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Review

Probing the Relative Importance of Molecular Oscillations in the Circadian Clock

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ABSTRACT

Circadian (~24 hr) rhythms of behavior and physiology are driven by molecular clocks that are endogenous to most organisms. The mechanisms underlying these clocks are remarkably conserved across evolution and typically consist of auto-regulatory loops in which specific proteins (clock proteins) rhythmically repress expression of their own genes. Such regulation maintains 24-hr cycles of RNA and protein expression. Despite the conservation of these mechanisms, however, questions are now being raised about the relevance of different molecular oscillations. Indeed, several studies have demonstrated that oscillations of some critical clock genes can be eliminated without loss of basic clock function. Here, we describe the multiple levels at which clock gene/protein expression and function can be rhythmically regulated—transcription, protein expression, post-translational modification, and localization—and speculate as to which aspect of this regulation is most critical. While the review is focused on Drosophila, we include some discussion of mammalian clocks to indicate the extent to which the questions concerning clock mechanisms are similar, regardless of the organism under study.

THE light:dark cycle generated by the earth's rotation is the driving force of daily behavioral and physiological rhythms exhibited by most organisms. However, these daily (~24 hr) rhythms are not just a passive response to the light:dark cycle; instead, an intrinsic timekeeping mechanism synchronizes physiological processes to the cyclic environment. The endogenous timekeeper is a self-sustained oscillator, termed the circadian clock, which can be entrained to environmental cues such as light and temperature (such environmental time signals are called zeitgebers), but more importantly, it free runs in constant conditions that lack environmental cues. In the past ~20 years, genetic analysis of circadian rhythms in model organisms such as Drosophila, Neurospora, Arabidopsis, cyanobacteria, and mice has yielded considerable insight into the molecular mechanisms of circadian oscillators. Despite these advances, the question of how exactly a rhythm is generated is getting some attention again because a number of recent studies have challenged the simple models proposed initially. This review traces these developments in the field and then proposes a revised model that incorporates the old and new findings. While the focus is on the molecular mechanisms of the *Drosophila melanogaster* circadian clock, advances in other circadian systems will also be discussed to illustrate conserved mechanisms. Readers interested in circadian clock mechanisms of other organisms are encouraged to read recent reviews (Hastings and Herzog 2004; Gardner *et al.* 2006; Ko and Takahashi 2006; Williams 2006; Woelfle and Johnson 2006; Heintzen and Liu 2007; Levi and Schibler 2007).

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THE BASIC CIRCADIAN FRAMEWORK: THE *per–tim* FEEDBACK LOOP

Genetic analysis has identified four proteins in Drosophila that are essential for, and largely dedicated to, circadian clock function: CLOCK (CLK), CYCLE (CYC), PERIOD (PER), and TIMELESS (TIM) (Konopka and Benzer 1971; Bargiello *et al.* 1984; Reddy *et al.* 1984; Zehring *et al.* 1984; Sehgal *et al.* 1994; Myers *et al.* 1995; Allada *et al.* 1998; Rutila *et al.* 1998). The manner in which a molecular clock is generated through the actions of these proteins has been investigated in some detail. During the day and early evening, CLK and CYC form a heterodimer, which activates *per* and *tim* expression through binding to specific enhancer elements (E-box) in their promoters (Darlington *et al.* 1998), resulting in a peak of *per* and *tim* transcripts during the early night. The PER and TIM proteins accumulate and associate with each other (Gekakis *et al.* 1995; Meyer

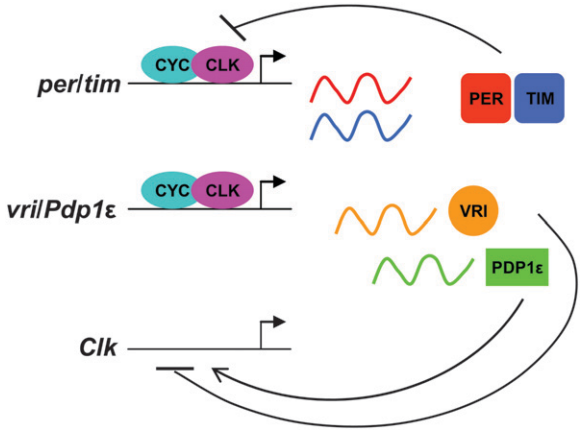

Figure 1.—Model of the Drosophila circadian clock based on interlocking transcriptional feedback loops. CLK and CYC form a heterodimer and bind to E-box elements of the circadian clock genes *per* and *tim* and activate their transcription during the day and early evening; as *per* and *tim* mRNAs peak, PER and TIM proteins accumulate, form a PER–TIM complex, and translocate into the nucleus to repress their own transcription during the late night. During the day, PER and TIM are degraded by light-dependent and independent pathways, thus allowing a new cycle of transcription to start. In another transcription-based loop, CLK–CYC activate transcription of *vri* and *Pdp1ε*; as VRI and PDP1ε proteins accumulate, they translocate into the nucleus to inhibit and activate *Clk* transcription, respectively. Both VRI and PDP1ε bind to E4BP4 sites in the *Clk* promoter. PDP1ε accumulation lags behind that of VRI, resulting in rhythmic *Clk* transcription.

et al. 2006) in the late night and translocate into the nucleus to repress the transcriptional activity of the CLK–CYC heterodimer (Hardin 2005) (Figure 1). Recent studies suggest that each of the two proteins can enter the nucleus alone (Shafer *et al.* 2002); however, nuclear TIM alone does not function as an efficient repressor of CLK–CYC activity (Ashmore *et al.* 2003; Chang and Reppert 2003). In contrast, PER alone can repress CLK–CYC activity (Rothenfluh *et al.* 2000; Chang and Reppert 2003; Nawathean and Rosbash 2004; Cyran *et al.* 2005), although the repression efficiency is greatly increased when TIM is present. After lights on, PER–TIM proteins are degraded, allowing a new cycle of transcription to start (Figure 1). The turnover of PER and TIM proteins during the daytime, the delay of their accumulation during early night, and their nuclear translocation during the late night appears to be crucial to maintaining the 24-hr cycle. These dynamic cyclic processes persist in constant dark conditions. Mammals have a similar framework, where the circadian clock consists of CLOCK, BMAL1 (mammalian ortholog of CYC), and PER and its partner, which is a molecule called cryptochrome (mCRY), rather than TIM (Ko and Takahashi 2006).

The mechanisms described above are usually synchronized to light:dark cycles through the process described below. However, they are sustained in constant darkness; indeed, they can even be initiated in the absence of light. When flies are raised under constant dark conditions, they are able to manifest rhythmic behavior (Sehgal *et al.* 1992; Tomioka *et al.* 1997), although individual flies are not in phase with each other.

The simplest model, then, is that rhythmic transcription produces rhythmic RNA expression, which leads to rhythmic protein expression. The protein, in turn, regulates transcription of its own gene, maintaining a 24-hr loop, which drives overt rhythms. However, a number of observations have challenged this model. Even when *per* and *tim* mRNA are held constant, the two proteins continue to cycle, and behavioral rhythms persist in a significant proportion of flies (Yang and Sehgal 2001). This contradicts the original model because the prediction was that abolishing rhythmic transcription would abolish the feedback loop, and thereby behavioral rhythms. Thus, mechanisms other than rhythmic transcription are able to maintain cyclic expression of the core clock proteins, and it would appear that cyclic expression of the two proteins is essential for clock function. Consistent with this idea, overexpression of either protein renders flies arrhythmic (Yang and Sehgal 2001). However, it may also be that overexpression of the proteins prevents necessary post-translational modifications (discussed further below).

In the mammalian circadian clock, even the significance of clock protein cycling has been questioned. Although overexpression of *mCry1* was reported to impair molecular oscillations in cultured fibroblasts (Ueda *et al.* 2005), infusion of constant levels of mCRY into cultured cells did not disrupt the molecular clock (Fan *et al.* 2007). One could argue that the overall levels of mCRY, or its post-translational modifications, were different in the two studies, but the latter study does suggest that robust cycling of mCRY is not necessary for a functional molecular oscillation, at least in the cell system used. Thus, it appears that rhythms can be generated in the absence of rhythmic mRNA expression, and perhaps even rhythmic protein expression, of one or more essential clock genes. In fact, as alluded to above, post-translational control of clock proteins is critical, if not sufficient for generating a rhythm.

LIGHT RESPONSE OF THE CIRCADIAN CLOCK

Light is the major entraining signal for the circadian clock. Since the clock's response to light is based largely upon the function of proteins introduced above, we will discuss it here before describing other aspects of the clock mechanism. The clock can be entrained to light by the visual system and by nonvisual, dedicated circadian mechanisms (Ashmore and Sehgal 2003). The dedicated circadian photoreceptor in Drosophila is cryptochrome (CRY) (Emery *et al.* 1998; Stanewsky *et al.* 1998), ortholog of the protein that in mammals is a component of the molecular clock. Upon light treatment, CRY is activated and transmits a signal that targets

TIM for degradation by the proteasome (Hunter-Ensor *et al.* 1996; Myers *et al.* 1996; Zeng *et al.* 1996; Naidoo *et al.* 1999). Light-dependent degradation of TIM is mediated by a specific E3 ligase protein termed JETLAG (JET) (Koh *et al.* 2006). The name was derived from the phenotype of mutant flies that fail to efficiently adjust their circadian behavior to a shift in the light:dark schedule, thus displaying extended "jetlag." *jetlag* (*jet*) mutants also have aberrent behavior in the presence of constant light. Unlike wild-type flies that are arrhythmic in the presence of constant light due to the constant degradation of TIM, *jet* flies are rhythmic under such conditions.

Although tyrosine kinase activity appears to be required for TIM degradation by light (Naidoo *et al.* 1999), the specific enzyme involved has not yet been identified. However, the serine/threonine kinase, glycogen synthese kinase [SHAGGY (SGG) in Drosophila], is involved in this process. Serotonin signaling increases SGG phosphorylation, thereby lowering its activity (SGG activity is lowered by phosphorylation at the Ser^{9} residue), and reduces TIM degradation by light (Yuan *et al.* 2005). On the other hand, a recent study showed that increased SGG stabilizes TIM and also reduces its response to light (Stoleru *et al.* 2007). This apparent contradiction cannot be simply explained by SGG activity toward TIM and may involve effects of SGG on CRY (Stoleru *et al.* 2007).

With respect to how the effect of light on TIM resets the clock, the association of TIM with CRY abrogates negative feedback by PER–TIM, and the subsequent degradation of TIM disrupts the PER–TIM complex (Lee *et al.* 1996; Ceriani *et al.* 1999). Thus, light alters the levels of a clock component, which resets the timing of all other events in the cycle. Interestingly, pulses of light delivered at night will reset the phase of the clock, but the effect is different depending upon the time of delivery: in the early night, a light pulse delays the clock (resetting to dusk) while in the late night it advances the clock (resetting to dawn). In molecular terms, a possible explanation may be provided by the levels of *tim* mRNA and the subcellular localization of PER and TIM. In the early night, the two proteins are cytoplasmic and mRNA levels are high and able to resynthesize the protein lost by degradation. Thus, the clock is delayed by the number of hours it takes to produce that amount of protein. In the late night, the PER–TIM complex is in the nucleus, repressing transcription. Thus, the protein cannot be replenished and the clock moves forward to the next cycle.

POST-TRANSLATIONAL REGULATION OF PER AND TIM

As may be evident from the description of the light response above, post-translational mechanisms are critical for the entrainment of the clock to light. Likewise, free-running clock function relies upon regulated post-translational events, even when *per* and *tim* mRNA are expressed with a robust rhythm. PER stability is regulated by phosphorylation carried out largely by a casein kinase I gene called *doubletime* (*dbt*). Mutations in *dbt* result in long or short period or arrhythmia, depending on the specific molecular lesion (Price *et al.* 1998). It is clear that in strong hypomorphic alleles of *dbt* PER levels are constantly high, consistent with the idea that DBT phosphorylates PER and destabilizes it. In the dbt^{s} mutant, PER accumulates more slowly in the nucleus in the early evening phase and is degraded faster in the late night and early morning (Bao *et al.* 2001). A mutation in a serine residue of PER (per^{s}) produced a similar late-night effect as dbt^{s} (Marrus *et al.* 1996). PER is also phosphorylated by casein kinase 2, and mutations in CK2 affect circadian periodicity most likely by affecting the timing of the nuclear entry of PER (Lin *et al.* 2002, 2005; Akten *et al.* 2003).

DBT phosphorylated PER is recognized by protein phosphatase 2A (PP2A). Elevated PP2A activity stabilizes PER and retains it in the nucleus throughout the day, resulting in arrhythmic behavior (Sathyanarayanan *et al.* 2004). Normally, PER phosphorylation displays a robust circadian oscillation (Edery *et al.* 1994). There is no obvious cycling of *dbt* RNA (Kloss *et al.* 1998) and protein (Preuss *et al.* 2004), but the PP2A regulatory subunit, *tws*, is expressed rhythmically, suggesting that cyclic PER phosphorylation and subsequent nuclear localization and degradation may be driven by cyclic phosphatase activity. Alternatively, cyclic expression of TIM may modulate the accessibility of PER to DBT, thereby affecting cyclic PER phosphorylation (Kloss *et al.* 2001). Indeed, PER is unstable, and its rhythmic phosphorylation is abolished in *tim* null mutants (Price *et al.* 1995). However, since there is no functional clock in *tim* null mutants, presumably cyclic *tws* expression is also abolished, as it is in *cyc* mutants (Sathyanarayanan *et al.* 2004); thus these two possibilities to explain rhythmic PER phosphorylation cannot be distinguished.

It is clear that TIM stabilizes PER although the mechanisms are not known. It is possible that TIM binding prevents DBT from phosphorylating PER (Kloss *et al.* 2001); without TIM, PER is hyperphosphorylated by DBT and subsequently degraded (Cyran *et al.* 2005). Alternatively, protein phosphatases may have better access to the TIM-bound PER (Sathyanarayanan *et al.* 2004; Fang *et al.* 2007). In fact, PER is dephosphorylated and stabilized by protein phosphatase 1 (PP1) in a TIM-regulated fashion (Fang *et al.* 2007). Thus, TIM does not affect PP2A action on PER, but it influences the stabilizing effect of PP1.

TIM stability and nuclear entry are likewise regulated by phosphorylation and dephosphorylation. In addition to its role in modulating light-dependent degradation of TIM, SGG also regulates TIM phosphorylation under constant dark conditions. Flies overexpressing SGG have short periods, while *sgg* mutants have long periods. SGG phosphorylation promotes TIM nuclear entry, which may account for the faster clock (Martinek *et al.* 2001).

Presumably, reduced expression of SGG decreases phosphorylation of TIM and delays its nuclear entry, thereby slowing down the clock. Although TIM levels are increased in *sgg* mutants, it seems that TIM degradation is not a direct consequence of SGG phosphorylation because TIM levels are not reduced in SGG overexpressing cells (Martinek *et al.* 2001; Stoleru *et al.* 2007). Since protein phosphatase 1 (PP1) dephosphorylates TIM (Fang *et al.* 2007), one might expect that inhibition of PP1 would produce similar effects on circadian period as SGG overexpression. However, inhibition of PP1 actually lengthens circadian period. Moreover, inhibition of PP1 does not affect the initiation of nuclear translocation although it delays the accumulation of TIM in the nucleus due to an effect on TIM stability. It is possible that SGG and PP1 target different sites and thus regulate different aspects of TIM nuclear entry and stability (Fang *et al.* 2007).

Hyperphosphorylated PER is a substrate for the ubiquitin–proteasome degradation machinery. Slimb, an F-box/WD40-repeat E3 ligase protein, is essential for the degradation of phosphorylated PER and perhaps TIM (Grima *et al.* 2002; Ko *et al.* 2002). In *Slimb* mutants, high levels of hyperphosphorylated PER and TIM are observed under constant dark conditions; in contrast, both PER and TIM continue to oscillate under light:dark conditions. Since PER stability depends upon TIM, the normal cycling of PER levels in *Slimb* mutants under light:dark conditions might be a secondary effect of light-dependent TIM degradation. Thus, light-dependent degradation of TIM does not rely on Slimb. As noted above, another ubiquitin E3 ligase, JET, targets TIM for degradation in response to light (Koh *et al.* 2006).

In the mammalian system also, post-translational regulation of clock proteins plays an important role. PER, CRY, and BMAL1 are phosphorylated by casein kinase 1 and PP1 dephosphorylates PER (Lowrey *et al.* 2000; Lee *et al.* 2001; Akashi *et al.* 2002; Eide *et al.* 2002; Gallego *et al.* 2006). A mutation in CK1ε as well as a mutation in a putative CK1ε phosphorylation site on PER2 have even been implicated in a human circadian disorder, *f*amilial *a*dvanced *s*leep *p*hase *s*yndrome (FASPS) (Toh *et al.* 2001; Xu *et al.* 2005). In addition, similar to Drosophila TIM and PER, CRY is targeted for proteasomal degradation by an E3 ligase F-box protein FBXL3. Loss-of-function alleles of this gene have long circadian periods, consistent with the role of mCRY as a repressor of CLOCK activity (Godinho *et al.* 2007; Siepka *et al.* 2007).

THE *Clk* FEEDBACK LOOP

Interaction of the PER–TIM complex with CLK not only represses CLK–CYC activity, but also brings DBT in close proximity to CLK. Thus CLK is phosphorylated by DBT and apparently dephosphorylated by PP2A (Kim and Edery 2006). Under normal light:dark conditions, *Clk* mRNA levels cycle with a robust circadian rhythm (Bae *et al.* 1998; Darlington *et al.* 1998). However, this robust mRNA cycling does not result in a corresponding cycle of CLK protein abundance: *Clk* mRNA levels change three to fivefold over the course of the day, while CLK protein levels remain constant (Houl *et al.* 2006; Yu *et al.* 2006). It is possible that the turnover of CLK has a rhythm that counters the effect of *Clk* mRNA cycling, although the purpose of such regulation would be difficult to explain. In fact, CLK is regulated in a circadian fashion at the level of phosphorylation, with the peak of phosphorylation occurring in the late night and early morning (Kim and Edery 2006; Yu *et al.* 2006), which is the same phase as the cycling of *Clk* mRNA. Since phosphorylated CLK is turned over by the proteasome degradation pathway, high levels of *Clk* mRNA at these times may allow sufficient CLK protein to be produced, thus keeping total CLK protein levels constant. However, the significance of this constant CLK protein level is unknown. One possibility is that constant CLK protein levels serve to jump-start transcription when repressors are removed, such as when flies are light pulsed in the late night. In response to such a pulse, TIM is degraded, releasing the repression of the PER–TIM complex on the CLK–CYC heterodimer and promoting a new cycle of transcription.

The CLK–CYC heterodimer regulates the expession of another two transcription factors, PAR domain protein 1 (*Pdp1*) and basic leucine zipper (*bZIP*) transcription factor *vrille* (*vri*), both *Pdp1* and *vri* are activated by CLK–CYC, so both have a robust circadian expression pattern. And both proteins feed back to regulate *Clk* expression although in opposing ways (Blau and Young 1999; Cyran *et al.* 2003). PDP1 binds to the *Clk* promoter via an E4BP4-binding site to activate *Clk* transcription while VRI competes with PDP1 for binding to the same site to repress *Clk* transcription. The PDP1 peak lags behind that of VRI, thus enabling sequential repression and activation of *Clk* and giving rise to rhythmic *Clk* mRNA expression (Blau and Young 1999; Cyran *et al.* 2003; Glossop *et al.* 2003) (Figure 1). Other factors may also be involved in regulating *Clk* mRNA cycling because expression of a *Pdp1* RNA interference construct or wild-type *Pdp1* in *tim*-expressing cells does not disrupt cycling of *Clk* mRNA or of VRI (Benito *et al.* 2007). However, the overall significance of *Clk* mRNA cycling and of the feedback loop generated through the mutual regulation of *Clk* and *vri/Pdp1* remains unclear. As noted above, the CLK protein does not cycle. In addition, its overexpression does not affect free-running rhythms, supporting the idea that levels of CLK do not constitute timekeeping cues (Kim *et al.* 2002). Expression of *Clk* under the control of the *per* promoter, which reverses the phase of mRNA expression, also has no significant effect on free-running behavioral rhythms although it affects the morning peak of locomotor activity in the presence of light:dark cycles (Kim *et al.* 2002). We speculate that the *Clk* feedback loop exists primarily to

allow interfaces between the clock and other pathways. For instance, *vri* and *Pdp1* may be regulated by inputs to the clock, and they may also cyclically activate/repress downstream genes. In this scenario, the cycling of *Clk* mRNA would be an epiphenomenon generated through the cyclic activity of VRI and PDP1.

In mammals, *Bmal1* is regulated through a feedback loop similar to the *Clk* loop in Drosophila. The nuclear receptors *Rev-erb* α and *Rora* are expressed cyclically under the control of CLOCK–BMAL1 activity, and they repress and activate *Bmal1* expression respectively. This feedback mechanism maintains robust oscillations of *Bmal1* mRNA (see reviews by Ko and Takahashi 2006; Levi and Schibler 2007).

RELEVANCE OF THE DIFFERENT MOLECULAR OSCILLATIONS IN THE CLOCK

We have just questioned the importance of the *Clk* feedback loop for the essential timekeeping mechanism. Similar concerns may apply to the *Bmal1* loop in mammals, given that a knockout of *Rev-erb*α, which loses *Bmal1* oscillations, is able to maintain basic clock function (Preitner *et al.* 2002). In addition, we pointed out studies that show that all components of the *per–tim* feedback loop, or of the *per–Cry* loop in mammals, need not necessarily cycle. The question, then, is what must cycle to generate a functional clock? While there is, as yet, no definitive answer to this question, it is worth examining the clock mechanism in the simplest organism known to have a clock—cyanobacteria.

Although a feedback loop similar to the one described above exists in cyanobacteria, a rhythm of autophosphorylation of the clock protein KaiC persists without cyclic RNA and protein expression (Tomita *et al.* 2005). Remarkably, cyclic phosphorylation of KaiC can be reconstituted in a test tube by incubating it with ATP and two other clock proteins, KaiA and KaiB (Nakajima *et al.* 2005). Thus the transcription–translation feedback loop is not necessary for this circadian clock. However, this clock drives rhythmic transcription of much of the cyanobacteria genome, perhaps in response to a cellular metabolism zeitgeber (Lakin-Thomas 2006; Woelfle and Johnson 2006). Interestingly, metabolic cues can also affect circadian clocks in other organisms. The redox state modulates mammalian CLOCK activity by regulating its DNA-binding efficiency (Rutter *et al.* 2001). Recently, we showed that oxidative stress affects the molecular circadian clock in Drosophila. Mutations in a FOXO transcription factor increase the sensitivity of the Drosophila clock to oxidative stress and result in degeneration of circadian rhythms (Zheng *et al.* 2007).

RHYTHMIC CLK–CYC ACTIVITY MAY BE ESSENTIAL FOR A FUNCTIONAL CLOCK

While it is tempting to speculate that circadian clocks in eukaryotic organisms are generated through mechanisms similar to those in cyanobacteria, this is not likely to be the case. It may be possible to dispense with rhythmic transcription for some genes, but we predict that some clock mRNAs continue to cycle. In the experiments described earlier where *per* and *tim* mRNA were kept constant (Yang and Sehgal 2001), mRNA levels of the PP2A regulatory subunit, *tws*, were probably still cycling and may have been sufficient to drive the rhythmic phosphorylation and thereby the cycling of PER. Cycling PER, in turn, would have rhythmically regulated activity of CLK–CYC. In the mammalian cell culture experiment where rhythms persisted despite constant levels of mCRY (Fan *et al.* 2007), some genes relevant to post-translational control of mCRY may have been expressed rhythmically. For example, mPER serves as a scaffold to mediate CKI ε phosphorylation of mCRY (Eide *et al.* 2002). This may have been sufficient for mCRY to rhythmically repress CLOCK–BMAL1. Experiments in the mammalian system have, in fact, demonstrated that repression of CLOCK–BMAL1 activity is essential for clock function (Sato *et al.* 2006).

As noted above, although levels of Drosophila CLK are constant throughout the day, there are robust daily oscillations of its phosphorylation (Houl *et al.* 2006; Yu *et al.* 2006). In addition, phosphorylation of CLK appears to directly affect its transcriptional activity (Kim and Edery 2006; Yu *et al.* 2006) [Likewise, transcriptional activity of mammalian BMAL1 and the cyanobacterial clock protein KaiC is regulated by phosphorylation (Eide *et al.* 2002; Nishiwaki *et al.* 2004; Xu *et al.* 2004).] Since the phosphorylation of CLK is PER dependent (Kim and Edery 2006; Yu *et al.* 2006), oscillations of PER could confer rhythmic regulation of CLK activity. Thus it seems that an oscillation of PER is a prerequisite for a functional clock. This oscillation has several components: cyclic PER protein expression and phosphorylation (Edery *et al.* 1994), rhythmic nuclear localization (Vosshall *et al.* 1994; Price *et al.* 1998), and subsequent binding to CLK–CYC (Lee *et al.* 1999) (Figure 2).

Overexpression of *per* or *tim* in the central clock cells abolishes protein cycling and results in arrhythmic behavior in many flies (Kaneko *et al.* 2000; Blanchardon *et al.* 2001; Yang and Sehgal 2001). The arrhythmicity may be due to increased levels of PER or TIM *per se* or due to the loss of rhythmic protein phosphorylation or due to a disruption in cyclic nuclear entry (these mechanisms are not mutually exclusive). Regardless of the precise mechanism, rhythmic repression of CLK–CYC by the PER–TIM complex, which involves PER-dependent phosphorylation of CLK by DBT, would be disrupted. This would lead to noncyclic expression of clock-controlled downstream target genes. On the basis of the mammalian study that indicates that the cycling of CRY is not essential, we predict that it is not the loss of PER or TIM cycling *per se* that causes the arrhythmia, but rather the loss of cyclic nuclear entry, which may, in turn, be regulated by phosphorylation. We propose that

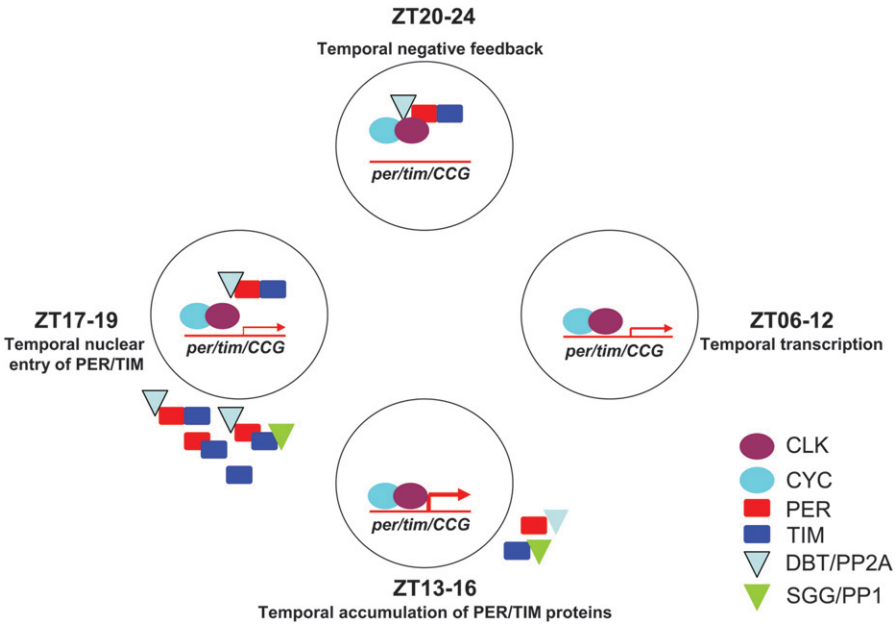

Figure 2.—Model of the Drosophila circadian clock depicting the importance of post-translational modifications. Clock genes such as *per* and *tim* and other clock-controlled genes (CCGs) are activated by CLK–CYC during the day, and their transcription peaks in the early night. The PER–TIM complex forms during the second half of the night and translocates into the nucleus to repress CLK–CYC activity. A balance of kinase and phosphatase activity regulates the stability of PER, TIM, and CLK and most likely the nuclear entry of PER and TIM. Casein kinases DBT and CKII phosphorylate PER and the glycogen synthesis kinase SGG phosphorylates TIM. PP2A and PP1 dephosphorylate both PER and TIM. For the sake of simplicity, each is shown here acting only on the primary target (PP2A on PER and PP1 on TIM). According to this model, critical steps of the timekeeping process are controlled by post-translational modifications of key clock proteins. Note that nuclear expression of SGG and PP1 has not been experimentally determined.

the critical function of PER–TIM in the clock is to rhythmically enter the nucleus and repress CLK–CYC, perhaps by providing DBT kinase activity.

Having argued that rhythmic activity of clock proteins, rather than rhythmic levels, is key to the timekeeping process, some aspects of clock function may require alterations in protein levels. For instance, in all organisms examined, light alters the levels of a clock component. Thus, it seems that the initial event in resetting a clock, or perhaps even in initiating a clock, is a change in the levels of a clock protein. In this context, it is interesting that ectopic expression of *Clk* is sufficient to generate molecular cycling of *tim* and *cry* under light:dark conditions (Zhao *et al.* 2003). It appears that CLK is able to activate and orchestrate the oscillation of necessary components when ectopically expressed. How this cycling is initiated is not clear. Since it is possible that light-driven TIM degradation jump-starts the feedback loop in nonclock cells, it would be interesting to see if ectopic CLK expression can start an oscillator in constant darkness.

Despite this emphasis on CLK, it is important to note that the transcriptional heterodimer at the center of the timekeeping mechanism does not have to include CLK in particular. Indeed, mice with a deficiency of *Clock* have functional clocks (Debruyne *et al.* 2006). Most mammalian circadian phenotypes are based on a dominant negative allele of *Clk*, which produces phenotypes more severe than those produced by a *Clk* deficiency, perhaps because dominant negative CLK interferes with BMAL1 binding to another partner. Indeed, BMAL1 can partner with the mCLK paralog NPAS2 that functions in the basal forebrain and other tissues (Reick *et al.* 2001). Recent findings demonstrate that CLK and NPAS2 act redundantly in the master pacemaker, the suprachiasmatic nucleus (SCN) (Debruyne *et al.* 2007a,b). In contrast, mCLK is necessary for circadian clock function in some peripheral tissues (Kennaway *et al.* 2006; Debruyne *et al.* 2007b).

Finally, it is important to note that while rhythmic transcription of some clock genes can be experimentally dispensed with, this is not to say that it is without function. Rhythms are less robust and penetrant, and periods are less precise when these genes are expressed noncyclically. Moreover, flies that express *per* and *tim* constitutively show defects in their response to pulses of light (Yang and Sehgal 2001). Overexpression of *mPer1* also impairs normal entrainment and molecular oscillations in mammals (Numano *et al.* 2006), supporting the idea that these responses depend upon cycling RNA. In further support of a role for transcription, new transcription factors continue to be identified as part of the clock mechanism. A bHLH ORANGE family protein CLOCKWORK ORANGE (CWO) is one such recently identified factor. *Cwo* is activated by CLK–CYC through the E-box in its promoter, and it also feeds back to synergize with PER to repress CLK–CYC activity. These feedback loops thus are able to amplify the oscillation and maintain a robust 24-hr cycle (Kadener *et al.* 2007; Lim *et al.* 2007; Matsumoto *et al.* 2007).

In summary, we are learning that the mechanism of the clock is much more intricate than previously thought. There are likely multiple feedback loops that lie at the heart of the clock, and some aspects of clock

function may be maintained through redundant mechanisms. While post-translational regulation is clearly critical to maintaining a clock, we believe it is unlikely that eukaryotic clocks will turn out to be entirely free of transcriptional control as is the cyanobacteria clock. Thus, while a subset of clock mRNAs, and even clock proteins, may be held at constant levels without complete loss of clock function, others are likely cycling under these conditions. In addition, even the dispensable oscillations probably serve functions that may sometimes be too subtle to detect.

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