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[REVIEW]

Molecular Cogs of the Insect Circadian Clock

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ABSTRACT—During the last five years, enormous progress has been made in understanding the molecular basis of circadian systems, mainly by molecular genetic studies using the mouse and fly. Extensive evidence has revealed that the core clock machinery involves “clock genes” and “clock proteins” functioning as molecular cogs. These participate in transcriptional/translational feedback loops and many homologous clock-components in the fruit fly *Drosophila* are also expressed in mammalian clock tissues with circadian rhythms. Thus, the mechanisms of the central clock seem to be conserved across animal kingdom. However, some recent studies imply that the present widely accepted molecular models of circadian clocks may not always be supported by the experimental evidence.

Key words: circadian rhythm, clock genes, clock proteins, transcriptional/translational feedback loops

INTRODUCTION

The physiological properties of most organisms, from cyanobacteria to human, display a circadian (Latin *circa dies*, or ‘about a day’) pattern of activity, which is regulated by an endogenous circadian clock. While rhythms controlled by circadian clocks are self-sustained and persist robustly in constant darkness and temperature with a period close to 24 hr, they are entrained by environmental time cues (Zeitgebers) such as light, temperature, and feeding (Soriano, 1981; Rusak *et al.,* 1993). The circadian clock allows organisms to anticipate periodic changes in environmental circumstance and to change their physiological status accordingly. The internal system that maintains circadian rhythms can be formally represented by three different components: namely, an input pathway, the pacemaker itself, and an output pathway (Dunlap, 1999). The input pathway transmits light and/or thermal, non-photic information from external stimuli to the clock (pacemaker). The pacemaker itself, which comprises the mechanism of the endogenous oscillator, is the source of circadian temporal modulation. The output pathway conveys rhythmic information from the central oscillator for eliciting the daily changes in behavioral activity and other physiological processes.

The molecular genetic study of circadian clocks was initiated in the fruit fly *Drosophila melanogaster*. When held in a light:dark (LD) 12:12 cycle, wild-type *Drosophila* individuals show a circadian pattern in their locomotor and eclosion behavior. Extensive evidence indicates that the circadian control of both behaviors depends upon several so-called “clock genes” and “clock proteins.” Many of the clock genes identified in *Drosophila* are also expressed in mammals as well as in other insects. In *Drosophila*, certain cell clusters in the brain, the lateral neurons (LNs) and dorsal neurons (DNs), express clock genes such as *period* (*per*) and *timeless* (*tim*) (Liu *et al.,* 1992; Saez and Young, 1996) (Table 1). In particular, the LNs are suggested to be the major site of circadian pacemakers that control daily locomotor activity and pupal eclosion rhythms. In adult flies, the ventral group of LNs (LNvs) have been subdivided into two subgroups of typically five small LNvs (s-LNvs) and four large LNvs (l-LNvs) projecting to the dorsal protocerebrum and to the medulla neuropile of the optic lobe, respectively (Stanewsky *et al.,* 1997; Kaneko and Hall, 2000). The l-LNvs also project to the LNvs and the medulla of the contralateral side. Both groups of LNvs express the *pigment-dispersing factor* (*pdf*) gene, which is often used to reveal the projection pattern of

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Table 1. Comparison of properties and functions of *Drosophila* and mouse clock proteins.

***Drosophila* Proteins**	**Properties and Funtions**	**Mouse Proteins**	**Properties and Funtions**
PERIOD (PER)	PAS protein. No DNA binding domain. Interacts with TIM. Inhibits CLK:CYC activity.	mPERIOD1, 2, 3 (mPER1, 2, 3)	PAS protein. Interacts with mCRY and mPER proteins and translocates into nucleus.
TIMELESS (TIM)	No DNA binding domain. Interacts with PER. Contains ALMADILLO-like domains. Inhibits CLK:CYC activity. Rapidly degrades in response to light.	mTIMELESS	Possibly has no clock role. Functions in development and/or differentiation system. Null mutation is embrionic lethal. Closest *Drosophila* relative is TIMEOUT protein.
CLOCK (CLK)	bHLH-PAS transcription factor. Interacts with CYC. Promotes transcription of *per* and *tim* via E-box. Inhibits transcription of its own gene.	mCLOCK (mCLK)	bHLH-PAS transcription factor. Interacts with BMAL1. Promotes transcription of *mPer* and *Cry* via E-box.
CYCLE (CYC)	bHLH-PAS transcription factor. Interacts with CLK and promotes transcription from E-box. Constitutively expresses.	BMAL1	bHLH-PAS transcription factor. Molecular relative of *Drosophila* CYC. Circadian mRNA expression. Heterodimerizes with mCLK and promotes *mPer* and *Cry*.
DOUBLE-TIME (DBT)	Phosphorylates PER and promotes its degradation. Translocates into nucleus together with PER.	Casein Kinase Iε (CKIε)	Phosphorylates mPER, and affects mPER stability and its intracellular localization.
CRYPTOCHROME (CRY)	Flavin binding protein. Circadian photoreceptor. Promotes light-dependent TIM degradation through light- dependent interaction with TIM.	CRYPTO-CHROME 1, 2 (CRY1, 2)	Core clock component in mouse. Binds to mPER and stabilizes it. Functions as mPER nuclear translocator. Inhibits *mPer* and *Cry*.
VRILLE (VRI)	bZIP transcription factor without PAR domain. Represses *Clk* transcription.	E4BP4	bZIP transcription factor without PAR domain. Represses *mPer1* transcription through DBP response element.
PAR domain-protein 1 (PDP1)	bZIP-PAR transcription factor. Activates *Clk* transcription.	DBP	bZIP-PAR transcription factor. Promotes *mPer1* transcription through DBPresponse element.
SHAGGY (SGG)	Phosphorylates TIM. Facilitates nuclear localization of PER:TIM.	Glycogen Synthase Kinase 3β (GSK3β)	Molecular relative of *Drosophila* SGG, but its clock function has not been elucidated.

LNvs (Kaneko and Hall, 2000). The *pdf* gene encodes the neuropeptide PDF, which is thought to function as a molecule signaling the clock's output to target tissues (see below).

Negative Pacemaker Components

The first identified genetic component of the clock was the *period* (*per*) gene discovered by Konopka and Benzer (1971). They systemically screened mutagen-exposed *Drosophila* for circadian clock mutants showing altered temporal pupal eclosion patterns in constant conditions (constant darkness, DD). Konopka's mutant strains had different circadian eclosion phenotypes showing abnormally long (per^L) or short (per^S) rhythms and some were arrhythmic (per^0) but all mapped to the single genetic locus *per*. Not only eclosion rhythms were affected by these mutations, but the locomotor activity rhythms were also altered in a similar way. In the mid-1980s, the *per* gene was cloned and shown to encode a protein, PERIOD (PER), which contains the PAS domain (Fig. 1). PAS is an acronym for the first three proteins found to share this domain: PER, the product SIM of the *Drosophila* gene *single-minded*, and a mammalian protein ARNT (aryl hydrocarbon receptor nuclear translocator) (Crews *et al.*, 1988). The PAS domain has since been known to be an important signaling module for protein-protein interaction, and for monitoring a number of environmental changes such as light, oxygen, and redox potential.

Both *per* mRNA and PER protein levels exhibit rhythmic abundance, reflecting the corresponding behavioral rhythm: the normal circadian molecular rhythm in wild-type flies, the short or long period rhythm in per^S or per^L mutants, and the lack of any rhythm in per^0 flies (Hardin *et al.,* 1990). Characterization of these mutant flies using genetic mosaic and transgenic approaches revealed that both the pupal eclosion and locomotor activity rhythms are dominantly controlled by only a small number of *per*-expressing LNs in fly brain (Zerr *et al.,* 1990; Liu *et al.,* 1992). The successful identification of *per* prompted subsequent genetic and biochemical screens to identify additional components of the molecular clock.

The second fly clock gene cloned was *timeless* (*tim*) (Sehgal *et al.,* 1994). tim^0 flies are arrhythmic, and *tim* mRNA and levels of its gene product TIMELESS (TIM) protein are almost coincident with those of *per* and PER, respectively (Hunter-Ensor *et al.,* 1996; Myers *et al.,* 1996). More importantly, tim^0 mutants exhibit an elevated level of cytoplasmic PER protein in the fly head, and TIM protein

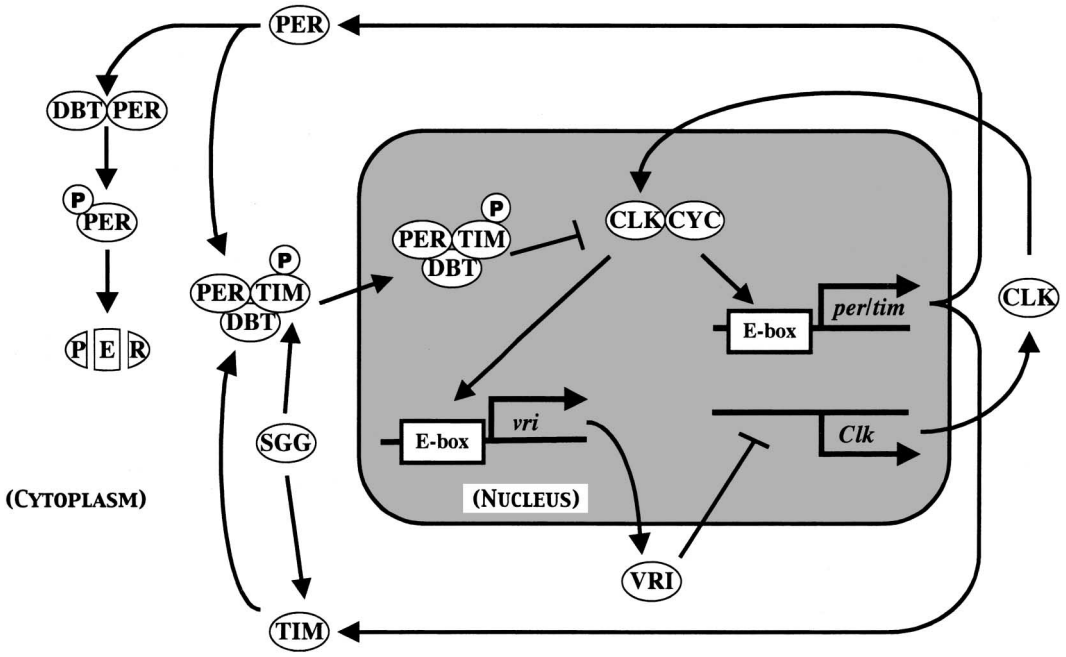

Fig. 1. Molecular interrelationship of clock proteins and clock genes in the circadian feedback loops in *Drosophila*. Gray box zone shows the nucleus and its outside the cytoplasm. Transcriptions of *per* and *tim* genes are activated by a heterodimer of bHLH-PAS containing transcription factors CLK and CYC. Phosphorylation of PER is dependent upon DBT, and phosphorylated PER is directed to degradation. Degraded PER is shown in hashed boxes. Cytoplasmic TIM heterodimerizes with PER to stabilize. Both PER and TIM enter the nucleus, possibly with DBT. SGG phosphorylates TIM, and facilitates nuclear translocation of TIM. Nuclear PER and TIM repress the CLK:CYC activity. When PER and TIM levels fall, repression is relieved to initiate a new cycle of transcription from the E-box. While *Clk* gene transcription is positively regulated by PER and TIM likely indirectly, it is negatively regulated by VRI transcription factor.

was also isolated as an interaction partner of PER using a yeast two-hybrid assay (Vosshall *et al.,* 1994; Gekakis *et al*., 1995). In wild-type flies, the spatial expression pattern of TIM is very similar to that of PER, and PER and TIM accumulate in nuclei of clock neurons in a circadian manner (Saez and Young, 1996). Thus, the circadian molecular clock is proposed to be composed of a negative feedback loop mechanism: PER and TIM heterodimerize and translocate to the nucleus, and the resulting nuclear PER:TIM heterodimer inhibits its own transcription (Fig. 2). PAS domains have been shown to be dimerization surfaces, found in proteins that contain a basic helix-loop-helix (bHLH) DNA-binding domain and that often function as heterodimeric partners. This suggested that PER could function as a transcriptional repressor of a bHLH-PAS heterodimeric pair, because PER lacks the bHLH domain (Huang *et al.,* 1993). The TIM sequence is unique and contains neither a known DNA-binding domain nor a PAS domain. Instead, TIM has three ARMADILLO-like domains (ALD) (Kyriacou and Hastings, 2001; Stanewsky, 2002). The ALD has been shown to participate in protein-protein interactions in some cell-adhesion- and cytoskeleton-associated proteins (Hatzfeld, 1999). Two of the TIM ALDs overlap with the region that can bind to PER (Kyriacou and Hastings, 2001; Saez and Young, 1996).

Positive Pacemaker Components

A behavioral screen in the mouse produced a semi-dominant long-period mutant called *Clock* (*mClk*) (Vitaterna *et al.,* 1994). Homozygotes generate either long-period locomotor activity rhythms or are arrhythmic. In 1997, positional cloning and sequencing analysis revealed that *mClk* gene product, mCLOCK (mCLK), is a bHLH-PAS-containing transcription factor (King *et al.,* 1997). Subsequent molecular genetic studies clarified that mCLK interacts directly with another bHLH-PAS family protein, BMAL1 (brain and muscle ARNT like protein 1, MOP3) (Gekakis *et al.,* 1998; Takahata *et al.,* 1998). Disruption of *BMAL1* gene causes an immediate loss of locomotor activity rhythms in mice. Furthermore, the mCLK:BMAL1 heterodimer was shown to bind to the specific DNA element E-box having a nucleotide sequence CACGTG found in the 5' flanking region of the mouse *period* homolog (*mPer1*), and this heterodimer enhances transcription of *mPer1* (Takahata *et al.,* 1998). Homology cloning approaches identified *Clock* (*Clk*) and *cycle* (*cyc*), as *Drosophila* counterparts to the mouse's clock genes *mClk* and *BMAL1,* respectively (Allada *et al.,* 1998; Darlington *et al.,* 1998). These two genes encode proteins containing a bHLH-PAS domain, and the gene products, CLOCK (CLK) and CYCLE (CYC) can dimerize and bind directly to the E-box enhancers present in the *per* and *tim* promoters, as well as stimulate transcription of both the *per* and *tim* genes, respectively. Moreover, it was found that if PER and TIM proteins are both produced in cultured *Drosophila* cells and allowed to undergo nuclear localization, transcriptional activation through the E-box by CLK:CYC dimer

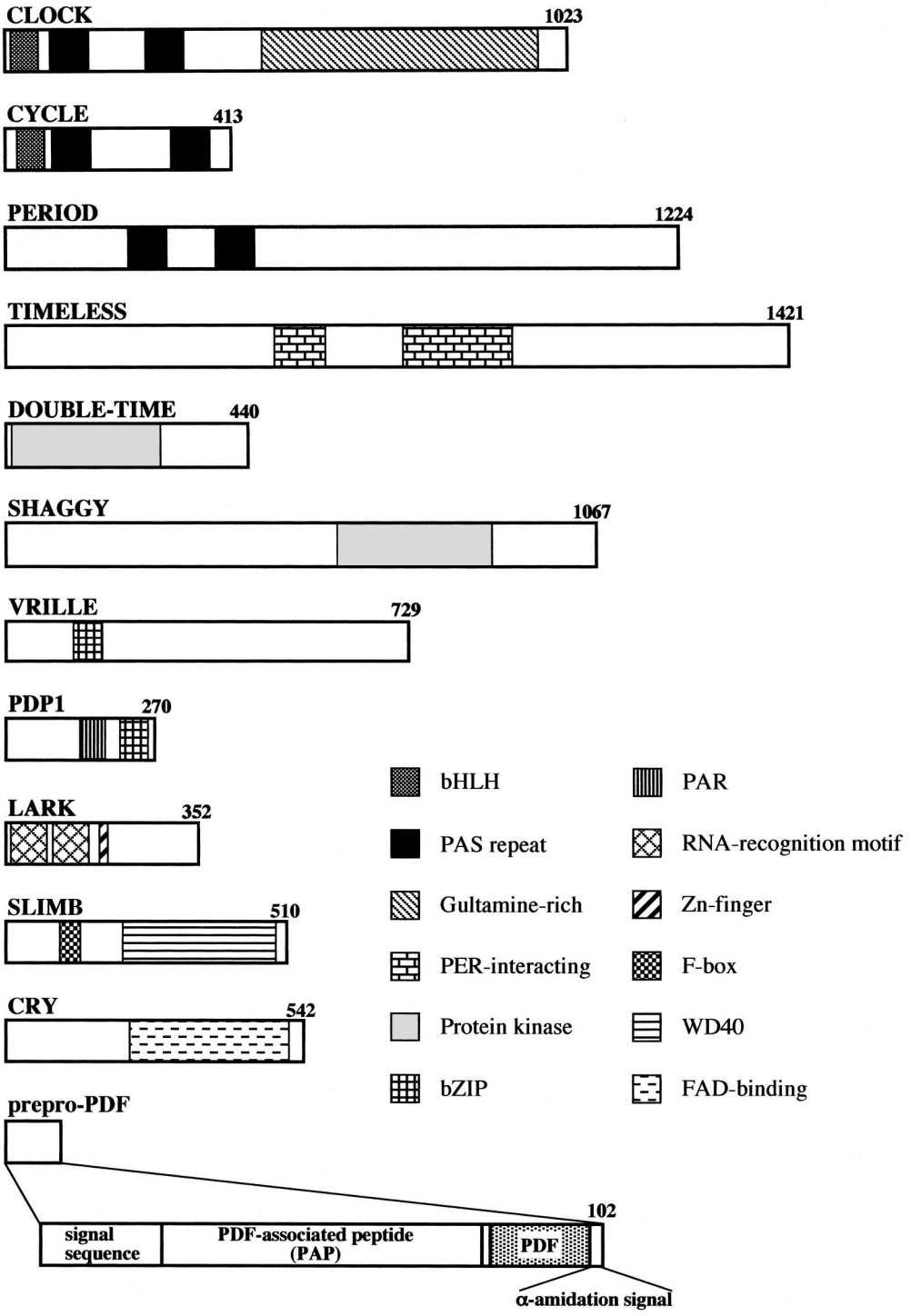

Fig. 2. Schematic rod illustration of clock proteins and clock-relevant proteins in *Drosophila*. Domains are shown in patterned decorative boxes, each of which is explained in the right-hand corner. Core clock proteins CLK, CYC, and PER are PAS domain-containing proteins. DBT and SGG are protein kinases which phospholyrate clock proteins and regulate their functions, localizations, and/or stabilities. VRI and PDP1 are a member of bZIP transcription factors, which participate in the interlocked feedback loop of *Drosophila* circadian clock. RNA binding protein LARK might modulate stabilities of mRNAs coding some clock-output proteins. SLIMB is a F-box protein and functions in the degradation pathway of PER and TIM. Fravoprotein CRY is a circadian photoreceptor in the *Drosophila* clock cells. PDF peptide plays an important role in the output system controlling circadian behavioral activities. Numbers on the rod indicate the total residue number of amino acids of respective proteins.

is remarkably suppressed (Darlington *et al.,* 1998). Thus, a transcription/translation autoregulatory feedback loop has been defined as a driving force for the clock's molecular mechanism. The mammalian circadian clock seems to be quite similar to this fly clock. The bHLH-PAS dimeric pair CLK:CYC resides in the nucleus where it occupies the E-box elements in the *per* and *tim* genes, positively regulating their transcription. PER and TIM protein levels continue to rise throughout the day to their peak levels in the early evening, a few hr after the peak level of *per* and *tim* mRNAs. The two proteins heterodimerize and translocate into the nucleus where they inhibit the transcriptional activity of the CLK:CYC dimer, thus repressing their own transcription. As both PER and TIM proteins are degraded before dawn, this process is relieved, lifting repression of the CLK:CYC dimer, and thereby starting another cycle of PER and TIM accumulation.

It has been demonstrated that PER/TIM's transcriptional inhibition of their own genes is due to their direct interaction with the CLK:CYC complex. This physical interaction prevents E-box-binding of CLK:CYC but does not disrupt the association between CLK and CYC (Lee *et al.,* 1999). This might be brought about by an allosterically induced conformational change in the CLK:CYC heterodimer or by a masking of the DNA-binding region in the CLK:CYC heterodimer, leading to a reduction in DNA-binding activity. PER and TIM could be in the CLK:CYC complex together, although the stoichiometry and their modes of interaction have not yet been elucidated.

Other Components for Posttranscriptional Regulation

Molecular genetic studies in *Drosophila* identify some additional circadian components. These circadian regulatory molecules act to refine the transcriptional/ translational feedback loops. Light changes the levels of these additional clock components, resetting the clock to different time of day. In *Drosophila*, the entrainment of the circadian clock relies on the degradation of TIM in response to light (Zeng *et al.,* 1996). CRYPTOCHROME (CRY) is the major mediator for the rapid light-induced degradation of TIM (Ceriani *et al.,* 1999), which has been demonstrated to be mediated by a ubiquitin-proteasome pathway (Naidoo *et al.,* 1999).

The *double-time* (*dbt*) gene encodes a *Drosophila* homolog of the mammalian casein kinase Iε (Kloss *et al.,* 1998). Phosphorylated forms of PER normally appear after PER protein levels reach a peak and begin to decline. In null mutant flies of *dbt* gene (dbt^P), even though *per* mRNA levels are normal, hypophosphorylated PER accumulates at higher than normal levels throughout the day, indicating that the effects on the protein are post-transcriptional (Price *et al.,* 1998). In fact, it has been demonstrated that DBT protein physically associates with both PER and the PER:TIM heterodimer, and phosphorylates PER (Kloss *et al.,* 2001). In late subjective day when monomeric PER is synthesized in the cytoplasm, DBT binds to PER and promotes its phosphorylation, leading to the degradation of PER and subsequent accumulation of TIM. High concentrations of TIM promote a formation of the stable complex of PER:TIM:DBT, that can enter the nucleus during early subjective night. Over an 8–10 hr period, the nuclear PER:TIM:DBT complex is converted to the PER:DBT complex, and concomitantly progressive repression of *per* and *tim* transcription results in a decreased accumulation of the PER:TIM:DBT complex. Nuclear DBT may progressively phosphorylate PER, leading to its nuclear (Kloss *et al.,* 2001).

The *syaggy* (*sgg*) gene encodes another kinase SGG that modulates a further circadian clock component. SGG is a homolog of glycogen synthase kinase-3β, and promotes TIM phosphorylation, which regulates the timing of nuclear entry of the PER:TIM complex (Martinek *et al.,* 2001). Recent studies reveal that *Drosophila* casein kinase 2β (CK2β) also plays an important role in the molecular clock (Akten *et al*., 2003). In the *Andante* fly, which is a mutant of the *casein kinase 2β* gene, PER and TIM accumulate to abnormally high levels and the nuclear translocation of PER and TIM is significantly delayed. This suggests that CK2β promotes nuclear entry of clock proteins by direct phosphorylation of clock proteins or by activation of a secondary kinase such as DBT and SGG.

Drosophila Slimb protein is a member of the F-box/WD40-repeat protein family of the SCF (Skp1/Cullin/F-box protein) ubiquitin E3 ligase complex that targets phosphorylated proteins for degradation. Recent research reveals that Slimb is an essential component of the circadian clock (Grima *et al.,* 2002; Ko *et al.,* 2002). Flies mutant for *slimb* are behaviorally arrhythmic, and can be rescued by targeted expression of wild-type Slimb in their clock cells. In head extracts from *slimb* mutants, highly phosphorylated PER and TIM are constitutively present throughout the day. Slimb interacts preferentially with DBT-mediated phosphorylated PER and targets it for rapid degradation by 26S proteasome. Slimb-dependent periodic degradation of PER and/or TIM is thought to be crucial for the maintenance of normal circadian rhythmicity.

Interlocked Feedback Loops

Several lines of evidence have revealed that PER and TIM can stimulate transcription of the *Clk* gene and that CLK:CYC heterodimers repress *Clk* transcription, forming interlocked feedback loops (Bae, *et al.,* 1998; Glossop *et al.,* 1999). These positive and negative aspects of clock proteins are important for the high amplitude modulation of circadian oscillation and for fine-tuning of the circadian clock. Recent studies reveal that VRILLE (VRI), the product of the *vrille* gene encoding a basic leucine zipper (bZIP) transcription factor, bridges between the interlocked feedback loops of the *Drosophila* circadian clock (Cyran *et al.,* 2003; Glossop *et al.,* 2003). VRI was first described as a transcription factor essential for signaling in the *decapentaplegic* pathway during *Drosophila* embryogenesis (George and Terracol, 1997). Differential-display screenings for clock-controlled transcripts identified *vri* as an important circadian compo-

nent (Blau and Young, 1999). Analysis of the promoter sequence revealed that *vri* contains an E-box, and that expression of *vri* is indeed driven by CLK:CYC. The *vri* gene expresses in the LNs, and its mRNA cycles in phase with those of *per* and *tim*. Overexpression of *vri* in clock cells by means of the GAL4-UAS system not only results in a longer period of or arrhythmia in locomotor activity, but also affects severely the expression of PER and TIM (Blau and Young, 1999). Glossop *et al*. (2003) and Cyran *et al*. (2003) independently reported that VRI directly regulates *Clk* transcription by specific binding to *Clk* promotor elements and functions as a negative component of the *Clk* feedback loop. In this context, the positive regulation of *Clk* by PER/TIM occurs indirectly, by preventing CLK:CYC function from activating *vri*. Such an interconnected feedback mechanism is also found in the mammalian circadian clock. In mice, the orphan nuclear receptor REV-ERBα plays an analogous role to VRI, repressing *BMAL1* expression by binding its response element within the *BMAL1* promotor.

Clock Output

In addition to the effect on the central clock's components, VRI affects the output system through a peptide named PDF (Blau and Young, 1999). PDF is an insect homolog of pigment-dispersing hormone, PDH, which was isolated through its ability to disperse pigment granules in the visual system of crustaceans, where it was first discovered (Rao and Riehm, 1993). The *pdf* gene of *D. melanogaster* encodes 102-amino acids and this precursor protein (prepro-PDF) consists of a signal peptide and a PDF-associated peptide (PAP) followed by the mature 18-amino acid PDF peptide with α-amidation signal (Park and Hall, 1998). The primary structure of *Drosophila* PDF is highly conserved among other members of the PDH/PDF family. In *Drosophila*, the spatial expression pattern of PDF is restricted largely to the LNs (Kaneko and Hall, 2000), and in insects, a number of pieces of evidence have revealed that PDF is a possible key output signal from the circadian clock. For example, microinjecting PDF into the cockroach optic lobe shifts the phase of its circadian rhythmicity (Petri and Stengl, 1997). PDF injection into the housefly's optic lobe enlarges the axons of monopolar cells in the first neuropile, or lamina, which normally change their caliber dynamically with a circadian rhythm (Pyza and Meinertzhagen, 1996). This suggests that daytime release of PDF might regulate the diurnal rhythmic size changes of these neurons *in vivo*. Furthermore, genetic studies on the *pdf* gene of *Drosophila* strongly suggest that PDF functions as a circadian transmitter.

From analysis of the null mutation for *pdf* (pdf^{01}), Renn *et al*. found that, although flies lacking PDF are well entraining under LD conditions, the evening peak in locomotor activity occurs about 1 hr earlier than that in wild-type flies (Renn *et al.,* 1999). The mutants did not anticipate the light-on signal as well either. After 2–3 days in constant dark conditions, many pdf^{01} mutants became arrhythmic. In pdf^{01} flies, LNvs are normal in number and morphology, and the mutant phenotypes can be rescued with exogenously introducing wild-type *pdf*. Although there is no circadian rhythm in the abundance of *pdf* mRNA in wild-type flies, mutations in the *Clk* or *cyc* genes reduced *pdf* expression and PDF-immunolabeling in the l-LNvs (Blau and Young, 1999). Disruption of *vri* oscillations also results in reduced PDF-immunoreactivity, but the abundance of *pdf* mRNA is not affected (Blau and Young, 1999). Notably, in wild-type flies, PDF peptide levels were found to be constant in the somata of LNvs, but to cycle at their axon terminals, indicating the rhythmic PDF release (Park *et al.,* 2000). In null mutants of *per* and *tim* genes, *pdf* mRNA levels are normal, although the circadian accumulation of PDF is eliminated. These results suggest that periodic release of PDF is important for circadian behavioral rhythmicity.

Ectopic expression of PDF in the vicinity of the ordinary targets of LNvs generates behavioral arrhythmia, but in more remote locations it does not (Helfrich-Förster *et al.,* 2000). This indicates that when PDF is released it acts locally as a signal rather than systemically. This was further supported in a recent study by Williams *et al.* (2001) on the *neurofibromatosis-1* (*Nf1*) gene. That study revealed that *Nf1* null mutations generate abnormalities in circadian locomotor activity and enhancement of activity in mitogen-activated kinase (MAPK). In wild-type flies, phospho-MAPK levels are higher during the night, although there is no significant difference in the *pdf* mutants. Immunocytochemical labeling has revealed a circadian oscillation of activated MAPK in the vicinity of nerve terminals of LNvs, suggesting a functional coupling of PDF to Ras/MAPK signaling (Williams *et al.,* 2001). These studies strongly suggest that the PDF peptide functions as neurotransmitter and exert its activity via a post- and/or pre-synaptic membrane receptor.

Although evidence supporting the possibility that PDF is a pacemaker peptide has also been accumulating, the entity with which PDF interacts, namely the PDF receptor, has yet to be characterized. Interestingly, Chuman *et al*. (2002) demonstrated that prepro-PDF in the cricket *Gryllus bimaculatus* contains a putative nuclear localization signal (NLS) instead of an obvious signal sequence for entry into a secretory pathway. Moreover, immuno-cytochemical analyses have revealed that *Gryllus* PDF can localize to the nucleus in a certain group of neurons (Chuman *et al.,* 2002). Growing evidence has revealed that neuropeptides and neurohormones such as angiotensin II (Ang II), dynorphin B, and fibroblast growth factor (FGF) can all function in the nucleus as intracrine molecules (Bouche *et al*., 1987; Ventura *et al*., 1998; Cook *et al*., 2001). In addition, microinjection of PDF to cockroach produces time-dependent phase shifts of locomotor activity rhythm, indicating that a transient change in PDF level could cause a stable change in molecular components of circadian clock as described above (Petri and Stengle, 1997). Considering these results and the fact that under some circumstances PDF affects components of the master clock, the site of action for PDF may be in the nucleus as well as at a membrane surface receptor.

Another important output gene is *lark*. The *lark* gene was identified in a mutagenesis screen for genes affecting the circadian eclosion pattern of *Drosophila* (Newby and Jackson, 1993). The circadian function of *lark* appears to be specific for eclosion, since *lark* mutants have no significant effect on locomotor rhythms. Molecular analysis has shown that the LARK protein is an RNA-binding protein containing RNA recognition motifs and a retroviral-type zinc finger, suggesting that LARK plays a role in regulating protein translation (Newby and Jackson, 1996; McNeil *et al*., 1999). LARK abundance changes in a circadian manner in *Drosophila* neurons expressing crustacean cardioactive peptide (CCAP), which is a neuropeptide required to initiate eclosion in several different insects (Zhang *et al*., 2000). Since *lark* *m*RNA does not cycle and LARK protein rhythmicity is abolished in the per^0 mutant, the rhythm in LARK is likely to be regulated by a circadian post-transcriptional mechanism (McNeil *et al*., 1998). The target of the LARK protein is not known yet, but *ccap* mRNA might be a potential target, in regard to its clock function.

Current Issues

The temporal expression pattern of clock genes and proteins and their interactions have been examined mainly based on the experiments that were carried out by using extracts from whole fly heads or expression systems with cultured cells. It should therefore be noted that the results obtained or the conclusions deduced from such studies may not necessarily reflect the processes *in vivo* in the clock neurons.

Shafer *et al*. (2002) have constructed a detailed time course of PER and TIM expression and their subcellular localization patterns in the LNvs by immunocytochemical analysis using a panel of antisera against PER and TIM. Surprisingly, they reveal that PER and TIM have different profiles of nuclear accumulation in the LNvs. PER accumulates in the nucleus during the early night, whereas TIM is restricted to the cytoplasm of the LNvs through much of that time. Moreover, l-LNvs and s-LNvs have a similar timing of PER nuclear localization, but differed by about 3 hr in the nuclear accumulation of TIM. These features are not predicted by the present model of the circadian molecular clock in *Drosophila* and are contradictory to the concept that PER and TIM function as an obligate heterodimer in their nuclear transport as well as in the suppression of CLK/CYC-dependent transcription. The result that PER appears in nuclei at least 3 hr prior to TIM suggests that most nuclear PER is not complexed with TIM at that time, but it does not preclude a catalytic role for TIM in PER nuclear entry. This could explain the apparent absence of nuclear TIM during the middle of the night, and that PER and TIM translocate into the nucleus as their PER/TIM heterodimer, and that subsequently TIM is exported to the cytoplasm. Such clock protein shuttling is known in mammalian PER proteins (Vielhaber *et al*., 2001; Yagita *et al*., 2002). Thus, this possibility needs to be tested in *Drosophila*.

Nakamura and coworkers investigating the circadian rhythm of spontaneous discharges among individual neurons in the SCN of *Clk* mutant mice, using slice cultures and dispersed cell cultures (Nakamura *et al*., 2002), have revealed that circadian rhythms exist in 77% of slice cultures and 15% of dispersed cell cultures. These results are also inconsistent with current hypotheses about molecular clocks. According to the model for the mammalian molecular clock, if any of the core clock components are lost, then circadian oscillations will also be abolished. Thus, even widely accepted molecular models of circadian clocks are not always able to explain all experimental evidence. Thus, it is clearly important for the elucidation of the molecular mechanisms of the clock to investigate the expression patterns of clock genes and also the localization and the interactions of clock proteins, and to do this within a single clock cell and/or clock tissue *in situ*.

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