Circadian Cycling of a PERIOD-β-galactosidase Fusion Protein in Drosophila: Evidence for Cyclical Degradation

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Abstract The authors analyzed circadian features of two period-lacZ (per-lacZ) fusion genes in transgenic strains of Drosophila. Both genes manifest circadian fluctuations of mRNA levels, but fluctuations of only the larger chimeric protein are apparent. Fusion protein cycling is indistinguishable from the behavior of wild-type per protein (PER), including apparent temporal regulation of phosphorylation state. Several arguments indicate that the difference in the two constructs is proper regulation at the level of protein turnover: the smaller protein has much higher levels; a β-galactosidase degradation product is visible in both strains but fails to manifest cycling, presumably due to a long half-life; and only the noncycling proteins accumulate as a function of adult age. The large cycling fusion protein also undergoes modest cycling in an arrhythmic per background. This is light dependent, resembles the regulation of the timeless protein (TIM) by light, and reflects a documented fusion protein-TIM interaction. The results are discussed with respect to the posttranscriptional regulation that is necessary for proper cycling of both PER and TIM as well as for clock function.

Key words: Drosophila, circadian, rhythm, period, timeless, β-galactosidase, proteolysis, degradation

INTRODUCTION

Circadian rhythms are virtually ubiquitous in eukaryotes and even exist in some prokaryotes. The generally accepted paradigm consists of a central pacemaker connected to upstream environmental time cues (zeitgebers). The clock also is connected to downstream outputs, which give rise to the biochemical, physiological, and behavioral oscillations that are commonly monitored (Edmunds, 1988; Hastings and Schweiger, 1976; Rosbash and Hall, 1989; Takahashi, 1991).

The period (per) gene product of Drosophila is the best studied, if not understood, clock component (Baylies et al., 1993; Edery et al., 1994a; Hardin et al., 1995; Lee et al., 1996; Zeng et al., 1996). Although its function remains largely enigmatic, characterization of the per gene and its products has led to a working model of how it contributes to clock function and how a circadian pacemaker keeps time in this organism. The model proposes that circadian timekeeping is intimately tied to the metabolism of both per protein (PER) and per mRNA in a manner that vaguely resembles the relationship of growth control and cell cycle timing to

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the metabolism of cyclins or the Retinoblastoma gene product (e.g., Murray, 1993; Nurse, 1992). The link between clock function and PER metabolism is based in part on robust circadian oscillations that both PER and per mRNA undergo (Edery et al., 1994b; Hardin et al., 1990; Zeng et al., 1994; Zerr et al., 1990).

In addition, both qualitative and quantitative features of these metabolic oscillations are altered in per mutants (Curtin et al., 1995; Hardin et al., 1990; Zerr et al., 1990), suggesting that some of these mutants affect PER metabolism (synthesis and turnover) directly and the output rhythms only more indirectly.

Recent studies have identified tim as a second bona fide clock gene of Drosophila (Sehgal et al., 1994). There are both arrhythmic and pericl-altering alleles (Rutlal et al., 1996; Sehgal et al., 1994), and the effects of tim mutations are similar to the effects of per mutations. For example, wild-type tim mRNA levels undergo circadian oscillations like per mRNA levels (Sehgal et al., 1995), and arrhythmic tim mutations eliminate the molecular oscillations of per and tim as well as the behavioral rhythms (Sehgal et al., 1994, 1995). There is evidence that the tim mutants not only affect per transcription but also the PER at a posttranscriptional level (Price et al., 1995; Vosshall et al., 1994). In this regard, very recent studies are revealing because they show that timless protein (TIM) and PER can interact in vitro (and in yeast [Cookakis et al., 1995]) and that these two proteins are heterodimeric partners in vivo (Lee et al., 1996; Zeng et al., 1996). If the PER-TIM dimer is a functional unit of a circadian clock, then it explains the similar phenotypic effects of the two genes as well as the posttranscriptional effects of TIM on PER.

There were independent prior indications that both transcriptional and posttranscriptional regulation were important for PER cycling, and there were indications that per coding DNA was important for this regulation. These studies used the classic approach of generating transgenic flies, containing per gene deletions or per gene chimeras (fusion genes), to explore which regulatory features persist and can be transferred to reporter gene constructs. The results indicated that, first, per is subject to circadian regulation at the level of transcription, and DNA upstream of the transcription start site is sufficient for normal or near-normal transcriptional regulation (Brandes et al., 1996; Hardin et al., 1990, 1992). Second, a number of strains containing per gene constructs with little or no upstream DNA were still rhythmic and continued to manifest PER cycling, suggesting that there also is regulatory information downstream of the transcription start site. Although the mode(s) of regulation could not be defined with certainty, a number of indirect arguments suggested that at least some of the information is likely present within the coding DNA, including perhaps a trans-acting enhancer (Frisch et al., 1994; Liu et al., 1991; Vosshall and Young, 1995).

Third, analysis of a set of per-lacZ fusion gene constructs also suggested that there is regulatory information within per coding DNA. At least a portion of the coding region was required for circadian cycling of β-galactosidase (β-GAL) enzyme activity (Zwiebel et al., 1991). Because fusion gene mRNA cycling was robust even without PER coding DNA (Zwiebel et al., 1991), the observations suggested that the coding DNA contributed to some important posttranscriptional mode of regulation. A contemporary view is that proper posttranscriptional regulation requires a proper interaction with TIM (Price et al., 1995; Zeng et al., 1996).

The original studies of fusion gene expression and cycling used histochemical and enzymatic assays, respectively (Liu et al., 1988; Zwiebel et al., 1991). We decided to apply an independent but potentially more informative assay to the fusion gene products. It relies on recent biochemical progress that has allowed the direct visualization of PER by Western blotting of bead extracts (Edery et al., 1994b). Because these experiments confirmed and extended the original histochemical observations of PER cycling (Zerr et al., 1996), we reasoned that this same approach should be applicable to PER fusion proteins. In addition, we decided to assay a larger fusion gene, termed BC, that previously had never been analyzed for cycling. As compared to the original fusion gene (SC), BC contains an additional 231 amino acids of PER (a total of 868 N-terminal amino acids for BC [see Fig. 1]) and so is more likely to mimic wild-type PER's circadian behavior.

The results confirm that the per coding region contains posttranscriptional regulatory information that is important for PER cycling. More important, they suggest that these additional 231 amino acids contain a region necessary for temporal instability and that this is an important mode of posttranscriptional regulation that contributes to PER's circadian cycling.

**METHODS**

**Constructs and Germ Line Transformation**

Two different PER-β-GAL fusion proteins were used in this study: SG (Liu et al., 1988; Zwiebel et al., 1991) and BG (Fig. 1). These different constructs gave...
riese to two different lines each: SG3 and SG10, located on the 2nd and the X chromosome, and BG6 and BG/TM2, located on the 2nd and 3rd chromosomes, respectively (the BG6 line was generated after mobilizing the original BG [homozynogous lethal] insert by crossing BG/TM2 to a transposase producing ΔΔ-5 strain [Robertson et al., 1980]).

The BG transforming plasmid was constructed by taking a 9.8-kb DNA fragment containing genomic per DNA from the BamHI site 4.2 kb upstream to the transcription start to the BamHI site in the 5th exon, subcloned in a modified pUC18 vector (an XhoI site was in front of the 5' end of the fragment) and digested with SphI at the 3' end of the insert in the polylinker. The single stranded ends were then treated with Klenow to generate blunt ends, to which a BgIII linker was ligated. The size of this linker was chosen to allow in-frame cloning of the per fragment to lacZ DNA in a later step. The XhoI/BgIII fragment containing the per DNA was then cloned into the Xhol/BamHI sites of a modified pUC19 vector (again an XhoI site was in front of the per fragment) to achieve a KpnI site at the 3' end. An approximately 4-kb EcoRI fragment containing E. coli lacZ DNA (isolated from a pC4 clone provided by V. Picotto) was cloned into PUC 18 to generate a KpnI site at the 5' end of this fragment, which also contains an internal XbaI site at the 3' end. Finally, the XhoI/KpnI per and the KpnI/XbaI lacZ fragments were cloned together into the SalI and XbaI sites of the cp201 P-element transformation vector (Simon et al., 1985). This construct, made by X. Liu, was injected into a perΔppyΔ strain and then crossed into a perΔ background. timΔBG/TM2 was generously provided by H. Zeng.

Flies Entainment and Collections

Flies were entrained at 25°C in light-dark (LD) cycles of 12 h each for a minimum of 48 h and collected at the appropriate times by immediately freezing them in dry ice. Heads for biochemical analysis were prepared as described subsequently. Newly eclosed flies were collected by clearing bottles of adults at Zeitgeber Time (ZT) 20 the day prior to the collection. On Day 1, flies were collected from individual bottles at the specific times. At ZT 20 of Day 1, the newly eclosed flies were transferred into new bottles and left to incubate for another day (for 2-day-old flies), for 2 more days (for 3-day-old flies), and so on; they were then collected at the appropriate times.

Protein Preparation and Western Analysis

Total protein extract of fly heads was prepared as described previously (Edery et al., 1994b) with the following modifications. Fly heads were homogenized in 1-2 volumes of ice-cold HEPES extraction buffer (Edery et al., 1994b), and 100-200 mg of total extract was separated on a 5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were stained with Poncova S to ascertain equal loading. Rat polyclonal BPF anti-PER and rabbit polyclonal anti-PER antibodies were used to detect PER-containing proteins; both sera were raised against full-length PER protein expressed in the baculovirus expression system (Liu et al., 1992). Mouse monoclonal anti-β-GAL antibodies (Promega) and mouse polyclonal anti-TIM antibodies (Zeng et al., 1996) were used for detection of β-GAL and TIM proteins. Appropriate secondary HRP-conjugated antibodies were used with the ECL chemiluminesence detection system (both from Amersham).

RNA Extraction and RNase Protection Assay

Fly heads (50-100 μl) were homogenized in 200 μl of extraction buffer (20 mM Tris, pH 8.3, 5 mM EDTA, 0.3 M NaOAc, 1% SDS) followed by a phenol-chloroform extraction. RNA samples were treated with RNase-free Dnase one (Promega), and 15-30 μg of
Figure 2. BG fusion protein in a wild-type per<sup>+</sup> background. Western blots of 12 time points of BGMTM2 (upper panels) and BG8 (lower panels), both in per<sup>+</sup> backgrounds, were visualized either by rabbit anti-PER antibody (A) or by anti-β-galactosidase (anti-β-GAL) antibody (B), both diluted 1:10,000. (A) Western blots visualized by anti-PER. The bottom arrow points to the PER band, and the top arrow points to the BG band. In the 1st lane, control fly (ry ZT 20) extracts are run. Lanes 2 through 13 contain BG extracts at appropriate time points (ZT 0 to ZT 22). (B) Western blots visualized by anti-β-GAL. There are two bands in each panel (upper panel is BGMTM2, lower panel is BG8); the top arrow points to the BG band, and the bottom arrow points to the 33-kD band absent in control fly extracts (Lane 2) but present in the molecular weight marker (Lane 1) at the position of the β-GAL marker. Lanes 3 through 14 correspond to the different time points (ZT 0 to ZT 22).
Figure 2. (C) Graph plots the quantitation of both BG lines over time. Open and closed squares represent BG/TH2 stained with anti-FER and anti-β-GAL, respectively; open and closed triangles represent BG6 stained with anti-FER and anti-β-GAL, respectively; and open circles represent PER in BG/TH2.

Total RNA was used to hybridize with the appropriate probes (RPA II kit, Ambion). Digestion of the RNA/RNA hybrids was carried out using 10 units per sample of RNase ONE (Promega).

Three sets of probes were used: RPA6, per6, and lacZ. RPA6 is a 120-nt transcript that protects a 58-bp fragment of an abundant noncycling ribosomal RNA and is used as an internal control of RNA loading (Hardin et al., 1990). per6 is a 210-nt transcript that protects a 163-bp fragment from nse6 and is transcribed from a subclone of nse6 (Zwiebel et al., 1991). The lacZ probe originates from a 240-nt transcript and protects a 207-bp fragment in the lacZ fusion, again being specific only to lacZ-containing sequences (Zwiebel et al., 1991).

Quantification of Protein and RNA Cycling

Quantification of both protein and RNA from membranes and gels was carried out using the BioRad GS 250 molecular imager with the appropriate screen for the detection of chemiluminescence or 32P.

Immunoprecipitation of β-GAL Fusion Proteins

Proteins were extracted from approximately 100 µl of fly heads in 1 ml total volume, using the same extraction buffer as already described with the addition of 20 mM β-mercaptoethanol and 100 mM Na3VO4 to inhibit phosphatase activity. A sample of packed 15 µl of Gamma Bind Plus beads (Pharmacia) to which 1 mg anti-β-GAL antibody had been bound (following instructions of the manufacturer, Promega) was incubated with the extract overnight at 4°C with rotation. Beads were then washed several times with extraction buffer, resuspended in 40 µl of SDS sample buffer, and boiled. Then one third of the sample was loaded onto an SDS polyacrylamide gel and transferred to nitrocellulose membranes, which subsequently were incubated with anti-FER and anti-TIM antibodies (Zeng et al., 1996).

RESULTS

To examine the behavior of the larger BG fusion protein during the circadian cycle (Fig. 1), two BG lines were examined by Western blotting with an anti-FER antibody (Fig. 2A). For both lines, fusion protein cycling appeared similar to that of endogenous PER. Indeed, quantitation indicated that the phase and amplitude of fusion protein cycling were indistinguishable from those of PER from the same blot (Fig. 2C). PER cycling in this strain also was indistinguishable from what has been reported previously for normal wild-type strains (Zeng et al., 1994); that is, the presence of the BG fusion gene had little or no effect on PER’s molecular oscillations. This is consistent with the behavioral assays of these strains, which indicate only subtle differences from wild-type behavior (Stanewsky et al., 1997; also see Discussion section). The fusion protein also undergoes temporal mobility changes, as does PER (Eddy et al., 1994b), suggesting that BG also is subject to temporal phosphorylation. The fusion protein also was detected with anti-β-GAL antibodies (Fig. 2B), and the cycling profiles were identical whether detected with anti-FER or anti-β-GAL antibodies (Fig. 2C). Unexpected, however, was the presence of a 116-KD band detected only with the anti-β-GAL antibody. It has the expected molecular weight for β-GAL, comigrates with a β-GAL marker protein, and is absent from wild-type strains (Fig. 2B). Therefore, it almost certainly is fusion gene derived. For both strains, the 116-KD band intensity is substantial, comparable to peak levels of fusion protein. In contrast to the fusion protein, however, the 116 KD protein undergoes little, if any, temporal cycling. Our working hypothesis is that the 116-KD band is generated by proteolysis from the fusion protein band,
Figure 3. Newly closed flies giving evidence that degradation of BG results in increase of the β-galactosidase (β-GAL) band. Panels A and B show newly closed BG flies, 1 through 5 days old, shown both as a Western blot and its quantification. At ZT 20, prior to the 1st day of collection, all the flies in the bottles were removed. Day 1 flies were then collected at ZT 2, 6, 14, and 28. At ZT 20 of Day 1, additional flies were transferred into new bottles and then collected on Days 2, 3, 4, and 5 at the appropriate times. (A) Western blot of perBG flies incubated with anti-β-GAL. The numbers above the lanes correspond to the hours after eclosion (2 corresponds to ZT 20 of Day 1, 26 corresponds to ZT 2 of Day 2, etc.). The top arrow points to the BG band, and the lower arrow points to the 116-kD band.

(B) Quantitation from two gels of the two different BG lines for both the top band (BG-β-GAL fusion) and the bottom 116-kD band over the first 5 days after eclosion. Open symbols represent the curves for the perBG/TM2 strain, and the closed symbols represent the curves for the perBG strain. Squares represent the BG-β-GAL fusion, and triangles represent the 116-kD band. Similar results were obtained at least twice for each line.

Figure 3. (C) Western blot of newly closed flies from BG over the 1st and 2nd days of eclosion assayed with anti-β-GAL. The top band represents the BG band, and the bottom band represents the 116-kD band. MW, the molecular weight marker, contains the Escherichia coli β-GAL band that is detected with the anti-β-GAL antibody (numbering as above).
but the former is too stable to manifest circadian cy-
clying (Wisotzki et al., 1992). As a consequence, there is
a large amount of protein inherited from previous
cycles. It decays slowly and obscures the generation of
new 116-kD protein during the cycle when flies are
collected.

To test this interpretation, we examined young flies
shortly after emergence (Fig. 3). The rationale was that
protein stability problems might be less severe in
young flies where there may be fewer previous cir-
cadian cycles during which the 116-kD band could
accumulate. Other possible sources of 116-kD protein
(e.g., internal transcription or translational initiation
sites) probably would be locked to fusion protein syn-
thesis and be age insensitive.

Consistent with the proteolysis interpretation, the
relative intensity of the 116-kD band was much lower
in young flies, and it increased dramatically during the
first 4 days posteclosion. By contrast, robust cycling of
the fusion protein band intensity was apparent in
young flies, and peak levels changed little, if at all,
between Cycles 1 and 4 (Fig. 3A,B). The observations
are consistent with the notion that the fusion protein
is the source of the 116-kD band, but only the former is
completely degraded at each cycle. A high-resolution,
1st-day posteclosion cycling experiment confirms the

Figure 3. (D) Quantification of the Western blot in Panel C.
Squares represent the BG band, and diamonds represent the
116-kD band. Both curves have been normalized to their respec-
tive highest values. The other BG line was assayed with very
similar results.

Figure 4. SG protein and BG protein comparisons. Panels A and B compare one high and one low time point of protein and RNA
expression, respectively, for the two different fusion genes with each other. (A) Western analysis comparing fusion protein levels of
BG and SG lines in a per−/− and per+/− background collected at low GTZ 1B and high GTZ 2D time points. The blot was incubated with
anti-β-galactosidase antibody. The top arrow points to the SG and BG fusion protein bands, and the bottom arrow points to the 116-kD
band.
Figure 4. (B) Histogram of the relative RNA abundance at low (ZT 13) and high (ZT 3) time points for the BG and SG flies. The average of two experiments with the standard error is shown.

Figure 5. Degradation of the PER/Galactosidase (β-GAL) fusion protein in newly eclosed SG flies. Western blots of newly eclosed per; SG3 flies from Days 1 through 5, incubated with anti-β-GAL antibody, are shown in Panel A and quantified in Panel B. The graph also shows the quantification of the per; SG30 line. The numbers shown represent the hours after eclosion (0 is ZT 0 for Day 1 of eclosion). Both SG fly strains and the 116-kD band are indicated on the blot (upper and lower bands, respectively) and on the graph (the SG band is represented by squares, and the 116-kD band is represented by diamonds; open symbols represent SG3, and closed symbols represent SG10 flies). The experiment was repeated for SG3 with very similar results.
more intense than the BG extracts. The SG extracts also contain the 116-kD band, which is present at comparable levels to the BG 116-kD band (Fig. 4A).

The lack of SG protein cycling is not due to problems at the RNA level, as SG fusion gene mRNA undergoes circadian cycling similar to BG mRNA and to per mRNA itself (Zwislocki et al., 1991; Fig. 4B). Moreover, peak SG mRNA levels are comparable to those from the BG strains, indicating that the high SG protein levels are not due to high mRNA levels (Fig. 4B). The lack of SG fusion protein cycling, therefore, could reflect a failure to undergo a temporal posttranscriptional event. As described previously, cycling might be more evident shortly after eclosion, when protein stability might make less of a contribution to steady state protein levels. But no temporal cycling is apparent even at Days 1-5 posteclosion (Fig. 5). There is, however, a substantial increase in fusion protein levels between ZT 14 and ZT 20, which probably follows the rise in fusion RNA that should take place shortly before this time. However, levels fail to decrease between 20 and 32 (i.e., between ZT 20 and ZT 8), consistent with the results from entrained but older flies.

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**Figure 5.** (B) [Graph showing relative protein abundance over time]

**Figure 6.** The BG fusion protein cycles with a lower amplitude in the per^res^ background compared to the per^res^ background. (A) Western blots of extracts from per^res^/BG/TM2 (top panel) and per^res^/BG (bottom panel). Lane 1 contains the molecular weight marker, and Lane 2 contains extracts from per^res^ flies (marked with ry^res^) at ZT 20. The top arrow points to the BG fusion protein band, and the bottom arrow points to the 116-kD band.
Figure 6. (B) Quantification of the per^{B6}::BG6 Western blots showing the relative protein abundance of BG band standardized with the 116-kD band. The quantification was performed twice more with the BG6 line and once more with the BG/7M2 line; the amplitude in the different experiments varied from 1.6 to 2.6.

To investigate in more detail BG fusion protein fluctuations in the arrhythmic per^{B6} background, complete time courses of both BG lines were examined. The fusion protein manifests apparent two- to threefold cycling, with a peak near dawn (ZT 0) and a trough near dusk (ZT 12) (Fig. 6A,B). Therefore, the per^{B6}:BG profile not only has a low amplitude but also has a delay in the phases of the peaks and troughs.

To verify these arrhythmic, low-amplitude oscillations of the per^{B6}:BG protein, we performed time courses on both BG lines during the first 5 days post-closure. Fusion protein cycling was readily apparent (Fig. 6C,D). The 116-kD band continued to accumulate during the 5 days with no evidence for cycling, consistent with the apparently stable character of this protein (Fig. 6C,D).

The low-amplitude BG cycling resembles that observed for TIM in an arrhythmic per^{B6} background (Myers et al., 1996; Zeng et al., 1996, Fig. 7). Indeed, both BG and TIM levels decrease two- to threefold after lights on, and then recover after lights off in this background. During the subsequent day in constant darkness (DD), there is no general decrease in BG

Figure 6. (C) Western analysis of newly ech^-4 flies from Days 1 through 5 for per^{B6}:BG/7M2 (top panel) and per^{B6}:BG6 (bottom panel). Numbers on top of lanes represent the hours after eclosion with 0 corresponding to ZT 0 of Day 1.
protein levels or in TIM levels (Fig. 7). This suggests that the fluctuations both in BG and in TIM are light driven in the absence of a functional clock.

Light-driven BG fusion protein cycling not only is similar to that of TIM but also requires the presence of the TIM protein. In an arrhythmic tim− background (in which there is no TIM), there are no circadian oscillations of BG under either LD or DD conditions (Fig. 8A). BG protein levels in these flies are low, comparable to the trough values observed in a wild-type background. This is similar to what has been observed for PER in tim− flies, namely, very low levels and no cycling (Price et al., 1995).

Both the light-driven cycling and the low levels in a tim− background suggest that the BG fusion protein interacts with TIM in a manner that reflects the PER-TIM interaction. This is reasonable given that both the BG and the SG fusion proteins contain the per PAS domain, which undergoes a direct interaction with TIM in vitro and in yeast (Gekakis et al., 1995). To test for a direct physical interaction between the fusion protein and TIM in vivo, fly extracts from various BG and SG strains were immunoprecipitated with anti-β-GAL antibodies. The immunoprecipitates were then assayed with anti-PER and anti-TIM antibodies by Western blotting. The results indicate that both fusion proteins do indeed interact with TIM (Fig. 8B); TIM was recovered with anti-β-GAL antibodies from the fusion protein strains similar to the recovery of TIM with anti-hemagglutinin (anti-HA) antibodies from a pseudo-wild-type (PER-HA-tagged) strain (Zeng et al., 1996). It appears that somewhat more TIM is immunoprecipitated from the arrhythmic per− strain. Although not investigated in detail, this suggests that in the absence of PER, more TIM might be available for interaction with the fusion protein. In any case, the data suggest that there is formation of a fusion protein-TIM complex in vivo, similar to what has been reported for the wild-type PER-TIM complex. They also suggest that complex formation is necessary but not sufficient for fusion protein cycling.
DISCUSSION

Previous comparisons of prr RNA and protein cycling indicated that the RNA fluctuations cannot account for the shape and amplitude of the protein curve; that is, there must be circadian changes in either the PER translation rate or the PER half-life (Zeng et al., 1994). We had also reported that a per-loz fusion gene gives rise to enzyme activity fluctuations and RNA cycling qualitatively similar to the protein and RNA level changes manifested by PER. RNA cycling was insufficient to generate protein cycling from a shorter fusion gene, providing independent evidence that both posttranscriptional and transcriptional mechanisms contribute to PER cycling (Zwiebel et al., 1991). The results of this current report, using Western blotting rather than enzyme activity assays, indicate that BC fusion protein levels and PER levels are very similar. This conclusion assumes that most of the dominant epitopes are present in the first 70% of the PER protein sequence. More significantly, the results also show that BG fusion protein cycling matches almost perfectly the phase and amplitude of PER cycling (Fig. 2). These quantitative experiments allow a stronger conclusion than was previously possible, namely, that all major regulatory mechanisms that govern PER cycling must also act on BG cycling.

Because this circadian regulation takes place only within flies, we have been unable to undertake definitive labeling experiments to measure protein half-lives. There are, however, several indirect arguments that favor protein instability as an important posttranscriptional regulatory mechanism, especially for the effect of light on TIM levels in the late night (Hunter-Ersoz et al., 1996; Myers et al., 1996; Zeng et al., 1996).

The data are less compelling for PER, but the analysis of the BG fusion protein in this report suggests that protein instability also is important for PER cycling. First, the shorter SG fusion gene manifests no protein cycling. The SG gene is identical to BG except for the absence of the information encoding PER amino acids 638-868; that is, SG contains the same 5' nontran-
scribed and nontranslated information as does BG. Although a biochemical function of these 231 amino acids is not defined, these include PEST subsequences that are well conserved among Drosophila species as well as between Drosophila and other insects (Repper et al., 1994). Because the segment of 231 amino acids is in the middle of the coding region, it is more likely that it contains an instability element than a signal for translational control. Consistent with instability, there is one PEST sequence at the beginning of the segment (Stanewsky et al., 1997) that might be a protease cleavage site or a phosphorylation site that signals proteolysis (Eder et al., 1994b; Kellerman et al., 1990; Yaglom et al., 1995). The presence of an important phosphorylation site could be related to the notable differences in temporal mobility between BG and SG; that is, the BG fusion protein but not SG probably undergoes temporal phosphorylation similar to PER. This focus on instability elements does not exclude the possibility that other regulatory elements also reside within this region of the per gene (cf. Frisch et al., 1994). We also cannot exclude the possibility that the key instability element lies within the SG fusion protein but is inactive because of some idiosyncratic feature of the construct, for example, a position-dependent inhibitory effect of the β-GAL sequence. This would lengthen the SG half-life to a point where it would be unable to manifest circadian cycling; that is, a short half-life is necessary for protein cycling.

A second argument is focused on the contrast between BG fusion protein cycling and the 116-kD band noncycling. The experiments with young flies make it likely that the latter is derived by proteolysis from the fusion protein, although we cannot exclude a more complicated mechanism such as internal translational initiation that varies as a function of adult age. β-GAL is very stable in Drosophila (Monsma et al., 1988), and this explains why the 116-kD band intensity increases over several cycles posteclosion rather than undergoing circadian fluctuations. The slightly different mobilities of the BG- and SG-derived 116-kD band also are explained by this hypothesis: they presumably reflect details of the proteolysis events that generate the band, that is, effects of the different flanking protein sequences in the BG and SG fusions. If the fusion protein band and the 116-kD band have a precursor-product relationship, then the cycling precursor must be short-lived relative to the noncycling product.

Assuming that the 116-kD band is generated by proteolysis from the fusion protein, there is a third argument that favors temporal proteolysis as a mechanism that contributes to BG (and PER) cycling. It focuses on the different levels of the SG and BG fusion proteins. The decay curves of the 116-kD bands vary in a manner similar to the 5- to 10-kD differences in protein levels. By contrast, the 116-kD band levels are quite similar. If there are differences, then it is the βD-derived 116-kD band that is more abundant than the SG-derived band. These comparisons argue that a translational control mechanism is unlikely to generate the reduced levels and cycling of BG (and endogenous PER) relative to SG; a lower rate of BG synthesis should give rise to a comparably reduced level of the 116-kD protein, the opposite of what is observed.

A logical time for temporal control of PER and BG half-life is between ZT 20 and ZT 6, that is, in the late night and early morning when PER levels decrease markedly. But this possibility is not exclusive; for example, there might also be important changes in PER stability during the early night, when PER levels rise. At this time, PER might be stabilized by rising TIM levels (Price et al., 1995). One can model the differences between the PER and per RNA cycling curves (Zeng et al., 1994) by assuming that they are due entirely to the temporal control of protein stability (Fig. 9). This underscores the extent to which a robust posttranscriptional control mechanism is likely required at both times of night to explain the offsets in the RNA and protein cycling.

Appearance of the 116-kD band should reveal when BG is degraded, but stability of this band precludes visualizing of when it appears. The analysis of young flies is less plagued with this problem, but the signal from the 1-day-old flies’ 116-kD band is quite weak. Nonetheless, the 1st day cycling experiment suggests that much of the 116-kD band might be derived from the fusion protein during its decrease after ZT 20 (Fig. 3C,D). In the absence of very strong data, it is important to note that BG should have a much shorter half-life than SG without defining a mechanism that is under temporal control.

A number of recent observations are inconsistent with our previous report that there is robust circadian cycling of β-GAL enzyme activity from the SG β-GAL fusion protein (Zwiebel et al., 1991): (a) another laboratory has failed to observe cycling of lacZ enzyme activity under identical circumstances (M. W. Young, personal communication); (b) subsequently, we failed
ourselves to repeat the cycling enzyme activity measurements (data not shown); and (c) there is no obvious cycling in staining intensity of brain sections from these per^SG flies, assayed with both anti-PER and X-GAL stainings (Stanewsky et al., 1997). It was in large part because of these failures that we began to assay both Sg and BG fusion protein cycling by Western blotting, which has significant advantages over the enzyme activity measurements. For example, PER cycling is assayed on the same blots (which makes it unambiguous that the background strain is per^ and rhythmic), and the fusion protein is distinguishable from lower molecular weight forms that might have enzyme activity. As described previously, Western blotting also failed to detect any circadian cycling of SG fusion protein levels. Based in part on the potent cycling of the larger BG fusion protein, we believe that these negative results are correct and that this aspect of the original report on the SG strain is in error.

We do not have a very satisfactory explanation for the discrepancy. It is possible that we introduced some temporal bias into the original assay so that random fluctuations in the enzyme activity measurements gave the appearance of circadian cycling. We are unable to categorically eliminate the possibility that we inadvertently assayed the BG strain rather than the SG strain, that is, that our original report of SG cycling was in fact a report of BG cycling. A third possibility is raised by the experiments with young SG flies (Fig. 5). There is a reproducible increase in SG protein levels that occurs between ZT 8 and ZT 23 during the first posteclosion day. Presumably, it is driven by the same transcriptional and posttranscriptional mechanisms that govern the daily accumulation of PER or BG fusion protein. Based on this observation, a high percentage of 1-day-old flies might have led to some apparent cycling of B-GAL activity. In any case, a major conclusion of the original report (Zwiebel et al., 1991) is confirmed here: RNA cycling clearly is insufficient to ensure protein cycling from these reporter constructs given that all fusion gene constructs used in both studies undergo robust RNA cycling in a per^ genetic background (Fig. 4B). With a variety of reporter transscripts, the per^5' nontranscribed region promotes robust RNA cycling in an insert-independent fashion (Brandy et al., 1996; Hardin et al., 1992; Zwiebel et al., 1991).

In an arrhythmic per^ background, the BG flies are behaviorally arrhythmic, indicating that the fusion gene does not have detectable biological activity in this assay (Stanewsky et al., 1997). Yet the BG fusion protein undergoes cycling in this background, albeit with an altered phase and amplitude. These parameters were very similar to those reported for the TIM protein under LD conditions in a per^ background (Zeng et al., 1996). It is very likely that the depressed cycling amplitude of BG also is due to the absence of RNA cycling in this background (cf. Hardin et al., 1990; Sehgal et al., 1994); that is, there is continued BG synthesis at inappropriate times, giving rise to high trough values (cf. Figs. 3A and 6A). Taken to-
gether with the recently described in vivo association of TIM and PER (Zeng et al., 1996), the observations suggested that the similar behavior of BG and TIM might reflect a physical interaction. Indeed, BG cycling, like TIM cycling, is light driven in this archetypic background, and there is a BG-TIM interaction as assayed by immunoprecipitation (Figs. 7 and 8). Presumably, the effects of light on TIM half-life (Myers et al., 1996; Zeng et al., 1996) also affect the stability of the TIM-BG dimer. An interaction between BG and TIM also explains the slight behavioral effects of the BG fusion gene in a wild-type background; a BG-TIM complex either contributes to or interferes with the normal function of the PER-TIM complex (Stanewsky et al., 1997).

Given that the SG fusion protein also contains the PAS region and the requisite TIM-interacting region (Gekakis et al., 1995), it is not surprising that there is a comparable interaction between SG and TIM. The histochromal results of Vosshall et al. (1994) also are consistent with a SG-TIM interaction, as SG fusion protein is mislocalized to the cytoplasm in a tim mutant background. Yet even in a wild-type background, SG levels fail to cycle and are much greater than those of BG. Given that BG levels are comparable to those of PER and TIM (Fig. 2), SG levels must be in excess of what can be accommodated in an SC-TIM dimer form (Zeng et al., 1996) and must be predominantly in monomer form at all times of the circadian cycle (or as a β-GAL tetramer or associated with other proteins). By contrast, BG and PER are only substantially in monomer form in the early morning after TIM’s disappearance. This reasoning suggests that TIM might be necessary for SG delivery to the nucleus but not for its stabilization or retention within nuclei. The biochemical approach precludes addressing other possibilities; for example, there may be differences between SG and BG that are related to differences between the three PER-positive cell types in Drosophila heads (cf. Stanewsky et al., 1997).

There now are good candidates, but still indirect arguments that protein instability is important for dynamic features of both PER and TIM cycling. Future progress almost certainly will require identifying and characterizing the protease system(s) responsible for degradation of these two important clock components.

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