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per mRNA Cycling Is Locked to Lights-Off under Photoperiodic Conditions That Support Circadian Feedback Loop Function

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Circadian fluctuations in *per* mRNA and protein are central to the operation of a negative feedback loop that is necessary for setting the free-running period and for entraining the circadian oscillator to light-dark cycles. In this study, *per* mRNA cycling and locomotor activity rhythms were measured under different light and dark cycling regimes to determine how photoperiods affect the molecular feedback loop and circadian behavior, respectively. These experiments reveal that *per* mRNA peaks in abundance 4 h after lights-off in photoperiods of ≤16 h, that phase shifts in *per* mRNA cycling and behavioral rhythmicity occur rapidly after flies are transferred from one photoperiod to another, and that photoperiods longer than 20 h abolish locomotor activity rhythms and leave *per* mRNA at a median constitutive level. These results indicate that the *per* feedback loop uses lights-off as a phase reference point and suggest (along with previous findings for per^{01} and tim^{01}) that *per* mRNA cycling is not regulated via simple negative feedback from the *per* protein.

Circadian rhythms in biochemical, physiological, and behavioral phenomena are a fundamental adaptation of both prokaryotic and eukaryotic organisms to environmental changes that occur over a 24-h period. These rhythms are driven by an endogenous clock that continues to operate under constant environmental conditions. The timekeeping component of the clock, or pacemaker, maintains a periodicity that can be hours longer or shorter than 24 h, and it is synchronized to local time by such environmental signals as light and dark. A stable phase relationship between the pacemaker and its Zeitgeber is clearly a prerequisite if preprogrammed biological changes are to be appropriately timed to daily environmental changes. Physiological and behavioral experiments have been used to determine how the clock adjusts its phase to circadian cycles composed of different proportions of light and dark. During these different photoperiodic conditions, the phase of the circadian pacemaker (and its physiological and behavioral output) is altered so that a stable phase relationship is maintained (26). Because of the lack of measurable pacemaker components, however, these physiological studies could not address the molecular mechanism by which the clock adjusts its phase to accommodate different environmental light-dark (LD) cycles.

Genetic screens for rhythm mutants have been used to identify components of the circadian pacemaker. Mutations in the *per* gene from *Drosophila melanogaster* can shorten (per^S and per^T), lengthen (per^L), or abolish (per^{01}) circadian rhythms of locomotor activity and eclosion during constant dark (DD) conditions (16, 17) and alter the phase of locomotor activity and eclosion rhythms during LD cycling conditions (3, 10, 11, 17, 30). These behavioral effects of the *per* mutants are paralleled at the molecular level by circadian fluctuations in the abundance of *per* mRNA and *per* protein (PER) (12, 41). These fluctuations in *per* mRNA and protein levels compose a negative feedback loop in which *per* mRNA serves as the template for PER synthesis and PER inhibits the synthesis of its own mRNA (12, 13, 39). Since disruption of this feedback loop by a pulse of PER causes a stable and long-lasting phase shift in locomotor activity (4) and this feedback loop (as indicated by PER cycling) operates in the pacemaker cells of the brain (7), it is thought to be an integral component of the *Drosophila* circadian pacemaker.

A second component of this feedback loop, called *timeless* (*tim*), was also recovered in a genetic screen for rhythm mutants (34). The original *tim* mutant, tim^{01}, abolishes locomotor activity and eclosion rhythms in DD and renders *per* mRNA arrhythmic (34). This arrhythmicity is thought to be due to a defect in PER nuclear localization, as a PER-LacZ fusion protein is blocked in tim^{01} (37). Recent findings show that native PER is only present at minute levels in tim^{01} mutants (29), suggesting that *tim* protein (TIM) may function to stabilize PER, which may allow PER to accumulate to levels required for nuclear entry. A direct effect of TIM on PER is supported by the finding that TIM can bind to PER in a yeast two-hybrid assay (8). The observation that *tim* mRNA cycles in phase with *per* mRNA suggests that TIM is also cycling in abundance, temporally restricting its interaction with PER (35). Given the effect of *tim* on *per* feedback loop function and the importance of this feedback loop for circadian rhythmicity, *tim* is another component of the circadian pacemaker in *D. melanogaster.*

In *Neurospora crassa*, mutations at the *frq* locus can also shorten (frq^1 and $frq^{2,4,6}$), lengthen (frq^3 and $frq^{7,8}$), or abolish (frq^9) rhythms in conidiation (reviewed in reference 22). Like that of *per* and *tim*, circadian expression of the *frq* gene is also regulated through a negative autoregulatory loop (1). The *frq* feedback loop contrasts with the *per* feedback loop, however, in that the *frq* mRNA cycle is roughly antiphase to that of *per* and *tim* (1, 12, 35). Perturbation of this negative autoregulatory loop with an inducible *frq* gene resets the phase of the clock in a predictable manner, indicating that fluctuations in the levels of *frq* gene products are required for, and central to, circadian oscillator function in *N. crassa* (1).

In this study, we asked what the phase relationship between the *Drosophila* circadian pacemaker and the Zeitgeber was

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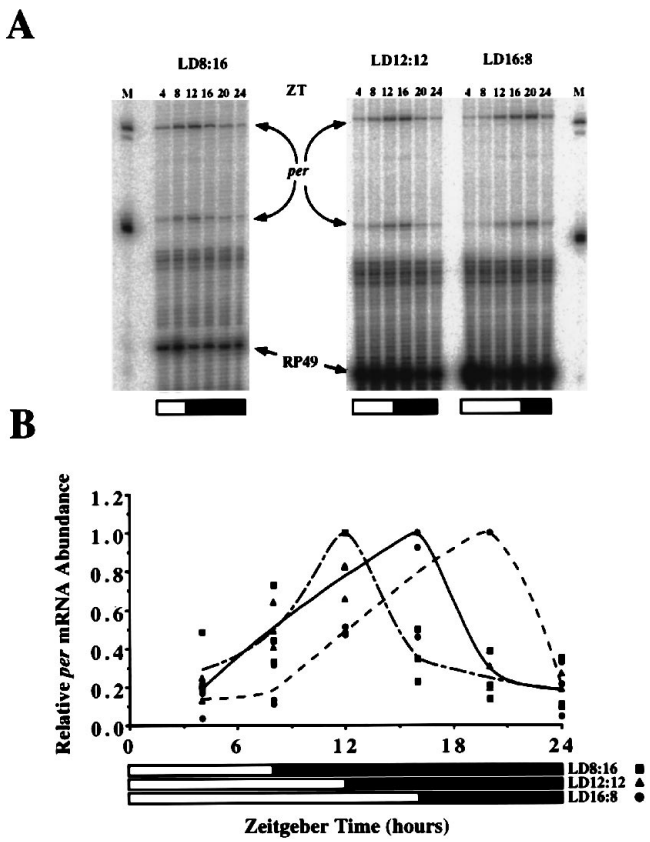

FIG. 1. The phase of *per* RNA cycling locks to lights-off in different photoperiods. (A) RNase protection assays were performed on total head RNA from flies entrained in LD8:16, LD12:12, or LD16:8 cycles as described in Materials and Methods. The numbers above each lane indicate the number of hours since the last lights-on in ZT. Molecular weight markers (M) are the 123-bp ladder. The *per*- and RP49-protected fragments are denoted by arrows. 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 was quantitated from the 259-nucleotide *per*-protected fragment. Relative abundance refers to the ratio of *per* RNA to RP49 RNA, for which the peak reading from each (n = 3) independent experiment in a given photoperiod was adjusted to 100. The curves are drawn through the average values of the datum points for each photoperiod. The open and solid bars represent lights-on (ZT0) and lights-off (ZT12), respectively.

under different photoperiodic conditions, how this relationship was achieved during the transition to a new photoperiod, and how the *per* feedback loop and locomotor activity rhythms react to constant light (LL)-induced arrhythmicity. By measuring *per* mRNA cycling and locomotor activity rhythms in different photoperiods, we show that there is a stable phase relationship between the *per* mRNA cycle and lights-off in photoperiods of ≤16 h. When photoperiodic conditions are altered by premature darkness or extended light, the circadian clock responds by adjusting its phase almost immediately, reaching a steady state by the next day. Photoperiods longer than ~20 h abolish both *per* mRNA cycling and locomotor activity rhythms and leave *per* mRNA levels at about half that of the peak seen in LD periods of 12 h of light and 12 h of dark (LD12:12). These results show that the *per* feedback loop uses lights-off as a phase reference point, indicate that phase resetting is not mediated by light-induced transcription, and suggest that an additional (non-PER) activator or repressor is operating within the *per* feedback loop. Thus, even though negative feedback loops are important for oscillator function in *D. melanogaster* and *N. crassa*, these organisms appear to use different mechanisms to entrain their oscillatory loops to LD cycles.

MATERIALS AND METHODS

Entrainment and fly sample collection. Adult wild-type (Canton-S) flies reared in LD12:12 cycles at 25°C were placed in fresh medium and entrained in different photoperiods for 3 days before collections commenced. Samples were collected every 4 h, immediately frozen, and stored at −80°C. In the photoperiod transition experiments, flies were entrained in LD12:12 cycles for 3 days and transferred to either LD8:16 cycles or LD16:8 cycles on the fourth day. Samples were collected every 2 or 4 h starting on the day of transfer for 3 days (LD8:16 and LD16:8) and were immediately frozen at −80°C. In the LL experiments, flies raised in LD12:12 cycles were transferred to LL for 3 to 4 days before collections started. In the LD12:12-to-LL experiments, flies were entrained in LD12:12 cycles for 3 days and were then transferred to LL between Zeitgeber time zero (ZT0) and ZT12. Fly samples were collected every 4 h (starting at the beginning of extended light) for 2 days. For the long photoperiod experiments, flies were entrained for 4 days in LD16:8, LD18:6, LD20:4, or LD22:2 cycles or LL, collected every 4 h on the fifth day, immediately frozen, and stored at −80°C.

Behavioral monitoring. Young (1 to 3 days posteclosion) adult wild-type (Canton-S) males reared in LD12:12 cycles at 25°C were assayed for locomotor activity as described previously (9). Briefly, individual flies were loaded into glass tubes, these tubes were clipped into an apparatus that continuously monitors activity via infrared light beam interruption, and activity events occurring in each tube were registered every 30 min under various photoperiodic conditions. For the photoperiod shift experiments, activity was monitored for 4 to 6 days in LD12:12 cycles and then in either LD16:8 or LD8:16 cycles for another 7 days at 25°C. For the LL experiments, activity was monitored for 4 to 6 days in LD12:12 cycles and then during LL for 7 to 10 days. Behavioral data were analyzed with the χ^2 periodogram and actogram programs (9). The rhythmic phenotype of each fly was classified as rhythmic (power, ≥10; width, ≥2) or arrhythmic (power, <10; width, <2) (39), with power referring to the height of the peak above the 5% significance line (9) and width indicating the number of period values within a peak that are statistically significant (6, 20); low-amplitude peaks and peaks having minimal widths correlate with weak rhythmicity (exemplified by a segment of the periodogram plot that contains a mere spike that crosses the significance line for only one or two bins).

LD activity data for individual flies were plotted as continuous-line actograms, and data for groups of flies were plotted as histograms as described by Hamblen-Coyle et al. (11). These LD activity data were filtered to remove light-transition-induced (i.e., non-clock-dependent) activity spikes as described previously (11).

RNA preparation. Fly heads were separated from bodies as described previously (24). Total RNA was prepared from heads immediately after isolation (21). RNA quantitation was done with an LKB Ultroscan III spectrophotometer.

RNase protections. 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 size standard used was the 123-bp ladder (Bethesda Research Laboratories). Quantitation was done with either a Fujix BAS 1000 or 2000 phosphorimager and MacBAS software.

RESULTS

A stable phase relationship between *per* mRNA cycling and lights-off exists. As a first step to understand how *per* mRNA cycles in different photoperiods, we compared the phases of *per* mRNA cycling in flies entrained under different photoperiodic conditions (Fig. 1A). Consistent with previous reports, *per* mRNA levels peaked around ZT16 in LD12:12 cycles (12, 13). In comparison, the phase of the *per* mRNA peak shifted to ~ZT12 in LD8:16 cycles and ~ZT20 in LD16:8 cycles, while little difference was seen in the cycling amplitudes or overall *per* mRNA levels (Fig. 1B). For these and other photoperiods (LD4:20 and LD10:14; data not shown), the peak of *per* mRNA abundance occurred 4 h after lights-off, indicating that there is a fixed phase relationship between the *per* mRNA cycle and the lights-off transition.

Changes in photoperiod rapidly shift the phase of *per* mRNA cycling and locomotor activity. To understand how long the clock takes to adjust the phase of *per* mRNA cycling in different photoperiods, we measured *per* mRNA cycling under conditions in which flies were entrained in LD12:12 cycles and transferred to either LD8:16 or LD16:8 cycles (Fig. 2). When flies were transferred from LD12:12 to LD8:16 cycles, the *per* mRNA peak quickly shifted (on the day of transfer) to ~ZT12.

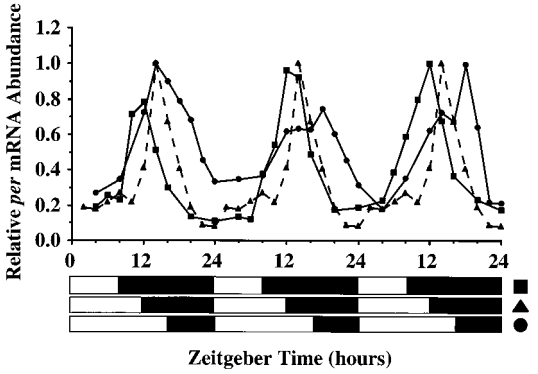

FIG. 2. Transitions in photoperiod elicit rapid changes in the phase of *per* mRNA cycling. RNase protection assays were performed on total head RNA from flies transferred from LD12:12 cycles to either LD8:16 or LD16:8 cycles (see Materials and Methods). The abundance of *per* RNA cycling in LD12:12 cycles and the new photoperiods were quantitated as described in the legend to Fig. 1. ■, LD12:12 to LD8:16; ●, LD12:12 to LD16:8; ▲, LD12:12. The cycling of *per* RNA in LD12:12 cycles was replotted for days 2 and 3 for the convenience of phase comparison. The axes and the open and solid bars are as defined in the legend to Fig. 1. This experiment was repeated once with similar results.

The phase of *per* mRNA cycling (i.e., rising and falling phases) adjusted to match the steady-state LD8:16 phase by the second day after transfer (Fig. 1). A shift from LD12:12 to LD16:8 cycles resulted in a delay of the falling phase of *per* mRNA abundance, thus prolonging high levels of *per* mRNA for several hours. The peak levels of *per* mRNA shifted to match that seen for flies entrained in LD16:8 cycles (Fig. 1) by the next day after transfer.

As a putative component of the pacemaker mechanism, the *per* gene not only affects the period of the locomotor activity rhythm but also affects its phase in LD cycles (3, 10, 11, 17). Since the phase of *per* mRNA cycling is altered in different photoperiods, we tested whether the phase of locomotor activity rhythms was also altered by photoperiod and, if so, whether these changes would parallel those seen for *per* mRNA. In LD12:12 cycles, the evening activity peak occurs ~1 h before the lights-off transition (ZT12) (11) (Fig. 3). During the transition from LD12:12 to LD8:16 cycles, locomotor activity immediately shifted to ZT8 and remained at that phase for the rest of the experiment (Fig. 3A and C). Upon transfer from LD12:12 to LD16:8 cycles, the evening activity peak immediately lengthened by 2 to 3 h, and the activity offset assumed its new phase (Fig. 3B and C). By the next day after the photoperiod transition, the peak had shifted to its new phase, but the onset of activity was intermediate between that of the old and new light cycles (Fig. 3C). By the second day after transition into the new photoperiod, activity onset, peak, and offset had reached a steady state (Fig. 3C). These results suggest that the pacemaker responds to changes in photoperiod very rapidly and equilibrates its phase and the phase of its behavioral outputs to a steady state in 1 or 2 days, depending on whether the new photoperiod advances or delays the phase of activity.

Locomotor activity rhythms and *per* mRNA cycling are abolished by LL. After flies are entrained, the locomotor activity and eclosion rhythms free-run in DD with a period of around 24 h (16) but are arrhythmic under constant bright (>10 lx) light (18, 28, 41). To determine whether this is an immediate response to light or a gradual decline in rhythmicity, we followed the locomotor activity of entrained flies as they entered LL. After flies (n = 60) are shifted from LD12:12 cycles to LL, their evening activity peak initially lengthens by several hours,

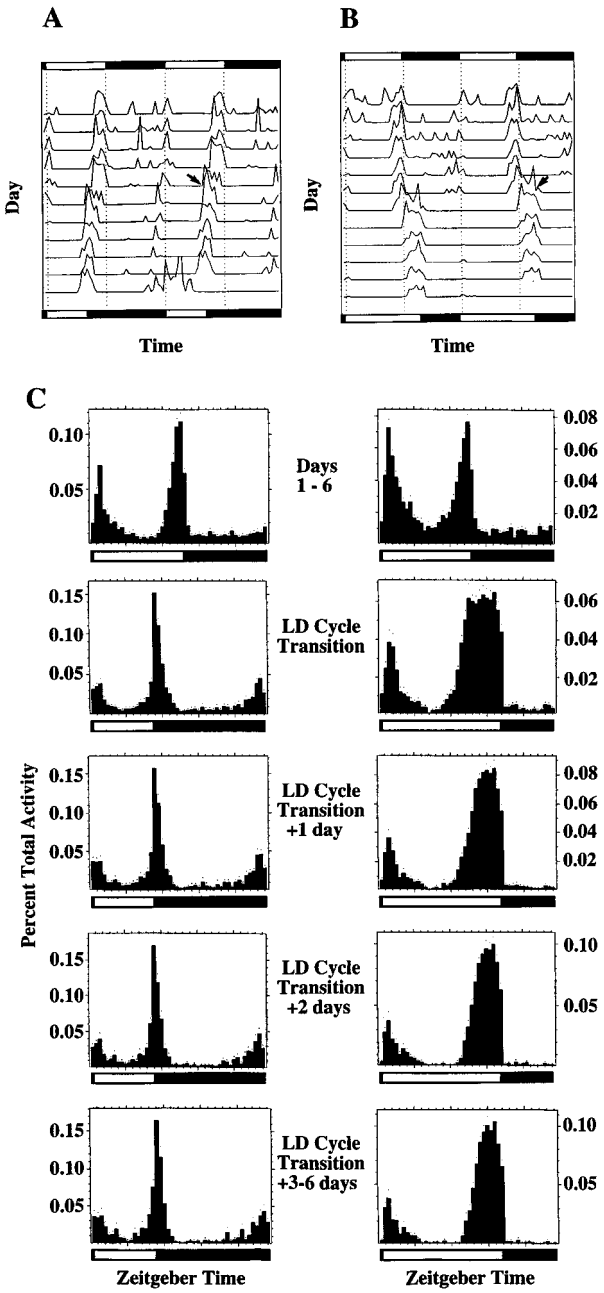

FIG. 3. Transitions in photoperiod elicit rapid changes in the phase of locomotor activity. (A) Locomotor activity of a fly transferred from LD12:12 to LD8:16 cycles (see Materials and Methods). Successive days of locomotor activity values are displayed both horizontally and vertically (hence, a double plot). Flies were initially entrained in LD12:12 cycles (depicted at the top of the plot as open and closed bars, respectively) for 6 days and were transferred to LD8:16 cycles (depicted at the bottom of the plot) for 6 days. The transition to the new photoperiod is indicated by the arrow. (B) Locomotor activity of a fly transferred from LD12:12 to LD8:16 cycles (see Materials and Methods). The locomotor activity plots, the light and dark cycles, and the transition point between photoperiods are as described above. (C) Locomotor activity of groups of 22 flies before, during, and after the transition from LD12:12 to LD8:16 cycles (plots on the left) and LD12:12 to LD16:8 cycles (plots on the right). Activity was tabulated each half hour (vertical black bars), and the proportion of total daily activity (total activity = 1) was calculated and plotted on the ordinate. Data were analyzed separately for the first 6 days in an LD12:12 cycle (top pair of plots), the transition to a new LD cycle (second pair of plots), the first day after transition (third pair of plots), the second day after transition (fourth pair of plots), and the third through the sixth days after transition (bottom pair of plots). The white and black bars below each plot represent times when lights were on or off, respectively.

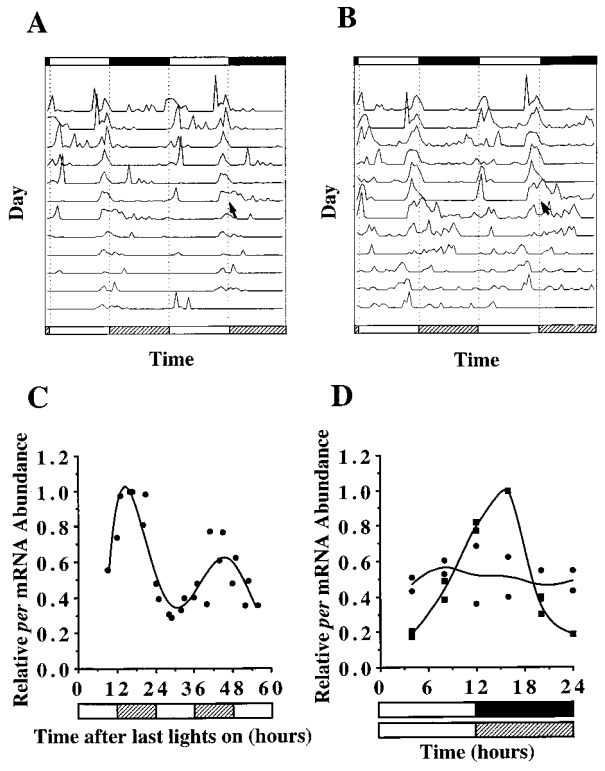

FIG. 4. LL leads to arrhythmic locomotor activity and loss of *per* mRNA cycling. (A and B) Locomotor activity was measured in flies entrained to LD12:12 cycles and transferred to LL (see Materials and Methods). The locomotor activity plots and the transition point between photoperiods are as described in the legend to Fig. 2. The locomotor activity plot in panel A is for a fly that is weakly rhythmic in LL, and the locomotor activity plot in panel B is for a fly that is arrhythmic in LL (see Materials and Methods for rhythmicity parameters and the text for proportions of rhythmic and arrhythmic flies). (C) RNase protection assays were performed on total head RNA from two independent sets of flies entrained to LD12:12 cycles and transferred to LL. The abundance of *per* mRNA was quantitated as described in the legend to Fig. 1. Relative abundance refers to the ratio of *per* RNA to RP49 mRNA, for which the peak level of *per* mRNA was set to 1.0. The open and hatched bars represent lights-on and subjective lights-off during LL, respectively. The numbers on the *x* axis represent times after the last lights-on. The curve was drawn on the basis of software-aided fitting of the datum points to polynomial functions. (D) RNase protection assays were performed on total head RNA from two independent sets of flies subjected to LD12:12 cycles or LL for 3 days. The abundance of *per* RNA in LD12:12 conditions (■) and LL (●) from each independent experiment ($n = 2$) was quantitated as described in the legend to Fig. 1. Relative abundance refers to the ratio of *per* RNA to RP49 mRNA, for which the peak level of *per* mRNA in an LD12:12 cycle was set to 1.0 and the level of *per* mRNA at other time points in an LD12:12 cycle and LL was calculated relative to the LD12:12 peak. The numbers on the *x* axis represent hours after lights-on in an LD12:12 cycle. Time points taken during LL were taken at the same time of day as those in the LD12:12 cycle. The curves were drawn as described in the legend to Fig. 1. In each panel, dark bars represent lights-off, open bars represent lights-on, and hatched bars represent times when lights would have been off during LL.

followed by arrhythmicity (49/60) or weak rhythmicity (11/60; average period, 24.57 h; average power, 23.34; average width, 3.36; see Materials and Methods) over the next 5 days (Fig. 4A and B). As with the arrhythmic per^{01} or tim^{01} mutants during DD (16, 34), LL-induced arrhythmicity does not alter overall activity over a 24-h period (data not shown). Consistent with this behavioral result is a lengthening of the *per* mRNA peak after transfer into LL and a later peak and substantial decrease in amplitude during the next cycle (Fig. 4C).

Correlated with the behavioral arrhythmicity of flies exposed to several days of LL is the observation that PER levels are constitutively low (29, 41). Since PER functions to repress its own transcript synthesis, *per* mRNA levels would be predicted to be relatively high under these conditions. *per* mRNA levels measured in wild-type flies exposed to LL for 4 days remained relatively constant at ~50% of the peak *per* mRNA level seen in LD12:12 cycles (Fig. 4D). This result is similar to what is seen in per^{01} flies (*per* mRNA is constitutively at 50% of the peak) (12, 34) and tim^{01} (11, 34) (*per* mRNA levels are somewhat erratic but average ~50% of the peak), suggesting that factors in addition to PER are necessary for high-level *per* mRNA accumulation (see below). A shift from LL to DD restores both locomotor activity and *per* mRNA rhythms starting at circadian time 12 (data not shown), consistent with earlier molecular (41) and behavioral (25, 26, 28, 31, 41) experiments that show a single L-to-D transition is sufficient to entrain the clock.

***per* mRNA cycling is progressively disrupted in long photoperiods.** Since LL abolished *per* mRNA cycling, it was of interest to determine the maximal photoperiod that would support *per* feedback loop operation. To do this, *per* mRNA cycling was measured in several photoperiods longer than LD16:8 cycles (Fig. 5), which were already shown to support high-amplitude *per* feedback loop function (Fig. 1). The amplitude of *per* mRNA cycling was calculated for each of the different photoperiods by dividing the average peak value by the average trough value. These values—13.1 for LD16:8 cycles ($n = 3$), 4.5 for LD18:6 cycles ($n = 2$), 1.7 for LD20:4 cycles ($n = 2$), 1.6 for LD22:2 cycles ($n = 2$), and 1.4 for LL ($n = 2$)—progressively decrease as the photoperiod increases, as is indicated by the *per* mRNA curves in Fig. 5. In addition, as *per* mRNA cycling dampened in longer photoperiods, the level of *per* mRNA progressively decreased, reaching a level of ~40 to 50% of that of the wild-type peak in LD22:2 cycles and LL (Fig. 5). These results demonstrate that a period of darkness is necessary for *per* feedback loop function and show that a period of darkness for ~6 to 8 h is required for wild-type feedback loop function (as judged by mRNA cycling amplitude).

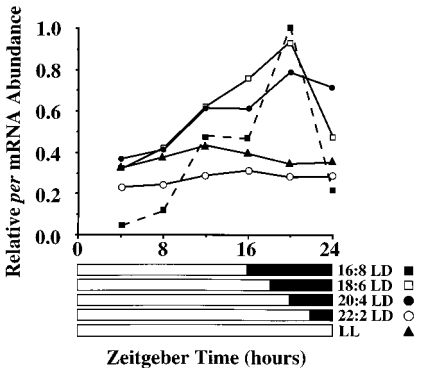

FIG. 5. Long photoperiods dampen *per* mRNA cycling to low constitutive levels. RNase protection assays were performed on total head RNA from flies entrained in LD16:8, LD18:6, LD20:4, and LD22:2 cycles and LL as described in Materials and Methods. The abundance of *per* mRNA was quantitated as described in the legend to Fig. 1. Relative abundance refers to the ratio of *per* RNA to RP49 mRNA, for which the peak level of *per* mRNA in an LD16:8 cycle was set to 1.0 and the level of *per* mRNA in other photoperiods was calculated relative to the LD16:8 peak. The open and solid bars represent light and dark phases, respectively. Time points taken during LL were taken at the same time of day as those during the other photoperiods. This experiment was repeated at least once for each photoperiod with similar results.

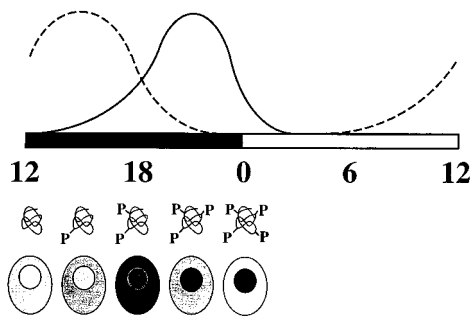

FIG. 6. Temporal relationship between *per* feedback loop events. During the dark phase, PER (represented as a solid curve, squiggly lines, and the shading within the ovals) increases to a peak level at ~ZT20 and declines to a trough level by ~ZT2 to ZT6 (6), becomes progressively phosphorylated (represented by P's) (6), and translocates from the cytoplasm (outer oval) to the nucleus (inner oval) over several hours centered on ZT18 (3). During the light phase (open bar), *per* and *tim* mRNAs (dashed curve) begin accumulating at ~ZT6, peak during the dark phase at ~ZT15, and decline to trough levels by ~ZT20.

DISCUSSION

In this report, we have analyzed the effect of photoperiods on *per* mRNA cycling and locomotor activity rhythms to better understand the molecular mechanism through which the circadian clock entrains to LD cycles. When flies are entrained in 24-h LD cycles consisting of ≤16 h of light and ≥8 h of dark (see below), a stable phase relationship between the pacemaker (as measured by *per* mRNA cycling) and the LD cycle is maintained such that *per* mRNA levels peak about 4 h after the lights-off transition. We infer that the phase of PER cycling is also maintained in these different LD cycles, since *per* mRNA levels, which are controlled via PER-mediated transcriptional repression (39), decline along similar time courses with respect to lights-off (Fig. 1B). An exception to this generalization occurs in extremely long photoperiods, in which the feedback loop progressively breaks down (Fig. 5), apparently limiting the *per* mRNA peak to no later than ~ZT20 (Fig. 5). Thus, the *per* mRNA cycle, and by extension the *per* feedback loop, is phase locked to the lights-off transition under these conditions.

With the steady-state cycling of *per* mRNA under different photoperiodic conditions being known, it was possible to assess how quickly the *Drosophila* oscillator reset its phase after a shift in photoperiod. When flies are shifted from LD12:12 cycles to either LD8:16 or LD16:8 cycles, the phase of *per* mRNA cycles is rapidly altered and assumes a stable phase with regard to the LD transition in 1 to 2 days (Fig. 2A). Although photoperiod-induced advances (LD12:12 to LD8:16) and delays (LD12:12 to LD16:8) in *per* mRNA cycling assume a stable phase in 1 to 2 days, they reach this new phase in different ways. Phase advances are characterized by a premature rise in the levels of *per* mRNA (Fig. 2), which, we infer, leads to an abnormally early accumulation of PER in the nucleus, resulting in a premature decrease in *per* mRNA and a stable phase advance by the next day (Fig. 6). Phase delays are characterized by a prolonged *per* mRNA peak, which, we infer, results in an extended transcriptional repression due to a prolonged presence of PER in the nucleus and a stable phase delay during the next cycle (Fig. 6). The extended *per* mRNA peak on the day of the phase delay is one instance in which the *per* mRNA peak is not phase locked to lights-off. This outcome is probably due to anticipation of lights-off at ZT12 (resulting in an accumulation of *per* mRNA to peak levels by ~ZT16) and a predicted delay in PER accumulation in the nucleus (resulting in turn in continued high levels of *per* mRNA until ~4 h after the new dark phase).

The speed at which the *per* oscillator reacts to and accommodates a new photoperiod is comparable to (or faster than) the time it takes locomotor activity rhythms to conform to a new photoperiod. For shifts from LD12:12 to LD8:16 cycles, activity onset, peak, and offset are shifted during the day of transition (Fig. 3A and C), just as the *per* mRNA onset, peak, and offset are shifted during the day of transition (Fig. 2). Likewise, for shifts from LD12:12 to LD16:8 cycles, the activity peak (Fig. 3B and C) and the *per* mRNA peak (Fig. 2) are each broadened during the transition, and both activity and *per* mRNA offset times are similar to those for flies entrained under LD16:8 conditions (Fig. 1 and 3B and C). During the second day after an LD12:12-to-LD16:8 shift, the peak has shifted for both activity (Fig. 3C) and *per* mRNA levels (Fig. 2), while the activity onset (Fig. 3C) and the *per* mRNA accumulation (Fig. 2) are both in transition between the LD12:12 and LD16:8 steady states. By the third day after the LD12:12-to-LD16:8 transition, the phases of activity (Fig. 3C) and *per* mRNA cycling (Fig. 2) have shifted to their steady-state phases. These results are similar to previous results in which a change in the same LD cycle (38) or a pulse of light (3, 32) results in a rapid resetting of behavioral activity. Thus, phase shifts of the negative feedback loop due to prolonged light or premature dark are translated into behavioral phase shifts along a similar time course.

Photoperiods longer than 16 h progressively dampen *per* mRNA cycling until arrhythmicity is induced in LD22:2 cycles and LL (Fig. 5). These results may be explained in the context of our current knowledge of the mechanisms which drive the *per* feedback loop (2, 14, 33). Since the *per* feedback loop and locomotor activity are phase locked to lights-off, the portion of the cycle in which phase resetting occurs starts just before *per* mRNA levels peak and PER begins to accumulate (Fig. 6).

When photoperiods are 16 h or less, we infer that the 8 h (or more) of darkness allows PER to accumulate (41), become progressively phosphorylated (5), translocate into the nucleus (2, 37), and regulate gene expression (13). If lights come on at 8 to 12 h after lights-off, the phase is advanced by a few hours to around the time lights come on. The decreasing mRNA cycling amplitude associated with photoperiods of >16 h may be explained by constraints on the timing of events within the *per* feedback loop. For instance, the shorter periods of darkness in photoperiods increasingly longer than 18 h would shift lights-on to a portion of the cycle in which little PER would be able to enter the nucleus and inhibit *per* gene transcription, thereby leading to a low-amplitude mRNA cycle (Fig. 6). Thus, extremely short (≤4 h) dark periods never allow the *per* feedback loop to be completed, leading to a breakdown of the rhythm.

The most extreme photoperiod, LL, may dampen or eliminate circadian behavioral rhythms in *D. melanogaster*, depending on the intensity of light (18). The 700- to 2,000-lx light intensity used in these experiments rendered more than four out of five flies behaviorally arrhythmic (Fig. 4), consistent with previous results (18). This light intensity also abolished *per* mRNA cycling, though cycling was not abolished immediately but over the course of 3 to 4 days (Fig. 4). These results are consistent with those of Pittendrigh and Minis (27), who showed that flies can entrain to complete photoperiods of ≥14 h in a 24-h cycle but not to the longer of two intervals (when the longer interval is ≥14 h) between light pulses in skeleton photoperiods (i.e., periods of time framed by light pulses) that constitute a 24-h cycle, indicating that light has a potent chronic effect on the clock. The results presented here suggest

that while LL does not immediately stop the clock, it does affect the activity or stability of some clock molecule, which leads to a gradual dampening of the rhythm to arrhythmicity (Fig. 4).

Several studies have recently shown that light rapidly destabilizes TIM protein, resulting in a reduction of TIM (15, 23) or TIM-PER complexes (19, 40). Since TIM acts to stabilize PER (29), light-induced destabilization of TIM would have a secondary effect of reducing PER levels. The light sensitivity of TIM could account for two of the principal results presented here: that the circadian feedback loop is locked to the lights-on–lights-off transition and that a minimal amount of darkness is required for feedback loop function. TIM would only accumulate in appreciable quantities once the lights were out, thereby stabilizing PER and allowing for PER nuclear translocation. However, if TIM underwent premature light-induced destruction (after only 4 to 6 h of darkness), insufficient quantities of PER and TIM would enter the nucleus to inhibit *per* gene transcription and the feedback loop would break down. In addition, TIM and TIM-PER complexes break down quickly in response to light (15, 19, 23, 40), which is consistent with our data which show that the clock and its locomotor activity output rapidly reset after photoperiod-induced advances and delays (Fig. 2 and 3).

In the circadian feedback loop, PER acts to repress its own mRNA synthesis. Since only minute amounts of PER are detected immunohistochemically (41) or by Western blotting (immunoblotting) (29) in LL, we would have predicted that *per* mRNA levels would rise to their peak levels; however, median levels of *per* mRNA were seen under these conditions (Fig. 4). These results are similar to those seen with per^{01} (12, 34) and tim^{01} mutants (11a, 34), in which PER is either not synthesized (5, 36) or does not accumulate (29), respectively, and suggest that the negative feedback is more complex than simple PER-dependent repression of transcription. To achieve the repression in *per* mRNA abundance caused by either LL or these arrhythmic mutants, perhaps an additional repressor or activator which is chronically active at a low level is present. Under normal (i.e., wild-type flies in LD12:12 cycles or DD) circumstances, the activity of this repressor or activator may fluctuate, thereby contributing to the generation of high-amplitude molecular and behavioral rhythms and explaining how *per* mRNA levels remain low after PER abundance decreases early in the light phase (14).

In contrast to the median levels of *per* mRNA in LL or mutant-induced arrhythmias in *D. melanogaster*, high constitutive levels of *frq* mRNA are observed in the arrhythmic frq^{9} mutant of *N. crassa* (1). This result suggests that *frq* protein (FRQ)-dependent repression is capable of producing the full wild-type *frq* mRNA cycling amplitude while the median levels of *per* mRNA in three different arrhythmic states (LL [Fig. 4], per^{01} [12, 34], and tim^{01} [34]) suggest that PER does not solely repress *per* mRNA expression. Thus, these negative feedback loops appear to differ in their complexity in that PER is not solely capable of producing high-amplitude *per* mRNA cycling while FRQ may well mediate high-amplitude *frq* mRNA cycling.

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