Multiple circadian-regulated elements contribute to cycling *period* gene expression in *Drosophila*

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A new regulatory element necessary for the correct temporal expression of the *period* (*per*) gene was identified by monitoring real-time *per* expression in living individual flies carrying two different *period*–*luciferase* transgenes. *luciferase* RNA driven from only the *per* promoter was not sufficient to replicate the normal pattern of *per* RNA cycling; however, a *per*–*luc* fusion RNA driven from a transgene containing additional *per* sequences cycled identically to endogenous *per*. The results indicate the existence of at least two circadian-regulated elements—one within the promoter and one within the transcribed portion of the *per* gene. Phase and amplitude analysis of both *per*–*luc* transgenes revealed that normal *per* expression requires the regulation of these elements at distinct phases and suggests a mechanism by which biological clocks sustain high-amplitude feedback oscillations.

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Introduction

Circadian rhythms are daily oscillations of biological processes that occur with a periodicity of ~24 h. These oscillations persist under constant environmental conditions, indicating that they are controlled by an endogenous pacemaker known as a biological clock. A large body of evidence implicates both the *period* (*per*) and *timeless* (*tim*) genes of *Drosophila melanogaster* as central components of the circadian clock (reviewed by Sehgal *et al.*, 1996). In constant darkness and temperature (DD), the null mutations *per*⁰¹ and *tim*⁰¹ each cause arrhythmic pupal eclosion and locomotor activity (Konopka and Benzer, 1971; Sehgal *et al.*, 1994). The mRNA and protein gene products of both *per* and *tim* show daily oscillations in abundance, and these fluctuations are also abolished by either the *per*⁰¹ or *tim*⁰¹ mutation (reviewed by Sehgal *et al.*, 1996). The molecular oscillations of both *tim* protein (TIM) and *per* protein (PER) appear similar. However, TIM is degraded rapidly in the presence of light; this observation suggests a mechanism which can explain how *per* and *tim* oscillations are reset by light (Hunter-Ensor

et al., 1996; Lee *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996).

The observation that *per* and *tim* mRNA cycle with a period and phase that is dependent upon the amino acid sequences of their coded proteins suggests that PER and TIM act in a negative feedback loop whereby they regulate their own transcription (Hardin *et al.*, 1990; Marrus *et al.*, 1996; Sehgal *et al.*, 1996). Further evidence for the existence of this feedback loop comes from the examination of the cellular distribution of PER and TIM (Liu *et al.*, 1992; Curtin *et al.*, 1995; Hunter-Ensor *et al.*, 1996). The timed nuclear localization of both PER and TIM is dependent on the presence of both proteins (Vosshall *et al.*, 1994; Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996) and is preceded by the formation of a heterodimeric PER–TIM complex (Lee *et al.*, 1996; Saez and Young, 1996; Zeng *et al.*, 1996). Thus, it appears that *per* and *tim* form interdependent feedback loops where PER and TIM interact *in vivo* to translocate into the nucleus at the appropriate time of day and directly or indirectly repress *per* and *tim* transcription.

Much of the evidence presented above suggests that *per* and *tim* comprise components of the biological clock. However, several inconsistencies exist that complicate this conclusion. First, assuming a simple negative feedback model for the oscillation of *per* and *tim*, one would anticipate that the lack of PER and TIM in the nucleus would lead to high *per* RNA levels. However, *per* RNA in a *per*⁰¹ mutant background shows relatively low levels of RNA when compared with *per*⁺ (Hardin *et al.*, 1990; Van Gelder and Krasnow, 1996). This observation suggests that the mechanism regulating *period* gene expression is more complex than a simple negative feedback system. Second, when *per* mRNA is monitored in DD by RNase protections, the mRNA rhythm dampens appreciably by the fourth to fifth day of monitoring (Hardin *et al.*, 1990). This observation is curious because *Drosophila* locomotor activity free-runs (remains cyclical) in DD for at least 3 weeks (e.g. Helfrich, 1986; Power *et al.*, 1995). This suggests three possibilities: (i) *per* mRNA cycling is not responsible for the robustness of these activity rhythms; (ii) absence of a light–dark (LD) cycle reduces the amplitude of *per* mRNA cycling in many tissues but not in the behaviorally relevant clock cells; or (iii) the variation among the endogenous periods of individual flies (~1–1.5 h) causes the flies to drift out of phase relative to one another and results in a dampened overall mRNA cycle.

We developed a novel *in vivo* luciferase assay that allows us to measure real-time gene expression in individual flies (Brandes *et al.*, 1996; Plautz *et al.*, 1997). Several refinements to this assay now permit us to address questions concerning the continuity of rhythmic *per* expression in single flies under free-running conditions. In addition, the ability repeatedly to measure individual flies with high

time resolution allows us to study the temporal regulation of *per* gene expression by comparing various *period-luciferase* fusion genes in a highly efficient and detailed manner.

Here we report the expression analysis of two *per-luc* transgenes using both our novel *in vivo* luciferase assay and high time resolution RNA quantitations. One construct is a newly generated protein fusion (designated BG-*luc*), whereas the second is the *per-luc* only (*plo*) promoter fusion construct (Brandes *et al.*, 1996). *In vivo* luciferase assays revealed no significant ultradian oscillations for *per*⁰¹ flies carrying the *plo* or BG-*luc* transgene. This indicates that the ultradian behavior observed in individual *per*⁰¹ flies is not correlated with molecular rhythmicity of *per* (reviewed by Dowse and Ringo, 1992). Our single-fly assays also indicated that expression of both *per-luc* reporter transgenes dampens to arrhythmicity in DD conditions. This finding, which differs from our earlier report (Plautz *et al.*, 1997), suggests that the molecular dampening in DD (cf. Hardin *et al.*, 1990) is due to dampening of *per* gene expression in individual flies and not exclusively to asynchrony among flies. Detailed comparisons of the amplitudes and phases associated with the two *per-luc* constructs revealed differences between the *per*-promoted reporter RNAs and that transcribed from the endogenous *per* gene. Furthermore, in the *per*⁰¹ genetic background, the relative level of the *plo* transgene's RNA is high whereas the BG-*luc* transgene's RNA level is low. Taken together, our observations reveal the existence of a circadian-regulated element within the transcribed portion of the *period* gene. The significant findings of this study are presented in a model which suggests a mechanism by which high-amplitude feedback oscillations are sustained.

Results

Real-time measurement of *period* gene expression in individual flies

To elucidate the mechanisms regulating temporal *period* gene expression, two different *per-luc* constructs were designed by fusing genomic regions of the *period* gene to coding sequences from the firefly *luciferase* gene. One construct (*plo*) contains only the *per* promoter fused to a *luciferase* cDNA (Brandes *et al.*, 1996). The second construct contains the same *per* and *luc* sequence as *plo*, plus additional *per* genomic DNA sufficient to encode the N-terminal two-thirds of PER (designated BG-*luc*) (Figure 1A). Figure 1B shows an average plot of a BG-*luc* line tested in LD in a *per*⁺ genetic background. All BG-*luc* flies showed a similar temporal expression pattern. Figure 1E shows a representative example of a single BG-*luc* fly's bioluminescence record and indicates that our improved assay conditions allow very clean single fly oscillations (see Materials and methods). These clean oscillations are also evident in flies from the *plo* lines (Figure 1C).

To compare the oscillations of BG-*luc* and *plo* transgenes, both data sets were normalized and plotted together (Figure 1D). In both cases, the amplitudes are ~2.5-fold; however, the phase of BG-*luc* is ~2 h later than *plo* (Figure 1D; see below for quantitative analysis). Additionally, a secondary bioluminescence peak is observed at the dark to light transition in the *plo* (Figure 1C; Brandes *et al.*,

1996) but not in the BG-*luc* transgenics. Comparison of BG-*luc* and *plo* flies in a *per*⁰¹ genetic background also revealed differences. The *per*⁰¹ *plo* average shows no evidence of cycling and maintains high levels of bioluminescence relative to the *plo* oscillations in a *per*⁺ genetic background (Figure 1C). In contrast, the *per*⁰¹ BG-*luc* average exhibits low-amplitude oscillations and maintains intermediate levels of bioluminescence when compared with BG-*luc* in a *per*⁺ genetic background (Figure 1B). These low-amplitude oscillations appear to be driven by protein interactions between BG-*luc* and TIM, the latter of which shows minor oscillations in a *per*⁰¹ mutant background (Zeng *et al.*, 1996; Dembinska *et al.*, 1997; see below).

Analysis of individual flies reveals significant differences between BG-*luc* and *plo* expression

The principal advantage of monitoring *period* gene expression by means of an *in vivo* reporter is the ability to monitor large numbers of individual flies from which one can make statistical conclusions. To examine whether the differences between *plo* and BG-*luc* bioluminescence are statistically significant, each bioluminescence record was subjected to a quantitative cosine fit analysis (see Materials and methods; also see Plautz *et al.*, 1997). From this analysis, amplitude, period and phase estimates were determined from each fly's record (Table I). The presence of a secondary bioluminescent peak in the *plo* transgenics prompted us to search for rhythmicity in two different control transgenes—*luciferase* driven from a heat-shock promoter (*hsp-luc*; Lockett *et al.*, 1992) and *luciferase* driven from a P-element promoter. Lines from both transgenes showed low-amplitude 12 or 24 h oscillations (see, for example, Figure 4A). Additionally, alterations in the testing conditions (e.g. monitoring flies that carried only one copy of the transgene) resulted in the appearance of secondary peaks for BG-*luc* (data not shown). Thus, all *luciferase* transgenes, irrespective of their promoter, showed evidence of low-amplitude oscillations. The widespread persistence of these oscillations indicates that they are unrelated to *per* gene expression and are a consequence of monitoring luciferase bioluminescence (see Discussion).

In order to remove the low-amplitude oscillations from the quantitative analysis, we developed a rhythmic cut-off by quantitatively analyzing bioluminescence fluctuations from three *hsp-luc* strains. Assuming *hsp-luc* expresses constitutively at constant temperature, any resulting cycling should not be due to gene transcription. Thus, the empirically determined cut-off for rhythmicity [relative amplitude errors (Rel-Amp errors) <0.7 are rhythmic] permits us to determine whether a given *per-luc* fly is rhythmic for gene transcription with a 95% confidence (see Materials and methods).

Using this cut-off, >99% of all *per*⁺ BG-*luc* and *per*⁺ *plo* flies tested in LD were rhythmic and showed periods in the circadian range (22–26 h; Table I). In agreement with the average plot (Figure 1D), quantitatively determined phases for the plotted lines showed a 2 h delay: Zeitgeber time 19.3 (ZT19.3) for BG-*luc* and ZT17.1 for *plo* (Table I; ZT0 = lights on, ZT12 = lights off). Even though both transgenes exhibited phase variation between lines, the average of the mean phases from all the BG-*luc* lines was ZT20.0 compared with ZT17.6 for the *plo*

Fig. 1. BG-*luc* and *plo* temporal bioluminescence in LD. (A) *plo* and BG-*luc* constructs are shown along with the *per* genomic region for reference. The *plo* construct contains *per* genomic DNA from -4.2 kb to $+32$ bp fused to the 1.8 kb *luciferase* cDNA (Brandes *et al.*, 1996). The BG-*luc* construct contains *per* genomic DNA from -4.2 to $+5.6$ kb (Citri *et al.*, 1987) fused to the *luciferase* cDNA. Closed bars indicate untranslated exons and open bars indicate translated portions of exons. *luciferase* cDNA sequences are indicated. (B) Comparison of averaged *per*⁺ BG-*luc* flies ($n = 71$) and averaged *per*⁰¹ BG-*luc* flies ($n = 37$) in LD. *per*⁺ BG-*luc* flies show rhythmicity with ~ 2.5 -fold amplitude and the phase of its peak at ZT19 (see Table I). Noticeable downward trend and amplitude reduction of the recordings are due to an artifact of the luciferase assay and can be removed (see Figure 4B–D). A secondary peak of *per* transcription cannot be discerned for the BG-*luc* strain (as opposed to the *plo* strain; see C). Low-amplitude oscillations of *per*⁰¹ BG-*luc* are most likely to be due to BG-*luc* interaction with TIM (see Results). Note the intermediate level of bioluminescence in the *per*⁰¹ background when compared with *per*⁺. (C) Comparison of averaged *per*⁺ *plo*1b-1 flies ($n = 67$) and averaged *per*⁰¹ *plo*1b-1 flies ($n = 36$) in LD. *per*⁺ *plo* flies also show an amplitude of ~ 2.5 -fold; the apparent lower amplitude of *plo* compared with BG-*luc* oscillations is due to the higher expression level of BG-*luc* (see D). In contrast to BG-*luc*, the phase of the *plo* peak occurs at ZT17 (see Table I). In addition, a secondary shoulder is evident just when the lights come on (see also Brandes *et al.*, 1996). *per*⁰¹ flies show no evidence of rhythmicity. Note the high level of bioluminescence in the *per*⁰¹ background when compared with *per*⁺. (D) The *per*⁺ BG-*luc* and *per*⁺ *plo*1b-1 averages were normalized and plotted together to reveal an ~ 2 h phase difference. Since the BG-*luc* bioluminescence levels are ~ 5 -fold higher than *plo* levels, the first evening peak of both data sets was normalized to 1. Quantifying the absolute level of RNA expression from both BG-*luc* and *plo* transgenes revealed that the ~ 5 -fold bioluminescence difference is due to an ~ 5 -fold difference in RNA levels (data not shown). Note the amplitude similarity and phase difference. (E) Example of a single *per*⁺ BG-*luc* fly from the average in (B). (F) Comparison of *per*⁺ BG-*luc* ($n = 14$) and *tim*⁰¹ BG-*luc* ($n = 10$) fly averages in LD. Flies are heterozygous for the transgene. Note the absence of the low-amplitude *per*⁰¹ BG-*luc* oscillations in *tim*⁰¹ BG-*luc*. Bioluminescence was measured in counts per second. ZT indicates Zeitgeber time. Open bars indicate lights on and closed bars indicate lights off.

lines. Statistical tests indicate that these phase differences are significant ($P < 0.01$).

Individual *per*⁰¹ flies do not exhibit *per* ultradian oscillations

Period analysis of single *per*⁰¹ *plo* flies revealed no significant ultradian or circadian oscillations in these individuals (Table I). One fly showed a 22.8 h period (Table I); however, using the assigned rhythmic cut-off, one would expect 5% of the tested flies to show rhythms (see Materials and methods). This result is significant considering the ultradian rhythms frequently seen in *per*⁰¹ locomotor activity records (Dowse and Ringo, 1992). Our results indicate that the behavioral rhythmicity observed for single *per*⁰¹ flies is unrelated to rhythmic expression from the *per* promoter.

As seen for the average plot of *per*⁰¹ BG-*luc* (Figure 1B), low-amplitude oscillations were detected from the

quantitative analysis of individual flies (Table I). These oscillations appear to be due to the BG-*luc* fusion's interactions with TIM protein. PER and TIM proteins interact via the PAS protein dimerization domain that is fully included in the BG-*luc* fusion protein (Gekakis *et al.*, 1995; Saez and Young, 1996). TIM is known to cycle in LD conditions in a *per*⁰¹ mutant but not in DD (Hunter-Ensor *et al.*, 1996; Myers *et al.*, 1996; Zeng *et al.*, 1996). Indeed, the low-amplitude bioluminescence cycling observed for *per*⁰¹ BG-*luc* in LD is absent in DD (compare Figures 1B and 5A). In addition, the BG-*luc* transgene's RNA in a *per*⁰¹ genetic background shows no evidence of cycling (Figure 3C and D). Lastly, when the BG-*luc* transgene was placed in the *tim*⁰¹ background, the low-amplitude cycling disappeared (Figure 1F). These findings demonstrate that the BG-*luc*'s low-amplitude oscillations in a *per*⁰¹ genetic background are not due to rhythmic gene transcription but appear to be a consequence of light-

Table I. Quantitative analysis of *plo* and BG-*luc* bioluminescent oscillations

Transgenic line	LD				DD		
	# Rhy/tested (% rhythmic)	Mean period ± SEM	Mean Rel-Amp Error ± SEM	Mean phase ZT ± SEM	# Rhy/tested (% rhythmic)	Mean period ± SEM	Mean Rel-Amp Error ± SEM
<i>per</i> ⁺ BG- <i>luc</i>	71/72 (99)	24.4 ± 0.0	0.20 ± 0.00	19.3 ± 0.1	53/61 (87)	25.1 ± 0.1	0.48 ± 0.01
<i>per</i> ⁺ BG- <i>luc</i> 3	15/16 (94)	24.2 ± 0.1	0.25 ± 0.02	20.2 ± 0.2	7/14 (50)	23.8 ± 0.3	0.58 ± 0.03
<i>per</i> ⁺ BG- <i>luc</i> 42	15/15 (100)	23.9 ± 0.1	0.31 ± 0.03	21.5 ± 0.5	10/15 (67)	25.0 ± 0.4	0.45 ± 0.02
<i>per</i> ⁺ BG- <i>luc</i> 56	32/32 (100)	24.4 ± 0.1	0.22 ± 0.01	19.4 ± 0.2	14/28 (50)	25.0 ± 0.4	0.54 ± 0.02
<i>per</i> ⁺ BG- <i>luc</i> 57	35/35 (100)	24.4 ± 0.1	0.23 ± 0.01	19.5 ± 0.1	21/32 (66)	25.8 ± 0.2	0.53 ± 0.02
<i>per</i> ⁺ <i>plo</i> 1b-1	67/67 (100)	24.4 ± 0.0	0.25 ± 0.01	17.1 ± 0.2	58/60 (97)	23.6 ± 0.1	0.45 ± 0.01
<i>per</i> ⁺ <i>plo</i> 1a-1	32/32 (100)	24.6 ± 0.1	0.38 ± 0.02	17.1 ± 0.4	17/31 (55)	24.0 ± 0.3	0.54 ± 0.03
<i>per</i> ⁺ <i>plo</i> 2a-2	15/16 (94)	24.2 ± 0.1	0.22 ± 0.01	18.1 ± 0.4	15/15 (100)	22.8 ± 0.2	0.33 ± 0.03
<i>per</i> ⁺ <i>plo</i> 2a-1	14/14 (100)	24.6 ± 0.1	0.30 ± 0.01	16.8 ± 0.3			
<i>per</i> ⁺ <i>plo</i> 2b-3	10/10 (100)	24.3 ± 0.2	0.26 ± 0.02	17.6 ± 0.6			
<i>per</i> ⁺ <i>plo</i> 3b-1	31/31 (100)	24.1 ± 0.1	0.22 ± 0.01	19.2 ± 0.3	30/31 (97)	23.2 ± 0.2	0.46 ± 0.01
<i>per</i> ⁰¹ BG- <i>luc</i>	27/37 (73)	25.1 ± 0.5	0.52 ± 0.02	20.5 ± 0.8	0/39 (0)		
<i>per</i> ⁰¹ BG- <i>luc</i> 3	12/15 (80)	24.2 ± 0.2	0.54 ± 0.02	20.6 ± 1.0	1/15 (7)	10.9	0.61
<i>per</i> ⁰¹ BG- <i>luc</i> 57	14/14 (100)	24.5 ± 0.2	0.36 ± 0.03	20.3 ± 0.7	1/16 (6)	31.3	0.52
<i>per</i> ⁰¹ <i>plo</i> 1b-1	0/36 (0)				0/33 (0)		
<i>per</i> ⁰¹ <i>plo</i> 1a-1	1/15 (7)	22.8	0.61		0/13 (0)		
<i>tim</i> ⁰¹ BG- <i>luc</i>	4/23 (17)	24.9 ± 1.0	0.62 ± 0.03				
<i>tim</i> ⁰¹ <i>plo</i> 2b-3	1/14 (7)	25.0	0.68				

Each fly tested by the luciferase *in vivo* assay was subjected to a quantitative analysis to determine rhythmicity, period and phase. All flies were tested in the homozygous condition with the exception of *per*⁺ BG-*luc*3 and *per*⁺ BG-*luc*57 which were tested in the heterozygous condition. For all flies, only periods between 4 and 35 h were included. 'Tested' flies are all flies analyzed. 'Rhythmic' indicates those flies with Rel-Amp errors <0.7 (see Results). For *per*⁺ flies tested in LD, an additional constraint was added for a fly to be determined rhythmic. 'Rhythmic' *per*⁺ flies in LD also required periods between 22 and 26 h (indicating steady entrainment to the Zeitgeber). 'Mean Period', 'Rel-Amp error' and 'Phase' are the means from the outputs of the quantitative analysis for every fly within a given line. The Rel-Amp error gauges the strength of a rhythm on a scale from 0 to 1; lower Rel-Amp errors indicate stronger rhythms. SEM = standard error of the mean.

driven TIM cycling and its interaction with the BG-*luc* fusion protein.

Normal period RNA expression requires two circadian-regulated elements

Since the BG-*luc* transgene encodes a PER-LUC fusion protein and the *plo* transgene contains no PER amino acids, the differences observed between *plo* and BG-*luc* bioluminescence reflect regulation of either *per-luc* RNA or PER-LUC protein. To resolve the origin of these differences, the temporal RNA expression from both constructs was analyzed by RNase protections. The *plo* transgene RNA shows both an amplitude reduction and an advanced phase when compared with endogenous *per* (Figure 3A and B). This observation suggests that either the *per* promoter lacks specific regulatory sequences necessary for proper RNA cycling or *luciferase* sequences have an aberrant effect on RNA cycling. To distinguish between these possibilities, temporal RNA expression was analyzed from both a BS-CAT and the BG-*luc* construct. The BS-CAT construct contains the same *per* sequences present in *plo* but is fused to the bacterial CAT gene (Hardin *et al.*, 1992). Our high time resolution RNase protections showed the CAT reporter RNA to exhibit a phase advance and amplitude reduction when compared with endogenous *per* RNA (Figure 2A), just as is seen for *plo* RNA (Figure 3A and B). Expression of both reporter RNAs started to increase strongly after ZT4, whereas *per* expression increased 2–4 h later (Figures 2A and 3A and B). Endogenous *per* RNA cycled with an ~15-fold amplitude (average of five experiments: see legend for amplitude calculations). In contrast, the amplitude of *plo* and BS-CAT RNA cycling was reduced ~3-fold (5-fold for *plo*, average of five experiments; 3.5-fold for

BS-CAT, average of two experiments; for each construct two independent transgene insertion lines were tested). The *plo* lines showed an average RNA peak phase of ZT12.2 (SEM = 0.3; six experiments) for the reporter transcript and ZT13.9 (SEM = 0.2) for the endogenous *per* transcript (see Materials and methods).

Temporal RNA expression analysis of flies transformed with the BG-*luc* transgene revealed that this reporter RNA cycled with the same phase and amplitude as the endogenous *per* transcript (Figure 2B). The BG-*luc* line showed an average peak phase of ZT16.1 and a 14-fold amplitude (two experiments) for the reporter transcript, and ZT15.3 and 14-fold for endogenous *per*. These similarities are also observed statistically; the correlation coefficient between BG-*luc* reporter RNA and endogenous *per* RNA is 0.96 (1.0 implying an exact linear relationship). The majority of the phase difference between the endogenous *per* phase of *plo* and BG-*luc* is most likely due to the lower number of experiments for the BG-*luc*, since the phases of locomotor activity between *plo* and BG-*luc* strains was 0.5 h or less (data not shown). In any case, the results demonstrate that *luciferase* sequences do not have an aberrant effect on RNA cycling and that a transcribed region of the *per* gene—present in BG-*luc* but not BS-CAT or *plo*—contributes to the generation of the temporal RNA expression pattern of *per*. Thus, the observed phase differences between BG-*luc* and *plo* bioluminescence appear to reflect differences in RNA regulation.

Bioluminescence levels of *plo* and BG-*luc* transgenes in a *per*⁰¹ background suggested that *per* expression is relatively high in *plo* and low in BG-*luc*. To ascertain whether these differences are due to RNA regulation, we compared RNA expression levels of both *plo* and BG-

Fig. 2. Temporal RNA expression pattern of BS-CAT and BG-*luc* transgenes compared with *per* RNA cycling. **(A)** RNase protection experiment, with 1 h time resolution, of flies carrying the BS-CAT transgene. Band intensities of the protected CAT fragments were quantified and standardized to the *rp49* signal. The average pattern of *per* expression (resulting from five independent experiments in which the *per* transcript was tracked; cf. Brandes *et al.*, 1996) is plotted in the same graph; for both transcripts, the maximum expression value was set to 1. CAT reporter RNA shows an earlier rise in abundance and also cycled with a lower amplitude (~3-fold) compared with the *per* transcript (~15-fold). The amplitudes were calculated after dividing the peak values of expression (between ZT8 and ZT15 for BS-CAT and ZT13 and ZT16 for *per*) by the trough values (ZT22 to ZT4 and ZT23 to ZT4, respectively). In a different BS-CAT transgenic line, the same earlier onset of transcription was observed and the amplitude was also reduced (~4-fold). **(B)** RNase protection experiment, with 1 h time resolution, involving BG-*luc* transgenic flies. Endogenous *per* and BG-*luc* reporter RNAs were assayed simultaneously using the *luc* and *per* 5/6 probes (Materials and methods). Band intensities were plotted after standardization to the *rp49* control and setting the absolute peak value for each cycling RNA to 1. In the lane corresponding to ZT13, much less RNA was loaded, explaining the dip in the rising portion of both RNA curves. The amplitudes (14-fold) were calculated as described above using the peak values from ZT15 to ZT18 and the trough values from ZT1 to ZT5 for both RNAs. The white and black portions of the bar represent times when the lights were on or off, respectively.

luc reporter RNAs with that of endogenous *per* RNA expression in the *per*⁰¹ genetic background. The direct comparison of *plo* and *per* RNA levels in the *per*⁰¹ mutant background revealed that the reporter RNA is expressed at constantly high levels (80% of its maximal expression in *per*⁺, Figure 3A and B), whereas endogenous *per*⁰¹ RNA is expressed at low levels (20% of its maximal expression in *per*⁺, Figure 3A and B). In contrast, BG-*luc* RNA expresses at a low level in the *per*⁰¹ background, similar to that of endogenous *per*⁰¹ RNA (Figure 3C and D). Thus, the expression level differences between *plo* and BG-*luc* appear to lie at the level of RNA. These results also reveal that sequences within the transcribed region of *per* are responsible for the constitutively low levels of *per*⁰¹ RNA.

Phase and amplitude comparisons of *per*⁺ *plo* and endogenous *per* RNA (or *per*⁺ BG-*luc*) indicate that the transcribed region of *per* contains a circadian-regulated element which acts to control the level of *per* RNA. The amplitude differences between *plo* and endogenous *per* could be explained by a shorter half-life for *per* RNA (as a result of an instability element); however, the phase differences cannot be explained. For two cycling RNAs transcribed coincidentally, the RNA with the shorter half-life will show an earlier phase and greater amplitude when compared with an RNA with a longer half-life (Wuarin *et al.*, 1992). Yet, *per* shows a greater amplitude (shorter half-life) but a later peak phase when compared with *plo* RNA (Figure 3A and B; also see Brandes *et al.*, 1996). Thus, we conclude that an element within the transcribed region of *per* regulates *period* gene expression in a circadian manner. This element is not simply an enhancer or suppressor regulated by light, since both *per*⁰¹ and BG-*luc* RNA fail to cycle in a *per*⁰¹ mutant background. Therefore, normal *per* expression requires the activity of at least two circadian-regulated elements, one within the promoter and one within the transcribed region of *per*.

***per*-driven bioluminescence dampens to arrhythmicity in individual flies in DD**

Bioluminescence emanating from individual *per-luc* flies was tracked under DD conditions. Using our improved assay conditions, which differ from the conditions used in our previous report (see Materials and methods), rapid dampening of the bioluminescence oscillations was observed for average plots of both *plo* and BG-*luc* (Figure 5A and B). This level of dampening is much greater than that observed in LD (compare Figure 1B and C with Figure 5A and B) but similar to the dampening reported when tracking *per* RNA in populations in DD by RNase protection (Hardin *et al.*, 1990; Hardin, 1994). To observe the dampening due to DD alone, a trend-adjusting method was developed to remove the downward trend artifact of the luciferase assay. The downward trend is an artifact because all *luciferase* flies show the downward trend, and replenishment of luciferin substrate results in signal restoration (Brandes *et al.*, 1996; Plautz *et al.*, 1997). To reveal the true oscillations of both *plo* and BG-*luc*, curve-fitting software was applied to *per*⁺ BG-*luc*, *per*⁺ *plo* and *hsp-luc* (control) LD data, and the downward trend was removed from each of the time courses (Figure 4B–D). Applying this method to representative *plo* and BG-*luc* individual flies tested in DD revealed clear dampening of

Fig. 3. RNA expression levels of reporter and *per* RNA, in *per*⁺ and *per*⁰¹ genetic backgrounds, of *plo* and BG-*luc* transgenic flies. (A) RNA expression of flies from the *plo1a-1* strain was measured in a *per*⁺ and *per*⁰¹ background in a 2 h time resolution RNase protection experiment using the *per* 2/3 and *luc* probes (Materials and methods). Protected luciferase and *per* RNA fragments in both backgrounds were separated on the same gel to allow a direct comparison between transcription levels in both genetic backgrounds. (B) Quantification of the data shown in (A) after standardizing the band intensities of the protected fragment against the *rp49* control and setting the maximum expression values of each transcript to 1. In a *per*⁺ background, luciferase and *per* RNAs cycled with their characteristically different phases and amplitudes (see also Figure 2 and Brandes *et al.*, 1996). In a *per*⁰¹ background, the level of *per* RNA is only at 20% of its peak level in *per*⁺ (here: ZT14 and ZT16), whereas the level of luciferase RNA in *per*⁰¹ is at 81% of its maximal level in *per*⁺ (which was at ZT9, ZT11, ZT13, ZT14 and ZT15 in this experiment). Similar results were obtained in two different experiments with 2 h time resolution (using the *plo1* strain) where *per*⁰¹ RNA was ~50% less abundant than two *per*⁺ control RNAs from *plo1* flies collected at ZT8 and ZT10. In the same experiments, the average luciferase RNA abundance in *per*⁰¹ was at the same level of the luciferase RNA isolated from the controls flies at ZT8 and ZT10. Recall that *per* expression is still rising between ZT8 and ZT10, whereas luciferase expression is reaching its maximum level at that time. (C) Same experiment as in (A) performed with BG-*luc* transgenic flies using the *per* 5/6 and *luc* probes. (D) Quantification of data shown in (C), after standardizing against *rp49* and setting the absolute peak expression value of each transcript to 1. For *per*, the exon 6 band is graphed, although exon 5 gave very similar results. In a *per*⁺ background, BG-*luc* and *per* RNAs cycled with nearly identical phase and amplitude (see also Figure 2B). In a *per*⁰¹ background, both transcripts are expressed at a constitutively low level (*per*, 33%; BG-*luc*, 40.1%) when compared with their peak times of expression in a *per*⁺ background (here ZT15 and ZT18 for both transcripts). The white and black portions of the bar represent 12 h time segments when the lights were on or off respectively.

their molecular oscillations (Figure 5C–F). Thus, the RNA dampening observed in DD (Hardin *et al.*, 1990; Hardin, 1994) is due predominantly to dampening *per* expression within each fly. To determine if the bioluminescence oscillations dampen to arrhythmicity, we performed a long-term DD experiment. To minimize the luciferase downward trend artifact, the experiment was started in the middle of a DD regime. Newly eclosed flies were placed in DD for at least 6 days; each fly was then loaded and tested for 5 days, while maintaining DD. Both *plo* and BG-*luc* flies tested in the *per*⁺ genetic background showed no evidence of rhythmicity by quantitative analysis, whereas the locomotor behavior of these flies was rhythmic (data not shown). These results suggest that either *per* is not required for the persistence of locomotor

behavioral rhythms in DD or *per* oscillations are maintained in only a subset of *per*-expressing cells that control rhythmic locomotor behavior.

Even though the bioluminescence oscillations dampened in DD, each fly was tested analytically for rhythmicity and period of gene expression (Table I). Tests of the hsp-*luc* strain in DD showed the same rhythmic distribution as LD; therefore, the same rhythmic cut-off used in the LD data was applied to the DD data. When compared with LD conditions, a much lower percentage of *per*⁺ flies were deemed rhythmic (Table I), and the strength of those rhythms was lower (presumably due to increased dampening). On average, the BG-*luc* lines showed longer periods than the *plo* lines. The average of the mean periods from all the BG-*luc* lines was 24.9 h compared with 23.4 h

Fig. 4. Downward trend removal from luciferase bioluminescence. (A) Average of *per*⁺ *hsp-luc* flies ($n = 54$) from three different lines with different genomic insert locations. In addition to the downward trend, low-amplitude, 12 h oscillations can be discerned (see Results). Note how the morning *hsp-luc* peaks coincide with the secondary peaks of the *plo* strains (see C). (B–D) The downward trend in the *per*⁺ *BG-luc* (B), *per*⁺ *plo* (C) and *hsp-luc* (D) have been removed to reveal the true cycling of *per-luc* expression. The *hsp-luc* trend-adjusted average reveals the relatively flat expression expected for flies tested at constant temperature. The same data manipulation has successfully removed both the amplitude reduction and downward trend of both *per-luc* constructs. See Materials and methods for the trend-adjusting procedure. Bioluminescence was measured in counts per second. ZT indicates Zeitgeber time. Open bars indicate lights on and closed bars indicate lights off.

for the *plo* lines. Statistical tests indicate that the period differences between *BG-luc* and *plo* transgenes are statistically significant ($P < 0.05$). The longer periods observed in *BG-luc* appear to be due to a subtle effect of the *BG-luc* transgene on the clock, since DD locomotor periods of *plo* strains are identical to controls whereas periods of *BG-luc* strains are longer (data not shown). As reported for LD, no evidence of molecular cycling was observed for the *plo* promoter strains in the *per*⁰¹ genetic background. In contrast to LD, the TIM-dependent oscillations of *per*⁰¹ *BG-luc* were absent in DD (see above).

Luciferase bioluminescence emanates predominantly from the body

The bioluminescence dampening observed in DD seems to occur faster than the dampening reported from RNase protections performed on fly heads (Hardin *et al.*, 1990). The speed of dampening appears to occur at the same rate as *per* RNA dampening in fly bodies (Hardin, 1994). This observation suggests that our luciferase assay is tracking a high percentage of *per* cycling in the body. Figure 6A shows a male *BG-luc* fly fed on luciferin-fortified food for 3 days and imaged with a photon-counting camera. Indeed, some signal emanates from the head and eyes, but the majority of bioluminescence is emitted from tissues in the abdomen (perhaps Malpighian tubules or the gut; cf. Hall, 1995). This spatial expression was similar for the *plo* strain (data not shown). Since the majority of the measured signal originates from the abdomen (Figure 6A), it is possible that in DD our assay has missed detecting *per* cycling that persists in head tissues.

Luciferase activity, not luciferase protein, oscillates in vivo

Given the level of luciferase bioluminescence from the body, we wished to determine the extent of luciferase protein expression in the head of both transgenics and determine whether this expression showed spatial and temporal similarity to endogenous PER. Head sections of both *plo* and *BG-luc* males were stained with anti-luciferase antibodies. Like PER, both proteins were expressed in photoreceptor cells R1–R8 and in *per* ‘lateral neurons’ (LNs) of the CNS (Figure 6C–J), which are the central pacemaker neurons of *Drosophila* (reviewed by Hall, 1995). In addition, the *BG-luc* fusion protein shows PER-like expression in glia cells of CNS cortical regions and in the region between the medulla and lamina (Figure 6D, arrowheads). Temporal examination of *BG-luc* protein staining revealed fluctuations in staining intensities (Figure 6C and D). At ZT9, a time where PER is usually expressed at low levels (see, for example, Zerr *et al.*, 1990), only weak signals are visible in photoreceptor cells and LNs (Figure 6C). At ZT21, when PER is high, staining is prominent in the photoreceptors and LNs (Figure 6D). In contrast to *BG-luc*, staining of the *plo* transgenic males did not reveal any temporal fluctuations in staining intensity (Figure 6E and F). This result was confirmed by Western blot analysis (Figure 6B), suggesting that only the activity of the luciferase enzyme cycles and that the half-life of the luciferase protein is too long to allow observable protein cycling. Application of luciferase in plants has revealed the same phenomenon (Millar *et al.*, 1992).

High-magnification views of the preparations revealed a punctate staining in all cells expressing the two luciferase proteins (Figure 6G–I, open arrows). These structures were not observed in anti-luciferase stainings of control flies, lacking a *luc* transgene (Figure 6J). Since the native luciferase enzyme is targeted to the peroxisomes (Keller *et al.*, 1987; Gould *et al.*, 1990) and since both *luc* transgenes contain the peroxisomal targeting sequence, these structures are most likely peroxisomes in the *Drosophila* brain.

Discussion

By coupling a novel method of measuring gene expression from individual living flies with high-resolution RNA

Fig. 5. BG-*luc* and *plo* temporal bioluminescence in DD. (A) Comparison of averaged *per*⁺ BG-*luc* flies (*n* = 71) and averaged *per*⁰¹ BG-*luc* flies (*n* = 37) in DD. As opposed to LD, *per*⁰¹ flies no longer show minor oscillations (Figure 1B). Although *per*⁺ flies show cycling, the amplitude dampens much faster (almost to the point of arrhythmicity). This dampening corresponds well with the dampening observed in RNase protections (Hardin *et al.*, 1990, 1994). (B) Comparison of averaged *per*⁺ *plo1b-1* flies (*n* = 58) and averaged *per*⁰¹ *plo1b-1* flies (*n* = 33) in DD. As seen in LD, *per*⁰¹ shows no rhythmicity whereas *per*⁺ shows cycling; however, the amplitude dampens as seen in the BG-*luc* (see A). (C and D) Examples of a single *per*⁺ BG-*luc* fly (C) and a single *per*⁺ *plo1b-1* fly (D) tested in DD. Note that the same degree of dampening is seen for both individuals and for the average. This result suggests that the majority of the dampening observed for the average in DD is not due to individual fly asynchrony but to dampening *per* expression within each fly (see Results). (E and F) The data from the two flies in (C) and (D) were trend-adjusted (E and F respectively) to remove the dampening due to the luciferase assay artifact (see Materials and methods). This manipulation reveals the degree to which *per* expression dampens in DD. The resulting curves may be compared with the LD averages (Figure 4B and C) to reveal the dampening due to DD alone. Bioluminescence was measured in counts per second. One day of LD is included in each plot. The x-axis indicates hours in darkness. Open bars indicate lights on, closed bars indicate subjective night and stippled bars indicate subjective day.

quantitations, we have identified a new regulatory element necessary for the proper temporal expression of the *period* gene. Comparison of the results from two different *per-luc* transgenes revealed significant phase differences in the *per*⁺ genetic background and level differences in the *per*⁰¹ genetic background. The origin of these differences was determined through the analysis of each transgene's RNA expression. The *plo* transgene RNA cycled with a different phase and amplitude when compared with endogenous *per*. This demonstrates that the *per* promoter is insufficient to replicate normal *per* RNA cycling and that additional sequence elements within the transcribed portion of the *per* gene are necessary. In contrast to *plo*, the BG-*luc* transgene RNA cycles with the same amplitude and phase as endogenous *per*. Thus, BG-*luc* contains all *cis*-acting sequences necessary for replicating *per*'s temporal RNA cycling and contains the regulatory element missing from *plo*. This element acts to regulate the level of *per* RNA and is circadianly regulated.

Functional properties of the novel regulatory element

A central question is whether this element alone is sufficient to: (i) generate cycling *per* expression; (ii) rep-

licate the same amplitude and phase of cycling observed for endogenous *per*; and (iii) rescue full clock function. Each of these questions has been addressed by transformation experiments and molecular studies of a *period* promoterless transgene (designated 7.2). The promoterless 7.2 construct contains a 7.2 kb segment of *per* DNA containing the entire coding region but lacking all 5'-flanking regulatory information, the first non-coding exon and most of the 2.3 kb first intron (Hamblen *et al.*, 1986). When placed in a *per*⁰¹ genetic background, the 7.2 RNA cycled with a lower amplitude (~2.5-fold) and later phase when compared with endogenous *per* (Frisch *et al.*, 1994). In addition, the 7.2 rescued the behavioral arrhythmicity of the *per*⁰¹ mutant, but the locomotor periods of these flies were significantly longer than those of wild-type controls (Frisch *et al.*, 1994). Therefore, the 7.2 construct was only able to partially replicate the RNA oscillations and function of wild-type *per*. By combining these results with our current ones, we conclude that proper temporal *period* gene expression requires the function of at least two circadian-regulated elements, and these elements are insufficient to replicate correct temporal *per* RNA expression when acting independently.

Since the 7.2 construct contains 3' sequences that are

Fig. 6. Protein expression and bioluminescence imaging of *plo* and *BG-luc* transgenics. **(A)** Pseudo-color image of *BG-luc* bioluminescence overlapped with a black and white reference image. A male *BG-luc* fly was fed on luciferin-fortified food (15 mM) for 3 days and imaged for 20 min with a Hamamatsu VIM camera system. The image was taken between ZT8 and ZT14. The fly is oriented dorsal side up with its head in the top right portion of the figure. Note the abdominal signal and presence of eye bioluminescence. **(B)** Temporal expression of PER and luciferase proteins in *plo* transgenic flies during a 12 h:12 h LD cycle was tracked using an anti-PER antibody (upper panel), followed by application of anti-luciferase antibody (lower panel). Endogenous PER protein undergoes characteristic temporal changes in abundance and mobility (Ederly *et al.*, 1994). The luciferase protein (62 kDa) shows no circadian fluctuations in abundance and mobility. Controls were extracts from *Df(1)w* flies (*per*⁺) and from *per⁰¹ w sn³* flies (*per⁰¹*). Numbers above the panels indicate the ZT time of fly collection; open portions of the bar represent the light, and closed portions the dark phase of the LD cycle. **(C–J)** Head sections of flies from both transgenic strains, entrained to a 12 h:12 h LD cycle, were stained with anti-luciferase antibody to reveal fusion protein and luciferase staining patterns. **(C)** *BG-luc* male collected at ZT9; weak staining of two *per* 'lateral neurons' (LNs) from the ventral group is shown (arrow). Photoreceptor cell (PR) staining is weak at this time point, but a punctate staining in the cytoplasm of the PRs is visible, most likely representing accumulation of the fusion protein in the peroxisomes (see Results); scale bar 25 μ m. **(D)** At ZT21, strong staining of the LNs can be observed in *BG-luc* males. The arrow points to a group of at least five neurons. In addition, PER-like glial staining is visible in the cortex and at the outer rim of the medulla (arrowheads). PRs appeared to be strongly stained in the nucleus as well as in the cytoplasm; scale bar 40 μ m. **(E)** Staining of a *plo1b-1* male at ZT9. The two LNs are labeled mainly in the cytoplasm (arrow); staining of the PRs also is predominantly cytoplasmic; the magnification is as in (C). **(F)** No difference in staining intensity and pattern is detectable in *plo2* males collected at ZT21, compared with ZT9 (above); magnification is as in (D). **(G)** Higher magnification of PR staining of a *BG-luc* male collected at ZT21. The open arrow points to one of the strongly labeled structures that give rise to the overall punctate staining pattern; scale bar 8 μ m. **(H)** Higher magnification of LNs (arrow) and glia cells (arrowheads) from the *BG-luc* male shown in (D). The punctate staining correlates with expression sites of the *BG-luc* transgene; **(I)** High-magnification view of PR staining in a *plo* male showing that the punctate staining pattern can also be observed in flies expressing luciferase only; magnification in (H) and (I) is as in (G). **(J)** Staining of a control non-transgenic [*Df(1)w*] male collected at ZT21 shows no PER-like or punctate staining; magnification as in (D).

not located in *BG-luc*, it is possible that the 3' end of the 7.2 contains additional regulatory elements. However, given the identical cycling of endogenous *per* RNA and *BG-luc* RNA, all temporally regulated elements must reside within the *BG-luc*'s *per* sequences. Thus, the element regulating the oscillations of the promoterless 7.2 is the same element identified within the transcribed region of *BG-luc*.

Why two circadian-regulated elements?

We hypothesize that the coordinated action of the promoter element and the transcribed region element is necessary

to maintain the high-amplitude feedback oscillations that are critical for a fully functional clock. For a given rhythmically expressed gene, the concentration fluctuations of the transcribed RNA will show a phase delay and amplitude reduction (dependent on the RNA half-life) when compared with the phase and amplitude of the transcription (Wuarin *et al.*, 1992). Similarly, proteins synthesized from cycling RNAs show an amplitude reduction and later phase (dependent on the protein half-life) when compared with the RNA (Wood, 1995). If a protein feeds back to effect transcription, the loss of amplitude during RNA and protein synthesis prevents the protein

Fig. 7. Role of two circadian-regulated elements in *per* gene expression. Circadian fluctuations of *per* RNA synthesize PER protein. The protein oscillations show an amplitude reduction when compared with the *per* RNA oscillations. PER protein (with TIM) feeds back to regulate transcription at the promoter element either directly or through additional factors. In addition, PER and TIM regulate a transcribed region element to control the level of *per* transcription or *per* RNA. The combined activities of the promoter element and the transcribed region element result in the proper phase and amplitude of *per* RNA. By combining two low-amplitude activities of similar phase, one is able to generate high-amplitude *per* RNA fluctuations.

oscillations from replicating the same amplitude of transcription without some additional forms of regulation. The *Drosophila* clock appears to have evolved several mechanisms for dealing with this 'loss of amplitude' problem. Some of this lost amplitude could be regained by temporally regulating PER half-life (Dembinska *et al.*, 1997). Moreover, temporally selective nuclear localization could increase the amplitude of concentration fluctuations in the nucleus (Curtin *et al.*, 1995). We believe that the discovery that *per* expression is regulated by at least two circadian elements suggests an additional mechanism by which lost amplitude can be regained (Figure 7).

Our observations suggest the following model: *per* gene expression requires at least two circadian-regulated elements, one within the promoter and one within the transcribed region. When the amplitude and phases of the promoter-only oscillations (Amp = 5-fold, early phase) and the promoterless oscillations (Amp ~2.5-fold, late phase) are combined, one obtains the phase and amplitude of endogenous *per* RNA cycling (Amp = 14-fold, normal phase; Figure 7). Since the cycling of both the promoter element and the transcribed region element requires PER and TIM, PER and TIM oscillations are able to act either directly or through additional factors separately to affect the cycling of both elements in a combined fashion. This type of regulation permits high-amplitude mRNA cycling to emerge from two low-amplitude oscillations (Figure 7).

DD dampening

Our previous report (Plautz *et al.*, 1997) has shown that *plo per-luc* flies are able to sustain cycling bioluminescence in DD for at least 10 days. In contrast, using our revised

assay conditions, both BG-*luc* and *plo per*⁺ flies tested in DD show a clear dampening of *per*'s oscillations within single flies. The differences reported in DD dampening appear to be due to the different assay conditions used (see Materials and methods). Given our large body signal (Figure 6A) and the degree of *per* mRNA dampening in the body in DD (Hardin, 1994), we believe that the current *luciferase* assay conditions accurately reflect dampening of *per* expression in individual flies.

Even though previous studies tracking *per* expression in DD by RNase protections have also observed such molecular dampening (Hardin *et al.*, 1990), these studies could not determine the degree to which *per* expression dampened. Our single-fly assay has allowed us to circumvent this problem. Overall, *per* expression dampens to arrhythmicity (see Results). One obvious implication of this result is that rhythmic *per* expression may be unrelated to locomotor behavior rhythms which persist in DD for at least 3 weeks (Helfrich, 1986; Power *et al.*, 1995). Although an intriguing interpretation, it is also possible that our assay misses cycling expression within critical clock tissues. The majority of the light we detect by imaging originates from the abdomen (Figure 6A). Thus, if some tissues in the head (e.g. the *per* LNs: Zerr *et al.*, 1990) retained cyclical expression in DD, it would be nearly impossible to detect this cycling given the constitutive expression from body tissues. It is also possible that the dampening we observe is not due to *per* dampening throughout the body, but to desynchronization of rhythms between various tissues within the fly. Indeed, initial experiments indicate that some tissues are able to retain cycling in DD (Emery *et al.*, 1997; Giebultowicz and Hege, 1997).

Secondary peak

Brandes *et al.* (1996) suggested that *period* gene expression has a secondary peak of transcription. Using our revised assay, we have observed the same 12 h rhythms (as a consequence of the secondary peaks) in the *plo* strains. However, we also observed 12 h rhythms in flies carrying two transgenes that contain no *per* sequences, i.e. *hsp-luc* and P-element promoter-driven *luc*. It has been suggested that these transgenes have landed near hypothetical cycling enhancers (Plautz *et al.*, 1997), but every *hsp-luc* line showed 12 or 24 h rhythms, as did >80% of lines carrying randomly distributed P-element promoter-driven *luciferase* insertions. These low-amplitude rhythms also show the same frequency (% showing rhythms/% tested) and rhythmic strength when compared between *plo* and *hsp-luc* lines. Moreover, the phases of the morning peaks in *hsp-luc* and *plo* show a high correlation (Figure 4C and D). Therefore, we conclude that the secondary peak of the *plo* strains is not due to *period* gene transcription.

In contrast to *per*⁺, both *per*⁰¹ *plo* and *per*⁰¹ *hsp-luc* do not show the same 12 and 24 h oscillations as *per*⁺ (data not shown); therefore, these rhythms require a functional clock. Even though much of the movement from the fly has been eliminated (see Materials and methods), we believe a clock-mediated behavior (e.g. movement or substrate ingestion) may be large enough to induce regular variations in the bioluminescence and produce these low-amplitude rhythms.

We have shown that our luciferase assay is able to

reveal temporal details of *per* expression and identify a novel regulatory element. We now intend to utilize this system to isolate the newly identified *per* regulatory element using *luciferase* fusion genes containing various portions of the *period* gene.

Materials and methods

Generation of the BG-luc construct

The pJD261 plasmid containing the *luciferase* cDNA (Luehrsen *et al.*, 1992) was cloned into the *Bam*HI–*Kpn*I sites of pBluescript II KS after removing the first ATG by mung bean nuclease treatment. This 1.8 kb *Bam*HI–*Kpn*I *luciferase* cDNA fragment and a 9.8 kb *Bam*HI *per* fragment, extending from –4200 to +5627 (Citri *et al.*, 1987), were then cloned into the *Bam*HI–*Kpn*I sites of the pCaSpeR 4 transformation vector (Thummel *et al.*, 1988); in the latter, a 1 kb *Eco*RI fragment, containing the SV40 polyadenylation site, previously had been incorporated. The final BG-*luc* construct contains 4.2 kb *per* upstream flanking material and encodes about two-thirds of the PER protein fused in-frame to the *luciferase* cDNA.

Stocks and P-element transformation

The BG-*luc* strain was generated after transforming *Df(1)w* embryos with the BG-*luc* construct, carrying the mini-*white*⁺ gene as selectable marker. Transformations were performed using standard techniques (e.g. Rubin and Spradling, 1986); transposase was supplied by co-injection of the helper plasmid pUChsΔ2-3 (Laski *et al.*, 1986). The P-element insertion of the one transformant line recovered was localized genetically to chromosome 3, and additional autosomal transgenic lines were isolated after crossing this line to a transposase-producing Δ2-3 strain (Robertson *et al.*, 1988). In order to analyze the BG-*luc* transgene in a *per*⁰¹ background, the *Df(1)w* chromosome (which carries *per*⁺) was replaced by an X chromosome carrying *per*⁰¹ *w* *sn*³ or *y per*⁰¹ *w* in a subset of those lines. All lines were tested in the homozygous condition unless stated otherwise in the text.

In vivo luciferase monitoring

Assays are similar to those described in Brandes *et al.* (1996). One hundred μl of a 5% sucrose 2% agar solution containing 25 mM luciferin (Promega) was added to every other well of a white 96-well microtiter plate (Optiplate, Packard). Newly eclosed flies were entrained to a 12 h:12 h LD cycle for 3 days. Flies were then etherized, added to each well and covered with a clear small plastic dome. The dome served to reduce fly movement in the Z-axis (closer to and further away from the photodetector). Without the dome, fly movement significantly increased the noise level of the data. Once loaded, five plates were placed in a Packard Topcount Multiplate Scintillation Counter and subjected to a 12 h:12 h LD cycle or DD conditions. The Topcount was programmed to read the bioluminescence level of each fly for ~20 s for each hour's time point. Since dark conditions are required to measure bioluminescence, each plate was in dark conditions for ~11 min of each hour. Flies were measured in this fashion for ~1 week. Background using these methods was typically <100 c.p.s. although cross-talk with high expressing lines sometimes resulted in <200 c.p.s. The first 12 h of data were excluded from the plots and quantitative analysis since flies newly placed on luciferin food require time to inactivate the concentration of luciferase enzyme already present (i.e. luciferase enzyme that is not newly synthesized).

The differences reported in DD dampening between this study and that of Plautz *et al.* (1997) appear to be due to different assay conditions. Either our current use of a higher luciferin concentration (25 versus 1 mM) or our current use of domes to minimize fly movement results in the apparent dampening difference. Flies tested with domes at both 1 and 25 mM still showed dampening oscillations, yet flies tested without domes were able to sustain their oscillations in DD for longer than flies tested with domes (data not shown). Therefore, we hypothesize that rhythmic locomotor behavior may result in the monitoring of rhythms that are not directly related to rhythmic *per-luc* bioluminescence. On the other hand, different concentrations of substrate (25 versus 1 mM) may result in different spatial bioluminescence patterns.

Quantitative analysis of data

All luciferase assay data were analyzed for phase, period and amplitude by an iterative, coupled Fourier transform–non-linear least squares (FFT–NLLS) multicomponent cosine analysis (Plautz *et al.*, 1997). Ninety-six

hours of data were analyzed from each record; the first day was excluded to allow inactivation of non-newly synthesized luciferase. Along with estimates for the period, phase and amplitude of the data, Rel-Amp errors were determined. Rel-Amp errors for each fly are obtained by dividing the 95% confidence interval by the amplitude estimate (ratio of amplitude error to most probable amplitude). This value ranges from 0 to 1, where 0 indicates a rhythm is known to infinite precision (zero error) and 1 indicates a rhythm that is not statistically significant (error exceeds most probable value) (Plautz *et al.*, 1997).

A cut-off for rhythmicity was set to eliminate non-transcriptional low-amplitude oscillations from the quantitative analysis. Since all hsp-*luc* lines show low-amplitude oscillations, the cut-off for rhythmicity was empirically determined by testing hsp-*luc* and setting a cut-off in terms of Rel-Amp error. Out of 54 hsp-*luc* flies tested quantitatively, 18 (33%) were determined as rhythmic; the average Rel-Amp error of these flies was 0.83 (SEM = 0.03). Theoretically, the Rel-Amp errors lie in a distribution. The 5% strongest rhythms (lowest Rel-Amp error) were below a Rel-Amp error of 0.7. The other 95% were >0.7; therefore, this value was used as a cut-off for rhythmicity. This cut-off permits one to determine whether a given fly is rhythmic for gene transcription (Rel-Amp error <0.7) with a 95% confidence.

Phase analysis of RNA data was accomplished by producing a full 24 h data set (points every hour). If a data set missed one hour of data, it was replaced by taking the mean of the point before and after the missing data point. This complete 24 h data set was replicated three times and concatenated to produce 4 days of data (96 h). These data were then analyzed by the FFT–NLLS method described above to estimate the phase of the record. Correlation coefficients were determined using Excel.

Mann–Whitney U tests (non-parametric) were used to perform statistical comparisons of the bioluminescence phases and periods between the transgenes. In both cases, the average phases or periods of individual strains were used to compare differences in constructs. These tests were performed using Statview software.

Trend-adjusting of the LD data sets was accomplished by first fitting the following cosine equation to each trend-adjusted data set [$y = DC + \text{amplitude} \times \cos((\text{phase} + \text{time})/\text{frequency}) \times e^{-kt}$]. This equation was initialized for DC, amplitude, phase and frequency, and best fit to the data using Kaelidagraph software. In all cases, *R* (correlation coefficient) was >0.98. The e^{-kt} component of each curve was then divided from all data points and plotted. Since the DD data contains two dampening components (the luciferase assay artifact and dampening *per* expression), trend-adjusting of DD data was performed using the following equation ($y = DC \times e^{-kt}$). The e^{-kt} component of each curve was then divided from all data points and plotted.

RNase protection assays

For the RNA determinations, flies were entrained at 25°C in a 12 h:12 h LD cycle for at least 96 h in each experiment. Collections were made every hour or every 2 h, over the course of a 24 h LD cycle. For each time point, heads were separated from fly bodies, and RNA was extracted from the former tissue as described (Lorenz *et al.*, 1989). The *luciferase* antisense RNA probe used for hybridization protects a 186 nucleotide fragment of *luc* RNA (Brandes *et al.*, 1996). The *per* 2/3 probe (Hardin *et al.*, 1990), which protects 259 nucleotides from exon 3 and 134 nucleotides from exon 2, was used in all experiments involving the *plo* transgene. The *per* 5/6 probe (Zwiebel *et al.*, 1991) was used to protect endogenous *per* RNA in BG-*luc* transgenic flies. This probe contains RNA from position 6054 to 6510 of the *per* gene and protects an exon 6 fragment of 213 nucleotides as well as an exon 5 fragment of 172 nucleotides (nucleotide positions are as in Citri *et al.*, 1987). Since the *per*-coding DNA present in the BG-*luc* construct extends only to position 5627 in exon 5, the *per* 5/6 probe does not protect this reporter RNA. Reporter RNA in BS-CAT transgenic flies (Hardin *et al.*, 1992) was protected by transcribing a 187 nucleotide CAT antisense probe from the pTRI-CAT plasmid (Ambion), which protects a 152 nucleotide fragment of the BS-CAT RNA. As a control for equal RNA loading in each lane, an antisense ribosomal protein probe (*rp49*) was included in each RNase protection assay which protects a 58 nucleotide fragment (Hardin *et al.*, 1990). RNA hybridizations, digestion and electrophoretic separations were performed as described (Lorenz *et al.*, 1989), except that the RPA II RNase Protection Assay kit (Ambion) and RNase ONE (Promega) were used. Each reaction contained 8 μg of total head RNA and ~1 × 10⁶ c.p.m. of each of the *luciferase* (or CAT), *per* 2/3 (or *per* 5/6) and RP49 probes. The protected fragments were separated on 8 M urea, 5% polyacrylamide gels. Protected bands of *per*, *luc* and CAT RNA were quantified using the Bio-Rad GS 250 Molecular PhosphorImager.

Immunoblotting

Flies from the *plo* transgenic strain were entrained for at least 3 days in 12 h:12 h LD cycles. Animals were collected and immediately frozen on dry ice at six different Zeitgeber times (ZT2, ZT6, ZT10, ZT14, ZT18 and ZT22). Total fly-head protein extracts were prepared from 100–200 heads of each collection as described in Edery *et al.* (1994). Electrophoresis and transfer of the proteins to nitrocellulose membranes was essentially as described in Stanewsky *et al.* (1997) except that a semi-dry electroblotter was used for protein blotting (60 min with 200 mA, in a buffer containing 20% methanol, 0.04% SDS, 44 mM Tris, 39 mM glycine). PER signals were visualized after incubating membranes with a polyclonal rabbit anti-PER serum followed by application of a secondary, horseradish-peroxidase (HRP)-coupled anti-rabbit antibody and developing the membranes with the Enhanced Chemi-Luminescence Kit (Amersham), as described in Stanewsky *et al.* (1997). After staining with anti-PER antibody, the membrane was stripped (in 100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7 for 30 min at 50°C), blocked, incubated with the polyclonal rabbit anti-luciferase antibody (1:1000) (Promega) and developed as described above.

Immunohistochemistry

Transgenic males carrying either the *plo* or *BG-luc* transgenes were exposed to at least three 12 h:12 h LD cycles at 25°C prior to sectioning. Flies were collected at two different Zeitgeber times, during which PER protein is expressed at high (ZT21) and low (ZT9) levels, respectively. Antibody stainings of 10 μ m frozen horizontal sections were performed as described in Stanewsky *et al.* (1997), except that the polyclonal rabbit anti-luciferase antibody (diluted 1:800) and a secondary biotinylated donkey anti-rabbit antibody (1:200) were used. Sections were photographed using Nomarski optics and a Zeiss Axiophot light microscope.

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