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THE MOLECULAR CONTROL OF CIRCADIAN BEHAVIORAL RHYTHMS AND THEIR ENTRAINMENT IN *DROSOPHILA*

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ABSTRACT

Molecular and genetic characterizations of circadian rhythms in *Drosophila* indicate that function of an intracellular pacemaker requires the activities of proteins encoded by three genes: *period* (*per*), *timeless* (*tim*), and *doubletime* (*dbt*). RNA from two of these genes, *per* and *tim*, is expressed with a circadian rhythm. Heterodimerization of PER and TIM proteins allows nuclear localization and suppression of further RNA synthesis by a PER/TIM complex. These protein interactions promote cyclical gene expression because heterodimers are observed only at high concentrations of *per* and *tim* RNA, separating intervals of RNA accumulation from times of PER/TIM complex activity. Light resets these molecular cycles by eliminating TIM. The product of *dbt* also regulates accumulation of *per* and *tim* RNA, and it may influence action of the PER/TIM complex. The recent discovery of PER homologues in mice and humans suggests that a related mechanism controls mammalian circadian behavioral rhythms.

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Introduction

Most forms of life show prominent adaptations to daily cycles of light and dark and have developed endogenous, temperature-compensated, circadian (from the Latin "about a day") clocks. Complex behaviors such as the wake/sleep cycle have come under the control of these clocks, and these physiological clocks allow temporal ordering of gene and protein expression throughout the day and night (1–3). Most circadian behavioral and molecular rhythms have become dependent on the activity of these biological clocks: Eliminating clock function by tissue or gene ablation produces behavioral and molecular arrhythmicities that cannot be reversed by provision of environmental cycles (4–7). Thus, cellular pacemakers use environmental cycles to establish the phase of a biological oscillation, which in turn regulates the behavioral and physiological response. Molecular and phylogenetic evidence suggests that intracellular mechanisms for circadian timekeeping arose before the divergence of the prokaryota and eukaryota (8). Mammals, insects, and fungi construct circadian oscillators, at least in part, from related proteins (discussed below), which suggests a common origin for eukaryotic clocks.

Circadian rhythms have certain shared properties, regardless of the organism studied. The rhythms persist with a species-specific period, usually 22–25 h, in constant darkness; this shows their endogenous origin. The phase of the rhythm can be reset (entrained) by pulses of daylight, and the period of the rhythm shows little tendency to vary with changes in temperature. Molecular mechanisms underlying these biological oscillators are being studied in humans (9, 9a), hamsters (10), mice (9, 9a, 11), *Drosophila* (12–14), *Neurospora* (15), *Arabidopsis* (16), and *Cyanobacteria* (17). For each of these systems, genetic screening for clock mutations has been directly or indirectly responsible for most progress. In this review I describe the organization of the *Drosophila* clock, for which there is now a fairly detailed understanding of the molecular origins of intracellular circadian oscillations and their entrainment by light.

Genetic Screens for Clock Mutants

Early screens in *Drosophila* were the first to prove that single gene mutations could affect circadian rhythms and alter fundamental properties of these oscillations such as period length. Konopka & Benzer (18), in a limited screen of the X chromosome, isolated three alleles of a single gene. The gene was named *period*, with allele per^0 giving arrhythmicity; per^L, long-period rhythms of

28 h; and *per*^{S}, short-period 19-h rhythms (18). Only recently have new genes been discovered that are also required for *Drosophila's* circadian rhythms. Mutations of the *timeless* (*tim*) locus, on chromosome 2, have produced arrhythmic, short-period, and long-period alleles (7, 19; A Rothenfluh-Hilfiker & MW Young, unpublished data), and a suppressor of the *per*^{L} mutation (20). Mutations of *doubletime* (*dbt*), chromosome 3, have provided arrhythmic, long-period and short-period alleles (J Price, B Kloss & MW Young, unpublished data). The most important feature of this collection of genes is that they all produce proteins that interact to regulate the progression and timing of a single intracellular circadian oscillator (19, 21–25; J Price, B Kloss & MW Young, unpublished data). Thus, *Drosophila* has likely adopted a single biochemical strategy for producing all circadian behavioral and physiological rhythms. I review the properties of each of these genes and their interactions in the following sections.

Molecular Characterization of per

The *per* locus was isolated independently by two groups. Chromosomal rearrangement breakpoints affecting *per* (26, 27) identified the gene in a chromosomal walk (28). Subsequently the gene was recovered by chromosomal microdissection (29). Behavioral rhythms were also restored through the transfer of cloned wild-type DNA to *per*^{0} *Drosophila* (30, 31). Behavioral studies of such transgenic flies, and genetic studies of *per* aneuploids, demonstrated that the period of *Drosophila* circadian rhythms is sensitive to *per* dosage, with lower doses of *per* increasing period length (27, 30, 32, 33).

Sequence analysis of wild-type and mutant alleles showed that *per*^{0} was null and that *per*^{L} and *per*^{S} were derived by single amino acid substitutions (33, 34). The gene encodes a protein of ~1200 amino acids (35–37) that is found predominantly in cell nuclei (38–40).

per has been cloned from representatives of four insect orders: *Diptera*, *Lepidoptera*, *Hymenoptera*, and *Blattodea* (41–43). Sequence comparisons indicate five discrete regions of high homology that are interspersed with sequences that are poorly conserved (41, 42). A high degree of sequence conservation is seen at the PER N-terminus (~75 aa), including a sequence forming the PER nuclear localization signal (NLS) (Figure 1; 19, 25, 42). Two more regions of high homology are referred to as PAS and CLD (together ~300 aa; Figure 1). Both domains mediate protein-protein interactions that are described in detail below. Downstream of CLD is found the short-mutable domain (~70 aa). Amino acid substitutions and deletions in a portion of this interval usually generate short-period circadian rhythms, and the original *per*^{S} mutation maps to this conserved interval (33, 34, 44). It has been suggested that this region regulates activity or stability of the protein (44). A fifth region

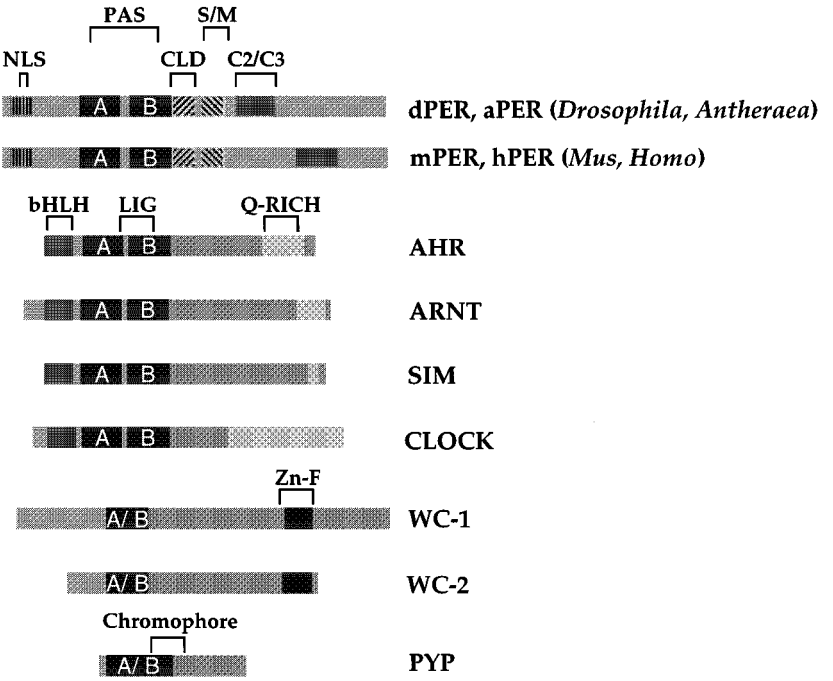

Figure 1 PAS family proteins include both products of clock genes and genes encoding blue-light phototransducers and photoreceptors. PER proteins encoded by *Drosophila*, mouse, and human genes can be related by five common sequence blocks: NLS, PAS, CLD, short-mutable domain (S/M), and C2/C3. NLS, nuclear localization signal; CLD, cytoplasmic localization domain; S/M, short-mutable domain. Arylhydrocarbon receptor (AHR) and AHR nuclear transporter (ARNT) heterodimerize to regulate transcription in response to xenobiotic factors. LIG stands for ligand-binding sequence of AHR. SIM, single minded, is required for aspects of neurectoderm development in *Drosophila*. Clock is required for circadian rhythmicity in the mouse. Q-RICH stands for possible transcriptional activator sequences, and bHLH, predicted DNA binding sequences in AHR, ARNT, SIM, and Clock. Zn-F stands for zinc finger linked to DNA binding of WC1 and WC2. *Neurospora* circadian rhythms and blue-light phototransduction depend on WC-1 and WC-2. PYP (bacterial photoactive yellow protein) is shown with the region of chromophore association.

of conserved sequence, C2/C3 (~130 aa), has not been functionally defined (41, 42). The level of conservation observed in these insect comparisons is sufficient for inter-order function, because a *per* transgene from the moth *A. pernyi* rescues rhythmicity in per^0 *Drosophila* (45).

per homologues were recently isolated from mouse and human DNA libraries (9, 9a). Homologies among the *Drosophila*, mouse, and human PER proteins are extensive: They involve all five conserved domains of the protein defined

by structural and functional studies in insects (9, 9a; Figure 1). The mouse gene is expressed in the suprachiasmatic nucleus (SCN), which controls behavioral rhythmicity in rodents (cf. 46). As in *Drosophila* (reviewed below), the mouse gene is expressed in clock tissues with a circadian rhythm (9, 9a). Thus, mammalian circadian pacemakers are probably organized after the fashion of the *Drosophila* clock.

PER (insect, mouse, and human) is linked to a family of proteins by inclusion of the ~260-aa protein interaction domain PAS (47; Figure 1). Although PER has not been shown to bind DNA or to contain a DNA binding protein motif, most PAS proteins include an established DNA binding sequence and are proven, or likely, transcription factors (Figure 1). The role of PAS was first revealed by studies of dioxin receptor assembly and activation and by in vitro studies of PAS protein associations. The two subunits of the dioxin receptor, AHR and ARNT, associate at least in part through their PAS domains in response to ligand binding through the PAS element of AHR (48–50). Dimerization of AHR and ARNT permits DNA binding and activation of target genes such as cytochrome P450 (51–53). The domain promotes heterodimerization of different PAS-containing proteins in vitro, which suggests that PER may interact with an unidentified PAS-containing protein (54). Although no PAS-containing partner has been identified, PER's PAS domain is an element of this protein's physical interaction with TIM (21, 25; described below), indicating a role for PAS in heterotypic as well as homotypic protein associations.

Recently PAS has been discovered in circadian clock proteins other than PERIOD in three noninsect species. In *Neurospora*, two proteins *white collar-1* (*wc-1*) and *white collar-2* (*wc-2*), are required for blue-light phototransduction (55–57). Both genes are also required for proper expression of the *Neurospora* clock gene *frequency* (*frq*), and loss of *wc-1*, *wc-2*, or *frq* results in arrhythmicity (55). Cloning of *wc-1* and *wc-2* revealed that both are PAS-containing (Figure 1) zinc-finger proteins that may dimerize to regulate transcription of *frq* and several previously identified light-responsive genes (56, 57). Both proteins bind DNA associated with regulated target genes (56, 57).

In the mouse, a gene referred to as *Clock* is required for circadian rhythmicity. Homozygotes produce long-period rhythms that grade into arrhythmicity following transfer from light/dark cycles to constant darkness (58). Cloning of *Clock* revealed a PAS-domain–containing protein (11, 58a; Figure 1). Like most PAS-family proteins, CLOCK carries a putative DNA binding sequence (bHLH), which suggests function in mouse timekeeping as a transcription factor.

Photoactive yellow protein (PYP) and certain Algal phytochromes have recently been added to the PAS family (Figure 1). Both of these proteins function as photoreceptors in conjunction with an associated chromophore (59). Inclusion of PYP, a phytochrome, wc-1, and wc-2 in the PAS family raises the

possibility that PER and CLOCK arose from a group of proteins originally dedicated to blue-light photoreception and phototransduction (55, 59).

Patterns of per *RNA and Protein Expression*

Studies of genetically mosaic *Drosophila* and of transgenic flies indicate that circadian behavioral rhythms are controlled by 20–30 neurons of the central brain (60–62). Some of the cells, referred to as lateral neurons (LN) (60), express the transcription factor GLASS, which is required for development of all known *Drosophila* photoreceptors (62–65). When the *glass* promoter is used to direct *per* expression to only these central brain cells in transgenic per^0 flies, behavioral rhythms are restored, even if the eyes and ocelli are removed (62). In a light/dark cycle, the phase of the rhythm produced by these eyeless, ocelliless flies is reset, which suggests that *glass*-expressing brain cells can mediate both rhythmicity and entrainment (62).

per RNA and proteins are expressed with a circadian rhythm. Mutations of *per* alter these molecular oscillations in a fashion that corresponds to their effects on behavioral rhythmicity. per^0 mutants eliminate *per* RNA cycling. per^L and per^S respectively lengthen and shorten the period of the RNA and protein cycles (5, 66). These rhythms have been documented in the eye and brain, including the LNs, and in tissues outside the nervous system of the fly (5, 39, 67–71). Oscillations in some of these tissues appear to be autonomously sustained. For example, circadian cycles of *per* RNA expression are observed in Malpighian tubules even when dissected from the carcasses of decapitated flies that have been maintained in culture for several days (70, 71). RNA and protein cycles are produced with a constant-phase relationship in all of these tissues. Highest levels of *per* RNA are observed about two hours after lights off in a 12-h/12-h light/dark cycle, and PER protein is subsequently detected in nuclei with a delay in peak accumulation of 4–6 h. The Malpighian rhythms can also be entrained following decapitation, suggesting involvement of a novel photoreceptor.

In addition to cycles of protein accumulation, cycles of PER phosphorylation have been observed (66). Phosphorylation appears to involve several sites on PER, and progressive, phosphorylation-dependent increases in mobility are seen until PER is degraded near dawn (66, 72).

Cycles of *per* RNA and PER protein expression have been reported in the silkmoth *Antheraea pernyi* (73, 74). In the eyes of the moth, *per* RNA and proteins cycle in a fashion that is indistinguishable from the *Drosophila* brain and eye rhythms (73). Yet in the moth brain, cytoplasmic rather than nuclear cycles of PER protein have been immunocytochemically observed. Biochemical studies of PER protein cycles in the moth are not available, and it is not known whether the immunocytochemically detected cycling is autonomously generated in any

of the moth tissues studied. Cells controlling behavioral rhythms also have not been identified in the moth. In some exceptional *Drosophila* tissues (e.g. ovaries), PER proteins accumulate cytoplasmically, as in *Antheraea* (38, 68), but no *per* RNA or protein rhythms are observed in these *Drosophila* tissues (68). As mentioned above, RNA from the mouse *period* gene is expressed with a circadian cycle in an established pacemaker tissue, the SCN (9, 9a). Thus, in both *Drosophila* and mice, cycling *per* expression can be linked to cells known to control circadian behavioral rhythms.

Although studies of *per* alone were unable to uncover a mechanism for generating clocklike cycles of gene expression, several observations suggested that PER proteins somehow inhibit *per* transcription:

1. Constitutively high levels of *per* transcription are found in per^0 mutants (75, 76).
2. *per* transcription declines as PER protein accumulates, and it does not rise again until PER proteins decay (66).
3. Constitutive overexpression of a *per* transgene that is limited to the eye blocks cycling of *per* transcription in that tissue but not in the brain (77).
4. Transient suppression of PER function leads to increased accumulation of *per* RNA (14, 23).

Cycles of clock gene expression are also associated with the circadian oscillator of *Neurospora* (6, 15). The clock gene *frequency* (*frq*) is required for the circadian regulation of conidiation (78), and *frq* RNA and proteins cycle with a circadian period (6, 79). Overexpression of FRQ protein suppresses *frq* transcription, and transitory reductions in the FRQ protein shift the phase of the conidiation rhythm and the molecular rhythms of *frq* RNA and protein accumulation (6, 15).

timeless *Promotes Cycles of* per *RNA and Protein Accumulation*

Null mutations of the second chromosome-linked clock gene *timeless* (tim^0) eliminate circadian behavioral rhythms, stop cycling of *per* RNA, and block nuclear accumulation of PER proteins (7, 19). tim^0 mutations also suppress accumulation of PER and lead to constitutive hypophosphorylation of residual PER proteins (72). tim^L mutants produce long-period behavioral rhythms and correspondingly lengthen the period of *per* RNA and protein rhythms (A Rothenfluh-Hilfiker & MW Young, unpublished data).

timeless encodes a large (~1400 aa) previously undescribed protein (80) that is well conserved in other *Drosophila* species (80a, 80b). tim^{01}, the most

completely characterized arrhythmic mutation, contains an intragenic deletion of 64 bp that blocks TIM protein production (80–82).

tim RNA and proteins cycle with a circadian rhythm (22, 24, 81, 82). The apparent molecular size of the protein also cycles as for PER (24, 82) because of rhythmic TIM phosphorylation (24). The phase of the *tim* RNA rhythm is similar to that of *per* (22), and TIM proteins accumulate in nuclei of lateral neurons and photoreceptors of the eyes and ocelli with kinetics that correspond to those of PER protein (81, 82). tim^0 and per^0 mutations block molecular cycles of *tim* RNA production (22). The per^S mutation shortens the period of *tim* RNA and protein cycles (22), whereas per^L and tim^L mutations lengthen the periods of these cycles (22; A Rothenfluh-Hilfiker & MW Young, unpublished data). Thus, molecular rhythms of *per* and *tim* are interdependent, with mutations at either locus eliciting corresponding changes in the cycles produced by both loci.

tim regulates PER's subcellular localization by encoding a protein that must heterodimerize with PER to permit movement to the nucleus. Coexpression of PER and TIM in cultured *Drosophila* cells (S2 cells) results in physical association and nuclear localization of both proteins, but expression of PER in the absence of TIM leads to cytoplasmic accumulation in S2 cells (25; see also further description below). PER also physically interacts with TIM in yeast (21), in vitro (21, 25), and in cells of the *Drosophila* head (23, 24). Just as TIM is required for PER nuclear localization, PER must be present for nuclear localization of TIM, because TIM accumulates cytoplasmically in per^0 mutants (82) and when expressed without PER in S2 cells (25). Although PER accumulation is suppressed in tim^0 mutants, high levels of TIM accumulate in the cytoplasm of per^0 flies (82). The defect in PER accumulation in tim^0 flies suggests that heterodimerization influences the stability of cytoplasmic PER proteins, and studies of truncated, PER–β-galactosidase fusion proteins have located sequences on PER that are likely to confer TIM-dependent accumulation (19, 76, 83). Because both *tim* and *per* RNAs accumulate with a circadian rhythm, the timing and rate of formation of PER/TIM dimers should be influenced by concentrations of *per* and *tim* RNA, by the affinity of PER for TIM, and by rates of decay of the PER monomer (14, 22).

The specificity of PER's physical interaction with TIM has been demonstrated in yeast. Gekakis et al (21) searched for proteins expressed in the *Drosophila* head that would associate with PER in a yeast two-hybrid assay. Of 20 million transformants, each expressing a PER bait and a prey protein from a *Drosophila* head cDNA library, 48 clones signaled a significant protein-protein interaction. When these clones were subsequently probed with *tim* cDNA, 16 clones, by far the largest subset, were found to carry a *tim* prey (21).

Studies of the PER/TIM interaction in yeast also support the expectation that rates of physical association of the two proteins can set the period of the

circadian rhythm. The mutation per^L lengthens the period of molecular and behavioral rhythms to ~28 h when *Drosophila* are maintained at 25°C, and the period is further increased through the elevation of temperature, with ~30-h rhythms observed at 29°C (84). By comparing the times of accumulation of PER proteins in lateral neurons of wild-type and per^L *Drosophila*, Curtin et al (85) showed that nuclear localization was delayed by the per^L mutation. The delays varied in per^L so that they corresponded to the changes in period length produced by the mutant at different temperatures. The per^L mutation is due to a single amino acid substitution in PER's PAS domain (33). Because PAS mediates PER's physical interaction with TIM (see below), Gekakis et al (21) compared wild-type and mutant protein interactions at varying temperatures in yeast. Higher temperatures reduced the affinity of TIM for PER^L, suggesting that the temperature-sensitive delays in nuclear localization were due to depressed association of TIM and the PER^L protein in vivo (21). These observations, and the finding that lowering PER dosage lengthens the period (27, 30, 32, 33), show that rates of PER/TIM association regulate the duration of a part of the circadian cycle.

Physical Association of PER and TIM Regulates Activity of Cytoplasmic Localization Domains

Sequences on PER and TIM responsible for their physical association have been mapped in vitro and by sequencing TIM fragments derived from yeast two-hybrid interactions with PER baits (21, 25; Figure 2). Two binding sites on PER involve PAS and an adjacent sequence referred to as CLD (cytoplasmic localization domain) . An N-terminal region referred to as PAS-A interacts with a region of TIM that includes its nuclear localization signal. CLD interacts with a second region of TIM downstream from the TIM NLS (Figure 2; 25).

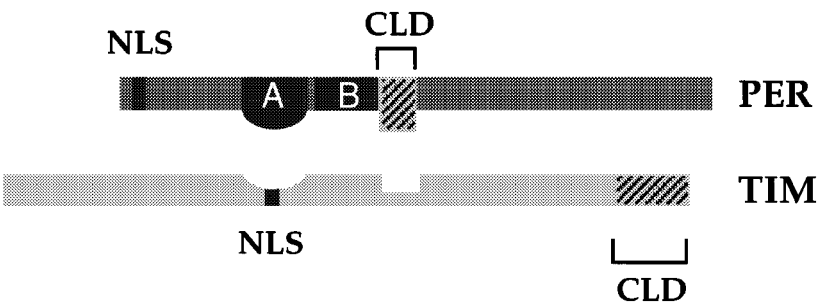

Figure 2 Binding sites for PER/TIM heterodimerization regulate cytoplasmic localization domains (CLDs). TIM binds two regions of PER, one of which, CLD, promotes cytoplasmic localization of PER monomers. A TIM CLD, of unrelated sequence, is also suppressed by formation of the PER/TIM complex.

These patterns of PER/TIM binding are pertinent to studies of PER and TIM sequences regulating subcellular localization (25). As first reported by Vosshall et al (19), PER contains a sequence that promotes cytoplasmic accumulation, and the activity of this sequence element is dominant to the PER NLS in monomeric proteins. Saez & Young (25) identified a comparable cytoplasmic localization domain (CLD) for TIM. The PER and TIM CLDs function in pacemaker cells of the fly head, but their activities have been studied most completely in cultured *Drosophila* cells. In S2 cells, full-length monomeric PER or TIM proteins accumulate in the cytoplasm, while certain truncations permit nuclear localization (25). The PER CLD was mapped to an ~60 amino acid region corresponding to the binding site for TIM that is adjacent to PAS-B (Figure 2). Thus, for PER a TIM binding site produces cytoplasmic localization of monomeric PER proteins. The TIM CLD was similarly mapped to ~160 C-terminal amino acids (25). Because coexpression of full-length PER and TIM proteins in S2 cells induced nuclear accumulation of both proteins, physical association of PER and TIM must inhibit the function of both the PER and TIM CLDs. Thus, the activities of the PER and TIM CLDs confine assembly of PER/TIM complexes to the cytoplasm.

Additional insights into CLD regulation have come from localization studies of reporter proteins carrying the PER and TIM nuclear localization signals coupled to a single CLD provided by either TIM or PER. In both cases localization of the reporter was cytoplasmic in S2 cells (25; L Saez & MW Young, unpublished data). The dominant functions of the PER and TIM CLDs, and their continued function in chimeric proteins, suggest that a cytoplasmic factor or factors may interact with monomeric PER and TIM proteins to inhibit nuclear localization. This regulation of subcellular localization may also influence rates of PER/TIM complex accumulation if monomeric PER and TIM proteins must compete with cytoplasmic factors for CLD binding.

Progression of a Self-Sustaining, Intracellular Oscillator

Sehgal et al (22) proposed a model to explain generation of self-sustaining circadian rhythms through the connected activities of PER and TIM. As indicated in Figure 3, transcription of *per* and *tim* is initiated 3-6 h after subjective dawn (time in constant darkness corresponding to lights on during entrainment) because of absence of nuclear PER and TIM proteins. Although *per* and *tim* RNA levels rise during the subjective day, unstable PER monomers fail to accumulate as a result of insufficient levels of TIM. Near subjective dusk, pools of *per* and *tim* RNA are sufficiently large to promote heterodimerization of PER and TIM. PER/TIM binding suppresses action of CLDs on each protein, allowing nuclear translocation. Nuclear PER/TIM complexes directly or indirectly suppress further *per* and *tim* transcription. As *per* and *tim* RNA levels fall, a threshold for PER/TIM heterodimerization is again reached, eliminating further nuclear

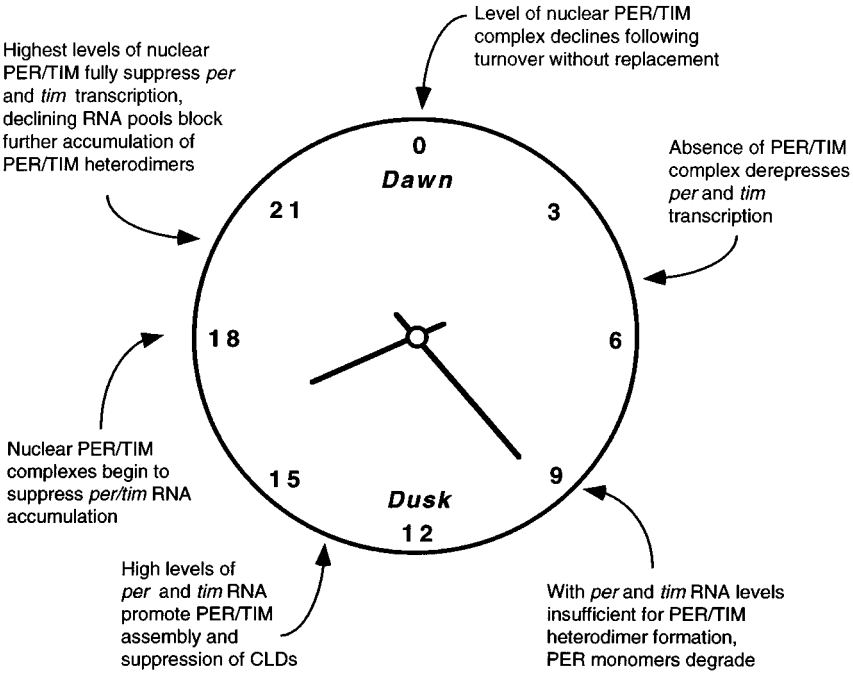

Figure 3 Model of the PER/TIM oscillator. Progression of molecular cycles is predicted for a self-sustained oscillator in constant darkness (after 22).

translocation and leaving a pool of nuclear PER/TIM complex that was amassed in the early subjective evening. Nuclear PER/TIM complexes will continue to suppress transcription until late subjective night, when the heterodimers are seen to turn over without replacement. As indicated above, period length can be significantly altered experimentally by changing the affinity of PER for TIM (21) or by reducing the concentration of PER (27, 30, 32, 33). Thus, self-sustained oscillations are promoted because times of RNA synthesis are separated from times of nuclear protein accumulation and function by an interval of PER/TIM heterodimerization. In the absence of a mechanism generating such temporal separations, autoregulation of *per* and *tim* transcription by PER and TIM proteins should not produce clocklike cycles of accumulation (22).

Light Entrains the Clock by Rapidly Eliminating TIM

Exposure of wild-type flies to constant daylight produces arrhythmicity (84, 86) and suppresses accumulation of the PER protein as in tim^{01} mutants (67, 72). Because the low levels of PER found in tim^{01} flies were not further suppressed through the exposure of the mutants to constant light, light's effects on wild-type flies might be mediated by TIM (72).

Several studies have now confirmed that light rapidly lowers the level of TIM protein (24, 81, 82). For example, a 10-min pulse of daylight is sufficient to eliminate immunocytochemical staining of TIM in the eyes and central brain (81, 82). The response of TIM-expressing brain cells (LNs) does not appear to require the eyes, as the protein is eliminated from these cells by exposing eyeless flies to the same light pulses (K Wager-Smith & MW Young, unpublished data). These effects of light on TIM do not require clock function, as TIM is rapidly lost when per^0 *Drosophila* are exposed to pulses of light (24, 82). Such clock-independent responses to light suggested that TIM's light sensitivity is responsible for entrainment of the phase of the circadian rhythm to the phase of the environmental cycle. TIM's light sensitivity could also explain realignment of the behavioral rhythm to a light/dark cycle with an altered phase, such as would be encountered on crossing time zones.

Drosophila maintained in constant darkness continue to show rhythmic behavior with a phase dictated by their prior experience in a light/dark cycle. However, pulses of daylight will reset the phase of these "free-running" rhythms. The response of a fly to light pulses provided at different times of day is reproducible and can be represented by a phase-response curve (PRC) (Figure 4, *top*). As can be seen for the *Drosophila* PRC, light pulses given between subjective dusk and midnight (CT 12 to CT 18) lead to phase delays in the rhythm whereas advances are produced by comparable pulses between subjective midnight and dawn (CT 18 to CT 24; Figure 4). Thus the response to the same stimulus differs in magnitude and direction, depending on the time of day.

Immunoblot analysis has shown that phase advances and delays also occur in the molecular rhythms of TIM protein in response to resetting light pulses (82). Light pulses delivered in the advance zone of the behavioral cycle cause premature loss of TIM protein. TIM accumulates on the following subjective day with an advanced phase. In contrast, light pulses in the delay zone cause a loss of TIM that is promptly followed by its reaccumulation. TIM subsequently decays, but with a new phase that is established by the secondary accumulation. Myers et al (82) proposed that advances are differentiated from delays by the different *tim* RNA titers found in the early versus late subjective night. Because *tim* and *per* RNA levels are highest during the first part of the subjective night (Figure 4, *bottom*), TIM proteins eliminated by a light pulse at this time are replaced by new translation in the current cycle. This would not be the case for light-induced loss of TIM near the end of the subjective night, as *tim* and *per* RNA levels are low. Thus, light late at night causes TIM to decay prematurely, and it advances the following day's cycle of *per* and *tim* RNA and protein accumulation (Figure 4; 82).

Three observations suggest that TIM protein changes mediate entrainment of behavioral rhythmicity through the above mechanism.

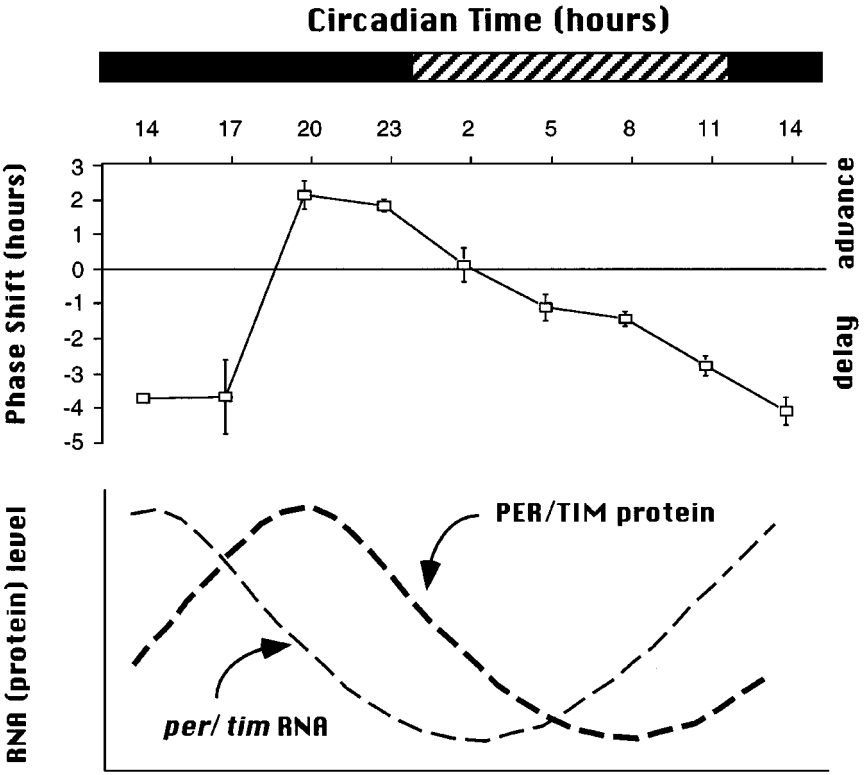

Figure 4 Time-specific levels of *tim* and *per* RNA may determine whether light pulses advance or delay the phase of the behavioral rhythm. *Top*: phase response curve (PRC) for 10-min pulses of daylight delivered at different times of subjective day or night. Pulses in early subjective night (CT 12 to CT 18) delay the behavioral rhythm by up to ∼4 h. Comparable light pulses in late subjective night (CT 18 to CT 24) can advance the rhythm by ∼2 h (after 82). *Bottom*: PER and TIM protein accumulation follows *per* and *tim* RNA accumulation by several hours in constant darkness. Behavioral delays occur when RNA levels are high at the time of the light pulse. TIM that is degraded by light is replaced by continued translation, further delaying the decline in TIM protein levels. Behavioral advances occur for light pulses given when RNA levels are low. Replacement of TIM requires new transcription. Premature loss of TIM without replacement in the current molecular cycle allows advanced *per* and *tim* RNA accumulation in the following cycle.

1. TIM levels drop when arrhythmic *per*^{0} flies are exposed to light but *tim* RNA levels are unaffected (22, 24, 82). In rhythmic flies (*per*$^+$ or *per*^{S}), light-dependent loss of TIM is followed by increased transcription of *per* and *tim* (14, 23, 87). Thus, acute effects of light are on TIM protein and not *tim* RNA.

2. As indicated above, TIM's light sensitivity is clock-independent because it occurs in *per*^{0} flies. Therefore changes in the accumulation and diminution profiles of the protein should dictate changes in the behavioral rhythm and not vice versa.

3. The new phase of the TIM protein rhythm is apparent within hours of the administered light pulse and thus accompanies the first evidence of a behavioral change in the flies (82, 88).

doubletime, *a Third Essential Component of the* Drosophila *Oscillator*

Further genetic screening might be expected to identify additional components of the fly clock. These could include genes controlling TIM's light sensitivity, PER's instability in the absence of TIM, and functions controlling PER and TIM phosphorylation. There is also evidence for posttranscriptional regulation of *per* and *tim* RNA accumulation. For example, *per* RNA coding sequences promote cycling of *per* mRNA in the absence of the *per* promoter (61). They also promote cycling when fused to a novel, constitutively active promoter (62). Sequences within the transcribed region of *per* delay the timing of *per* RNA accumulation. These findings suggest effects on rates of *per* transcription or RNA stability (76).

Another gap in our understanding involves the biochemical activity of the PER/TIM complex. Accumulation of the complex results in suppression of *per* and *tim* transcription, but there is no evidence that PER, TIM, or the complex directly interacts with DNA or a mediating transcription factor or factors. There is some evidence that effects on transcription are indirect: Phase-advancing or -delaying light pulses that transiently eliminate TIM give rise to brief increases in *per* and *tim* RNA pools that appear 4–5 h after administration of the pulse (14, 23).

A third clock gene, *doubletime* (*dbt*), encodes a factor required for behavioral rhythmicity and progression of the PER/TIM cycle. Three classes of mutant alleles have been recovered at *dbt*. The mutation originally defining the locus, *dbt*^{S}, produces flies with short-period, 18-h rhythms when homozygous (J Price & MW Young, unpublished data). Thus, *dbt*^{S} produces a more extreme period-shortening phenotype than *per*^{S}. Gene dosage studies suggest that increasing

the level of wild-type *dbt* function shortens the period (J Price, B Kloss & MW Young, unpublished data). A long-period mutant has also been isolated, dbt^{L} (~27.5 hours) (A Rothenfluh-Hilfiker & MW Young, unpublished data). Both dbt^{S} and dbt^{L} are semidominant. $dbt^{S}/+$ heterozygotes produce ~21-h rhythms, whereas $dbt^{L}/+$ flies have ~25-h periods (J Price, A Rothenfluh-Hilfiker & MW Young, unpublished data). The third class of *dbt* mutations, dbt^{0}, are null. However, unlike per^{0} and tim^{0} mutations, dbt^{0} alleles confer recessive lethality, establishing a double role for the gene. Although adult behavior cannot be assessed, molecular studies of developing dbt^{0} *Drosophila* indicate that they are arrhythmic (B Kloss, J Blau & MW Young, unpublished data).

dbt mutations affect *per* and *tim* RNA cycling. For example, dbt^{S} shortens rhythms of *tim* RNA expression to 18 h (B Kloss and MW Young, unpublished data). The effects of dbt^{0} mutations are of the most interest, as these mutations block production of *dbt* RNA and *tim* RNA. In addition to these effects on RNA levels, PER proteins accumulate abnormally in dbt^{0} *Drosophila* (B Kloss, J Blau, J Price & MW Young, unpublished data).

The *dbt* locus has been cloned (B Kloss, J Price & MW Young, unpublished data), and *dbt* RNA localization studies in situ have shown that *per*, *tim*, and *dbt* are expressed in the same cells in the *Drosophila* head (J Blau & MW Young, unpublished data). Therefore *dbt*, *per*, and *tim* appear to represent three essential components of a single intracellular oscillator.

How Does Cycling of an Intracellular PER/TIM Oscillator Regulate Behavior?

Tissue transplantation studies in silkmoths (89, 90) and *Drosophila* (91) have shown that cells composing the clock for the moth and fly can control rhythmic behavior through action of a diffusible substance. Handler & Konopka (91) transplanted brains from dissected heads of per^{S} adults into abdomens of adult per^{0} recipients. Recipient flies showed transformed locomotor activity rhythms with periods expected for the donor strain (19 h). per^{0} recipients that received brain transplants from per^{0} donors continued to show behavioral arrhythmicity (91).

A diffusible signal or signals similarly couple cells of the hamster suprachiasmatic nucleus to control of circadian locomotor rhythms. Silver et al (46) transplanted encapsulated SCN tissues from wild-type donors into SCN-ablated, *tau*/+ recipient hamsters. The *tau* mutation shortens the locomotor activity rhythm so that *tau*/+ hamsters produce ~22- rather than 24-h rhythms (10). Transplant recipients were recovered that displayed donor (24-h) rather than recipient rhythms. Because the transplanted cells were encased in semipermeable membranes, it was concluded that cell contact was not required for establishment of the behavioral rhythm (46). The suprachiasmatic nucleus is composed

of cells that autonomously produce circadian oscillations in culture (92), which suggests that diffusible signals controlling behavior may be products of SCN pacemaker cells themselves.

These and other factors that must integrate cellular responses to circadian molecular oscillators are likely to be products of clock-controlled genes (CCGs) (2). Clock-controlled genes produce transcripts that accumulate with a circadian rhythm. But these genes can be distinguished as targets of the circadian pacemaker rather than cycling clock components because CCG loss fails to block pacemaker cycling. Some of the earliest descriptions of CCGs emerged from studies of plants (cf. 1, 93) and fungi (2), and known or likely CCGs are now well characterized in *Drosophila* (3, 94), *Cyanobacteria* (17), and cold- and warm-blooded vertebrates (cf. 95–100).

CCGs that cycle with stable phase differences can be identified in the same organism (cf. 2, 3, 94). This regulation of CCGs indicates that a cell can evaluate progression of the molecular oscillator over much of its cycle. In *Drosophila* this suggests hierarchical control by the PER/TIM oscillator. It is as yet unknown how the pattern of expression of any CCG is determined, but possibly the same regulatory activity of the PER/TIM complex that mediates cycling transcription of *per* and *tim* will be found to time expression of *Drosophila*'s CCGs.

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