THE ROYAL SOCIETY

doi 10.1098/rstb.2001.0961

Flies, clocks and evolution

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The negative feedback model for gene regulation of the circadian mechanism is described for the fruitfly, *Drosophila melanogaster*. The conservation of function of clock molecules is illustrated by comparison with the mammalian circadian system, and the apparent swapping of roles between various canonical clock gene components is highlighted. The role of clock gene duplications and divergence of function is introduced via the *timeless* gene. The impressive similarities in clock gene regulation between flies and mammals could suggest that variation between more closely related species within insects might be minimal. However, this is not borne out because the expression of clock molecules in the brain of the giant silk moth, *Antheraea pernyi*, is not easy to reconcile with the negative feedback roles of the *period* and *timeless* genes. Variation in clock gene sequences between and within fly species is examined and the role of co-evolution between and within clock molecules is described, particularly with reference to adaptive functions of the circadian phenotype.

Keywords: circadian; clock; evolution; *Drosophila*; insects

1. INTRODUCTION

How good is a model organism in elucidating a biological phenomenon? Circadian biology represents as good an example as any for discussion, and this review focuses on comparative aspects of molecular chronobiology. Historically, *Drosophila* has taken centre stage in the circadian saga since 1971, when Konopka & Benzer (1971) identified the *period* (*per*) gene. From the mid-1980s, when *per* was first cloned, until the mid 1990s, *Drosophila* provided the only molecular model for circadian timing in the animal kingdom, although it was accompanied by the equally compelling fungal work with the *frequency* (*frq*) locus in *Neurospora*. However, when *per* was identified in *Antheraea pernyi*, some initial surprises in the expression patterns of silk moth clock genes began to suggest that the *Drosophila* template for clock gene regulation was not followed slavishly in species after species. Perhaps we should not have been surprised. Certainly, recent work with clock genes in mammals shows that variations on a theme tend to be the rule rather than the exception.

2. GENES AND LOOPS

(a) *Negative regulators*

It seems reasonable to assume that rhythmic phenotypes are the endpoint of the rhythmic expression of genes encoding for the molecular gears of the clock. In this respect, it is quite fortunate that the first fly clock genes to have been identified and cloned, i.e. *per* (Konopka & Benzer 1971; Bargiello *et al*. 1984; Reddy *et al*. 1984) and *timeless* (*tim*, Sehgal *et al*. 1994; Myers *et al*. 1995; Gekakis *et al*. 1995), actually conform to this expectation. These two genes are rhythmically expressed and cycle in abundance more-or-less in synchrony at the RNA and protein levels. Both *per* and *tim* RNAs peak early in the evening (ZT 13–16, ZT = Zeitbeger Time, ZT 0 = light on, ZT 12 = light off; Hardin *et al*. 1990; Sehgal *et al*. 1995), whereas the protein products reach a maximum late at night (ZT18–24; Zeng *et al*. 1996; Lee *et al*. 1996; Hunter-Ensor *et al*. 1996; Edery *et al*. 1994). The meaning of the RNA cycle is not clear since it may not be necessary for overt rhythmic behaviour (Vosshall & Young 1995), but the fact that the RNA levels decline as soon as the protein levels rise suggests that the expression of both genes is under control of their protein products. Indeed, in per^0 and tim^0 mutants the RNA cycling is abolished for both genes (Hardin *et al*. 1990; Seghal *et al*. 1994). The negative effect that PER and TIM exert on their own transcription creates a negative feedback loop that has been the central theme of any clock model.

In order to autoregulate gene expression, PER and TIM must enter the nucleus, and they obligingly do so as a complex (Saez & Young 1996). Deprived of a DNA-binding domain but able to engage in protein–protein interactions, PER and TIM exert their nuclear influence by physically associating with positive transcription factors and forming a complex unable to attach at the DNA target (Darlington *et al*. 1998; Lee *et al*. 1998, 1999; Bae *et al*. 2000). Some of this repression may be effected by PER on its own at times in the day when the light-sensitive TIM molecule has already disappeared. Rothenfluh *et al*. (2000) have recently described a new *tim* mutant, tim^{UL}, which, when kept in constant darkness (DD), shows abnormally high levels of *per* and *tim* RNA during the subjective day (the time of day corresponding to the light phase of the previous light–dark (LD) cycle), as well as abnormal persistence of the PER/TIM^{UL} complex. Removing TIM^{UL} with light brings the RNAs to a lower level, consistent with the view that PER alone may be sufficient for transcriptional repression of the *per* and *tim* genes. This

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interpretation is particularly appealing given that, in mammals, TIM (arguably) lacks any significant role in the clock machinery (see § 3a), and that in *Drosophila* there is another *timeless* gene, *tim2* (Benna *et al.* 2000; Gotter *et al.* 2000), that appears to be the true orthologue of mammalian *tim*. So far, *tim1* (the original *tim* gene) has been found only in insects, whereas *tim2* genes are also present in the worm *Caenorhabditis elegans* and in mammals (Benna *et al.* 2000; Gotter *et al.* 2000). It is possible that the duplication of *tim* (from the ancestral *tim2* to *tim1*) is a relatively recent evolutionary event that has occurred in the insect lineage, in which TIM1 has assumed a role in the clock by specializing from a more general nuclear transporter into the specific translocator of PER. Clearly, more comparative work needs to be done in order to assess whether *tim1* genes are found actually outside the insects, although a recent search of the human genome suggests that *tim1* sequences are probably absent in mammals (Clayton *et al.* 2001). It is also important to investigate the role of *tim2* in *Drosophila*, in particular whether or not TIM2 is also a nuclear translocator that contributes to the clock mechanism. In mice, *tim2* knockouts (that lack a functional *tim2* gene) are embryonic lethals, so *tim2* has a vital function, perhaps as a more general nuclear transporter (Gotter *et al.* 2000).

(b) *Dedicated clock genes?*

A critical feature in the molecular dissection of the circadian clock has been that *per* and *tim* not only constitute an intrinsic part of the oscillator, but at the same time they are under clock control. However, it is not necessary for a gene to be rhythmically expressed in order to encode a clock component, and *dbt* (*double-time*) is such an example (Price *et al.* 1998). Furthermore, *dbt* is not a dedicated clock gene in that lethal mutations reveal other vital functions (not unexpected for a kinase; Price *et al.* 1998; Kloss *et al.* 1998). One might make a case for *per* and *tim* being dedicated clock genes, in that lethal mutations in *tim* have yet to be described, and *per* can be deleted without any obvious loss in viability. Certainly, all the early phenotypes ascribed to *per* mutations have an element of 'clockishness' in them, be they the lovesong cycle defects (Kyriacou & Hall 1989; Alt *et al.* 1998) or the developmental timing changes seen in the classic *per* mutants (Kyriacou *et al.* 1990). More recently, however, the role of *per* in sensitization to cocaine has suggested that classifying it only as a regulator of genes involved with rhythmic phenotypes may be too restrictive (Andretic *et al.* 1999). Interestingly, *tim* mutants did not affect the sensitization phenotype whereas mutations in the positive regulators *dClock* and *cycle* (see § 2c) produced the same defects as in *per* mutants (Andretic *et al.* 1999). Therefore *tim* seems to be set apart from the other three clock genes with respect to this phenotype. In the case of drug-craving, *dClock*, *cycle* and *per* may be functioning as regulators of tyrosine decarboxylase, the enzyme that is normally induced on exposure to cocaine.

(c) *Positive regulators*

The rhythmic expression of *per* and *tim* has provided a molecular benchmark for investigating whether other mutations that generate behavioural abnormalities are acting at the highest levels in the circadian regulatory hierarchy or in the output pathway. Put simply, if an arrhythmic mutant shows normal *per* and *tim* cycling, it is likely to be downstream of the clock, whereas other mutants that alter these molecular cycles will be working at the same level or upstream of the oscillator. Low and non-cycling expression of *per* and *tim* in flies mutant for *dClock* (*dClk*, also known as *Jrk*; Allada *et al.* 1998) and/or *cycle* (*cyc*, Rutila *et al.* 1998) indicates that these genes are positive regulators of *per* and *tim* transcription. dCLK and CYC share with PER a protein–protein interaction domain called PAS (Huang *et al.* 1993; Allada *et al.* 1998; Rutila *et al.* 1998) but, unlike PER, they also contain a basic helix–loop–helix (bHLH) region which allows further protein–protein interactions and, most importantly, binding to DNA (Allada *et al.* 1998; Rutila *et al.* 1998). bHLH transcription factors bind, usually as heterodimers, to a short DNA sequence known as an E-box. There are E-boxes in the *per* and *tim* promoter regions and the dCLK/CYC dimer binds to them, activating transcription (Darlington *et al.* 1998). There is some debate as to whether E-boxes are the only sequences required for activation of *per* and *tim*, cycling of their transcripts and normal spatio-temporal expression (Lyons *et al.* 2001; Darlington *et al.* 2001). However, it is clear that the E-box is a key circadian regulatory element, both in flies and mammals (reviewed in Kyriacou & Rosato 2000). Binding of dCLK/CYC to the E-box will eventually generate high levels of the PER/TIM complex, which moves into the nucleus and interacts with dCLK/CYC. This results in the transcriptional repression of *per* and *tim* and the closure of the first circadian loop (Darlington *et al.* 1998). Such interaction probably takes place via dCLK but leaving the dCLK/CYC dimer intact (Lee *et al.* 1998, 1999; Bae *et al.* 2000). Indeed, *in vitro* studies have shown the formation of a dCLK/CYC/PER/TIM tetramer unable to bind DNA (Lee et al., 1999), although equally defective dCLK/CYC/PER and dCLK/CYC/TIM complexes can also be formed (Lee *et al.* 1999). Since PER persists longer than TIM in the nucleus during the early day (Hunter-Ensor *et al.* 1996; Lee *et al.* 1996; Zeng *et al.* 1996), and as a monomer is an efficient transcriptional repressor of the *per* and *tim* genes (Rothenfluh *et al.* 2000, see § 2a), the role of TIM in the first negative feedback loop is at present very controversial.

(d) *Negative regulators with positive effects and* vice versa

Another molecular loop in the circadian machinery is opened by the rhythmic expression of *dClk* (*cyc* instead is constitutively expressed) and is in antiphase with *per* and *tim*. *dCLK* mRNA peaks late at night to early in the morning (ZT 23 to ZT 4, Darlington *et al.* 1998; Bae *et al.* 1998) at times when the levels of PER/TIM are high. This suggests that PER/TIM may activate dCLK transcription, and this idea is supported by the observation that *dClk* RNA levels are low in per^{01} and tim^{01} mutants (Bae *et al.* 1998). Mutants that lack functional dCLK and CYC, however, express *dClk* mRNA at constitutively high levels (Glossop *et al.* 1999), suggesting that PER/TIM inhibits repression of (derepresses) *dClk* transcription, and that dCLK/CYC dimer may be the repressor. So, in the overall scheme of things, the high levels of PER/TIM in the nucleus at night block the action of the positive

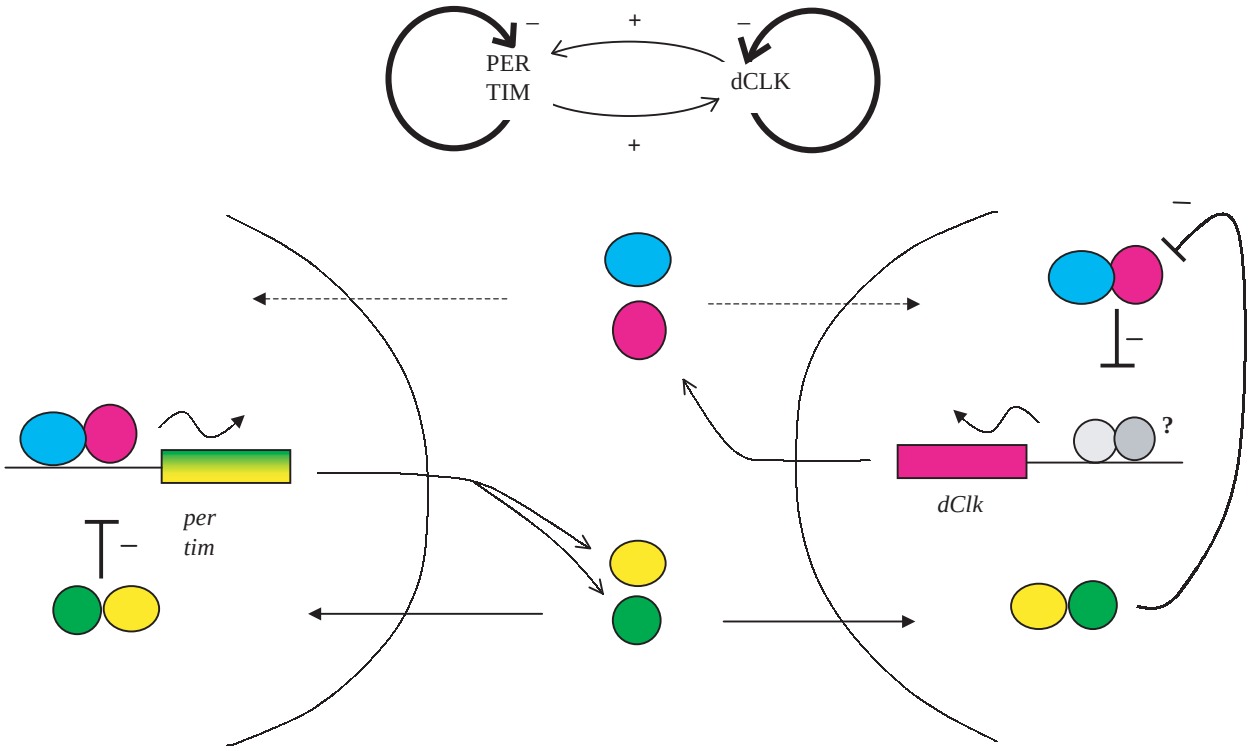

Figure 1. Interlocked feedback loops. For ease of description some elements of the *Drosophila* clockworks, whose function is less clear, are not depicted. Left: dCLK (purple) and CYC (blue) directly activate *per* and *tim* transcription in a rhythmic fashion (∼). Then the PER (green) and TIM (yellow) proteins move back into the nucleus as a dimer and repress their own production. Right: The transcription of d*Clk* is regulated by unknown transcription factors (?), which are inhibited by the dCLK/CYC dimer. It is not known how dCLK and CYC enter the nucleus, and this is indicated by dashed arrows. The PER/TIM complex represses dCLK/CYC and therefore acts as a positive factor in *dClk* negative loop.

transcription factors dCLK/CYC on *per* and *tim*, but derepress *dClk*. Thus, as *per* and *tim* mRNA levels go down, *dClk* levels start to come up. PER/TIM levels fall early in the morning, first as TIM, then as PER; they are then degraded, releasing dCLK/CYC dimers to repress *dClk* transcription. By the end of the day *dClk* levels are low, but the dCLK/CYC dimers have reactivated *per* and *tim* transcription. After a delay (as a consequence of DBT-mediated degradation of PER), PER and TIM dimerize, enter the nucleus and derepress *dClk*, and the relentless cycle begins again. These results show that two interlocked negative feedback loops are at the core of circadian oscillator function in *Drosophila* (figure 1).

(e) *Somewhere in the loops*

The more we know about the circadian clock, the more difficult it becomes to distinguish between 'central' pacemaker components and cycling input factors (see Roenneberg & Merrow 2001). The rhythmically expressed *cryptochrome* (*cry*) gene is such an example. Biochemically, CRY is a flavoprotein related to 6,4-photolyases; it is able to absorb light and there is evidence that it acts as a photoreceptor (Stanewsky *et al.* 1998; Emery *et al.* 1998; see Foster & Helfrich-Förster 2001). Flies carrying the mutant allele cry^b, in which an amino-acid substitution disrupts one of the flavin binding sites (Emery *et al.* 1998), show several defects in light perception. They respond abnormally to a short resetting light pulse (Emery *et al.* 1998) and they are rhythmic (wild-type flies are arrhythmic) under constant illumination (Emery *et al.* 2000). In cry^b mutants the stability of PER and TIM is also affected (Stanewsky *et al.* 1998). PER stability depends on TIM, which, after light exposure, is degraded by the ubiquitin-proteasome pathway (Naidoo *et al.* 1999). In cry^b lateral neurons (LN), the pacemaker cells regulating *Drosophila* behavioural rhythmicity, TIM is much more stable during the light phase of the light–dark cycle, indicating that CRY may be involved in TIM light sensitivity (Stanewsky *et al.* 1998). Indirect evidence in support of this view comes from experiments in S2 cells that show a direct physical interaction between CRY and TIM, and the ability of CRY to inhibit PER/TIM repression of dCLK/CYC-mediated E-box transcription (Ceriani *et al.* 1999). Interestingly the effect of CRY on transcription requires constant illumination of the cells, indicating that within the nucleus CRY activity is dependent upon light. However, in the cytoplasm CRY is able to bind TIM in constant darkness (Ceriani *et al.* 1999), suggesting that CRY has a 'light' function and a 'dark' function, and can act both as a circadian photoreceptor and a clock component. This might explain some of the results reported in the literature that are difficult to reconcile with the view that CRY is only involved in light perception (Stanewsky *et al.* 1998; Ishikawa *et al.* 1999).

Another gene awaiting to be fully ennobled into the 'true' clock genes circle is *vrille*, which encodes a bZIP transcription factor (*vri*, Blau & Young 1999; George & Terracol 1997). Like *dbt*, *vri* is an essential developmental gene indicating a pleiotropic demand for *vrille* function. *vri* shows the right credentials to be considered part of the

clock: it is rhythmically transcribed in phase with *per* and *tim* in the LNs; its overexpression alters behavioural and molecular phenotypes that are consistent with a block in *per* and *tim* expression; and finally, it is under control of the same transcriptional loop that regulates *per* and *tim* mRNA levels (Blau & Young 1999). The full investiture now needs only to await biochemical data demonstrating interactions with the other clock components.

(f) *Definitively out*

One of the biggest challenges in circadian biology is to link what we know about the circadian pacemaker with what we see as rhythmic phenotypes. Several genes have emerged as part of the clock output; one is *lark*, a gene whose product encodes an RNA-binding protein that is required for rhythmic eclosion but not adult locomotor behaviour (Newby & Jackson 1993; McNeil *et al.* 1998). LARK protein cycles in abundance in a restricted number of neurons in the central nervous system (CNS) and the ventral nervous system (VNS) of late pupae, and colocalizes with a neuropeptide CCAP, which plays a role in insect ecdysis (see McNeil *et al.* 1998, and references therein). LARK levels are high in the day, suggesting that it acts as a repressor of eclosion, restricting the emergence of flies to a specific gate. As expected of an output gene, the LARK cycle is eliminated in *per*-null mutants, but how the RNA-binding protein regulates the eclosion system is yet to be unravelled.

Another output gene is *pigment dispersing factor* (*pdf*), probably the principal transmitter of circadian time information for adult locomotor behaviour (Renn *et al.* 1999; Park *et al.* 2000). The gene is expressed in a subset of the larval and adult brain pacemaker cells but the transcript does not cycle. Instead, the peptide cycles at specific nerve terminals, and this can be obliterated by *per* and *tim*-null mutations. The *pdf* transcript is positively regulated by dCLK and CYC, but only within a subset of the neurons in which it is expressed, yet an E-box found in the promoter of *pdf* does not appear to be relevant to dCLK/CYC mediated transcription. A mutation in *pdf* has similar rhythmic defects as a transgenic ablation of the relevant pacemaker cells, suggesting that these neurons are dedicated to circadian behavioural expression. Finally, in *dClk* and *cyc* mutants, the *pdf*-expressing axons show unusual projection patterns, revealing an additional developmental defect. As yet, the effects of the *pdf* mutation on the *per* and *tim* mRNA cycle have not been investigated, so there is an unlikely but formal possibility that *pdf*, like *vrille*, could be more than just an output gene.

A recently identified output gene is *take out*. Both the transcript and protein cycle, and are localized in the cardia, crop and antennae (Sarov-Blat *et al.* 2000). The gene is induced by starvation and is a member of a family of novel insect proteins that have sequence similarities to juvenile hormone binding protein as well as the product of the gene adjacent to *per* in *Drosophila*, the famous 0.9 kb transcript (Lorenz *et al.* 1989). This neuropeptide appears to convey temporal information for feeding activity and mutations in the gene show an abnormal behavioural response to starvation. Clearly, significant progress has been made in the identification of output genes; an excellent review on the subject of output genes can be found in Jackson *et al.* (2001).

3. WHAT ABOUT OTHER ORGANISMS?

(a) *Clock gene in mammals*

This brief trip around the clockworks in *Drosophila* begs the question of how conserved are these mechanisms? We can begin by taking a look at the clock of the mouse, as this is the next best understood animal model. The last two years have seen remarkable progress in the identification of the murine equivalents of the fly clock genes. There are, at present, three *mPer* genes, two *mCry* genes, an *mClk*, *mCyc* (called *Bmal1*), and an *mDbt* (*Casein kinase I*ε). There is also an *mTim* (actually a *tim2*-like gene—see § 2a). In addition, further putative members of the mammalian *Per* and *Casein kinase I*ε*?* gene families have been identified in the human genome (Clayton *et al.* 2001). Mammals often have several copies of genes that are represented as singletons in the fly, due to ancient genomic duplications in the mammalian lineages. However, for the *mPer* genes, *mPer2* appears to have the most important in that a knockout produces behavioural arrhythmicity in constant conditions (Zheng *et al.* 1999). The role of *mTim* appears to be negligible for the clock as mentioned earlier, but the *Cry* genes act very differently in the mouse. They appear to have lost their photoreceptor function and instead act as true negative components of the circadian mechanism (reviewed in Reppert & Weaver 2000; Shearman *et al.* 2000*a*). In the mouse, *Bmal1* cycles, whereas *mClk* does not (remember that in the fly it is the opposite: *dClk* is rhythmic, *cyc* is not). The mouse model has *mPer* and the *mCry* transcripts cycling, with dimerization between the mCRYs and mPERs important for nuclear translocation. mPER2 protein then positively regulates *Bmal1*, and BMAL1 and mCLK proteins then activate transcription of the *mPer* and *mCry* genes (Shearman *et al.* 2000*a*). The mCRY proteins, however, are also the negative regulators of *mPer* and *mCry*, by antagonizing BMAL1-mCLK mediated transcription. We can see that, as in *Drosophila*, positive regulation of *Bmal1* by mPER2 generates an interlocking of the *mPer* and *Bmal1* loops (in the fly, the interconnection is between the *per/tim* and *dClk* loops). Finally, the mammalian homologue of fly *doubletime*, casein kinase Ie (CK1e) is encoded by the *tau* locus in the hamster. The classic *tau* mutation shortens the circadian period, and the mutant protein is defective in its phosphorylation of mPER protein (Lowrey *et al.* 2000).

Of course, there are any number of loose ends that require further investigation. For example, Yagita *et al.* (2000) have shown that in cell lines, mPER3 is required for the nuclear transportation of mPER1 and mPER2, and this can occur even in a *mCry1/mCry2* double mutant background. Nuclear localization of mPER1 also occurs in the suprachiasmatic nuclei (SCN) and peripheral tissues in *Cry* mutant mice (Shearman *et al.* 2000*a*; Yagita *et al.* 2000), but mPER2 stability appears to be compromised in the SCN of these mutants, although in other brain regions it is nuclear. This is reminiscent of the finding that in *Drosophila* photoreceptors, PER-TIM are cytoplasmic in cry^b mutants, whereas they do translocate into the nucleus in the LNs (Stanewsky *et al.* 1998). Thus, in both organisms, there appear to be different mechanisms for the movement of clock proteins into the nucleus when pacemaker cells are compared with other tissues. We can

Table 1. Circadian clock genes in the fly and mammals. Note that for the mammalian clock there is controversy about the expression and function of several genes.

Drosophila			mammals		
gene	expression	function	gene	expression	function
per	rhythmic	repressor	*Per1*	rhythmic	repressor
			Per2	rhythmic	repressor (activator?)[a]
			Per3	rhythmic	uncertain
tim	rhythmic	repressor			
tim2	unknown	unknown	*Tim*	constant	unknown
dbt	constant	casein kinase	*Tau*	constant	casein kinase
dClk (*Jerk*)	rhythmic	activator	*Clk*	constant	activator
cyc (*dbmal1*)	constant	activator	*Bmal1*	rhythmic	activator
cry	rhythmic	photoreceptor (transcription regulator?)[b]	*Cry1*	rhythmic	repressor (photoreceptor?)[d]
			Cry2 ?[c]		repressor (photoreceptor?)[d]
vri	rhythmic	transcription factor			

[a]The current data suggest a role for PER2 also as a transcriptional activator.
[b]There is no incontrovertible proof that dCRY is a transcription regulator in flies.
[c]There is controversy regarding the rhythmic or constant expression of *Cry2*.
[d]The current data cannot exclude a possible role of mammalian *Cry* genes as photoreceptors.

conclude that even in an arrhythmic *Cry* mutant mouse, there remain mechanisms for the nuclear transport of mPERs that may be largely intact. The role of CRY must therefore be related to the circadian regulation of PER nuclear entry, rather than providing the vehicle for nuclear translocation itself. To complicate matters further, in *mPer3* knockout mice there is only a very subtle effect on behavioural circadian rhythmicity (Shearman *et al.* 2000*b*) so, in the absence of mPER3 protein, we assume that nuclear translocation of mPER proteins must be reasonably normal. There is also evidence from mammalian cell lines that mPER2 and CKIε? retard, and therefore regulate the rate of entry of mPER1 into the nucleus (Vielhaber *et al.* 2000). The full significance of all these results is yet to be appreciated.

Nevertheless, we can see that by-and-large, the same players are involved in generating the murine and fly oscillators (table 1). There is some apparent switching of roles between mouse and fly (e.g. *Bmal1* and *dClk*), and because no *tim1*-like molecule has yet been identified in the mouse, it is not clear what role, if any, *mTim* may play. Given the large evolutionary distance between mouse and fly, one could therefore be forgiven for thinking that interspecific comparisons of these regulatory networks among more closely related species (for example, insects), would yield little variation on the basic theme. One would be wrong.

(b) *Clock molecules in other insects—the giant silk moth*

The giant silk moth, *Antheraea pernyi*, has provided a number of surprises in the analysis of clock molecules. The *per* gene from the silk moth has a rather odd truncated sequence compared with *Drosophila*, but its expression patterns in the central brain, retina and embryonic/larval midgut reveal cycles of mRNA and protein (Reppert *et al.* 1994; Sauman & Reppert 1996; Sauman *et al.* 1996). In the photoreceptors and midgut, PER cycles from the cytoplasm to the nucleus as in the fly. However, central brain expression, as revealed by PER and TIM antibodies, resides in eight neurosecretory cells, but the two antigens are detected only in the cytoplasm and axonal projections, and they cycle. At face value, this would suggest that autoregulatory negative feedback of PER and TIM is unlikely. Furthermore, a cycling *per* antisense transcript was also detected in these cells, but the rhythm of this mRNA was in antiphase to that of *per*, raising the possibility that RNA duplexes might be involved in the regulation of cycling (Sauman & Reppert 1996).

The antisense transcript corresponds to the sequence of a small fragment from the 3′ end of the PAS domain, but has no open reading frames and so will not be translated (Gotter *et al.* 1999). Further analysis revealed that the antisense did not originate from *per* but from another *per*-like gene. In addition, a third *per*-like gene was discovered that also shares this PAS region, and both this gene and the antisense gene are only found in females. The original silk moth *per* was present in both males and females and because Lepidoptera have an unusual sex-determining system, in that the females are the chromosomally heterogametic sex (ZW) whereas the males are homogametic (ZZ), it meant that the two new *per* variants were located on the female W chromosome. The novel sense gene, *perW*, encodes a truncated version of the PER protein which stops abruptly just past the PAS region, and from its organization appears to be an incompletely duplicated and rearranged version of *perZ*. The *perW* transcript does not cycle in the brain, but PERW appears to be translated. Finally, there are many copies of a *perW*-like gene on the female W chromosome, only one of which, *perW*, is transcribed and translated, whereas another encodes the rhythmically expressed yet untranslated antisense *perW* (Gotter *et al.* 1999).

This extremely interesting system also exposes the evolutionary dynamics of the sex chromosomes in Lepidoptera. Usually, when sex is determined chromosomally, the heterogametic chromosome (Y in mammals or flies, W in Lepidoptera) progressively degenerates due to the restriction of recombination. This leads to the evolution of mechanisms of dosage compensation for sex-linked genes (see Gotter *et al.* 1999, and references therein). In moths, the dosage compensation system has not developed, suggesting that the Z/W sex-determining system is a relatively recent phenomenon. *perZ* is more similar in amino-acid sequence to *perW* than it is to the *perZ* genes of other moth species (Regier *et al.* 1998), giving the credible scenario that *perW* duplicated recently from *perZ*, after silk moth species divergence. *perW* then got caught up in the degeneration that accompanies the development of a chromosomal sex-determining system. This may explain why antisense *per* is also associated with a retrotransposon (because degeneration is often linked to inactivation of genes by insertion of mobile elements), and why *perW* is an incomplete duplication and rearrangement of *perZ* (perhaps it was originally a complete duplication that became rearranged almost immediately).

As males do not carry either of the *perW* genes, yet are perfectly rhythmic in behaviour and eclosion, the *perW* genes are clearly not required for rhythmicity. Therefore the oscillations of *perZ* RNA do not require the antisense because, in males, there is no *per* antisense. However, this still leaves the little matter of cycling cytoplasmic PER and TIM in the central brain cells. Perhaps this cycling is mediated by silk moth CRY, in a manner similar to the negative regulation provided by mCRY (see § 3a). What the silk moth has revealed is the evolutionary flexibility of a circadian gene regulation mechanism that diverged more than 200 million years ago from the common ancestor of Lepidoptera and Diptera. No doubt the silk moth has more surprises in store for us.

(c) *Clock gene variation within larger flies*

The molecular evolution of clock genes has also been approached from within the Diptera. In the sheep blow fly, *Lucilia cuprina*, *per* mRNA and protein cycle with a 3 h phase lag between them in a LD cycle, compared with the 4–6 h delay in *Drosophila* (Warman *et al.* 2000). This may be due to constraints imposed on the feedback loop in a 12 L:12 D cycle, because this large dipteran has a 22 h free-running circadian cycle. This altered phase angle between mRNA and protein may also mean that PER could be released from the control of DBT, as little additional delay would be needed to maintain a feedback loop. In a related dipteran, the housefly, *Musca domestica*, the *per* sequence shows some unusual characteristics. Phylogenetic analysis reveals that the PAS domain and cytoplasmic localization domains (CLD) are more closely related to those of *D. melanogaster* than are those of *D. pseudoobscura* and *D. virilis* (Piccin *et al.* 2000). This is surprising given that the time of divergence from the common ancestor of muscid and fruitflies was about 100 million years (Myr) ago, whereas that of *D. melanogaster* from the other fruitfly species was 30–60 Myr ago. This rather odd evolutionary profile nevertheless correlates extremely well with functional data. Transformation of *D. pseudoobscura per* into *D. melanogaster per*-null mutants produces a poor rescue of rhythmic behaviour (Petersen *et al.* 1988; Peixoto *et al.* 1998), whereas the *Musca per* transgene restores an extremely robust rhythmicity (Piccin *et al.* 2000). One simple possible explanation for these observations is based on the notion that the PAS domain interacts with TIM (Gekakis *et al.* 1995). As the PAS domain of *Musca* PER is phylogenetically more closely related to that of *D. melanogaster* than is *D. pseudoobscura*, then we might expect PER–TIM interactions to be more efficient in transformants when the *Musca* transgene is introduced, compared with *D. pseudoobscura*, leading to the enhanced rescue of rhythmicity. This was indeed observed experimentally. However, a chimeric transgene, in which the 5′ half of the *per* coding sequences including PAS and CLD regions of *D. melanogaster* were joined to 3′ sequences from *D. pseudoobscura*, resulted in an almost perfect rescue of rhythmicity in *per*-null transformants (Peixoto *et al.* 1998). This scenario implies that PER and TIM may evolve together to ensure their mutual interaction, a hypothesis that can easily be tested in a number of ways, biochemically, behaviourally and phylogenetically.

(d) *Interspecific variation in* tim

The *tim* gene has also been identified in a number of *Drosophila* species. The original sequence of Myers *et al.* (1995) was based on cDNA analysis and has been amended by the work of Ousley *et al.* (1998), who discovered an additional, functionally important coding region within the transcription unit. Furthermore, *tim* shows an interesting species-specific polymorphism in the putative translational start site. In one allelic variant in *D. melanogaster*, this polymorphism results in either two sites being available for translational initiation, whereas the second allele has a start site 23 residues downstream of the first (Rosato *et al.* 1997*a*). In these latter variants, a mutation generates a stop codon between the first and second initiation sites. The sequence around each site gives some clues as to the likely efficiency of translation and suggests that the second site, with its slightly truncated version of TIM, will provide the predominant form of the protein (Rosato *et al.* 1997*a*). Although only a limited number of individuals have been assayed for this polymorphism in other *Drosophila* species, so far none has revealed this type of variation (Rosato *et al.* 1997*a*). A similar form of clock protein truncation has been observed with *Neurospora* FREQUENCY (FRQ), where at high and low temperatures, a different translation initiation start site is preferred, giving rise to long and short FRQ proteins that differ in their *N*-terminal 99 residues (Liu *et al.* 1997; Garceau *et al.* 1997). The significance of this is that each isoform of FRQ is particularly adept at rescuing *frq*-null arrhythmicity at different temperature extremes, thereby extending the temperature range at which fungal rhythmicity can be generated. One wonders whether the polymorphism in *D. melanogaster tim* might serve a similar temperature-related function?

(e) *Variation in clock genes and adaptation*

In fact, how the clock copes with temperature variation has been the focus of a number of studies in *Drosophila*. Majercak *et al.* (1999) revealed how thermosensitive splicing of a 3′ untranslated sequence of *per* generates an

earlier upswing in the mRNA cycle at colder temperatures, which correlates with the behavioural changes in locomotor activity. *Drosophila* species have different locomotor activity patterns, and interspecific transformation experiments with *D. pseudoobscura* and *D. melanogaster* reveal that the *per* gene is responsible for these species-specific profiles (Petersen *et al.* 1988). Could it be that different 3′ splicing events are responsible for the differences in behaviour?

Interspecific sequence comparisons of *Drosophila* species *per* genes also led to the discovery of a coding polymorphism within *per* that was relevant to circadian thermal responses (Costa & Kyriacou 1998). The Thr-Gly/Ser-Gly repeat region encoded within *per* shows polymorphism in length within *D. melanogaster* and *D. pseudoobscura* (Costa *et al.* 1991). The length of this repetitive region varies widely between species and statistical analysis of the relevant sequences revealed that interspecific changes in the length of this tract (irrespective of sequence) appeared to require compensatory amino-acid changes in the short non-repetitive immediate flanking regions, presumably to stabilize the protein (Peixoto *et al.* 1993; Nielsen *et al.* 1994). This hypothesis was tested with chimeric genes between *D. melanogaster* and *D. pseudoobscura*, two species having different Thr-Gly lengths, in which the positioning of the chimeric junction was manipulated (Peixoto *et al.* 1998). Small alterations in the junction gave remarkably different results in transformants. One set of transformants gave ostensibly wild-type phenotypes, another set gave a dramatic temperature sensitivity of the free-running circadian period, whereas another produced an arrhythmic phenotype (Peixoto *et al.* 1998). These results fully supported the suggestion that compensatory mutations are required to stabilize PER when the repetitive tract expands (or contracts).

In *D. melanogaster*, a length polymorphism in this Thr-Gly repeat, which is not compensated by flanking amino-acid changes, alters the ability of the flies to maintain a constant period when challenged with changes in temperature (Sawyer *et al.* 1997). The different variants are furthermore distributed as a latitudinal cline in Europe, with the more robustly temperature compensated variants predominating in the more thermally hostile northern European locations (Costa *et al.* 1992). Their southern cousins are less able to withstand changes in temperature on their circadian period, yet at high temperatures they keep a period very close to 24 h, thereby resonating with the environmental circadian cycle (Sawyer *et al.* 1997). This suggests that balancing selection could be maintaining the polymorphism, where each variant is particularly suited to its own thermal niche (Costa & Kyriacou 1998). The variation observed in the DNA sequences around the Thr-Gly region is not inconsistent with a balancing selection scenario (Rosato *et al.* 1996, 1997*b*). In addition, structural analyses of the Thr-Gly peptides in both *D. melanogaster* and *D. pseudoobscura* reveal that the length polymorphisms observed differ by single conformational units. In *D. melanogaster*, the major variants found in the wild have 14, 17, 20 or 23 pairs of Thr-Glys (Costa *et al.* 1992). Three pairs of Thr-Glys form a β-turn, so each PER variant differs by this structural unit (Castiglione-Morelli *et al.* 1995). Remarkably, the thermal stability of the circadian period in these *per* variants is linear, whereas very rare variants, whose Thr-Gly length falls outside the $(\text{Thr-Gly})_3$ interval, for example, $(\text{Thr-Gly})_{15}$, show much poorer temperature compensation (Sawyer *et al.* 1997). Thus there is a correlation between behaviour, polymorphism, protein structure and natural selection—a rare combination.

In *D. pseudoobscura*, the length polymorphism is based not only on Thr-Gly pairs but also on a related degenerate pentapeptide sequence (Nielsen *et al.* 1994). Conformational analysis reveals that in this species, the pentapeptide forms the same β-turn structural unit (Guantieri *et al.* 1999) and the polymorphism in this species involves changes in the numbers of these higher-order units (Costa *et al.* 1991). It remains to be seen whether variation in this repetitive region of *D. pseudoobscura per* has any implications for circadian temperature adaptation as in *D. melanogaster.*

4. SUMMARY

This brief review has attempted to summarize some of the more prominent comparative aspects of clock gene research. The focus of work in this area has clearly switched from being exclusively concerned with insects to now include vertebrates. From the work described above, it seems clear that natural selection appears to 'tinker' with the nuts and bolts of the circadian oscillator and reuses the same components, but in slightly different ways. Just how extensive this change in the use of identified clock components will be will depend on a number of alternative model systems being developed. At present, these include Coleoptera, Lepidoptera, Orthoptera and Diptera in the insects, and mice, rats and zebrafish among the vertebrates. An interesting report recently extended this range of organisms to the Hymenoptera, in which *per* mRNA levels seemed to correlate with the age-related division of labour in worker bees (Toma *et al.* 2000). Older worker bees that forage for food show more pronounced circadian behavioural rhythms and have higher levels of oscillating *per* mRNA than younger workers, whose activities are mainly restricted to the hive. While we can make a reasonable guess at what the role of *per* in bees might be, the difference in *per* levels between different types of worker, and how this is regulated (splicing?), will make an interesting addition to the comparative literature.

In conclusion, we anticipate that there will be plenty of unexpected observations that will require amendments to the basic negative feedback model as the clock community continues to probe the comparative aspects of clock gene regulation.

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