (Figure 6, legend). This suggests that the circadian rhythms generated in *glass*–PERc transgenics were entrained by the light–dark cycle.

Discussion

Biological clocks are acutely sensitive to the phase-resetting properties of light. In Drosophila pseudoobscura, a 1 min pulse of light administered during the dark portion of the cycle can reset the phase of eclosion (pupal hatching) rhythms by up to 12 hr (reviewed in Saunders, 1982; Pittendrigh, 1960, 1967). Although Drosophila melanogaster may be less sensitive to phase-resetting light pulses, a 15–30 min light pulse given at the appropriate point in the cycle can alter phase by up to 4 hr (Saunders, 1982; Dushay et al., 1990; Saunders et al., 1994; Edery et al., 1994a; J. Price, personal communication).

What are the cellular targets of these light pulses? The obvious input pathway for light would be photoreceptor cells responsible for receiving visual stimuli. However, the results of this study (see Table 1) and work by others (Engelmann and Honegger, 1966; Helfrich and Engelmann, 1983; Helfrich, 1986; Dushay et al., 1989; Wheeler et al., 1993) suggest that conventional photoreceptors are dispensable for both entrainment and circadian function in Drosophila. Therefore, there must be light-sensitive cells outside of the eye capable of interpreting light–dark cues.

Pacemaker activity and photoreceptor function are tightly linked in several multicellular organisms. In birds, circadian rhythms are probably generated by single cells with photoreceptor activity: dissociated pineal cells produce melatonin in culture with a circadian rhythm that can be phase shifted by pulses of light (Deguchi, 1979; Robertson and Takahashi, 1988; Zatz et al., 1988). Recent work has identified nonvisual opsins expressed specifically in the pineal cells that may be associated with their circadian function (Okano et al., 1994; Max et al., 1995). In Xenopus, pacemaker activity has been associated with photoreceptor cells composing the retina (Cahill and Besharse, 1993). For the mollusk Bulla, basal retinal neurons contain a circadian pacemaker expressed at the level of single cells in culture (Michel et al., 1993), again indicating a close relationship of circadian and photoreceptor function.

In the present study, we reasoned that a similar, tight linkage between photoreception and clock function might hold for Drosophila. *glass* is required for differentiation of retinal and ocellar photoreceptor neurons of the adult (Moses et al., 1989; Moses and Rubin, 1991; Ellis et al., 1993), and we found that *per*, when expressed under the control of the *glass* promoter, restores circadian rhythmicity to *per^{0}* flies. Robust behavioral rhythms were observed, and PER protein cycling occurred with a circadian period.

In this study we have also shown that the *glass* promoter is active in small clusters of cells bilaterally represented in the central brain of the adult, in addition to its activity in the external photoreceptors. In earlier stages of development, *glass* is expressed in the developing visual system and in a small subset of central nervous system neurons that may include precursors to the adult brain cells described in this work (Moses and Rubin, 1991; Ellis et al., 1993). Given the clear dependence of all known photoreceptor function on *glass* expression, *glass*-producing cells in the central brain seem likely to contribute to the light sensitivity of visual system–deficient flies. Further support for this suggestion is provided by our finding that *per* expression limited to *glass*-expressing cells in the central brain seems to be sufficient to establish entrainable circadian behavioral rhythms (see Table 3; Figure 6). Nevertheless, our studies also establish that *glass*-expressing cells cannot

uniquely provide pacemaker function when *per* is expressed more widely in the brain under the control of its endogenous promoter. This conclusion follows from our observation that per^+ flies deficient for *glass* continue to show circadian rhythms (see Table 1).

Relation of *glass*-Expressing Cells to Previously Described LNs and PDH-Immunoreactive Cells of the Central Brain

Ewer et al. (1992) concluded from their extensive genetic mosaic studies that *per* expression in two bilaterally represented clusters of neurons, the LNs, was unlikely to be required for circadian rhythmicity. These studies did show that *per* expression in glial cells, which in the mosaics studied occupied a region of the brain distinct from those containing *per*-expressing neurons, could establish weak behavioral rhythmicity. In light of prior work on the arrhythmic mutation *disco*, which affects the presence and location of the LNs, Ewer et al. (1992) suggested that the role of *per*-expressing glia might be dependent on the presence (and therefore some function) of LNs, regardless of their *per* genotype. Accordingly, even LNs that were genotypically per^o would support behavioral rhythmicity provided *per* were expressed in the glia. Interestingly, the LNs were implicated as potential pacemaker cells by experiments of a different sort, involving expression of a promoterless *per* transgene in per^o flies (Frisch et al., 1994). This truncated gene, which lacks the *per* promoter, transcription start site, first exon, and most of the first and largest intron, is expressed at some chromosomal locations, perhaps through acquisition of a novel promoter. As might be expected, its pattern of expression varies in a strain-dependent fashion. A single line was identified that showed weak behavioral rhythmicity, while possibly limiting PER expression to LNs. Another rhythmic line carrying the promoterless transgene at an unrelated chromosomal location produced PER in a wider variety of cell types. These cells corresponded to a more extensive subset of cells expressing the endogenous *per* gene, including the LNs (Frisch et al., 1994).

Some LNs appear to be located at positions equivalent to cells that are immunoreactive for PDH. This PDH reactivity may be significant, as release of this hormone is associated with migration of retinal screening pigments during light adaptation in some Crustacea (Rao and Riehm, 1989), and homologous peptides from several insects exhibit related activity (reviewed by Helfrich-Forster and Homberg, 1993). There is some evidence that regeneration of PDH-expressing cells may be linked to the reestablishment of circadian rhythms in neural ablation experiments with the cockroach (Stengl and Homberg, 1992, 1994).

Helfrich-Forster (1995) has reported that PDH-expressing neurons in Drosophila contain PER protein. Two apposed cell clusters, including 4 PDH-immunoreactive cells each, have been observed in lateral positions on either side of the brain (Helfrich-Forster, 1995). Immunocytochemical studies indicate that these PDH-expressing cells are identical to the ventral-most *per*-expressing LNs (Helfrich-Forster, 1995). Antibodies to PDH reveal neuronal processes extending from these cells into the medulla of the optic lobe, along the posterior optic tract (which intersects the esophagus), and projecting dorsally to the calyces of the mushroom bodies (Helfrich-Forster and Homberg, 1993).

Indeed, some of the *glass*-expressing central brain cells identified in this study appear to correspond to a subset of the LNs. The dorsal-most *glass*-expressing cells are found in a position similar to that described for the dorsal-most *per*-expressing LNs (Ewer et al., 1992; Helfrich-Forster, 1995). Also, *glass*-expressing cells found at the level of the esophagus occupy a position within the central brain that coincides with the PDH- and PER-immunoreactive LNs (Ewer et al., 1992; Frisch et al., 1994; Helfrich-Forster, 1995). However, most *glass*-expressing central brain cells lie well below the level of the esophagus and near the ventral border of the central brain. It may be important that *per* expression in the ventral-most regions of the central brain was shown to be sufficient for weak pacemaker activity in prior mosaic studies (Ewer et al., 1992). While a role for glia was originally implied by this mapping, a more pertinent feature of this region of the brain may be one that unites it with areas containing the LNs—the common presence of *glass*-expressing cells. We suggest that earlier mosaic and transgene studies may have been influenced by activities emanating from the different clusters of *glass*-expressing cells.

While we were unable to determine whether all *glass*-expressing cells of the central brain contribute equally to circadian rhythmicity, our investigations do indicate that they share a common developmental pathway involving function of the *glass* protein, and all of the identified cells occupy closely related positions in the central brain with respect to their proximity to the optic lobes. In future work, expression of an alternative cytoplasmic marker under the control of the *glass* promoter might be useful in determining whether ventral cells that express *glass* project into areas of the brain containing the LNs or their fibers (Helfrich-Forster and Homberg, 1993).

Why Does Expression of the *glass*-PERc Transgene Lead to Cycling Levels of PER Protein?

It has been demonstrated that fusion of the *per* promoter to an unrelated transcription unit can confer cycling gene expression (Hardin et al., 1992). In this paper, we expressed *per* under the control of a heterologous promoter, *glass*. In contrast to *per*, cycling regulation of *glass* RNA was not seen. Thus, the *glass* promoter may allow us to uncouple molecular and behavioral rhythms. Surprisingly, cycling of PER immunoreactivity continued to be observed in photoreceptors of both transformed lines examined, *glass*–PERc1 and *glass*–PERc2. Our results demonstrate that PER protein cycling can occur without feedback on the *per* promoter. Earlier work with a promoterless transgene suggested that *per* mRNA cycling can occur in the absence of the *per* promoter (Frisch et al., 1994). As well, a recent study of cycling PER–β-gal fusion proteins suggests that at least one postranscriptional control point involves temporal control of PER proteolysis (M. Dembinska, R. Stanewsky, J. Hall, and M. Rosbash, personal

communication). In the present study, PER cycling was observed in each of two independent lines assayed immunocytochemically. This indicates that the regulation of cycling was dependent on transcriptional or posttranscriptional regulatory sequences within the transgene or encoded PER protein.

Conclusions

Transplantation and tissue ablation studies have been used to map circadian pacemaker cells to discrete areas of the rodent and avian brains and to the retinae of Xenopus and certain mollusks. The results of the present work indicate that expression of *per* in a small group of central brain cells that also expresses *glass*, a gene essential for known photoreceptor function, is sufficient to provide pacemaker function in adult Drosophila.

As mentioned earlier, strong evidence for cellular autonomy of circadian pacemaker activity has been obtained in birds (Deguchi, 1979; Robertson and Takahashi, 1988; Zatz et al., 1988) and particularly in the mollusk Bulla (Michel et al., 1993). Recent studies demonstrating that circadian rhythms of *per* transcription can be modulated by altering the abundance or subcellular location of the PER protein are consistent with cell-autonomous pacemaker activity in Drosophila (Zeng et al., 1994; Sehgal et al., 1994; Vosshall et al., 1994). The results of the present study and related work by Frisch et al. (1994) are more direct, as they suggest that expression of PER in a total of 10–20 cells of the central brain is sufficient to establish robust circadian behavioral rhythms, increasing the likelihood that pacemaker activity is expressed in the Drosophila brain at the level of single cells.

Do *glass*-expressing cells in the central brain contribute to the circadian rhythmicity of wild-type Drosophila? As indicated in this study, the *glass*–PER transgene is expressed in a subset of those cells expressing *per* under the control of its own promoter. As well, biochemical and genetic studies of *per* have indicated likely direct interactions of the PER protein and other cellular factors, some of which are themselves essential for circadian rhythmicity (Edery et al., 1994b; Vosshall et al., 1994). Thus, circadian behavioral rhythms should be restored only if *per* expression is directed to cells expressing further molecular components of Drosophila's clock. Evidently *per* and these additional factors are normally coexpressed in *glass*-producing cells. The results strongly suggest that *glass*-expressing cells can provide circadian pacemaker function in wild-type flies.

Experimental Procedures

glass–PERc Construct

The *glass* promoter was obtained by screening a Drosophila Canton-S genomic library in Charon 4 (Maniatis et al., 1978) with the insert from plasmid pBS6.5R (Moses et al., 1989). DNA was prepared from purified positive phage and digested with EcoRI to release the insert. The insert was digested with SalI and BanII, and the expected 5116 nucleotide fragment representing upstream sequences previously shown to be sufficient to rescue *glass* mutant phenotypes (Moses et al., 1989) was blunt ligated into the XbaI site of plasmid pSL1180 (Pharmacia). Although the entire *glass* promoter isolated from the Maniatis genomic library was not sequenced, the restriction map matched that reported by Moses et al. (1989) (data not shown), and the sequence of both ends was identical to the reported genomic sequence of *glass* (Moses et al., 1989) (data not shown). The minimal region containing the regulatory and coding sequences necessary to obtain rescue of the *glass* mutant phenotype extends from a SalI site designated *glass* nucleotide 1 to a SalI site designated *glass* nucleotide 9949 by the mapping of Moses et al. (1989). For the purposes of expressing *per* from the *glass* promoter, we used a fragment extending from the SalI site at *glass* nucleotide 1 to a position 29 nucleotides 5′ of the AUG initiation codon for *glass* (BanII site at *glass* nucleotide 5116; Moses et al., 1989). *per* genomic coding sequences and termination and polyadenylation sequences contained in an ~8 kb XbaI–XhoI fragment were cloned into the EcoRV site of the pSL1180 polylinker; 36 bp of the pSL1180 polylinker lie between the end of the *glass* promoter and the *per* XbaI site at *per* nucleotide 2449. A 345 nucleotide portion of the first *per* intron (+2270 to +2661 relative to the transcription start site) is included in this *glass*–PERc construct. Fusions of *per* promoter sequences to the CAT gene by Hardin et al. (1992) indicated that the minimal region containing the promoter and upstream regulatory sequences for *per* expression and oscillation is −1300 to +1 (transcription start), although normal levels required a region from −4000 to +1. The *glass*–PERc construct was excised from pSL1180 by digesting with HpaI and ApaI, blunt ending, and ligating into the XbaI site of Casper (Pirrotta et al., 1985; Thummel et al., 1988) for P element–mediated transformation of *y w* embryos. Seven independent transformed lines were generated, and maintained and tested as heterozygotes after crossing into a *y per*^{o} w background. All *per* nucleotide numbers used in this study were derived from the complete *per* genomic sequence (Jackson et al., 1986; Citri et al., 1987) as corrected for Canton-S by M. K. Baylies (personal communication).

β-gal Fusion Constructs

PER1–95β-gal

An XhoI linker (5′-CCTCGAGG-3′; New England Biolabs) was inserted at nucleotide 3183 after cutting a plasmid containing a *per* XbaI–XmaI (*per* nucleotides 2449–4370) fragment with AccI and blunt ending. The SphI–XbaI *per* promoter fragment was added to the above plasmid; the entire *per* fragment was excised with XhoI and subcloned into the pCaSpeR-βgal3 vector (Thummel et al., 1988) altered to create an XhoI site in the polylinker (Vosshall, 1993). This produces a fusion protein encoding the first 95 amino acids of PER (PER amino acid numbering was from the Canton-S strain; Baylies et al., 1993), followed by β-gal. Three independent lines were generated and analyzed.

glass–PER1–95β-gal

The *glass* promoter was exchanged for the *per* promoter in the PER1–95β-gal construct by digesting PER1–95β-gal with XbaI, which cuts the DNA 5′ of the *per* promoter at the XbaI site in the pCaSpeR-βgal3 polylinker and at *per* nucleotide 2449. The *per* promoter fragment, extending from the XhoI site to the XbaI site at *per* nucleotide 2449, was removed. The *glass* promoter fragment was excised from pSL1180 by digesting with HpaI and BglII and blunt end ligated into the plasmid described above. Four independent transformed lines were generated and analyzed.

Germline Transformation

Germline transformation of Drosophila was carried out essentially as described (Rubin and Spradling, 1982; Spradling, 1986) using 400 μg/ml cesium-banded construct DNA and 100 μg/ml wings clipped helper DNA (Karess and Rubin, 1984). Constructs were injected into a *y w* strain. Multiple independent transformed lines were generated, crossed into an arrhythmic *y per*^{o} *w* background, and maintained and tested as heterozygotes.

RNase Protection Assays

RNA was prepared by a modification (Puissant and Houdebine, 1990) of the method of Chomczynski and Sacchi (1987) from mass-isolated adult fly heads. For each assay, 40 μg of total RNA was used. The reference probe was Drosophila tubulin. A genomic 1.4 kb XhoI fragment (glass nucleotides 3949–5344; Moses et al., 1989) was used to generate an RNA probe that protects *glass* exon 1 (nucleotides 4189–4410; 221 bp fragment) and exon 2 (nucleotides 5057–5193; 136 bp fragment). *per* RNA was detected with an EcoRI–PstI probe from the *per* cDNA that protects a 340 bp fragment consisting of 198 bp of exon 1 (nucleotides 301–499) and a 142 bp fragment from exon 2 (nucleotides 2794–2936). Radioactive RNA probes were synthesized

using SP6, T3, or T7 RNA polymerase following the recommendations of the manufacturer (Promega). Reference tubulin probes used 10-fold less [^{32}P]rUTP than experimental probes. Probes were annealed to RNA for 16 hr at 45°C and processed thereafter according to standard procedures (Sambrook et al., 1989). Protected fragments were separated on a sequencing gel and exposed to X-ray film and, subsequently, to phosphorimager screens. Quantification was by phosphorimager analysis (Molecular Dynamics).

Immunocytochemistry

Adult fly heads were prepared for sectioning by anesthetizing flies with diethyl ether. Heads were removed with razor blades and embedded in TissueTek OCT Compound (Miles Inc., Diagnostic Division). Frozen sections (14 μm) were cut and collected on silanized slides (Vectabond Reagent; Vector Labs). Sections were fixed immediately in 2% paraformaldehyde in PBS for 15 min, washed in PBS, and permeabilized with PBS and 0.1% Triton X-100. Blocking solution containing 0.1% Triton X-100 and 10% heat-inactivated normal goat serum in PBS was applied to the sections for 30 min. The sections were incubated with primary antibody diluted in blocking solution for 16 hr at room temperature. Antibody staining was continued as described (Vosshall et al., 1994) by incubating sections with biotin-conjugated goat anti-rabbit IgG, followed by Vectastain ABC kit (Vector Labs) incubation using the horseradish peroxidase substrate 3′,3′-diaminobenzidine (Polysciences). Diaminobenzidine staining was for 10 min in 0.2 mg/ml diaminobenzidine, 0.024% hydrogen peroxide. Sections were mounted with Aquamount (Lerner Laboratories). Anti-β-gal polyclonal antibody (Cappel/Organon Technika) was preabsorbed with fly acetone powder and used at a final dilution of 1:1000. Polyclonal anti-PER antiserum (kindly provided by L. Saez) was generated in rabbits against full-length PER protein as described (Saez and Young, 1988; Vosshall et al., 1994). This antiserum was preabsorbed against a lysate prepared from *y per^{o} w* heads and used at a final dilution of 1:30.

Locomotor Assays

Locomotor behavior was tested as described (Sehgal et al., 1994). Animals were entrained for at least 5 days to LD12:12 at 25°C, and locomotor activity was monitored for at least 7 days in constant darkness. Period length was analyzed as described (Sehgal et al., 1994) using χ^2 periodogram analysis (Sokolove and Bushell, 1978) (software obtained from Mini-Mitter). The phase of each fly's activity rhythm in Figure 6 was taken as the time of activity offset at the end of day 7 in constant darkness. Degree of synchrony and strength of phasing were calculated as described (Sehgal et al., 1992) (see also Figure 6, legend).

Fly Stocks

Flies were raised on cornmeal–yeast–agar medium supplemented with Tegosept to retard mold growth at 25°C. The *glass*–PERc1 P element (second chromosome insertion) was recombined onto the *eya^{ph}* chromosome using a *w; eya^{ph}*/CyO strain kindly provided by Nancy Bonini. To generate *eya^{ph}* (eyeless), *oc, per^{o}* triple mutants carrying the *glass*–PERc1 P element, the following cross was designed: *y per^{o}* w oc/*y per^{o} w*; *eya^{ph}/eya^{ph}* females × *y per^{o} w* oc/Y; *eya^{ph} glass*–PERc1/CyO males. Locomotor behavior was monitored for *y per^{o} w oc*; *eya^{ph}* male progeny for 7 days, after which genomic DNA was prepared from individual flies. Triple mutants were genotyped using PCR with a forward *glass* primer (LV81: CAAGATGAAGCGTAGGAAAAGCAG; *glass* genomic sequence nucleotides 5073–5096) and a reverse *per* primer (LV82: TTCGAGGAGATTCCGTGACTA CTG; *per* genomic sequence nucleotides 2665–2642) to yield a 316 bp product specific for the *glass*–PERc transgene. Each experiment included an internal positive control 393 bp fragment amplified from per exon 8 (LV22: GAGCAAGATCATGGAGCACC forward; *per* genomic sequence nucleotides 6841–6860; LV24: GCTTGGCTTGAGATCTACAT reverse; *per* genomic sequence nucleotides 7234–7215). Fly stocks were obtained from the following investigators: S. E. Celniker and E. M. Meyerowitz (*Mc*); R. Mestel and S. L. Zipursky (*chp* alleles); S. Britt and C. S. Zuker (*eya^{1}*); N. Bonini and S. Benzer (*eya^{ph}*); K.-F. Fischbach (*mud*); W. S. Stark (*rdo* and *none*); R. Buchanan and S. Benzer (*drd*); K. Moses and G. M. Rubin (*gl* alleles); and Kathy Matthews of the Indiana University Drosophila stock center (all other stocks).

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References

Aronson, B. D., Johnson, K. A., Loros, J. J., and Dunlap, J. C. (1994). Negative feedback defining a circadian clock: autoregulation of the clock gene *frequency*. Science *263*, 1578–1584.

Bargiello, T. A., and Young, M. W. (1984). Molecular genetics of a biological clock in *Drosophila*. Proc. Natl. Acad. Sci. USA *81*, 2142–2146.

Baylies, M. K., Bargiello, T. A., Jackson, F. R., and Young, M. W. (1987). Changes in abundance or structure of the *per* gene product can affect periodicity of the *Drosophila* clock. Nature *326*, 390–392.

Baylies, M. K., Vosshall, L. B., Sehgal, A., and Young, M. W. (1992). New short period mutations of the Drosophila clock gene *per*. Neuron *9*, 575–581.

Baylies, M. K., Weiner, L., Vosshall, L. B., Saez, L., and Young, M. W. (1993). Genetic, molecular, and cellular studies of the *period* locus and its products in Drosophila melanogaster. In Molecular Genetics of Biological Rhythms, M. W. Young, ed. (New York: Marcel Dekker), pp. 123–153.

Bonini, N., Leiserson, W. M., and Benzer, S. (1993). The *eyes absent* gene: genetic control of cell survival and differentiation in the developing Drosophila eye. Cell *72*, 379–395.

Cahill, G. M., and Besharse, J. C. (1993). Circadian clock functions localized in Xenopus retinal photoreceptors. Neuron *10*, 573–577.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

Citri, Y., Colot, H. V., Jacquier, A. C., Yu, Q., Hall, J. C., Baltimore, D., and Rosbash, M. (1987). A family of unusually spliced biologically active transcripts encoded by a *Drosophila* clock gene. Nature *326*, 42–47.

Colley, N. J., Baker, E. K., Stamnes, M. A., and Zuker, C. S. (1991). The cyclophilin homolog ninaA is required in the secretory pathway. Cell *67*, 255–263.

Deguchi, T. (1979) A circadian oscillator in cultured cells of chicken pineal gland. Nature *282*, 94–96.

Dunlap, J. C. (1993). Genetic analysis of circadian clocks. Annu. Rev. Physiol. *55*, 683–728.

Dunlap, J. C., Liu, Q., Johnson, K. A., and Loros, J. J. (1993). Genetic and molecular dissection of the Neurospora clock. In Molecular Genetics of Biological Rhythms, M. W. Young, ed. (New York: Marcel Dekker), pp. 37–54.

Dushay, M. S., Rosbash, M., and Hall, J. C. (1989). The *disconnected* visual system mutations in *Drosophila melanogaster* drastically disrupt circadian rhythms. J. Biol. Rhyth. *4*, 1–27.

Dushay, M. S., Konopka, R. J., Orr, D., Greenacre, M. L., Kyriacou, C. P., Rosbash, M., and Hall, J. C. (1990). Phenotypic and genetic analysis of *Clock*, a new circadian rhythm mutant in *Drosophila melanogaster*. Genetics *125*, 557–578.

Edery, I., Rutila, J. E., and Rosbash, M. (1994a). Phase shifting of the circadian clock by induction of the *Drosophila period* protein. Science *263*, 237–240.

Edery, I., Zwiebel, L. J., Dembinska, M. E., and Rosbash, M. (1994b). Temporal phosphorylation of the *Drosophila* period protein. Proc. Natl. Acad. Sci. USA *91*, 2260–2264.

Edmunds, L. E., Jr. (1988). Cellular and Molecular Bases of Biological Clocks (New York: Springer Verlag).

Ellis, M. C., O'Neill, E. M., and Rubin, G. M. (1993). Expression of *Drosophila glass* protein and evidence for negative regulation of its activity in non-neuronal cells by another DNA-binding protein. Development *119*, 855–865.

Engelmann, W., and Honegger, H. W. (1966). Circadian eclosion rhythm of an eyeless *Drosophila melanogaster* mutant. Naturwissenschaften *53*, 588.

Eskin, A. (1979). Circadian system of the *Aplysia* eye: properties of the pacemaker and mechanisms of its entrainment. Fed. Proc. Fed. Am. Soc. Exp. Biol. *38*, 2573–2579.

Ewer, J., Frisch, B., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1992). Expression of the *period* clock gene within different cell types in the brain of *Drosophila* adults and mosaic analysis of these cells' influence on circadian behavioral rhythms. J. Neurosci. *12*, 3321–3349.

Feldman, J. F., and Hoyle, M. N. (1973). Isolation of circadian clock mutants of *Neurospora crassa*. Genetics *75*, 605–613.

Fischbach, K. F., and Technau, G. (1984). Cell degeneration in the developing optic lobes of the sine oculis and small-optic-lobes mutants of *Drosophila melanogaster*. Dev. Biol. *104*, 219–239.

Fleissner, G. (1982). Isolation of an insect circadian clock. J. Comp. Physiol. *149*, 311–316.

Frisch, B., Hardin, P. E., Hamblen-Coyle, M. J., Rosbash, M., and Hall, J. C. (1994). A promoterless *period* gene mediates behavioral rhythmicity and cyclical *per* expression in a restricted subset of the Drosophila nervous system. Neuron *12*, 555–570.

Garen, S. H., and Kankel, D. R. (1983). Golgi and genetic mosaic analyses of visual system mutants in *Drosophila melanogaster*. Dev. Biol. *96*, 445–466.

Hall, J. C., and Rosbash, M. R. (1993). Oscillating molecules and how they move circadian clocks across evolutionary boundaries. Proc. Natl. Acad. Sci. USA *90*, 5382–5383.

Handler, A. M., and Konopka, R. J. (1979). Transplantation of a circadian pacemaker in *Drosophila*. Nature *279*, 236–238.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1990). Feedback of the *Drosophila period* gene product on circadian cycling of its messenger RNA levels. Nature *343*, 536–540.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1992). Circadian oscillations in *period* gene mRNA levels are transcriptionally regulated. Proc. Natl. Acad. Sci. USA *89*, 11711–11715.

Hardin, P. E., Hall, J. C., and Rosbash, M. (1993). Circadian cycling in the levels of protein and mRNA from Drosophila melanogaster's *period* gene. In Molecular Genetics of Biological Rhythms, M. W. Young, ed. (New York: Marcel Dekker), pp. 155–169.

Harris, W. A., Stark, W. S., and Walker, J. A. (1976). Genetic dissection of the photoreceptor system in the compound eye of *Drosophila melanogaster*. J. Physiol. *256*, 415–439.

Helfrich, C. (1986). Role of the optic lobes in the regulation of the locomotor activity rhythm of *Drosophila melanogaster*: behavioral analysis of neural mutants. J. Neurogenet. *3*, 321–343.

Helfrich, C., and Engelmann, W. (1983). Circadian rhythm of the locomotor activity in *Drosophila melanogaster* mutants "*sine oculis*" and "*small optic lobes*." Physiol. Entomol. *1*, 3–15.

Helfrich, C., Cymborowski, B., and Engelmann, E. (1985). Circadian activity rhythm of the house fly continues after optic tract severance and lobectomy. Chronobiol. Int. *2*, 19–32.

Helfrich-Forster, C. (1995). 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. USA *92*, 612–616.

Helfrich-Forster, C., and Homberg, U. (1993). Pigment-dispersing hormone-immunoreactive neurons in the nervous system of wild-type *Drosophila melanogaster* and of several mutants with altered circadian rhythmicity. J. Comp. Neurol. *337*, 177–190.

Hofbauer, A., and Buchner, E. (1989). Does *Drosophila* have seven eyes? Naturwissenschaften *76*, 335–336.

Hotta, Y., and Benzer, S. (1972). Mapping of behaviour in *Drosophila* mosaics. Nature *240*, 527–535.

Huang, Z. J., Edery, I., and Rosbash, M. (1993). PAS is a dimerization domain common to *Drosophila* Period and several transcription factors. Nature *364*, 259–262.

Jackson, F. R. (1993). Circadian rhythm mutants of Drosophila. In Molecular Genetics of Biological Rhythms, M. W. Young, ed. (New York: Marcel Dekker), pp. 91–121.

Jackson, F. R., Bargiello, T. A., Yun, S. H., and Young, M. W. (1986). Product of *per* locus of *Drosophila* shares homology with proteoglycans. Nature *320*, 185–188.

Johannsen, O. A. (1924). Eye structure in normal and eye-mutant *Drosophila*. J. Morphol. Physiol. *39*, 337–349.

Karess, R. E., and Rubin, G. M. (1984). Analysis of P transposable element functions in Drosophila. Cell *38*, 135–146.

Klein, D. C. (1978). The pineal gland: a model of neuroendocrine regulation. In The Hypothalamus, S. Reichlin, R. J. Baldessarini, and J. B. Martin, eds. (New York: Raven Press), pp. 303–327.

Konopka, R. J., and Benzer, S. (1971). Clock mutants of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *68*, 2112–2116.

Lickey, M. E., Block, G. D., Hudson, D. J., and Smith, J. T. (1976). Circadian oscillators and photoreceptors in the gastropod, *Aplysia*. Photochem. Photobiol. *23*, 253–273.

Lindsley, D., and Zimm, G. G. (1992). The Genome of *Drosophila melanogaster* (San Diego, California: Academic Press).

Liu, X., Lorenz, L., Yu, Q., Hall, J. C., and Rosbash, M. (1988). Spatial and temporal expression of the *period* gene in *Drosophila melanogaster*. Genes Dev. *2*, 228–238.

Liu, X., Zwiebel, L. J., Hinton, D., Benzer, S., Hall, J. C., and Rosbash, M. (1992). The *period* gene encodes a predominantly nuclear protein in adult *Drosophila*. J. Neurosci. *12*, 2735–2744.

Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K., and Efstratiadis, A. (1978). The isolation of structural genes from libraries of eucaryotic DNA. Cell *15*, 687–701.

Max, M., McKinnon, P. J., Seidenman, K. J., Barrett, R. K., Applebury, M. L., Takahashi, J. S., and Margolskee, R. F. (1995). Pineal opsin: a nonvisual opsin expressed in chick pineal. Science *267*, 1502–1506.

Meyerowitz, E. M., and Kankel, D. R. (1978). A genetic analysis of visual system development in *Drosophila melanogaster*. Dev. Biol. *62*, 112–142.

Michel, S., Geusz, M. E., Zaritsky, J. J., and Block, G. D. (1993). Circadian rhythm in membrane conductance expressed in isolated neurons. Science *259*, 239–241.

Monsma, S. A., Ard, R., Lis, J. T., and Wolfner, M. F. (1988). Localized heat-shock induction in *Drosophila melanogaster*. J. Exp. Zool. *247*, 279–284.

Moore, R. Y., and Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res. *42*, 201–206.

Moses, K., and Rubin, G. M. (1991). *glass* encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing *Drosophila* eye. Genes Dev. *5*, 583–593.

Moses, K., Ellis, M. C., and Rubin, G. M. (1989). The *glass* gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. Nature *340*, 531–536.

Okano, T., Yoshizawa, T., and Fukuda, Y. (1994). Pinopsin is a chicken pineal photoreceptive molecule. Nature *372*, 94–97.

O'Tousa, J. E., Baehr, W., Martin, R. L., Hirsch, J., Pak, W. L., and Applebury, M. L. (1985). The Drosophila *ninaE* gene encodes an opsin. Cell *40*, 839–850.

Page, T. L. (1982). Transplantation of the cockroach circadian pacemaker. Science *216*, 73–75.

Page, T. L. (1994). Time is the essence: molecular analysis of the biological clock. Science *263*, 1570–1572.

Pirrotta, V., Steller, H., and Bozzetti, M. P. (1985). Multiple upstream regulatory elements control the expression of the *Drosophila white* gene. EMBO J. *4*, 3501–3508.

Pittendrigh, C. S. (1960). Circadian rhythms and the circadian organization of living systems. Cold Spring Harbor Symp. Quant. Biol. *25*, 159–184.

Pittendrigh, C. S. (1967). Circadian systems. I. The driving oscillation and its assay in *Drosophila pseudoobscura*. Proc. Natl. Acad. Sci. USA *58*, 1762–1767.

Puissant, C., and Houdebine, L.-M. (1990). An improvement of the single-step method of RNA isolation by acid guanidinium-thiocyanate phenol-chloroform extraction. BioTechniques *8*, 148–149.

Quiring, R., Walldorf, U., Kloter, U., and Gehring, W. J. (1994). Homology of the *eyeless* gene of *Drosophila* to the *Small eye* gene in mice and *Aniridia* in humans. Science *265*, 785–789.

Ralph, M. R., and Menaker, M. (1988). A mutation of the circadian system in golden hamsters. Science *241*, 1225–1227.

Rao, K. R., and Riehm, J. P. (1989). The pigment-dispersing hormone family: chemistry, structure activity relation, and distribution. Biol. Bull. *177*, 225–229.

Ready, D. F. (1989). A multifaceted approach to neural development. Trends Neurosci. *12*, 102–110.

Reddy, P., Zehring, W. A., Wheeler, D. A., Pirrotta, V., Hadfield, C., Hall, J. C., and Rosbash, M. (1984). Molecular analysis of the *period* locus of Drosophila melanogaster and identification of a transcript involved in biological rhythms. Cell *38*, 701–710.

Roberts, M., and Block, G. (1983). Mutual coupling between the ocular circadian pacemakers of *Bulla gouldiana*. Science *221*, 87–89.

Robertson, L. M., and Takahashi, J. S. (1988). Circadian clock in cell culture. I. Oscillation of melatonin release from dissociated chick pineal cells in flow-through microcarrier culture. J. Neurosci. *8*, 12–21.

Rubin, G. M., and Spradling, A. C. (1982). Genetic transformation of *Drosophila melanogaster* with transposable element vectors. Science *218*, 348–353.

Rusak, B. (1977). The role of the suprachiasmatic nuclei in the generation of circadian rhythms in the golden hamster, *Mesocricetus auratus*. J. Comp. Physiol. *118*, 145–164.

Saez, L., and Young, M. W. (1988). *In situ* localization of the per clock protein during development of *Drosophila melanogaster*. Mol. Cell. Biol. *8*, 5378–5385.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

Saunders, D. S. (1982). Insect Clocks, Second Edition (Oxford: Pergamon Press).

Saunders, D. S., Gillanders, S. W., and Lewis, R. D. (1994). Light pulse phase response curves for the locomotor activity rhythm of *period* mutants of *Drosophila melanogaster*. J. Insect Physiol. *40*, 957–968.

Sehgal, A., Man. B., Price, J., Vosshall, L. B., and Young, M. W. (1991). New clock mutations in Drosophila. In The Temporal Control of Drug Delivery, W. J. Hruskesky, R. Langer, and F. Theeuwes, eds. (New York: Annals of the New York Academy of Sciences), pp. 1–9.

Sehgal, A., Price, J., and Young, M. W. (1992). Ontogeny of a biological clock in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA *89*, 1423–1427.

Sehgal, A., Price, J. L., Man, B., and Young, M. W. (1994). Loss of circadian behavioral rhythms and *per* RNA oscillations in the *Drosophila* mutant *timeless*. Science *263*, 1603–1606.

Shieh, B.-H., Stamnes, M. A., Seavello, S., Harris, G. L., and Zuker, C. S. (1989). The *ninaA* gene required for visual transduction in *Drosophila* encodes a homologue of cyclosporin A-binding protein. Nature *338*, 67–70.

Siwicki, K. K., Eastman, C., Petersen, G., Rosbash, M., and Hall, J. C. (1988). Antibodies to the *period* gene product of Drosophila reveal diverse tissue distribution and rhythmic changes in the visual system. Neuron *1*, 141–150.

Smith, H. K., and O'Kane, C. J. (1991). Use of a cytoplasmically localized P-lacZ fusion to identify cell shapes by enhancer trapping in *Drosophila*. Roux's Arch. Dev. Biol. *200*, 306–311.

Sokolove, P. G., and Bushell, W. N. (1978). The chi square periodogram: its utility for analysis of circadian rhythms. J. Theor. Biol. *72*, 131–160.

Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila*: A Practical Approach, D. B. Roberts, ed. (Oxford: IRL Press), pp. 175–197.

Stengl, M., and Homberg, U. (1992). Pigment-dispersing hormone-immunoreactive neurons as possible candidates for the circadian pacemaker in orthopteroid insects. In Rhythmogenesis in Neurons and Networks, N. Elsner and D. W. Richter, eds. (Stuttgart, Germany: Thieme), p. 89.

Stengl, M., and Homberg, U. (1994). Pigment-dispersing hormone-immunoreactive neurons in the cockroach *Leucophaea maderae* share properties with circadian pacemaker neurons. J. Comp. Physiol. *175*, 203–213.

Takahashi, J. S., and Menaker, M. (1979). Physiology of avian circadian pacemakers. Fed. Proc. Fed. Am. Soc. Exp. Biol. *38*, 2583–2588.

Takahashi, J. S., and Zatz, M. (1982). Regulation of circadian rhythmicity. Science *217*, 1104–1111.

Thummel, C. S., Boulet, A. M., and Lipshitz, H. D. (1988). Vectors for *Drosophila melanogaster* P-element-mediated transformation and tissue culture transfection. Gene *74*, 445–456.

Tomlinson, A., and Ready, D. F. (1987). Neuronal differentiation in the *Drosophila* ommatidium. Dev. Biol. *120*, 366–376.

Tomlinson, A., Kimmel, B. E., and Rubin, G. M. (1988). *rough*, a Drosophila homeobox gene required in photoreceptors R2 and R5 for inductive interactions in the developing eye. Cell *55*, 771–784.

Truman, J. W. (1976). Extraretinal photoreception in insects. Photochem. Photobiol. *23*, 215–225.

Vitaterna, M. H., King, D. P., Chang, A.-M., Kornhauser, J. M., Lowrey, P. L., McDonald, J. D., Dove, W. F., Pinto, L. H., Turek, F. W., and Takahashi, J. S. (1994). Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. Science *264*, 719–725.

Vosshall, L. B. (1993). Regulated nuclear localization of the *period* protein of *Drosophila melanogaster* and its role in controlling circadian rhythms. Ph.D. thesis, Rockefeller University.

Vosshall, L. B., Price, J. L., Sehgal, A., Saez, L., and Young, M. W. (1994). Block in nuclear localization of *period* protein by a second clock mutation, *timeless*. Science *263*, 1606–1609.

Wheeler, D. A., Kyriacou, C. P., Greenacre, M. L., Yu, Q., Rutila, J. E., Rosbash, M., and Hall, J. C. (1991). Molecular transfer of a species-specific behavior from *Drosophila simulans* to *Drosophila melanogaster*. Science *251*, 1082–1085.

Young, M. W., ed. (1993). Molecular Genetics of Biological Rhythms (New York: Marcel Dekker).

Zatz, M., Mullen, D. A., and Moskal, J. R. (1988). Photoendocrine transduction in cultured chick pineal cells: effects of light, dark, and potassium on the melatonin rhythm. Brain Res. *438*, 199–215.

Zeng, H., Hardin, P. E., and Rosbash, M. (1994). Constitutive overexpression of the *Drosophila period* protein inhibits *period* mRNA cycling. EMBO J. *13*, 3590–3598.

Zerr, D. M., Hall, J. C., Rosbash, M., and Siwicki, K. K. (1990). Circadian fluctuations of *period* protein immunoreactivity in the CNS and the visual system of *Drosophila*. J. Neurosci. *10*, 2749–2762.

Zuker, C. S., Cowman, A. F., and Rubin, G. M. (1985). Isolation and structure of a rhodopsin gene from D. melanogaster. Cell *40*, 851–858.

Note Added in Proof

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