Analysis of Period mRNA Cycling in *Drosophila* Head and Body Tissues Indicates that Body Oscillators Behave Differently from Head Oscillators

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The period (*per*) gene is thought to be part of the *Drosophila* circadian pacemaker. The circadian fluctuations in *per* RNA and protein that constitute the *per* feedback loop appear to be required for pacemaker function, and have been measured in head neuronal tissues that are necessary for locomotor activity and eclosion rhythms. The *per* gene is also expressed in a number of neuronal and nonneuronal body tissues for which no known circadian phenomena have been described. To determine whether *per* might affect some circadian function in these body tissues, *per* RNA cycling was examined. These studies show that *per* RNA cycles in the same phase and amplitude in head and body tissues during light-dark cycles. One exception to this is the lack of *per* RNA cycling in the ovary, which also appears to be the only tissue in which PER protein is primarily cytoplasmic. In constant darkness, however, the amplitude of *per* RNA cycling dampens much more quickly in bodies than in heads. Taken together, these results indicate that circadian oscillators are present in head and body tissues in which PER protein is nuclear and that these oscillators behave differently.

Circadian rhythms influence many biochemical, physiological, and behavioral processes in plant, animal, and microbial systems (10). In any organism, different rhythms must be coordinated with respect to each other and to the time of day. This coordination results from the action of an endogenous, genetically driven circadian clock that persists under constant environmental conditions, is reset by environmental parameters such as light and temperature, and is relatively temperature independent.

To understand the molecular circuitry underlying circadian-clock function, genetic screens have been performed to isolate mutants having altered circadian rhythms. Mutations in the period (*per*) gene of the fruit fly *Drosophila melanogaster* can shorten (*per^S*), lengthen (*per^L*), or abolish (*per⁰¹*) circadian rhythms in eclosion and locomotor activity (17). As *per* function also appears to be necessary for entrainment (to light-dark cycles) of the circadian clock (7), it is likely that *per* expression is required for flies to either measure or tell time. An important aspect of *per* expression is that its mRNA and protein products undergo daily fluctuations in abundance (12, 36). These fluctuations constitute a feedback loop in which *per* mRNA is the template for *per* protein (PER) synthesis and PER is necessary for the circadian synthesis of its own mRNA (12, 36). Since PER is nuclear in most tissues (22) and is able to repress its own RNA's synthesis (35), it is thought to function by directly repressing its own gene's activity (13). The regulatory features of the *per* feedback loop parallel formal theoretical models of self-sustaining circadian oscillators and might also accommodate the effects of PER on circadian behavior (13). Thus, the *per* feedback loop is thought to be a critical component of the *Drosophila* circadian clock.

The *per* feedback loop was initially shown to function in many neuronal tissues of the head (12, 36) where the circadian pacemaker controlling locomotor activity and eclosion rhythms resides (6, 18). However, *per* is also expressed in a number of

neuronal (thoracic ganglia) and nonneuronal (salivary glands, Malpighian tubules, ovaries, testes, and gut) body tissues (21, 29, 31) for which neither *per* molecular rhythms (13) nor behavioral rhythms have been described. The virtual lack of *per* molecular rhythms in bodies is somewhat surprising since PER's function within the feedback loop is dependent on its nuclear localization (30, 33) and PER appears to be localized in the nucleus in all tissues except the ovary (21, 22, 29, 31). Therefore, either PER has other (non-feedback loop) functions in the nuclei of these body tissues or *per* mRNA cycling in nonovarian body tissues might be masked by noncycling *per* RNA from ovaries.

To distinguish among these possibilities, I have measured *per* mRNA cycling in various body fractions from male and female flies. These experiments show that with the exception of ovaries, *per* body RNA cycles with a phase and amplitude similar to those of *per* head RNA during light-dark cycling conditions but that this high-amplitude body cycling dampens much more rapidly than that in heads during constant darkness. These results show that *per* feedback loop-associated oscillators are present in head and body tissues in which PER is nuclear and indicate that these oscillators behave differently under free-running (in constant darkness) conditions.

MATERIALS AND METHODS

Circadian light regimens. Wild-type (Canton-S) flies were reared in cornmeal-molasses-agar-yeast medium at 25°C during 12-h light–12-h dark (LD) cycles. For all time course experiments, adults were placed in fresh media and entrained for 3 days before collections commenced. When only males or only females were used in a given experiment, they were separated before being entrained, collected, and frozen. LD samples were collected every 4 h, immediately frozen, and stored at –80°C. Samples collected during constant darkness (DD) were placed in a dark incubator after the entrainment period, collected every 4 h starting at 21 h circadian time (CT21) or CT23, and immediately frozen at –80°C.

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FIG. 1. Circadian cycling of *per* RNA in whole males and whole females during LD. (A) RNase protection assays were performed on total whole-fly RNA from 3- to 7-day-old wild-type male and female adults collected every 4 h during LD cycles. Approximately 10 μ g of male RNA and 20 μ g of female RNA were used in each protection assay. The number above each lane indicates the number of hours since the last lights on. Molecular weight markers (M) are the 123-bp ladder. Arrows denote the positions of the *per* RNA-protected fragments and the RP49-protected fragment. RP49 was included as a measure of RNA loading in each lane. The open and solid bars represent lights on and off, respectively. (B) The abundance of *per* RNA in whole males and females was densitometrically quantitated from the 259-nt *per*-protected fragment. Relative RNA abundance refers to the ratio of *per* RNA to RP49 RNA, where the peak reading from whole males and whole females was adjusted to 100. The open and solid bars represent lights on (ZT0) and off (ZT12), respectively.

Tissue isolation and RNA extraction. Various fractions, including heads, whole bodies, thoraces, abdomens, bodies minus ovaries, and ovaries, were isolated from frozen flies. Heads were separated from bodies as described previously (24). Bodies were subdivided into thoraces and abdomens by manual dissection with a razor blade. Body-minus-ovary and ovary fractions were obtained by manually dissecting ovaries under a dissecting microscope. RNA was prepared from all tissues immediately after their isolation (23). RNA quantitation was done on an LKB Ultrosan III spectrophotometer.

RNase protection assays. RNase protection assays were performed as described previously (12). In all cases, antisense *per* 2/3 probe was used to measure *per* RNA abundance, and antisense ribosomal protein 49 (RP49) was used as a measure of the relative amount of RNA in each sample. The *per* 2/3 probe was linearized either with *Spe*I (see Fig. 3 to 6), which gives 259- and 143-nucleotide (nt) protected fragments, or with *Nco*I (see Fig. 1 and 2), which gives 259- and 93-nt protected fragments. The size standard used was either the 123-bp ladder (Bethesda Research Laboratories) or *Msp*I-cut pBR322 DNA (New England Biolabs). Quantitation was done by either densitometrically scanning autoradiographs with an Apple color scanner with Ofoto 2.0 and NIH Image 4.1 software or directly counting the samples with a Fujix BAS 2000 phosphor-imager with MacBAS software. Each protection experiment was repeated independently at least twice, with similar results.

Calculating cycling amplitude. The values for the relative levels of *per* mRNA for each overlapping set of six consecutive 4-h time points (24-h bins) were averaged. The difference between the value for each time point in the bin and the average value for that bin was calculated and used as a measure of cycle amplitude. Thus, a 3-day experiment in which

4-h time points were used would result in 13 separate amplitude values corresponding to each overlapping 24-h bin of six time points. The relative amplitude for the first set of time points, which started 21 or 23 h past the last lights on, was set to 1.0. Linear regression lines were calculated for each set of amplitude values with MacCurveFit version 1.0.3 and Minitab.

RESULTS

Analysis of *per* RNA cycling in whole males and females. Little if any *per* RNA cycling is seen in fly bodies from a mixed (male and female) population (14), even though, with the exception of the ovary, PER appears to be located in nuclei (21, 22, 29, 31). Since a lack of PER nuclear localization appears to block circadian function at both the molecular and behavioral levels (30, 33), *per* RNA should manifest high-amplitude cycling in males and low-amplitude cycling in females because the noncycling *per* ovarian RNA resulting from nonnuclear PER would mask the high-amplitude *per* RNA cycling from other tissues only in females.

To address this possibility, *per* RNA levels from whole male and female flies were measured every 4 h during LD cycles (Fig. 1). In males, *per* RNA cycled with an ~5-fold amplitude, which is similar to the 5- to 10-fold cycling amplitudes seen in heads (12). In females, *per* RNA levels were relatively constant, having a cycling amplitude of only approximately twofold. The fact that high-amplitude *per* RNA cycling is observed in males indicates that PER has a circadian function in body nuclei. The lack of high-amplitude cycling in female flies suggests that noncycling *per* ovarian RNA masks high-amplitude cycling predicted to occur in other tissues in which PER is nuclear.

FIG. 2. *per* mRNA cycling in male bodies during LD. (A) RNase protection assays were performed on total wild-type male head, thorax, and abdomen RNA collected every 4 h during LD cycling. Approximately 5 μ g of head RNA and 10 μ g of body RNA were used in each protection assay. The lanes, protected fragments, molecular weight markers, and open and solid bars are as defined in the legend to Fig. 1. (B) The abundance of *per* RNA in male heads, thoraces, and abdomens was densitometrically quantitated from the 259-nt *per*-protected fragment. The axes are as defined in the legend to Fig. 1.

Comparison of *per* RNA cycling in male heads with those in thoraces and abdomens. Previous studies indicate that *per* protein is localized to nuclei in a number of neuronal and nonneuronal tissues in male bodies (22, 29). To test whether *per* RNA is cycling in body parts where *per* is expressed in both neuronal and nonneuronal tissue (thorax) or nonneuronal tissue only (abdomen), the abundance of *per* RNA in the heads, thoraces, and abdomens of flies kept in LD cycles was monitored every 4 h (Fig. 2). All three dissected fly parts exhibited circadian cycling in the levels of *per* RNA. The phase and amplitude of cycling in thoraces and abdomens were similar to those seen in heads, though the overall abundance of *per* RNA in both thoraces and abdomens was approximately fivefold less than that in heads as determined by comparison of equal amounts of RNA from the three body parts. The fact that *per* RNA cycles similarly in male heads, thoraces, and abdomens suggests that this cycling is manifest in body tissues of neuronal and nonneuronal origin and that *per* oscillators are present in different tissues.

Analysis of *per* RNA cycling in female bodies. The only tissue-specific differences in *per* gene expression among males and females occur in the gonads, where *per* is expressed in the testes of males and in the follicle and nurse cells which surround the oocyte in females (21, 29). These sex-specific differences in *per* expression may account for the observation that *per* RNA cycling amplitude is approximately threefold higher in whole males than in whole females (Fig. 1). If this is the case, then *per* RNA should exhibit little if any cycling in isolated female bodies.

When *per* RNA levels in female bodies were measured, low-amplitude cycling (less than or equal to twofold) was detected (Fig. 3). This low-amplitude cycling was not simply due to the inability to entrain the LD cycles, as *per* RNA from the heads of these same flies cycled with an $\sim$ 10-fold amplitude, $\geq$ 5-fold higher than that of RNA from the body (Fig. 3).

The low-amplitude *per* RNA cycling observed in female bodies is consistent with the hypothesis that a large proportion of noncycling RNA from ovaries overwhelms the underlying high-amplitude rhythms in body tissues common to both males and females.

Comparison of *per* RNA cycling in ovaries and in bodies without ovaries. To directly determine whether noncycling *per* RNA in ovaries masks high-amplitude cycling in the rest of the body, females collected every 4 h during LD were separated into ovary and body-minus-ovary fractions and RNA from these fractions was used for RNase protection assays. In nonovarian body tissues, *per* RNA cycles with an approximately fourfold amplitude, while in ovarian tissue, *per* RNA shows little if any (less than or equal to twofold) cycling (Fig. 4). These results indicate that common male and female body tissues (i.e., salivary glands, Malpighian tubules, and the gut) support *per* RNA cycling whereas reproductive tissues either do (male testes) or do not (female ovaries) support *per* RNA cycling.

To determine if the lack of *per* RNA cycling in ovaries could account for the low-amplitude cycling seen in whole females and female bodies, I quantitated the amounts of total RNA and *per* RNA in heads, bodies minus ovaries, and ovaries (Fig. 5). Spectrophotometric measurements of RNA isolated from dissected female body parts show that heads contain $\sim$ 5% of total fly RNA, bodies minus ovaries contain $\sim$ 25% of total fly RNA, and ovaries contain $\sim$ 70% of total fly RNA (data not shown). Based on these amounts of total RNA, the percentages of *per* RNA at its peak level (16 h zeitgeber time [ZT16]) are as follows: $\sim$ 18% in female heads, $\sim$ 56% in bodies minus ovaries, and $\sim$ 26% in ovaries (Fig. 5B). From these ratios, an estimate of whether low-amplitude *per* ovarian RNA cycling could account for lower amplitudes seen in whole females (Fig. 1) and female bodies (Fig. 3) can be made. If $\sim$ 26% of *per* RNA (from ovaries) is not cycling, $\sim$ 18% is cycling with a

FIG. 3. Circadian cycling of *per* RNA in female bodies. (A) RNase protection assays were performed on total body RNA from 3- to 7-day-old wild-type female heads and bodies collected every 4 h during LD cycling. Approximately 5 μ g of head RNA and 30 μ g of body RNA were used in each protection assay. The lanes, protected fragments, molecular weight markers, and open and solid bars are as defined in the legend to Fig. 1. (B) The abundance of *per* RNA in heads and bodies was densitometrically quantitated from the 259-nt *per*-protected fragment. The axes are as defined in the legend to Fig. 1.

10-fold amplitude (from female heads), and $\sim 56\%$ is cycling with a 4-fold amplitude (from bodies minus ovaries), then a whole-fly cycling amplitude of <3 -fold and a female body cycling amplitude of ~ 2 -fold would be expected. These expect-

tations are very close to the whole-female and female body measurements presented in Fig. 1 and 3, respectively. Thus, masking of high-amplitude cycling in females appears to result from the large quantity of noncycling *per* RNA in ovaries.

FIG. 4. *per* RNA cycling in ovaries versus bodies minus ovaries. (A) RNase protection assays were performed on total ovary and body-minus-ovary RNA from 3- to 7-day-old wild-type females collected every 4 h during LD cycling. Approximately 10 μ g of body-minus-ovary RNA and 30 μ g of ovary RNA were used in each protection assay. The lanes, protected fragments, and open and solid bars are as defined in the legend to Fig. 1, except that the molecular weight markers (M) are *Msp*I-cut pBR322 DNA. (B) The abundance of *per* RNA in female ovaries and bodies minus ovaries was densitometrically quantitated from the 259-nt *per*-protected fragment. The axes are as defined in the legend to Fig. 1.

FIG. 5. Levels of *per* RNA in different female tissues. (A) Head RNA (2 μ g) body-minus-ovary RNA (12 μ g) and ovary RNA (36 μ g) extracted at ZT16 were used for RNase protection assays. Head RNA, body-minus-ovary RNA, and ovary RNA are designated above each lane. Molecular weight markers (M) are *Msp*I-cut pBR322 DNA. Arrows denote the positions of the *per* RNA-protected fragments and the RP49-protected fragment. RP49 was included as a measure of RNA loading in each lane. (B) Quantitation of *per* RNA levels in panel A from the 259-nt *per*-protected fragment. "Percent of total *per* RNA" refers to the amount of *per* RNA in each body fraction as a percentage of the total amount of *per* RNA in the fly.

Cycling of *per* RNA in male heads and bodies during constant darkness. As shown above, the levels of *per* RNA undergo circadian cycling in heads and bodies of male flies kept in LD cycles (Fig. 1). To determine whether these body RNA oscillations are due to the action of an endogenous pacemaker or are driven by LD cycles, *per* RNA was measured from the heads and bodies of male flies that had been entrained to LD cycles, placed under free-running (in DD) conditions, and collected every 4 h for 3 days. Three peaks in RNA abundance were seen in heads and bodies during the course of this experiment (Fig. 6), indicating that circadian cycling of *per* RNA persists in male bodies under free-running conditions. All three peaks occurred just after subjective lights off (the time at which lights would be off if the LD cycles had continued), in phase with peaks measured in LD, while the amplitude of these fluctuations damped from fivefold to less than twofold by the third day in DD. Thus, the periodicity and phase of *per* RNA cycling in male bodies are comparable to what are seen in heads under the same conditions, but the body cycling amplitude dampens much more quickly than that of heads (12).

A metric was devised to quantitate the amplitude of *per* RNA cycling in head and body fractions under free-running conditions. This metric compares the relative RNA values

from each set of overlapping 24-h bins (see Materials and Methods), and these values were used for quantitative comparisons. Regression lines were also calculated from the amplitude values for each head or body datum set for qualitative comparisons. From calculations based on the samples from Fig. 5, the head amplitude dropped from 1 to 0.70 in 3 days whereas the body amplitude dropped ~ 2.5 -fold faster, from 1 to 0.28 (Fig. 7). Other independent experiments showed similar differences in amplitudes between heads and bodies. In fact, if the head data (as a whole) are compared with the body data (as a whole), *per* head RNA amplitude does not significantly change ($r^2 = 0.288$; $P < 0.05$, not significant) whereas *per* body RNA amplitude significantly decreases ($r^2 = 0.886$; $P < 0.05$), indicating that the head and body amplitudes differ through time. This increased dampening of *per* RNA in bodies suggests that *per* body oscillators either rapidly decrease in amplitude or become asynchronous under constant environmental conditions.

DISCUSSION

Even though the *per* gene is expressed in a number of adult head and body tissues (21, 29, 31), circadian fluctuations in the levels of *per* RNA were initially observed in fly heads (12). Heads were examined in these early studies because (i) they contain the locomotor activity and eclosion pacemakers (6, 18), (ii) little if any circadian cycling was seen in RNA from whole flies (27, 34), and (iii) circadian fluctuations in PER immunoreactivity from heads had already been demonstrated (31). These original studies have now been extended to show that *per* RNA from bodies also cycles in a circadian manner. These *per* body RNA fluctuations occur in the same phase (peaking between ZT12 and ZT18) and have an amplitude (approximately fivefold) similar to that of *per* head RNA fluctuations (12). This *per* body RNA cycling does not occur in all body tissues and is notably absent in the ovary, which accounts for $\sim 70\%$ of total RNA in female bodies. The noncycling *per* RNA in ovaries ($\sim 26\%$ of total *per* RNA) can account for the low-amplitude cycling seen in mixed (male and female) flies and in females (27, 34), while the lack of ovaries correlates with robust *per* RNA cycling in males (Fig. 2).

The current model for how the *per* feedback loop operates predicts that PER protein is involved in negatively regulating its own gene's transcription (13). Three lines of evidence indicate that PER's regulatory role is rather direct: PER has been localized to the nucleus in head and body tissues exclusive of the ovary (22), PER contains a protein-protein dimerization domain (PAS) common to several other transcription factors (15), and the *timeless* clock mutation blocks both PER nuclear localization and the *per* feedback loop (30, 33). Since PER lacks any known DNA binding motifs, negative regulation could be achieved by inactivating a transcriptional activator or by activating a transcriptional repressor, perhaps through protein-protein-mediated dimerization involving the PAS domain (15). These proposed regulatory functions of PER within the *per* feedback loop are dependent upon the nuclear localization of this protein; thus, *per* feedback loop function would not be predicted to occur in tissues in which PER is cytoplasmic. The only tissue for which this prediction can be tested under normal circumstances (in wild-type flies) is the ovary, where PER itself (29) or a PER-LacZ fusion protein (21, 22) is almost completely cytoplasmic. The lack of *per* RNA cycling in ovaries (Fig. 4) indicates that the *per* feedback loop is not operating and fits with the prediction that PER must be localized to the nucleus to carry out its circadian feedback loop function. It will be of interest to determine whether the lack of

FIG. 6. *per* mRNA cycling in male heads and bodies during DD. (A) RNase protection assays were performed on total head or body RNA from wild-type males collected every 4 h during constant darkness. Approximately 10 μ g of head RNA and 20 μ g of body RNA were used in each protection assay. The number above each lane indicates the number of hours since the last lights on. Arrows denote the positions of the *per* RNA-protected fragments and the RP49-protected fragment. RP49 was included as a measure of RNA loading in each lane. (B) The abundance of *per* mRNA in heads and bodies of free-running male flies was densitometrically quantitated from the 259-nt *per*-protected fragment. "Relative *per* RNA abundance" refers to the ratio of *per* RNA to RP49 RNA, where the peak reading from male heads was adjusted to 100. The open, solid, and hatched bars below the numbers of hours represent lights on, lights off, and subjective lights on, respectively.

PER nuclear localization in ovaries is due to the absence of *timeless*.

The phases of *per* RNA cycling were very similar in all of the body fractions measured (Fig. 2). This synchrony among *per* oscillators in different body parts suggests that they are coordinated, or coupled, in some way. The mechanism underlying this coupling is largely dependent upon the nature of these oscillators, which could range from autonomous pacemakers that are entrained by light and set the phase of the rhythm to "slave" oscillators whose entrainment and phase are dependent upon a "master" oscillator (25). There is precedent for

both autonomous pacemakers and slave oscillators. In vitro cultures of gypsy moth testes (8), *Xenopus* eyes (4), chicken pineal glands (32), and *Aplysia* eyes (16) show that autonomous, light-entrainable pacemakers control free-running rhythms of sperm release (moth testes), melatonin release (*Xenopus* eyes and chicken pineal glands), and electrical activity (*Aplysia* eyes). Transplantation experiments show that an autonomous pacemaker in the *Drosophila* brain (11) and the hamster suprachiasmatic nucleus (26) sets the period of free-running activity rhythms. In contrast, the mammalian pineal gland, which produces melatonin in a rhythmic manner,

FIG. 7. Dampening of *per* RNA cycling amplitude in heads and bodies during DD. Data from three independent free-run (in DD) *per* head RNA cycling and *per* body RNA cycling experiments (see the legend to Fig. 6 and Materials and Methods for details) were analyzed to determine cycling amplitude. For each set of six overlapping 4-h time points within a time course (x axis), a relative amplitude was calculated, and for each time course, a regression line was calculated (see Materials and Methods). Asterisks indicate the samples shown in Fig. 6A.

can be removed without affecting activity rhythms (2), indicating that it is a slave oscillator. The relative independence of *per* body oscillators is not known. However, the relatively rapid dampening of body oscillators (Fig. 5 and 7) compared with that of head oscillators in DD argues that body oscillators are relatively more dependent on light for coupling of their phase and/or boosting of their amplitude, but whether this represents a dependence on head oscillators is not known.

Although the function of *per* RNA cycling in at least some head tissues is associated with the pacemaker that controls locomotor activity and eclosion rhythms, no such correlation between *per* RNA cycling and circadian outputs in fly bodies exists. The two main sites of *per* gene expression in bodies are the digestive and reproductive tracts. Cyclic *per* gene expression in these tissues may be related to circadian rhythms in locomotor activity. For instance, digestive enzymes may have to be made in anticipation of food intake during the active phase. Free-running circadian rhythms in food intake have been demonstrated for mammals (5) and are independent of the main pacemaker in the suprachiasmatic nucleus (28). Likewise, sperm may have to be generated and released in anticipation of courtship and mating during the active phase. Sperm release rhythms in gypsy moths have been described (8). On the other hand, the function of noncycling *per* ovarian RNA, which is presumably not involved in circadian function, remains obscure. Although a daily rhythm in oviposition has been reported, this rhythm is apparently driven by LD cycles, as it fails to persist in constant conditions (1, 3). Perhaps PER protein serves some ultradian or infradian function in the ovary. Such a possibility is not inconceivable, since *per* has been shown to affect a 60-s ultradian rhythm in the courtship song of male *D. melanogaster* (19) and an ~10-day infradian rhythm in the development of *D. melanogaster* (20). The *per*-expressing cells necessary for courtship song rhythms, however, map to the thorax (9) rather than the ovary, and the cells necessary for the ~10-day developmental rhythm have not been mapped.

The discovery of synchronous *per* RNA cycling in body

tissues indicates that a number of coupled circadian oscillators are present in *D. melanogaster*. Since mechanisms for coupling autonomous oscillators (e.g., direct connection to entrainment pathways) may be somewhat different from those for nonautonomous oscillators (e.g., electrical or humoral connections to a pacemaker), the nature of these *per* body oscillators must first be determined. Once this is accomplished, experiments to probe the molecular mechanisms that underlie coupling can then be designed. Through these sorts of studies, the organization of the *Drosophila* circadian system can be defined.

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