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Circadian oscillations in period gene mRNA levels are transcriptionally regulated

(circadian rhythms/transcriptional control/*Drosophila*)

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ABSTRACT **The period (*per*) gene is involved in regulating circadian rhythms in *Drosophila melanogaster*. The *per* gene is expressed in a circadian manner, where fluctuations in *per* mRNA abundance are influenced by its own translation product, which also cycles in abundance. Since *per* gene expression is necessary for circadian rhythmicity, we sought to determine how certain features of this feedback loop operate. The results of this study reveal that fluctuations in *per* mRNA are primarily controlled by fluctuations in *per* gene transcription, that *per* mRNA has a relatively short half-life, and that sequences sufficient to drive *per* mRNA cycling are present in 1.3 kilobases of 5′ flanking sequences. These and other results indicate that the *per* feedback loop has all of the basic properties necessary to be a component of a circadian oscillator.**

A wide array of behavioral and physiological phenomena are expressed as circadian rhythms in eukaryotic (1) and even some prokaryotic organisms (2). These daily rhythms result from the action of an endogenous circadian clock, which persists under constant environmental conditions, is reset by environmental parameters such as light and temperature, and is relatively temperature independent (3). Although a wealth of descriptive chronobiology has been amassed on a variety of rhythmic phenomena, comparatively little is known about how the clock controls these rhythms.

Much of what is known or suspected about clock mechanisms has been discovered through the use of pharmacological agents and molecular genetics. Pharmacological agents have been used to show that RNA and protein synthesis (i.e., gene expression) are necessary for circadian rhythmicity in several organisms (4). In fact, transcription during a limited portion of the circadian cycle has recently been shown to be necessary for the generation of circadian rhythms in *Aplysia* (5). Correlated with this restricted period of gene activity has been the discovery of RNAs and proteins expressed at specific times during the circadian cycle in a number of organisms (6–18). Most of these cycling molecules, however, probably control rhythmic outputs downstream from the pacemaker and, therefore, are not immediately useful in probing the mechanisms underlying circadian pacemaker function.

Several pieces of evidence suggest that the period (*per*) locus of *Drosophila melanogaster* is intimately associated with the pacemaker (19–24). Mutations due to single amino acid substitutions in the *per*-encoded protein can shorten (per^s) or lengthen (per^L) the free-running circadian period (in constant darkness), while loss of functional *per*-encoded protein (per^{01}) abolishes the free-running rhythms (19, 25, 26). These effects on circadian period are seen in locomotor activity behavior of individual flies and in eclosion (emergence of adults from their pupal cases) profiles of fly populations (19). Since *per* function also appears to be necessary for the entrainment of the circadian pacemaker (22), we believe that *per* gene expression is required for flies to either measure or tell time.

A recently discovered aspect of *per* gene expression is that its mRNA and protein products undergo daily fluctuations in abundance (20, 21, 23, 24). These fluctuations constitute a feedback loop whereby the per protein affects the oscillations of its own mRNA (21). To determine how this feedback loop works, we have first focused on how the circadian cycling of *per* mRNA is regulated. Experiments presented here show that *per* mRNA fluctuations are due to parallel fluctuations in *per* gene transcription. This result predicts that a circadian clock-regulated transcription factor is responsible for *per* mRNA cycling. This prediction is particularly interesting in light of the suggestion that the per protein may act as a transcription factor (27).

MATERIALS AND METHODS

Construction of Transformation Plasmids. Four different *per*–chloramphenicol acetyltransferase (CAT) fusion genes were constructed (see Fig. 2). The CAT portion of these constructs consisted of a *Hin*dIII–*Xba* I fragment containing the CAT protein coding region, 31 base pairs (bp) of upstream sequences, and 941 bp of downstream sequences [including a simian virus 40 intron and poly(A) addition sequences] (28). This CAT gene-containing fragment was cloned into Bluescript KS− (Promega) at the *Hin*dIII and *Xba* I sites to form KS-CAT. The *per* portion of these constructs consisted of the following DNA fragments: BS (approximately −4000 bp to +29 bp), R+1 (−1310 bp to +1 bp), Spe+1 (−330 bp to +1 bp), HS (−179 bp to +29 bp), and H+1 (−179 bp to +1 bp) (see ref. 29 for numbering system). These fragments, generated via PCR or subcloning, were inserted at the *Sal* I site in the KS-CAT polylinker and the *Hin*dIII site upstream of the CAT gene. The entire *per*–CAT insert was cut out with *Sal* I and *Xba* I and ligated into the unique *Sal* I and *Xba* I sites of the transformation vector Cp20.1 (30).

Germ-Line Transformation. Germ-line transformants were made by using standard procedures (31). The recipient strain used for germ-line transformation was per^{01}; ry^{506} (the Cp20.1 vector is marked with ry^+). Each transformant line was generated from a different injected embryo. Each line involved an autosomal insert that was balanced by using *In(2LR)CyO, Cy* flies (second chromosome) or *In(3LR)TM2, ry Ubx* flies (third chromosome).

RNase Protection Assays. Flies used for time courses were entrained at 25°C in a 12-hr light/12-hr dark cycle for at least

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Abbreviations: CAT, chloramphenical acetyltransferase; nt, nucleotide; ZT, Zeitgeber time.

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72 hr before collections were taken. The wild-type strain employed in these studies was Canton-S. For each time point, heads were isolated and RNA was extracted (21). The probes employed in these studies were *per* precursor, *per* 2/3, and CAT. The *per* precursor probe contains RNA from nucleotides −179 to +366 and protects a 320-nucleotide (nt) mature RNA fragment, a 384-nt precursor RNA fragment, and a 563-nt genomic DNA fragment. The *per* 2/3 probe covers a 259-nt portion of exon 3 and a 134-nt portion of exon 2, as described (21). The CAT probe contains *per* RNA from −179 to +29, Bluescript KS− polylinker sequence from the *Sal* I to the *Hin*dIII sites, and CAT RNA from the *Xba* I site to the *Hin*dIII site. This probe protects either 298-nt (constructs ending at +29) or 250-nt (constructs ending at +1) fusion gene RNA fragments. As a control for RNA loading in each lane, a ribosomal protein probe (RP49) was included in each protection assay (21). RNA hybridization, digestion, and separations were performed as described (21) with the following modification: contaminating DNA was eliminated from total RNA samples (10 μg) by treatment with 2 units of RNase-free DNase (Promega) for 20 min at 37°C before hybridization. This modification was necessary because the CAT probe protects the same size RNA and DNA fragment. Radiolabeled 123-bp markers (BRL) were run on each gel as size standards. Quantitation was done using a Microtek MSF-300GS image scanner. Protection analyses were performed on RNA extracted from flies of at least two independent lines for each construct (except Spe+1–CAT); little, if any, interline variation in the RNA abundance patterns was detected.

RESULTS

Control of *per* mRNA Cycling. Two distinct possibilities regarding the control of *per* mRNA cycling are that this gene's transcript is synthesized in a circadian manner or that *per* mRNA is broken down in a rhythmic manner. These two control mechanisms predict that different types of molecules would be involved in *per* mRNA cycling (e.g., transcription factors versus ribonucleases), that they would function at different times during the circadian cycle {i.e., during *per* mRNA accumulation [8–12 hr after lights on = Zeitgeber time (ZT) 8–12] or decline [16–20 hr after lights off = ZT 16–20]}, and that they would function in different intracellular locations (i.e., in the nucleus or the cytoplasm).

In circadian systems, nuclear run-on experiments have been used to determine if a gene is under transcriptional control (11, 12, 14, 17, 18, 32). However, two aspects of *per* mRNA expression made this type of analysis impossible: the low abundance of *per* mRNA (even at its peak) and the head specificity of high amplitude *per* mRNA cycling. Since large numbers of heads could not be isolated such that intact transcription complexes were maintained (as measured by radiolabel incorporation into run-on transcripts), two alternative approaches were taken to define the level at which *per* mRNA cycling is regulated: testing (*i*) whether *per* precursor RNA levels cycle and (*ii*) whether *per* upstream sequences are sufficient to drive the cycling of a heterologous transcript. Transcriptional regulation would be indicated if cycling were seen for both *per* precursor and *per*-driven heterologous transcripts, whereas noncycling in both cases would support a posttranscriptional control mechanism.

Cycling of *per* Precursor RNA. The levels of *per* precursor RNA were measured in wild-type flies collected and frozen at 4-hr intervals during a 12-hr light/12-hr dark cycle (ZT 0–12/ZT 12–24). Total RNA isolated from the heads of these flies was used for RNase protection with a probe that could distinguish between *per* precursor transcripts, mature *per* transcripts, and contaminating genomic DNA (see *Materials and Methods*).

Both *per* precursor and mature transcripts were found to oscillate with respect to ZT (Fig. 1). The cycling amplitude of the two transcripts was indistinguishable, even though the precursor was much lower in abundance (5–10% the level of the mature transcript). The phase of cycling was almost identical in that transcript levels rise dramatically between ZT 6 and 10, remain relatively high for about 8 hr, and then

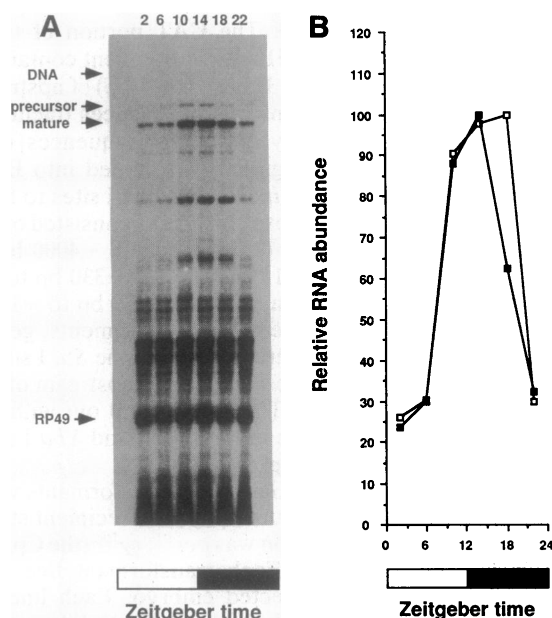

FIG. 1. Twelve-hour light/12-hr dark cycling of *per* precursor RNA. (*A*) An RNase protection assay was performed on head RNA from wild-type flies collected every 4 hr during a 12-hr light/12-hr dark cycle. The numbers above each lane refer to the number of hours past the last time lights went on (ZT 0). The *per* DNA (DNA), *per* precursor (precursor), *per* mature (mature), and RP49 protected fragments are denoted by arrows. (*B*) Quantitation of the data in *A*. □, Mature *per* RNA; ■, *per* precursor. Relative RNA abundance refers to *per*/RP49 values, where the peak value was set to 100 (21). The open and solid bars represent times when lights were on (ZT 0–12) or off (ZT 12–24), respectively.

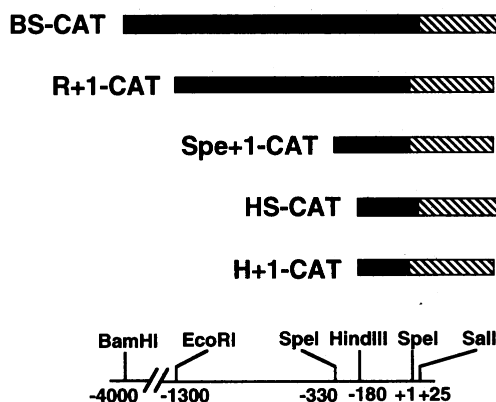

FIG. 2. *per*–CAT constructions used for germ-line transformations. For each construct, *per* (black bars) and CAT (hatched bars) DNA was inserted into the transformation vector Cp20.1. The *per* sequences included in each construct are −4000 bp to +25 bp (BS–CAT), −1330 bp to +1 bp (R+1–CAT), −330 bp to +1 bp (Spe+1–CAT), −179 bp to +25 bp (HS–CAT), and −179 bp to +1 bp (H+1–CAT). The *per* sequences for each construct were fused to CAT protein coding sequences. A map depicting *per* gene sequences from 4000 bp upstream (−4000) to 25 bp downstream (+25) of the transcription start site (+1) is shown. Enzymes used to make the constructions and their positions relative to the start of *per* transcription are as denoted in the figure.

drop precipitously by ZT 22. The similar phase and amplitude suggest that *per* mRNA cycling is transcriptionally regulated and that *per* mRNAs, like *per* pre-mRNAs, have short half-lives.

Cycling of *per*–CAT Fusion Gene Transcripts. To test whether the *per* promoter is sufficient to confer RNA cycling to a heterologous transcript, flies were transformed with a series of five *per*–CAT fusion genes (Fig. 2) and assayed for CAT RNA cycling. The largest of these fusion genes, BS–CAT, consists of *per* genomic sequences from approximately −4000 bp to +29 bp fused to the CAT gene coding region. Genomic *per* constructs containing these same upstream sequences and the *per* gene's complete coding region are expressed normally; i.e., there are robust levels and correct spatial distributions of *per* mRNA (23, 33). In addition, they can restore virtually normal circadian rhythms to flies whose genetic background includes the per^{01} mutation (29, 34). The other *per*–CAT fusion genes (Fig. 2) contain shorter stretches of *per* upstream sequences (from −1310 bp, −330 bp, or −179 bp upstream to +1 bp or +29 bp downstream), which have not previously been tested for their effects on *per* expression. Since functional per protein is required for *per* RNA cycling and behavioral rhythmicity (21, 24), *per*–CAT fusion gene-derived transcript cycling was assayed in a wild-type, per^{+}, rhythmic background. As with other *per*–reporter gene fusions (24), even extra copies of *per*–CAT fusion genes had no effect on either circadian activity or endogenous *per* mRNA cycling of wild-type flies (data not shown). The normal behavioral rhythms and *per* mRNA cycling indicate that the per protein is functioning properly in these *per*–CAT transformants. Cycling of fusion gene-derived transcripts was assayed by RNase protection of head RNA extracted from *per*–CAT transformants collected and frozen at 4-hr intervals during the 12-hr light/12-hr dark cycle.

Transcripts from the largest of these constructs, BS–CAT, cycled with almost identical phase, amplitude, and peak levels as did the endogenous *per* transcript (Fig. 3). This result is similar to what was seen with *per*–β-galactosidase fusion genes having the same amount of *per* upstream region (24); however, the BS–CAT fusion gene used here contains only 29 bp of *per* transcribed sequences compared to several hundred nucleotides in the *per*–β-galactosidase fusion genes assayed previously. To further delimit sequences sufficient to drive *per* mRNA cycling, the other four *per*–CAT transformant fusion genes having shorter regions of *per* upstream sequence were tested for fusion gene mRNA cycling. All four constructs produced considerably lower transcript levels than BS–CAT, indicating that additional *per* sequences enable the BS–CAT construct to express mRNA at levels very similar to wild-type *per* mRNA. In fact, transcripts from the three shortest *per*–CAT constructs (Spe+1–CAT, HS–CAT, and H+1–CAT) could not be detected above background

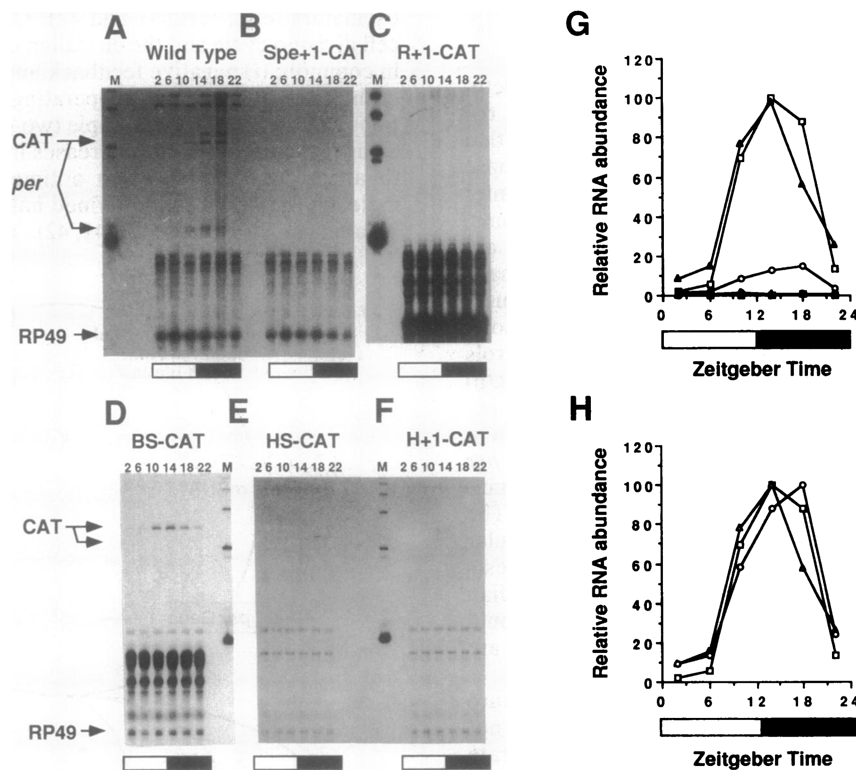

FIG. 3. Cycling of *per*–CAT-derived transcripts. RNase protection assays were performed on head RNA from *per*–CAT transformants collected every 4 hr during a 12-hr light/12-hr dark cycle. The number above each lane indicates the number of hours since the last time lights went on. Arrows denote the positions of the *per*–CAT fusion gene RNA (CAT) protected fragments [the two arrows for *D–F* indicate the long (BS and HS) and short (H+1) CAT protected fragments], the endogenous *per* RNA protected fragments (*per*), and the RP49 RNA protected fragment (RP49). RP49 was included as a measure of RNA loading. In *C*, there is a strong background band above the RP49 protected fragment. The actual RP49 protected fragment is aligned with the "RP49" arrow to the left of *A*. Size markers (M) were a radiolabeled 123-bp ladder. RNase protections were performed on Spe+1–CAT (*A* and *B*), R+1–CAT (*C*), BS–CAT (*D*), HS–CAT (*E*), and H+1–CAT (*F*) transformants. The *per* 2/3 RNase protection probe was used to measure endogenous wild-type *per* mRNA levels in *A*, whereas the CAT RNase protection probe was used to measure *per*–CAT fusion gene-derived RNA levels in *B–F*. (*G*) Quantitation of data shown in *A–F*. Relative RNA abundance refers to the ratio of either *per* or *per*–CAT fusion RNA to RP49 RNA, where the peak reading from *A* was adjusted to 100. □, Endogenous wild-type *per* RNA; △, BS–CAT fusion gene RNA; ○, R+1–CAT fusion gene RNA; ■, Spe+1–CAT fusion gene RNA; ▲, HS–CAT fusion gene RNA; ●, H+1–CAT fusion gene RNA. (*H*) Quantitation of data shown in *A*, *C*, and *D*. Relative RNA abundance refers to the ratio of either wild-type *per* or *per*–CAT fusion RNA to RP49 RNA, where the peak reading in each panel was adjusted to 100. The symbols for the relevant transcripts are the same as in *G*. The open and solid bars in each panel symbolize times when lights were on or off, respectively.

(Fig. 3), thereby making it impossible to determine if they cycle. Although R+1–CAT (−1300 to +1)-derived transcripts are reduced 5- to 10-fold compared to those of BS–CAT, they could be detected above background, and they cycled with a similar phase and amplitude as BS–CAT-derived transcripts (Fig. 3).

The partial (R+1–CAT) or complete (Spe+1–CAT, HS–CAT, and H+1–CAT) loss of expression in flies carrying these constructs may result from the elimination of sequences responsible for high level expression, sequences responsible for proper spatial expression, or both. Unfortunately, it has not been possible to distinguish between these two possibilities because CAT transcript levels were too low for *in situ* hybridizations, and anti-CAT antibody (5 Prime → 3 Prime, Inc.) was unable to detect CAT protein in tissue sections (data not shown). In any case, the R+1–CAT construct contains no *per* transcribed region yet drives cyclic RNA expression, indicating that *per* RNA cycling is almost certainly under transcriptional control and that one or more cycling elements are contained within the first 1.3 kilobases of *per* gene upstream sequence. This result does not preclude the presence of additional cycling elements within or downstream of the gene. Indeed, promoterless *per* gene constructs that rescue circadian behavior (34, 35) also express cycling transcripts, suggesting that internal or downstream *per* gene sequences may contribute to *per* mRNA cycling (data not shown).

DISCUSSION

The experiments presented here show that circadian fluctuations in the abundance of *per* mRNA are controlled at the transcriptional level. This conclusion is based on the fact that *per* precursor RNA cycles with the same amplitude and phase as *per* mRNA and that *per* promoter sequences can confer cycling to a heterologous mRNA. In a variety of organisms, circadian fluctuations in the levels of several RNAs are controlled transcriptionally (6–18), though most and perhaps all of these cycling transcripts are likely to encode products that function downstream of the clock. The *per* gene, however, is vital to circadian clock function as it controls qualitative (presence of) and quantitative (period length) aspects of circadian rhythms in individuals (locomotor activity) and populations (eclosion) (19). The phase (in a light/dark cycle) and period (in constant darkness) of *per* mRNA cycling are controlled by its own protein product, thereby constituting a molecular feedback loop (21). The observation that *per* mutants alter or eliminate this molecular feedback loop and behavioral rhythmicity in parallel suggests that the loop may be important for the fly's circadian rhythms. If so, the dissection of this feedback loop should lead to a better understanding of how circadian rhythms are generated.

The virtual identity in phase and amplitude of the mature and precursor transcripts (even on 2-hr time courses; data not shown) indicates that there is little difference in synthesis rate or turnover and that there is no detectable lag due to posttranscriptional processing. Since mRNA precursors generally turn over quite rapidly (on the order of minutes; ref. 36), it is also likely that *per* mRNA has a short half-life. Such a short-lived mRNA would be necessary in a system where steady-state levels of mRNA must be able to quickly respond to changes in transcription, perhaps in response to phase-shifting stimuli.

Although circadian cycling of the *per* precursor and mature transcripts can be superimposed, this does not hold true for the per protein. per protein cycles such that its peaks in abundance occur ≥8 hr later than those of *per* mRNA (21, 23). Current evidence indicates that this lag in accumulation is posttranscriptionally regulated (24). Therefore, elements of both transcriptional and posttranscriptional control may play a part in the *per* feedback loop, though the relative contributions these mechanisms make are not known.

The per protein is involved in regulating both molecular rhythms (the *per* feedback loop) and behavioral rhythms (eclosion and locomotor activity). How per protein regulates these various processes can only be addressed once the biochemical function of per protein is known. Little knowledge in this area has been forthcoming, but several pieces of evidence suggest that the per protein's effect on the transcription process may be rather direct. First, immunoelectron microscopic studies show that per is a nuclear protein in the brain (27), the site of the fly's circadian pacemaker (37). Second, per has sequence similarity to two proteins that are known or suspected transcription factors: the singleminded (*sim*)-encoded protein from *Drosophila* (38, 39) and the aryl hydrocarbon receptor nuclear translocator (ARNT) from rats and humans (40). However, the region of sequence similarity, ≈270 amino acids, is of unknown function; moreover, unlike sim and ARNT, per contains no known DNA-binding motifs. If per protein is affecting the amount or activity of a transcription complex, then the phase difference between the levels of *per* mRNA and per protein is most easily accommodated by proposing that per protein negatively affects its own transcription.

The regulatory features of the *per* feedback loop have direct parallels to formal theoretical models of self-sustaining oscillators (e.g., refs. 41 and 42). Oscillator models for the cell division cycle and the circadian clock have two elements in common: (*i*) negative feedback and (*ii*) time delays. When both of these elements are operating, self-sustaining oscillations can be modeled in a simple two-component form, where a particular molecule (A) increases in abundance, giving rise to another molecule (B) in a time-delayed fashion; then molecule B, which has a defined half-life, is responsible for breaking down molecule A (41, 42). Thus, the time needed to

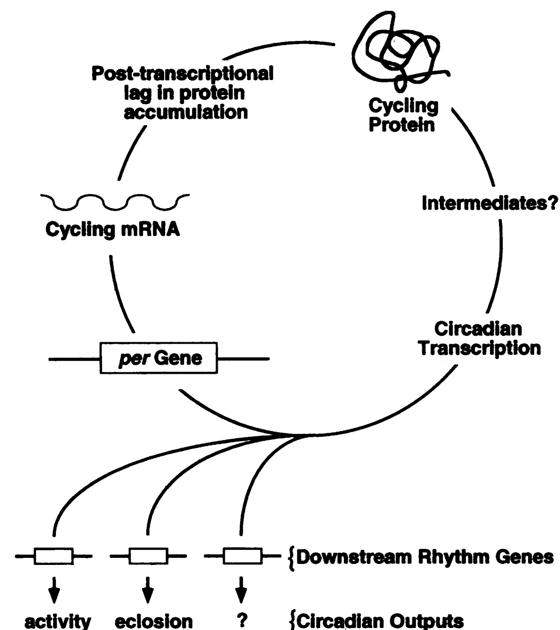

FIG. 4. Model for the regulation of the *per* feedback loop. The *per* gene is transcribed in a circadian manner such that *per* RNA accumulates during a particular time of day. The lag in accumulation of per protein is due to some sort of posttranscriptional mechanism, whereby the peak level of per protein occurs ≥8 hr out-of-phase with that of *per* RNA. The per protein then acts to affect transcription from its own gene either directly or through intermediates and may regulate the expression of genes controlling other circadian outputs in a like manner.

accumulate A, the time lag involved in generating B, the efficiency and speed at which B breaks down or inhibits the synthesis of A, and the half-life of B all contribute to the periodicity, amplitude, and phase of the oscillation.

The *per* feedback loop has all of these features (Fig. 4). The *per* mRNA (molecule A) rises steadily during the last half of the lights-on phase (in a 12-hr light/12-hr dark cycle), peaking between ZT 13 and ZT 15. *per* mRNA then gives rise to per protein (molecule B), which lags in its accumulation such that its peak does not occur until the end of the dark phase (ZT 21–24). per protein then affects *per* transcription, probably by decreasing transcription to relatively low levels at the end of the lights-off phase per protein then breaks down over the first half of the lights-on phase, and the cycle begins anew with *per* mRNA accumulating over the last half of the lights-on phase. Mutations that alter either *per* gene (truncated *per* gene transformants; refs. 34 and 35) or per protein structure (25, 26) may affect the time delay, the ability of per protein to feedback inhibit its own transcription, or the ability of the gene to properly respond to per protein levels, thereby leading to alterations in the period, amplitude, or phase of circadian oscillations. This sort of scenario would also accommodate the effects of per protein on circadian behavior, since other downstream genes could be directly affected transcriptionally by varying levels of the per protein at different times of the day (Fig. 4).

Although these results indicate that *per* RNA is under transcriptional feedback control from its own protein product, a number of important questions remain. For instance, does per protein act directly and, if so, how does per protein work to control transcription [e.g., by competing with positive factors for binding sites (43, 44), by inactivation of positive factors via protein–protein interactions (45, 46), etc.]? What mechanism accounts for the posttranscriptional regulation of per protein cycling (e.g., translational delay or stabilization delay)? Are both mRNA and protein cycling absolutely required for behavioral circadian rhythmicity? The answers to these questions and others will enable us to further refine the role of the *per* gene as a component of the circadian pacemaker.

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