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.

Keywords: circadian rhythms/Drosophila/luciferase reporter/period

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 per01 and tim01 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 per01 or tim01 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 per01 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 perD flies carrying the plo or BG-luc transgene. This indicates that the ultradian behavior observed in individual perD 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 perD 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 perD 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 transgensics. Comparison of BG-luc and plo flies in a perD genetic background also revealed differences. The perD plo average shows no evidence of cycling and maintains high levels of bioluminescence relative to the plo oscillations in a perD genetic background (Figure 1C). In contrast, the perD BG-luc average exhibits low-amplitude oscillations and maintains intermediate levels of bioluminescence when compared with BG-luc in a perD 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 perD 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 1). The presence of a secondary bioluminescent peak in the plo transgensics 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 transgensics, 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 perD BG-luc and perD 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
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-
The BS-CAT construct contains the same analyzed from both a BS-CAT and the BG-luc tested in the homozygous condition with the exception of per 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).

Regulation of period gene expression

### Table 1. Quantitative analysis of plo and BG-luc bioluminescent oscillations

<table>
<thead>
<tr>
<th>Transgenic line</th>
<th>LD</th>
<th>DD</th>
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<tbody>
<tr>
<td></td>
<td># Rhy/tested (% rhythmic)</td>
<td>Mean period ± SEM</td>
</tr>
<tr>
<td>per+ BG-luc</td>
<td>71/72 (99)</td>
<td>24.4 ± 0.0</td>
</tr>
<tr>
<td>per+ BG-luc-3</td>
<td>15/16 (94)</td>
<td>24.2 ± 0.1</td>
</tr>
<tr>
<td>per+ BG-luc-s</td>
<td>15/15 (100)</td>
<td>23.9 ± 0.1</td>
</tr>
<tr>
<td>per+ BG-luc-5s</td>
<td>32/32 (100)</td>
<td>24.4 ± 0.1</td>
</tr>
<tr>
<td>per+ BG-luc-57</td>
<td>35/35 (100)</td>
<td>24.4 ± 0.1</td>
</tr>
<tr>
<td>per+ plo1b-1</td>
<td>67/67 (100)</td>
<td>24.4 ± 0.0</td>
</tr>
<tr>
<td>per+ plo1a-1</td>
<td>32/32 (100)</td>
<td>24.6 ± 0.1</td>
</tr>
<tr>
<td>per+ plo2a-2</td>
<td>15/16 (94)</td>
<td>24.2 ± 0.1</td>
</tr>
<tr>
<td>per+ plo2a-3</td>
<td>10/10 (100)</td>
<td>24.3 ± 0.2</td>
</tr>
<tr>
<td>per+ plo3b-1</td>
<td>31/31 (100)</td>
<td>24.1 ± 0.1</td>
</tr>
<tr>
<td>per+ BG-luc</td>
<td>27/37 (73)</td>
<td>25.1 ± 0.5</td>
</tr>
<tr>
<td>per+ BG-luc-3</td>
<td>12/15 (80)</td>
<td>24.2 ± 0.2</td>
</tr>
<tr>
<td>per+ BG-luc-57</td>
<td>14/14 (100)</td>
<td>24.5 ± 0.2</td>
</tr>
<tr>
<td>per+ plo1b-1</td>
<td>0/36 (0)</td>
<td>0/33 (0)</td>
</tr>
<tr>
<td>per+ plo1a-1</td>
<td>1/15 (7)</td>
<td>22.8</td>
</tr>
<tr>
<td>tim-1 BG-luc</td>
<td>4/23 (17)</td>
<td>24.9 ± 1.0</td>
</tr>
<tr>
<td>tim-1 plo2a-3</td>
<td>1/14 (7)</td>
<td>25.0</td>
</tr>
</tbody>
</table>

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 and per+ BG-luc-57 which were tested in the heterozygous condition. For all flies, only periods between 35 and 4 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 and 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+ 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-
luc reporter RNAs with that of endogenous per RNA expression in the per\textsuperscript{01} genetic background. The direct comparison of plo and per RNA levels in the per\textsuperscript{01} mutant background revealed that the reporter RNA is expressed at constantly high levels (80% of its maximal expression in per\textsuperscript{+}, Figure 3A and B), whereas endogenous per\textsuperscript{01} RNA is expressed at low levels (20% of its maximal expression in per\textsuperscript{+}, Figure 3A and B). In contrast, BG-luc RNA expresses at a low level in the per\textsuperscript{01} background, similar to that of endogenous per\textsuperscript{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\textsuperscript{01} RNA.

Phase and amplitude comparisons of per\textsuperscript{+} plo and endogenous per RNA (or per\textsuperscript{+} 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 \textit{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 \textit{et al.}, 1996). Thus, we conclude that an element within the transcribed region of per regulates \textit{period} gene expression in a circadian manner. This element is not simply an enhancer or suppressor regulated by light, since both per\textsuperscript{01} and BG-luc RNA fail to cycle in a per\textsuperscript{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 \textit{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 \textit{et al.}, 1996; Plautz \textit{et al.}, 1997). To reveal the true oscillations of both plo and BG-luc, curve-fitting software was applied to per\textsuperscript{+} BG-luc, per\textsuperscript{+} 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
Regulation of \textit{period} gene expression

**Fig. 3.** RNA expression levels of reporter and \textit{per} RNA, in \textit{per}\textsuperscript{+} and \textit{per}\textsuperscript{01} genetic backgrounds, of \textit{plo} and \textit{BG-luc} transgenic flies. (A) RNA expression of flies from the \textit{ploA-1} strain was measured in a \textit{per}\textsuperscript{+} and \textit{per}\textsuperscript{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 \textit{per}\textsuperscript{+} background, luciferase and per RNAs cycled with their characteristically different phases and amplitudes (see also Figure 2 and Brandes et al., 1996). In a \textit{per}\textsuperscript{01} background, the level of per RNA is only at 20\% of its peak level in \textit{per}\textsuperscript{+} (here: ZT14 and ZT16), whereas the level of luciferase RNA in \textit{per}\textsuperscript{01} is at 81\% of its maximal level in \textit{per}\textsuperscript{+} (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 \textit{plo1} strain) where \textit{per}\textsuperscript{01} RNA was ~50\% less abundant than two \textit{per}\textsuperscript{+} control RNAs from \textit{plo1} flies collected at ZT8 and ZT10. In the same experiments, the average luciferase RNA abundance in \textit{per}\textsuperscript{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 \textit{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 \textit{per}\textsuperscript{+} background, \textit{BG-luc} and per RNAs cycled with nearly identical phase and amplitude (see also Figure 2B). In a \textit{per}\textsuperscript{01} background, both transcripts are expressed at a constitutively low level (\textit{per}, 33\%; \textit{BG-luc}, 40.1\%) when compared with their peak times of expression in a \textit{per}\textsuperscript{+} 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.

The RNA expression levels were obtained 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. A 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 \textit{per}\textsuperscript{+} background, luciferase and per RNAs cycled with their characteristically different phases and amplitudes (see also Figure 2 and Brandes et al., 1996). In a \textit{per}\textsuperscript{01} background, the level of per RNA is only at 20\% of its peak level in \textit{per}\textsuperscript{+} (here: ZT14 and ZT16), whereas the level of luciferase RNA in \textit{per}\textsuperscript{01} is at 81\% of its maximal level in \textit{per}\textsuperscript{+} (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 \textit{plo1} strain) where \textit{per}\textsuperscript{01} RNA was ~50\% less abundant than two \textit{per}\textsuperscript{+} control RNAs from \textit{plo1} flies collected at ZT8 and ZT10. In the same experiments, the average luciferase RNA abundance in \textit{per}\textsuperscript{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. A 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 \textit{per}\textsuperscript{+} background, \textit{BG-luc} and per RNAs cycled with nearly identical phase and amplitude (see also Figure 2B). In a \textit{per}\textsuperscript{01} background, both transcripts are expressed at a constitutively low level (\textit{per}, 33\%; \textit{BG-luc}, 40.1\%) when compared with their peak times of expression in a \textit{per}\textsuperscript{+} 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.

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 \textit{per}\textsuperscript{+} flies were deemed rhythmic (Table I), and the strength of those rhythms was lower (presumably due to increased dampening). On average, the \textit{BG-luc} lines showed longer periods than the \textit{plo} lines. The average of the mean periods from all the \textit{BG-luc} lines was 24.9 h compared with 23.4 h
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 transgensics 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 6I). 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
Regulation of period gene expression

quanti\textit{t}ations, 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\textsuperscript{+} genetic background and level differences in the per\textsuperscript{D} 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.

\textbf{Functional properties of the novel regulatory element}

A central question is whether this element alone is sufficient to: (i) generate cycling per expression; (ii) replicate 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\textsuperscript{D} 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\textsuperscript{D} 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

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{BG-luc and plo temporal bioluminescence in DD. (A) Comparison of averaged per\textsuperscript{+} BG-luc flies (n = 71) and averaged per\textsuperscript{D} BG-luc flies (n = 37) in DD. As opposed to LD, per\textsuperscript{D} flies no longer show minor oscillations (Figure 1B). Although per\textsuperscript{+} 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\textsuperscript{+} plo1b-1 flies (n = 58) and averaged per\textsuperscript{D} plo1b-1 flies (n = 33) in DD. As seen in LD, per\textsuperscript{D} shows no rhythmicity whereas per\textsuperscript{+} shows cycling; however, the amplitude dampens as seen in the BG-luc (see A). (C and D) Examples of a single per\textsuperscript{+} BG-luc fly (C) and a single per\textsuperscript{D} 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 respectfully) 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.}
\end{figure}
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. 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 (Edery 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 per31 w sm2 flies (per0). 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 μm. (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 μm. (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 μm. (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).
Regulation of *period* gene expression

**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 *luciferase*. It has been suggested that these transgenes have landed near hypothetical cycling elements in *Drosophila* (Helfrich, 1986; Power *et al.*, 1995). It appears to be due to the different assay conditions used 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).

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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 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).

Regulation of *period* gene expression
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 pD261 plasmid containing the luciferase cDNA (Luehrsen et al., 1992) was cloned into the BamHI–KpnI sites of pBlueScript II KS after removing the first ATG by mung bean nuclease treatment. This 1.8 kb BamHI–KpnI luciferase cDNA fragment and a 9.8 kb BamHI per fragment, extending from +4200 to +5627 (Citi et al., 1987), were then cloned into the BamHI–KpnI sites of the pCasper 4 transfection vector (Thummel et al., 1988); in the latter, a 1 kb EcoRI 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. Transformants were performed with standard techniques (Rubin and Spradling, 1986); transposase was supplied by co-injection of the helper plasmid pUCHStΔ2-3 (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 perX background, the Df(1)w chromosome (which carries per+) was replaced by an X chromosome carrying perX w sn or y perX w in a subset of those lines. All lines were tested in the homoygous 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 12:12 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 LD cycle for at least 96 h in each experiment. Collections were made at 12 h intervals, with an antisense ribosomal protein probe (rp49) used for hybridization. The 68 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 protected a 58 nucleotide fragment of the BS-CAT RNA. In all cases, R (correlation coefficient) was >0.98. The e+ component of each curve was then divided from all data points and plotted. Since the DD data contains two damping components (the luciferase assay artifact and dampening per expression), trend-adjusting of DD data was performed using the following equation $	ext{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, the e+ 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 12:12 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 was generated by hybridizing remethylated EcoRI and KpnI sites of the pCaSpeR 4 transformation construct, carrying the mini–white gene and encoding about two-thirds of the PER protein fused in-frame to the luciferase cDNA and encodes about two-thirds of the PER protein fused in-frame to the luciferase cDNA. The differences reported in DD dampening between this study and others may result in different spatial bioluminescence patterns.
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 a 12% 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 Hamblen, M.J. et al. (1995) Tripping along the trail to the molecular mechanisms of biological clocks. Trends Neurosci., 18, 230–240.

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 μm frozen horizontal sections were performed as described in Stanewsky et al. (1997), except that the 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.

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