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Genetics

A post-transcriptional mechanism contributes to circadian cycling of a per–β-galactosidase fusion protein

(gene expression/period gene/*Drosophila*/circadian rhythms)

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ABSTRACT **The period gene (*per*) of *Drosophila melanogaster* affects circadian rhythms. Circadian fluctuations in per mRNA levels are thought to contribute to circadian fluctuations in per protein levels in the heads of adult flies. To address the mechanisms underlying these oscillatory phenomena, we have analyzed RNA and protein cycling from two *per*–β-galactosidase fusion genes. These studies demonstrate that 5′ noncoding sequences from *per* are sufficient to cause the fusion mRNA levels to cycle in a wild-type (rhythmic) background. Protein cycling requires additional sequences derived from the *per* coding region. The data suggest that there is a *per*-dependent post-transcriptional mechanism that is under circadian clock control required for per protein levels to fluctuate in a rhythmic fashion.**

Circadian rhythms are endogenous biological cycles that have been extensively studied in a wide variety of organisms. These rhythms, which persist under constant environmental conditions, have been observed at the biochemical, behavioral, and physiological levels and can be entrained by numerous external cues, including light and temperature (1).

In *Drosophila melanogaster* the period (*per*) gene affects the generation and/or maintenance of circadian rhythms (2). This is exemplified by the fact that mutations at this locus have been shown to alter or eliminate various circadian and/or ultradian rhythms (for review, see ref. 2). Although the biochemical function of the *per* gene is not known, these and other observations (3) suggest that the *per* gene product contributes to or influences oscillator function in the fruit fly.

More recent studies have also shown that the levels of per mRNA and perhaps the per protein undergo circadian fluctuations in the heads of adult flies (4–6). The RNA cycling was assayed biochemically, whereas the protein cycling was inferred from immunohistochemical observations. The *per* gene products, therefore, behave similarly to transcripts and proteins in other systems that are subject to circadian cycling—e.g., luciferin-binding protein and other proteins in *Gonyaulax* (7, 8), vasopressin mRNA in the rat suprachiasmatic nucleus (9), chlorophyll a/b mRNA in plants (10), and the mammalian liver transcription factor DBP (11). It is likely that these other molecules lie downstream of a circadian oscillator and contribute to one or more output pathways. The per protein is unique in that it affects not only behavioral rhythms but also fluctuations in the levels of its own mRNA (4).

To further understand the mechanisms involved in this feedback loop, we have undertaken a biochemical study of the various elements that contribute to both RNA and protein cycling. To this end, we have analyzed RNA and protein cycling from two *per*–β-galactosidase fusion genes. We have been able to localize elements necessary for RNA cycling to the 5′ noncoding region of the *per* gene. The results also indicate that the previously observed changes (5, 6) in per protein immunoreactivity are due to changes in per protein levels. Fusion protein cycling requires the presence of per coding sequences as well as the noncoding sequences required for RNA cycling, indicating that the protein cycling is not simply a consequence of fluctuations in mRNA levels. This suggests that there is a post-transcriptional mechanism under circadian clock control that contributes to the observed oscillations in per protein levels.

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MATERIALS AND METHODS

DNA Constructions and Germ-Line Transformations. The SG construct employed in this study has been reported (12). The NG DNA fragment was constructed by fusing the *per* DNA fragment in Fig. 1 (13) to a bacterial β-galactosidase DNA fragment in-frame at an *Nco* I site at the *per* translation start codon. *per*- and β-galactosidase-containing DNA fragments were ligated into the *P*-element-containing vector cp20.1 (14) at the *Xho* I and *Xba* I sites, respectively. Four separate autosomal insert lines (all of which are immunohistochemically indistinguishable; data not shown) of each transformant were generated in per^{01} hosts and crossed into X chromosome-bearing per^{+} backgrounds.

RNase Protection Assays. For each time point, heads and bodies were separated and RNA was extracted (4). The per 5/6 probe contains RNA from the *Pst* I site at position 6510 to the *Pst* I site at position 6054 (13) and protects an exon 6 fragment of 213 nucleotides (nt) as well as an exon 5 fragment of 172 nt. The β-galactosidase probe includes RNA from the *Hpa* I site at nt 437 to the *Sau*3a site at nt 230 (13) and protects a fragment of 207 nt. As a control for the amount of RNA in each lane, an antisense ribosomal protein probe (RP49) was included in each RNase protection assay (4). RNA hybridizations were performed as described (15), except that the protected fragments were separated on 8 M urea/5% polyacrylamide gels. Quantitation was done by either densitometrically scanning or directly measuring radioactivity in the *per* exon 6, β-galactosidase, and RP49 bands using a Microtek MSF-300GS image scanner or an Ambis radioanalytic imaging system, respectively. All protections were repeated at least three times with similar results; representative examples are shown in Figs. 2 and 3.

β-Galactosidase Assays. Extracts were prepared from fly heads collected and frozen at each time point. Approximately 50 heads from each time point were homogenized in 300 μl of BW buffer [20 mM Hepes, pH 7.5/80 mM KCl/50 mM $(NH_4)_2SO_4$/10 mM EDTA/0.1% Triton X-100/0.1% Nonidet P-40/0.05% SDS/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride/aprotinin (1 mg/ml)/leupeptin (1 μg/ml)/pepstatin (0.02 mg/ml)/10% (vol/vol) glycerol] followed by sonication for 30 sec. Extracts were cleared of debris by gentle centrifugation at setting 1 in an Eppendorf microcentrifuge for 2 min at 4°C, and the supernatants were then

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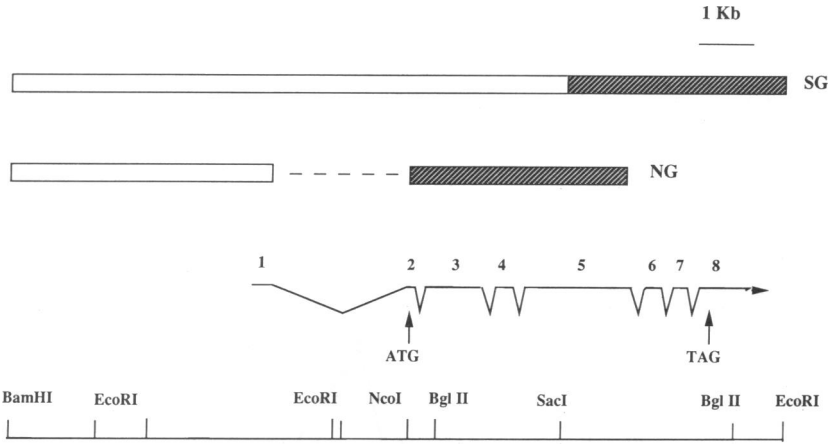

FIG. 1. DNA constructions and germ-line transformations. Plasmid constructions used for germ-line transformation are shown. The bars represent the various constructs containing *per* DNA (open bars) and β-galactosidase DNA (hatched bars) inserted into the cp20.1 vector. The dotted lines indicate deleted first intron sequences. These constructs are SG, a 9-kilobase (kb) *per* DNA fragment that covers the 5′ half of *per* transcribed DNA plus 4 kb of *per* 5′ upstream sequences fused in-frame to the bacterial β-galactosidase gene at the *Sac* I site (12), and NG, a 4.5-kb *per* DNA fragment with the same 5′ end as SG fused to the β-galactosidase gene at the *Nco* I site, which is at the translation initiation codon in the second exon of *per*. The first intron of *per* was deleted from the NG construct. The structure of the major form of per mRNA (13) and a restriction map of *per* gene are also depicted.

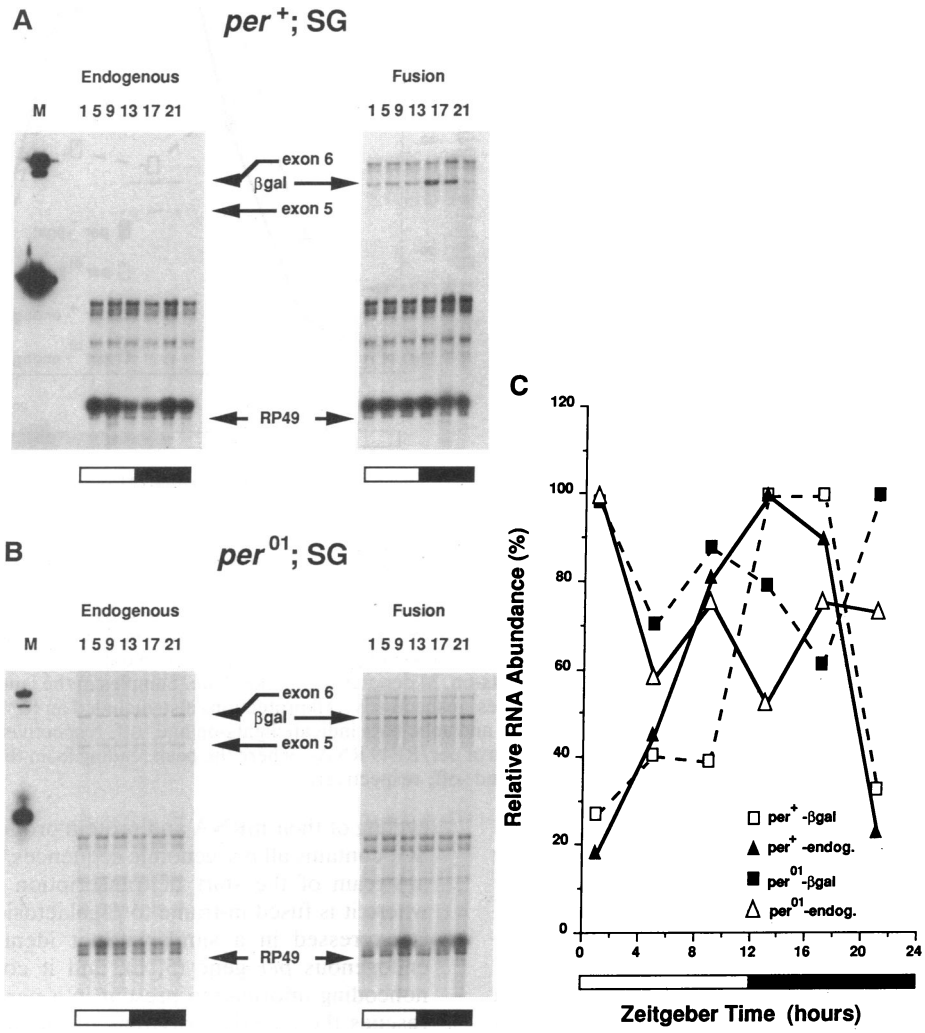

FIG. 2. Cycling of the endogenous per and the per–β-galactosidase transcripts in SG transformants. (*A*) RNase protection assay of per$^+$;SG RNA samples taken during 12-hr light/12-hr dark cycles. Numbers above each lane indicate the number of hours past the last lights-on period. M denotes the lane containing labeled 123-base-pair markers (BRL). Samples in the six lanes marked endogenous were protected with the per probe 5/6. Samples in the six lanes labeled fusion were protected with the β-galactosidase probe. Each protection also included the RP49 probe as a measure of RNA loading. Arrows denote the positions of the *per* exon 5-, *per* exon 6-, β-galactosidase (βgal)-, and RP49-protected fragments. The open and solid bars represent lights-on and -off, respectively. (*B*) RNase protection assay of *per*^{01};SG samples taken during 12-hr light/12-hr dark cycles. The numbers above each lane indicate the number of hours past lights-on. M denotes the marker lane. Samples in the lanes marked endogenous and fusion were protected with the per 5/6 and β-galactosidase probes, respectively. All protections also included an RP49 probe as above. The arrows represent the positions of protected fragments and the open and solid bars indicate lights-on and -off, respectively. (*C*) Quantitation of data shown in *A* and *B*. Relative RNA abundance refers to the values of per/RP49 RNAs, where the peak reading from the per$^+$;SG time course was adjusted to 100. Zeitgeber time refers to hours after lights-on in the 12-hr light/12-hr dark cycle; the open and solid bars represent lights-on and -off, respectively. βgal, β-galactosidase probe; endog., endogenous (per probe 5/6).

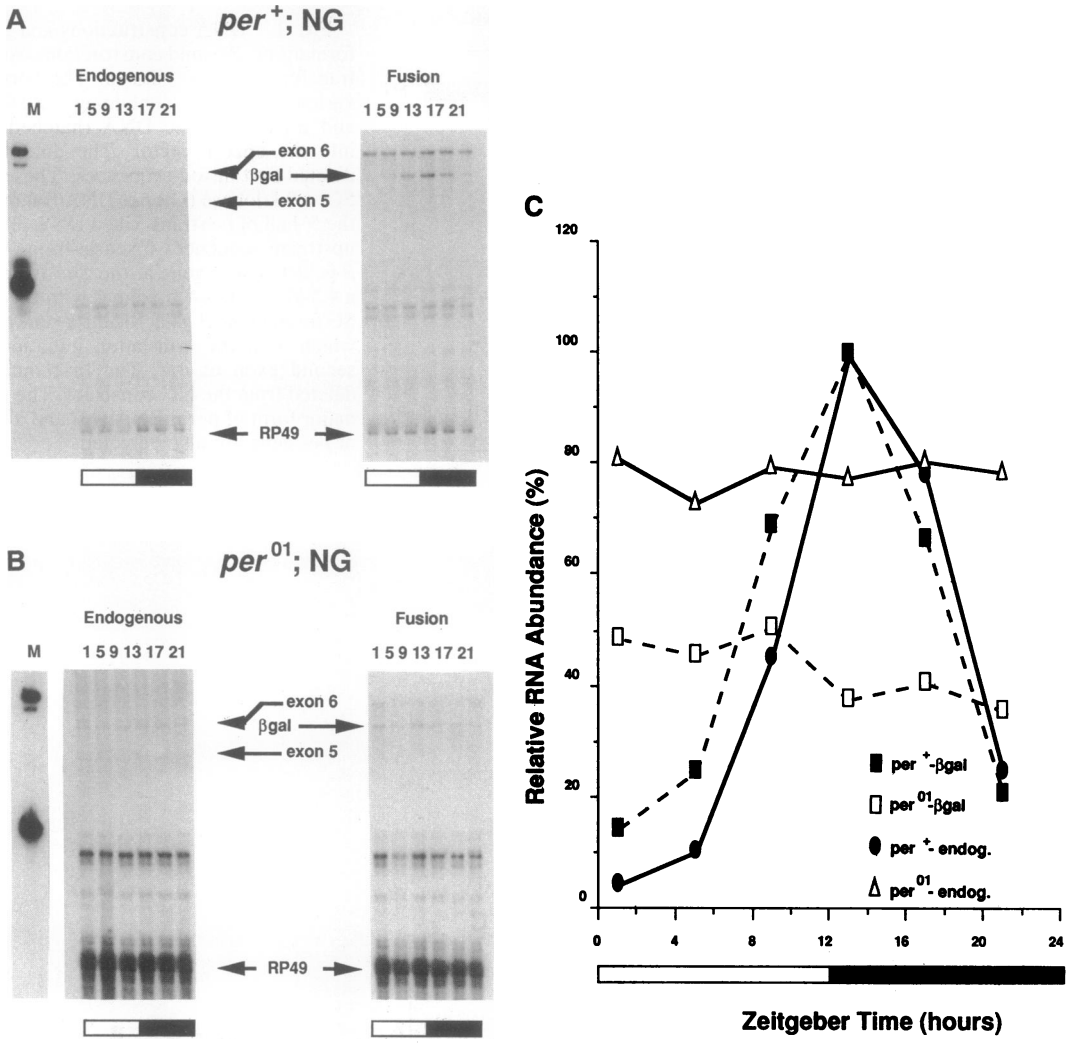

FIG. 3. Cycling of the endogenous per and the per β-galactosidase transcripts in NG transformants. RNase protection assay of per$^+$;NG RNA samples taken during 12-hr light/12-hr dark cycles. The number above each lane indicates the number of hours after the last lights-on. M denotes the lane containing labeled 123-base-pair markers (BRL). Samples in the six lanes marked endogenous were protected with the per probe 5/6. Samples in the six lanes labeled fusion were protected with the β-galactosidase probe. Each protection also included the RP49 probe as a measure of RNA loading. Arrows denote the positions of the *per* exon 5-, *per* exon 6-, β-galactosidase-, and RP49-protected fragments. The open and solid bars represent lights-on and -off, respectively. (*B*) RNase protection assay of per^{01};NG samples taken during 12-hr light/12-hr dark cycles. The number above each lane indicates the number of hours past lights-on. M denotes the marker lane. Samples in the lanes marked endogenous and fusion were protected with the per 5/6 and β-galactosidase probes, respectively. All protections also included an RP49 probe as above. The arrows represent the positions of protected fragments and the open and solid bars indicate lights-on and -off, respectively. (*C*) Quantitation of data shown in *A* and *B*. Relative RNA abundance refers to the values of per/RP49 RNAs, where the peak reading from the per$^+$;SG time course was adjusted to 100. The open and solid bars represent lights-on and -off, respectively.

transferred to a fresh tube. Protein concentrations were calculated from the absorbance at 280 nm. Typically, 100 μg of extract was used in each assay; β-galactosidase activity was assayed with the substrate chlorophenol red β-D-galactopyranoside (Boehringer Mannheim) according to Simon and Lis (16). Reaction mixtures were incubated at 37°C and the OD_{574} was read at 0.5, 1, 2, and 4 hr after extract addition. The rate of color development was linear throughout this time period. After subtraction of endogenous background (i.e., the value of a *per*$^+$ or a *per*^{01} extract that does not contain a *per*–β-galactosidase fusion gene), β-galactosidase expression was measured as the change in OD units per min per μg of extract. All graphs correspond to 2-hr incubations at 37°C in assay buffer.

RESULTS

Cycling of per–β-Galactosidase Fusion Transcripts. Two *per*–β-galactosidase fusion genes were tested for circadian cycling of their mRNA and protein products. One construct, SG, contains all *per* genomic sequences from ≈4.0 kilobases upstream of the start of transcription to amino acid 607, where it is fused in-frame to β-galactosidase (12). This gene is expressed in a similar if not identical pattern to the endogenous *per* gene (5, 6), and it contains all of the 5′ noncoding information present in a *per* gene construct that rescues the circadian rhythms of the arrhythmic *per*^{01} host strain (13). The other construct, NG, contains the same 5′ nontranscribed sequences as SG but is fused to β-galactosidase at the initiating ATG; i.e., it encodes no per amino acid sequence. Also, it is missing the large first intron that is present in SG (Fig. 1). Both fusion genes fail to restore the missing locomotor activity rhythms of *per*^{01} flies, and they have little or no effect on circadian locomotor activity rhythms in wild-type (*per*$^+$) backgrounds (ref. 12 and data not shown; also see *Discussion*).

To test for SG fusion gene-derived transcript cycling in a rhythmic background, *per*$^+$;SG flies were collected and fro-

zen at 4-hr intervals during a 12-hr light/12-hr dark cycle. RNA was isolated from the frozen heads and the fusion transcript was assayed by RNase protection. The endogenous per transcript was similarly assayed and served as an internal control (Fig. 2). Both endogenous and fusion transcripts cycled with similar phases and amplitudes, indistinguishable from what has been described for the per transcript in wild-type flies (4). The results indicate that the SG fusion gene contains cis-acting sequences that are sufficient for circadian cycling of its mRNA. Thus, the 3′ half of the per transcript is not necessary for mRNA cycling, nor does the presence of the β-galactosidase portion of the fusion RNA detectably affect its cycling.

In an arrhythmic per^{01} genetic background (Fig. 2), both the SG fusion transcript and the endogenous per^{01} transcript failed to manifest detectable cycling, as reported for the per^{01} transcript (4). Thus, the SG fusion gene was unable to rescue per RNA cycling in a light/dark cycle environment, consistent with its failure to rescue behavioral rhythms in constant darkness (ref. 12 and data not shown).

The NG fusion transcript was assayed in an identical fashion with indistinguishable results; namely, it cycled in a wild-type background with the same amplitude and phase as the endogenous per^{+} transcript. Also, the NG transcript failed to cycle in an arrhythmic per^{01} background (Fig. 3). This result shows that sequences necessary for cycling are contained within the NG fusion gene, indicating that intron 1, the 3′ half of the *per* gene, and per protein coding sequences are not necessary for RNA cycling. As shown above for the SG transcript, NG transcript cycling is dependent on per^{+}-mediated rhythmicity; i.e., there was no detectable cycling in a per^{01} background.

Cycling of per–β-Galactosidase Fusion Proteins. As described above, per protein cycling has been observed histochemically, but no biochemical experiment has verified this observation, probably because the level of per protein is too low to detect by Western blot analysis (ref. 6; L.J.Z. & I. Edery, unpublished observations). Consequently, we sought to address this issue by determining whether β-galactosidase activity undergoes circadian cycling in the fusion-gene-bearing transformants. Extracts were prepared from adult heads because the level of endogenous enzyme activity is considerably higher in the body than the head (ref. 12 and data not shown). Also, the use of head extracts was indicated since genetic mosaic analysis has identified the head as the focus of the effect of per on adult activity rhythms (17) and since per mRNA cycling and protein immunoreactivity cycling are most apparent in these tissues (4–6).

As for the RNA cycling experiments reported above, flies were collected and frozen at 4-hr intervals during a 12-hr light/12-hr dark cycle. Protein extracts were prepared from heads and assayed for β-galactosidase activity. In a wild-type background, the SG transformants showed a strong cycling of β-galactosidase activity. The amplitude ranged from 4- to 9-fold in several independent experiments (Fig. 4*A*), and the times of peak and trough values were similar if not identical to those reported for the oscillations in per immunoreactivity (5). We note that peak values of the per transcript precede the peak values of both immunoreactivity (5) and SG β-galactosidase activity (Fig. 4) by approximately 6 hr. As was found for the RNA cycling, no significant fluctuations were apparent in an arrhythmic (per^{01}) background.

In contrast, the NG transformants manifested no β-galactosidase activity cycling, even in wild-type backgrounds (Fig. 4*B*). Because the NG fusion gene transcript levels cycled normally (Fig. 3), we conclude that mRNA cycling is not sufficient for protein cycling. Similar negative results were obtained with *per*–chloramphenicol acetyltransferase reporter gene constructs (P.E.H., unpublished results), indicating that the results obtained for the NG β-galactosidase fusion protein were not simply an artifact of this reporter gene.

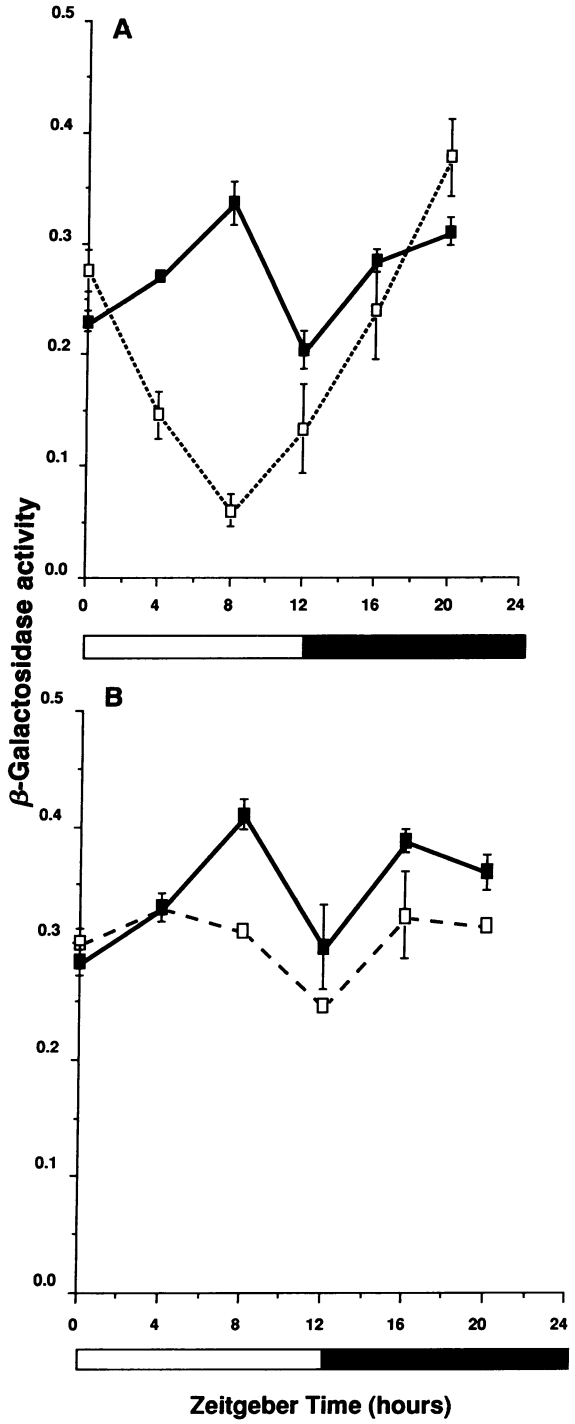

FIG. 4. Cycling of β-galactosidase activity in NG and SG transformants. (*A*) β-Galactosidase assay of per^{+};SG (open squares) and per^{01};SG (solid squares) protein extracts from flies collected during LD cycles. Numbers below the horizontal axis denote the time (in hours past the last lights-on) samples were collected. Activity was measured in terms of OD units ($\times 10^{-4}$) per μg per min after a 2-hr incubation at 37°C in assay buffer. All values were in the linear range of the assay and were adjusted for endogenous activity by subtraction of values for a per^{01};13.2:34 extract that carries a wild-type copy of the *per* gene but no *per*–β-galactosidase fusion gene. (*B*) β-Galactosidase assay of per^{+};NG (open squares) and per^{01};NG (solid squares) protein extracts from flies collected during LD cycles. Calculations were performed as in *A*. Data presented in *A* and *B* represent averages of four experiments; error bars indicate the standard error of these averages.

DISCUSSION

In this report, we present data suggesting that there are at least two mechanisms that contribute to the observed cycling of mRNA and protein from a *per*–β-galactosidase fusion gene. Although it is possible that a single transcriptional control point could account for both mRNA and protein cycling, the failure of the NG fusion protein to manifest circadian cycling makes it more likely that there are two mechanisms that operate at the RNA and protein level, respectively. As no RNA or protein cycling is apparent in arrhythmic (per^{01}) strains, both mechanisms are dependent, directly or indirectly, on *per* gene activity.

The SG fusion gene transcripts manifested circadian fluctuations in a manner indistinguishable from those of the wild-type *per* gene transcripts, suggesting that they are subject to the same regulatory mechanisms. This is consistent with previous reports indicating that both genes are expressed in similar if not identical cellular locations (5, 6, 15). The NG RNA cycling indicates that cis-acting signals sufficient to confer this property to a largely β-galactosidase-encoding gene lie on the 5′ side of the initiation codon of the *per* gene. These observations and other more recent experiments (P.E.H., unpublished results) suggest that the regulation is transcriptional. Only the SG fusion gene manifested circadian fluctuations at the protein level, suggesting that some per protein sequences are required for this regulation.

Similar data were obtained by Western blot analysis. Quantitation of the SG protein band showed that the amplitude of the SG fusion protein cycling fluctuated approximately 5-fold and was dependent on per^{+} since SG protein levels remained constant in a per^{01} background. For the NG transformant, there were no obvious fluctuations of β-galactosidase protein levels in either genetic background (data not shown). Thus the fusion protein data presented herein suggest that the cycling observed in immunohistochemical studies of the per protein (5, 6) reflects real changes in protein levels.

A transcriptional control mechanism that governs RNA cycling and a second post-transcriptional mechanism necessary for protein cycling might also help to explain the substantial lag (≈6 hr) between the SG RNA peak and the fusion protein peak. As the fusion RNA fluctuations are indistinguishable from those of per mRNA (4) and as the immunohistochemical per protein fluctuations (5, 6) are similar to the SG fusion protein fluctuations, a similar lag probably exists between the per mRNA peak and its protein peak. This is presumably due to the same post-transcriptional mechanism that governs the SG fusion protein cycling. Because the NG β-galactosidase activity levels are relatively high and because the noncycling SG levels in an arrhythmic (per^{01}) background are also high, this mechanism probably operates to reduce SG β-galactosidase activity to the low trough values present once a day.

There are at least two post-transcriptional mechanisms that could account for cycling of the SG fusion protein and the failure of the NG fusion protein to undergo cycling. The first is translational and dictates that per protein synthesis is modulated as a function of circadian time. Our data indicate that this would require the translation of some sequences in the N-terminal half of the per coding region. The second mechanism involves protein turnover and implies that features of the per amino acid sequence within the N-terminal half of the fusion protein target the protein for degradation. Either the activity of the proteolysis system or the activity of a post-translational system that marks the per protein-containing sequences for degradation or delivery to an appropriate cellular compartment could vary as a function of circadian time. Whatever the mechanism, the regulation must be under clock control as no fluctuations occur in a per^{01} background.

Although these observations reinforce the validity of our previous observations on per RNA and protein cycling (4–6), a number of important questions remain unanswered. Can protein cycling occur in the absence of RNA cycling? In other words, the NG fusion results indicate that RNA cycling is not sufficient for protein cycling, but is it necessary for protein cycling? Perhaps more important is determining the extent to which RNA and protein cycling are necessary for the behavioral rhythms that the *per* gene affects. For example, recent reports have described (3) a heat shock promotor *per* gene construct that lacked almost all the 5′ flanking DNA present in the NG fusion gene but retained some ability to rescue rhythmicity at elevated temperatures. This observation suggests that some biological activity may not require per RNA cycling.

The results presented herein recall observations concerning genes critical for cell cycle progression in eukaryotes. Several of these gene products undergo cell-cycle-mediated alterations at both the RNA and protein level; e.g., there are indications (18–22) that transcriptional as well as post-transcriptional regulatory mechanisms underlie the oscillations. Many of these mechanisms are central to the cell cycle, and future experiments must address whether the daily fluctuations of *per* gene product levels are equally central to circadian rhythms.

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