A Comparative Analysis of the Circadian Clock in Diptera

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University of Leicester

Veryan Codd

Department of Biology, University of Leicester

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Abstract

A Comparative Molecular Analysis of the Circadian clock in Diptera

Veryan Codd

The circadian central oscillator of *Drosophila melanogaster* consists of at least two interlocked negative transcriptional feedback loops. This has been taken to be a general model for higher eukaryotes with the core components conserved but their regulation altered. The work presented here indicates that in *Musca domestica*, a dipteran closely related to *Drosophila*, one of these regulatory loops, involving PERIOD (PER) and TIMELESS (TIM), functions in a completely different manner. This study shows that in contrast to *Drosophila*, *Musca* PER remains constant in western studies in any lighting condition, whereas like *Drosophila* TIM cycles in both LD and DD and is constantly degraded in LL. In addition within the central brain immunostaining revealed that even in the small set of cells thought to contain the central pacemaker PER staining was restricted exclusively to the cytoplasm. However following the *Drosophila* model PER was observed to cycle in the cytoplasm of these cells. Although TIM co-localises with PER in these cells, unlike PER, TIM does become nuclear. This indicates that the negative feedback model illustrated by analysis of the *Drosophila* is inadequate to explain clock function in *Musca*. A putative *Musca* PER nuclear export sequence which functions in other species was tested in GFP constructs but not shown to be involved in altered localisation. In contrast in peripheral tissue such as photoreceptor cells both PER and TIM cycle and both proteins become nuclear late at night as in *Drosophila*.

Stability of *Musca* PER in LL and an altered relationship between transgenic *Musca* PER and *Drosophila* DOUBLETIME indicates an altered relationship between PER and the DBT kinase that may be responsible for PER stability.

Thus although it can be seen that a different model is required for other insect species how these proteins act remains to be elucidated.
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Chapter 1

Introduction
1.1 The Circadian Clock

The circadian clock is the mechanism by which most organisms regulate their physiology and behaviour in anticipation of predictable changes in their environment. The most obvious of these is the daily cycling of light, dark and temperature, which in turn correspond to selective characteristics such as food availability and predator avoidance. Other biological cycles include adaptations to yearly (circannual) oscillations, monthly (circalunar, for example the female menstrual cycle) and twice daily (circatidal) cycles.

The best characterised of these oscillations is the circadian (about 24 h) cycle. For a cycle to be classed as circadian it must fulfil several requirements (Reviewed in Young, 1998). Firstly, the rhythm must be maintained under constant darkness and constant temperature in order to reveal its endogenous nature. Secondly, the rhythm must be capable of being reset by environmental stimuli, such as light, in order to be synchronised with the solar day, thus allowing seasonal adaptations. The rhythm must also be relatively insensitive to changes in temperature (within the normal physiological range for the organism) and have a period within the range of 22-25 h.

Whilst it may seem an obvious advantage for an organism to possess a functional circadian clock, as reflected by the fact circadian clocks have been found in all eukaryotes and some prokaryotes (Dunlap, 1999), increased fitness linked to a functional clock has only been experimentally proven in a couple of cases. Firstly a functional clock with a period corresponding to that of the environmental light-dark (LD) cycle confers a selective advantage in competition experiments performed in cyanobacteria (Ouyang et al., 1998). More recently a functional clock has been linked to increased male reproductive fitness in the fruit-fly, Drosophila melanogaster (Beaver et al., 2002).

Photosynthesis and leaf movement are among the circadian phenomena observed in plants, and in insects the most common examples are adult locomotor and eclosion cycles (emergence of the adult fly from the pupal case). Daily rhythms in body temperature and the sleep/wake cycle are the most familiar phenotypes in humans.

For humans the most obvious effects of the circadian clock can be experienced with "jet lag" and shift work. Jet lag is the term used to describe the effects experienced due to a desynchronised clock when travelling between different time zones. Shift workers have to overcome the body’s signals for sleep when working at
night. It is now also known that certain sleep disorders can be directly linked to clock
dysfunction. These include Familial Advanced Sleep Phase Syndrome (FASPS, Toh
et al., 2001) and Delayed Sleep Phase Syndrome (Archer et al., 2003). The circadian
clock is also assumed to be linked to seasonal depression.

The clock consists of three components, an input pathway, a central oscillator
and an output pathway. The input pathway represents the mechanism by which
environmental stimuli are sensed and the signal brought to the oscillator. Anything
that is described as oscillating will move away from equilibrium before returning to it.
This can be achieved if the product of the process acts as a negative element, feeding
back to slow down the process itself (Dunlap, 1999). A delay in the negative
regulation is required for the oscillation. The system would then need a positive
element to activate the oscillation again and preventing it from stopping.

It is known that both the negative and the positive elements in the oscillator
act on gene transcription. Not only is the transcription of clock genes regulated, it is
thought that components of the oscillator also act to regulate transcription of other
genes, termed “clock controlled genes” (Dunlap, 1999). These genes function in the
output pathway, which mediates physiology and behaviour by temporal ordering of
gene expression.

Research has been conducted in many model organisms for molecular studies,
including mammals, plants, fungi and cyanobacteria. For the main part of this chapter
the molecular mechanism, as it is currently understood, will be discussed for the fruit-
fly Drosophila melanogaster and then compared to that of other species.

1.2 The period gene

The gene period (per) was the first clock locus to be identified (Konopka and
Benzer, 1971) in a screen for mutants in the eclosion rhythm. Three variants were
discovered. One line showed a short period of 19h (per^s), another showed a long
period of 29h (per^l) and the third was arrhythmic (per^0). Genetic mapping revealed
that these mutants arose from three alleles of the same X-linked locus. Each of these
mutants arises from a single point mutation (Baylies et al., 1987, Yu et al., 1987). A
missense mutation replacing Val 243 with Asp produces per^s, Ser 589 is replaced
with Asn in \( \text{per}^s \) and \( \text{per}^0 \) is the result of a nonsense mutation of codon 464 (Figure 1.1).

The gene encodes a protein of 1218 amino acids (Citri et al., 1987). Sequence comparisons between \textit{Drosophila} species reveal five regions of homology (Colot et al., 1988), the N-terminus (including nuclear localisation signal – NLS), PAS, CLD (cytoplasmic localisation domain) and C-domain (Figure 1.1). One of the non-conserved regions is a region of Threonine-Glycine repeats, and thus is termed the "TG" region and is located downstream of the C-domain. This region is known to convey species-specific characteristics of the ultradian male courtship song cycle (Wheeler et al., 1991) and has also been shown to play a role in temperature compensation (Sawyer et al., 1997). Another important region of PER has recently been determined, termed the CCID, which is responsible for the transcriptional repression of its own transcriptional activators (discussed later in this chapter). The CCID contains a second classical bipartite NLS that is more potent than the one found at the N-terminus (Chang and Reppert, 2003).

PAS is an acronym of PER, ARNT and SIM, the three proteins in which this domain was first identified. The domain has approximately 270 residues and contains two degenerate 51aa repeats, and is a site for protein – protein interaction. SIM is the product of \textit{single-minded} and is a basic-helix-loop-helix PAS containing transcription factor. It is required for normal development of midline cells of the \textit{Drosophila} CNS (Nambu et al., 1991). ARNT is the human aryl hydrocarbon receptor translocator (Hoffman et al., 1991). The aryl hydrocarbon receptor (AHR) is a ligand activated transcription factor (Burbach et al., 1992) that forms a heterodimer with ARNT through their PAS domains mediating translocation of AHR to the nucleus (Hoffman et al., 1991). Both proteins contain a bHLH motif, which suggests that the dimer acts as a DNA binding complex as is seen in other HLH heterodimers (Burbach et al., 1992). Unlike the other PAS proteins, PER does not contain a bHLH motif or any other DNA binding domain and therefore is unlikely to have a direct transcriptional function.

Expression of both \textit{per} mRNA and PER protein oscillates with a 24 h period (Hardin et al., 1990; Edery et al., 1994). The mRNA peaks during the early night at ZT14-16 and reaches its minimum during the early day at ZT4. Zeitgeber Time (ZT) is the time set by an environmental stimulus, in the case of light, ZTO represents lights on, ZT12 lights off in a 12hr light, 12hr dark (12:12 LD) regime. The peak in protein
Figure 1.1 Diagrammatic representation of the *Drosophila* PER protein
The known domains of PER are shown as coloured boxes. A and B refer to the PAS domains. C1 – C5 represent the conserved regions of PER proteins as described in Colot et al., 1988. The positions of the point mutations described are shown with an asterix and the regions of interaction with other clock proteins are shown as brackets underneath. Adapted from Chang and Reppert, 2003.
level occurs late at night, 4-6 hours after the mRNA peak at ZT20-22 (Edery et al., 1994). Both the cycling of mRNA and protein levels and the delay in protein accumulation after the mRNA peak suggest that PER acts as a negative element to regulate its own transcription so that mRNA levels fall as the protein levels rise (see Figure 1.2). As the amounts of PER rise, a dramatic increase in the apparent size of PER is also observed (Edery et al., 1994), suggesting that PER undergoes significant post translational modification as a function of time. This increase in size has been attributed to phosphorylation of PER.

It is difficult to derive the protein curve from the RNA curve with a transcriptional model considering the robust amplitude of the protein curve and the substantial delay observed (Zeng et al., 1994), which also suggests translational or post-translational control as an important mechanism for this cycling. This delay is thought to probably only be necessary to generate the 24h period as two long period double-time mutants remove the delay between the mRNA and protein peaks whilst maintaining rhythmic behaviour (Suri et al., 2000). The cycling of the RNA and protein are part of the circadian mechanism. Both the per$^S$ and per$^L$ mutants are rhythmic in LD cycles, but the peaks in mRNA levels occur earlier and later respectively to the peak in per$^+$ (Hardin et al., 1990). Phosphorylation of PER in per$^S$ also occurs earlier and the protein disappears earlier as expected (Edery et al., 1994).

1.3 timeless

The timeless (tim) gene was identified by P-element mutagenesis that gave rise to mutants that were arrhythmic for eclosion (Sehgal et al., 1994). These mutants were also shown to be arrhythmic for locomotor activity under free-running conditions although they were rhythmic in LD. Genetic mapping placed tim on chromosome 2 and positional cloning of tim (Myers et al., 1995) revealed it encoded a protein of 1389 aa. Later it was shown that a fragment of 32aa was missing from the original cDNA (Ousley et al., 1998). Moreover, it was shown that a second methionine was the real start of transcription (Rosato et al., 1997), shortening the product by 23 residues. Myers et al. also showed that the original null mutant termed tim$^{01}$, to be the result of a 62bp deletion that leads to the truncation of TIM to 758aa.
Sehgal et al. (1994) found that in \textit{tim}^0\ mutants, \textit{per} mRNA failed to cycle. Nuclear localisation of \textit{PER} is also blocked in the \textit{tim}^{01} mutants and \textit{PER} accumulation is suppressed (Vosshall et al., 1994). In concert with these observations the \textit{per}^0 mutation blocks cycling of the \textit{tim} transcript and also blocks the nuclear localisation of TIM (Sehgal et al., 1995; Hunter-Ensor et al., 1996). It was therefore concluded that \textit{PER} and TIM require each other to facilitate nuclear entry and that their molecular rhythms are interdependent.

TIM is also temporally expressed with a pattern of mRNA and protein cycling almost identical to that of \textit{PER}. However, the increase in TIM very slightly anticipates the increase in \textit{PER} and the decline of TIM is much more rapid and precedes the decline of \textit{PER} (Zeng et al., 1996). In \textit{tim}^{01} mutants \textit{PER} accumulation is suppressed and residual \textit{PER} proteins are constitutively hyperphosphorylated (Vosshall et al., 1994; Price et al., 1995). Together these observations point towards a role for TIM in \textit{PER} stability as well as its role in nuclear localisation. Phosphorylation is often used to target a protein for degradation and TIM is also phosphorylated, but to a much lower extent that \textit{PER} (Zeng et al., 1996).

There is no reciprocal dependence on \textit{PER} for TIM stability. TIM levels are relatively high in \textit{per}^0 and cycle under LD conditions but not under DD. Low levels are present during the day with levels rising after dark (Zeng et al., 1996). The decline in TIM is much more rapid than that for \textit{PER} in flies, and corresponds to the onset of light, suggesting that TIM is regulated by light (Zeng et al., 1996). Due to the fact that \textit{tim} mRNA does not cycle in \textit{per}^0, the effect of light on TIM must be mediated by a post-translational mechanism such as degradation or translational inhibition. Light has been shown to trigger rapid degradation of TIM via the ubiquitine-proteosome pathway (Myers et al., 1996; Hunter-Ensor et al., 1996; Naidoo et al., 1999). Therefore TIM is a strong candidate for mediating the light resetting of the clock.
Rhythmic cycling of the clock components is illustrated. \( dClk/dCLK \) mRNA (black line) peaks during the early night (black bar) and after an approximately 6h delay their protein products peak in abundance (red line). There is no temporal delay in translation of \( dClk \) and so both the mRNA and protein cycle in phase. When \( \text{per/tim} \) mRNA is at its highest levels \( dCLK \) levels are at a minimum.

1.4 \textbf{\textit{dCLOCK and CYCLE, activators of \textit{per/tim} transcription}}

Both \textit{dCLOCK} (\textit{dCLK}) and \textit{CYCLE} (\textit{CYC}) are bHLH-PAS transcription factors that, as heterodimers, activate \textit{per} and \textit{tim} transcription (Darlington \textit{et al.}, 1998). Activation is mediated by the specific binding of the E-box elements (CACGTG) within the promoters of both genes (Hao \textit{et al.}, 1997; Darlington \textit{et al.}, 1998).

Mutants for the \textit{Clock} gene (\textit{Clk}^{\textit{erk}}) show a semidominant phenotype (Allada \textit{et al}, 1998). Flies homozygous for the mutation show period alterations and half are arrhythmic, whereas homozygotes are uniformly arrhythmic. The mutation is the result of a premature stop codon that eliminates much of the C-terminal where the activation domain of CLK resides. As the DNA binding domain and PAS domains
are maintained, but the truncated protein is unable to activate transcription this explains the dominant phenotype observed. The effects of the cycle
mutants are very similar to those observed for Clk
. Homozygotes are arrhythmic but heterozygotes show robust rhythms with an altered period of approximately one hour longer than the per genotype would predict (Rutila et al, 1998). The cyc
mutation is the result of a premature stop codon also, eliminating 60% of the C-terminus of the protein, including all of the PAS-B domain.

PER and TIM expression in both of these mutants are effected in the same way with very low protein titres due to reduced levels of transcription, comparable to the low trough values in WT (Allada et al., 1998; Rutila et al., 1998). The continual, low level expression of TIM is responsible for rendering both of these mutants insensitive to light on transition in LD cycles. These mutants are even less light sensitive than per
and tim
.

CLK shows temporal changes in both abundance and phosphorylation pattern, like PER and TIM (Lee et al, 1998). There is no temporal delay between the mRNA and protein levels as seen with per and tim. CLK peaks at ZT23.5 but it is not clear whether it peaks until ZT4 or whether it declines and peaks again at ZT4 (Lee et al, 1998; Rutila et al, 1998). Temporal phosphorylation of CLK has been shown to have a key role in its function, apparently negating effects of changing the levels and timing of clock expression (Kim et al., 2002).

CLK also appears to have a role in mediating light responses (Kim et al., 2002) as increasing expression levels causes differences in locomotor activity pattern. Flies show a substantially longer phase of the morning activity and show longer bouts of increased activity following administration of a light pulse. This effect is seen regardless of the time at which the light pulse is administered.

A novel arrhythmic allele of clock has recently been reported that shows reduction in CLK activity as a result of disrupted splicing of the transcript (Allada et al., 2003). Flies carrying this allele show arrhythmic behaviour despite the persistence of weak molecular rhythms. It is therefore likely that the amplitude of PER and TIM cycling is a key factor in establishing behavioural rhythms. These flies also give support to CLK having a role in light responses as in LD there is a lights-off startle response instead of the lights-on startle response shown by wild type flies (Allada et al., 2003).
Clk also appears to be a master regular of circadian rhythmicity. Not only has microarray data suggested that all rhythmic gene expression is dependent on Clk (McDonald and Rosbash, 2001) but misexpression of Clk generates ectopic clocks in cells where there is not normally rhythmic gene expression (Zhao et al., 2003). These ectopic clocks potently affect behavioural rhythms, suggesting that they form functional connections with output pathways.

1.5 The first feedback loop – PER:TIM repression of per/tim transcription

In order for both PER and TIM to feed back and negatively regulate their own transcription they would first have to enter the nucleus. PER and TIM have been shown to physically associate in in-vitro and in ex-in-vivo studies (Saez and Young, 1996; Gekakis et al., 1995) and that formation of the heterodimer facilitated its nuclear entry (Saez and Young, 1996). It was also thought that formation of this dimer stabilised PER from premature degradation (Price et al., 1995; Vosshall et al., 1994; Zeng et al., 1996). It was suggested that nuclear entry of the heterodimer was facilitated by binding masking the cytoplasmic localisation domain (CLD) present in each protein allowing the nuclear localisation domains (NLS) to signal nuclear transport (Saez and Young, 1996).

PER nuclear entry is essential for the repression of dCLK:CYC mediated transcription (Chang and Reppert, 2003). The region of PER responsible for this repression has been mapped to amino acids 764-1034, termed the dCLK:CYC inhibition domain (CCID, Chang and Reppert, 2003). Interestingly, the authors also found within this region (813-840) a second NLS that is more potent than the original NLS in the N terminus, suggesting that it is this signal that is responsible for nuclear entry.

Shafer and co-workers (2002) have shown that during the early night TIM is restricted to the cytoplasm of lateral neurons whilst PER is situated in both cellular fractions, which is inconsistent with the obligate heterodimer theory. Nuclear entry of PER is first detected at the time of initial repression revealed in total head extracts (Hardin et al., 1990; Edery et al., 1994), yet TIM is still confined to the cytoplasm. It may be that TIM enters the nucleus with PER but is then exported back to the cytoplasm or that formation of the heterodimers triggers a post-translational event for
PER, enabling PER nuclear transport. This is supported by recent data that shows that TIM is indeed shuttled between cellular compartments until it is retained in the nucleus by PER late at night (Ashmore et al. 2003). Although both PER and TIM can enter the nucleus independently they each have influence on each other's nuclear entry.

S2 cell culture experiments have shown that PER and TIM when co-expressed reduce CLK:CYC activation (Darlington et al., 1998). The presence of CLK was also shown to be essential for this. Both PER and TIM co-purify with CLK, and PER and CLK have been shown to interact in vitro (Lee et al., 1998). Further data reveals that CLK is present in limiting amounts and is found almost exclusively in complex with CYC throughout the day (Bae et al., 2000). CYC is present in substantially larger quantities (200-fold higher than CLK) but does not interact with PER or TIM unless it is in complex with CLK, suggesting that CLK is required in order for CLK:CYC to associate with PER and TIM. It has also been shown that PER alone can repress per/tim transcription (Rothenfluh et al., 2000a), suggesting a more important role for PER than TIM in negative feedback. More recent data has shown that TIM on its own cannot mediate negative feedback (Ashmore et al., 2003).

1.6 PER and TIM phosphorylation and degradation

The doubletime (dbt) gene encodes a protein (DBT) closely related to the human Casein Kinase 1ε (Kloss et al., 1998). Six mutants have been discovered to date (Kloss et al., 1998; Rothenfluh et al., 2000b; Suri et al., 2000). Two of these, termed dbt^d (26.8h), dbt^s (18h) show altered period lengths. Both dbt^d and dbt^s are semidominant alleles and are the result of single amino acid changes in conserved regions of the kinase. The third mutant, dbt^p is the result of a P-element insertion and abolishes most dbt expression. This mutation is embryonic lethal, which is not suppressing as dbt, also termed discs-overgrown, is an important developmental gene (Zilian et al., 1999). The fourth allele dbt^p results in arrhythmia in homozygotes and long periods in heterozygotes (Rothenfluh et al., 2000b). All of these alleles alter the pattern of PER phosphorylation and degradation.
Although $dbt^p$ confers pupal lethality it is possible to look at its effects on PER in known clock cells in the brain of third instar larvae. In $dbt^p$ PER is expressed constitutively in both LD and DD and the high, uniform electrophoretic mobility of the protein indicates that it is hypophosphorylated (Price et al., 1998). As levels of per transcription are the same as those observed in WT flies the accumulation of PER in $dbt^p$ indicates higher stability rather than increased transcription. This is supported by the observation that in $dbt^p$ flies PER accumulates 2 h earlier and disappears 6 h earlier than in WT flies, further suggesting that stability of PER is affected. PER also accumulates in constant light and therefore no longer requires TIM for stability. DBT has been shown to bind PER both in vitro and in Drosophila cells (Kloss et al., 1998). PER also contains many consensus sequences for casein kinase phosphorylation, with nested clusters of these enriched in the N terminus, further linking DBT to a role in the phosphorylation of PER.

$tim$ mRNA is seen to oscillate in LD but not in DD in $dbt^p$ (Price et al., 1998). As light degrades TIM (Myers et al., 1996; Hunter-Ensor et al., 1996) the PER/TIM complex is no longer formed which releases the repression of $tim$ transcription, allowing cycling to occur. The elimination of cycling under DD infers that even very low levels of TIM are sufficient to form complexes with the now stabilised PER and continually repress transcription. Stabilisation of PER now removes the temporal constraints imposed on PER by phosphorylation and subsequent degradation in WT flies.

$dbt$ itself is constitutively expressed at both the mRNA and protein levels (Kloss et al., 1998 and 2001) although it shows robust changes in sub-cellular localisation (Kloss et al., 2001). It appears to be a predominantly nuclear protein by default, always being detected in the nuclei of $per^0$ flies. DBT is always found within the nucleus but accumulates in the cytoplasm as a function of time. Timing of cytoplasmic accumulation corresponds to times of PER production, implying that cytoplasmic PER is required for cytoplasmic DBT accumulation. It is plausible that PER interacts with newly produced DBT within the cytoplasm and restricts its nuclear translocation. Movement of DBT exclusively to the nucleus occurs at the time of PER:TIM nuclear translocation, suggesting that the three proteins probably translocate as a complex (Kloss et al., 2001). DBT is found in complex with PER at all times throughout the day and this association is independent of TIM. However cycles in PER phosphorylation reflect cyclic changes between PER:TIM and DBT,
suggesting that the complex may effect both phosphorylation of PER and nuclear translocation.

DBT affects the stability of PER both within the cytoplasm and the nucleus (Price et al., 1998). It is likely that TIM prolongs the light independent phosphorylation of PER as increases in PER phosphorylation occur after TIM is degraded in response to light (Kloss et al., 2001). DBT is either responsible for the gating of PER nuclear stability or nuclear entry as in dbf\textsuperscript{d} flies PER accumulates within the nucleus later than in wild-type (Bao et al., 2001). This is not a result of reduced PER levels as higher levels of PER accumulate faster than in wild-type but are restricted to the cytoplasm. Despite this there is a later accumulation and faster decline in per mRNA, thus shortening the lag between mRNA and protein peaks in these flies. It is therefore likely that dbf\textsuperscript{d} decreases the positive feed-back loop, which will be discussed later in this chapter.

Although DBT plays a key role in PER turnover two other proteins are known to be involved. Once PER has become progressively phosphorylated by DBT it interacts with phosphorylated SLIMB (Grima et al., 2002). SLIMB is an F-Box/ WD40-repeat protein of the ubiquitin ligase SCF complex and stimulates PER degradation via the ubiquitin-proteasome pathway (Grima et al., 2002; Ko et al., 2002). Over expression of SLIMB increases PER turnover, whilst knocking out SLIMB by RNAi in S2 cell culture assays prevents degradation of PER (Ko et al., 2002). Removal of the F-box region of SLIMB leads to accumulation of phosphorylated PER and behavioural arrhythmia. Although mutant flies show increased levels of highly phosphorylated PER and TIM in DD, both proteins cycle normally in LD, suggesting that SLIMB acts in the circadian control of degradation but not in light-controlled degradation and that these mechanisms are different (Grima et al., 2002).

It is known that DBT, as other Casein Kinases requires a phosphoserine in its target site, suggesting that other kinases are required to act before DBT. One such candidate for this role is Casein Kinase 2 (CK2, Lin et al., 2002; Atken et al., 2002). Mutations in either the alpha or beta subunit of this enzyme result in reduced enzyme activity leading to long behavioural periods and delayed nuclear accumulation of PER and TIM. Mutants in the beta subunit, termed Andante, also show accumulation of PER and TIM to abnormally high levels (Atken et al., 2002). CK2 directly phosphorylates PER in-vitro (Lin et al., 2002), suggesting that CK2 plays an important role in PER turnover.
Like PER, TIM also undergoes rhythmic changes in phosphorylation (Martinek et al., 2001). TIM is phosphorylated by SGG, an orthologue of glycogen synthase kinase-3 (Martinek et al., 2001). SGG has the opposite role of DBT in that phosphorylation of TIM advances rather than delays nuclear entry.

A diagrammatic representation of the feedback loop is given in Figure 1.3.

1.7 Cryptochrome and light entrainment of the clock

Cryptochromes are blue light photoreceptors that were first discovered in *Arabidopsis* and have since been identified in many other species, including *Drosophila* and mammals (Reviewed in Cashmore et al., 1999). They show high sequence homology with the 6-4 photolyase family but have no role in DNA repair. Sequence alignment of cryptochromes and 6-4 photolyases shows high homology in the two regions responsible for binding of a chromophore (flavin) but differ greatly in their carboxy-termini (Yang et al., 2001).

The *Drosophila* *cry* gene was identified through the *cry*^b^ mutation, a single amino acid mutation resulting in an Asp → Asn substitution at amino acid 410, a highly conserved position on the flavin binding site (Stanewsky et al., 1998; Emery et al., 1998). Behavioural experiments show that these mutants entrain normally to 12:12 LD cycles, have normal 24hr rhythmicity under DD conditions and can re-entrain to a second light regime (Stanewsky et al., 1998). However, unlike wild type flies, *cry*^b^ flies do not phase shift in response to a 10min light pulse administered during the subjective night. Flies hemi/heterozygous for the *norpAP41* (no receptor potential A) mutation are visually blind but entrain to 12:12 LD cycles normally (Stanewsky et al., 1998). Double mutants for both *norp*^A^ and *cryb* show severely compromised light entrainment, revealing that the *cry*^b^ mutation dramatically disrupts the circadian flow of light information via CRY. As the *cry*^b^ mutation is located at such a highly conserved position within the flavin (the catalytic chromophore) binding site, it is feasible that the reduced efficiency is due to the reduced ability of CRY^b^ to activate any hypothetical signalling partner. Finally these results may also indicate that the light input pathways to the clock, both ocular and extra-ocular, may
Figure 1.3 Diagrammatic representation of the first auto regulatory feedback loop. During the day dCLK:CYC heterodimers activate transcription of per and tim. TIM is destroyed by light and PER is subjected to phosphorylation by DBT, triggering its degradation. At night TIM is stable and forms a complex with PER:DBT and is also phosphorylated by SGG. This complex moves to the nucleus where PER acts to repress CLK:CYC activation of transcription.
converge on CRY, which is responsible for transducing the signal to the pacemaker through light dependent redox reactions.

Like other clock genes, *cry* cycles at both the mRNA and protein levels (Emery *et al.*, 1998). In LD cycles the mRNA peaks at ZT1-ZT7 with a trough at ZT17-ZT19. CRY levels are low during the day when the mRNA levels are high and increase during the night to peak at ZT23. The increase in protein therefore occurs ahead of the increase in mRNA. When the mRNA peaks two hours later at ZT1 CRY is already reduced to 50% of its peak value, suggesting that a translational or post-translational mechanism is important in CRY cycling.

In constant darkness the cycling of both mRNA and protein are observed to be very different from that observed in LD. The mRNA amplitude is much lower and the pattern different. Levels of CRY increase continually throughout the subjective day and night with an initial level corresponding to the ZT23 peak of the day before DD entrainment was initiated. This is indicative that CRY cycling is largely light dependant. CRY expression was also studied in arrhythmic mutants for the other clock genes *per*, *tim*, *Clock* and *cycle*. In all of these, little or no mRNA cycling was observed, with levels being relatively low in *per*0 and *tim*0, and relatively high levels in *Clock* and *cycle*0 (Emery *et al.*, 1998). The levels were also relatively high in *per*0;*Clock* and *per*0;*cycle*0 double mutants, indicating an epistatic effect of *Clock* and *cycle* over *per*. Relative CRY levels correlated with the relative mRNA levels, being higher in *Clock* and *cycle*0 than in *per*0 and *tim*0. Robust cycling of CRY was also observed in LD cycles. These results together indicate that *cry* is regulated at the transcriptional level by the clock and regulated at the translational or post translational level by light, as demonstrated by over expressed CRY, which still shows robust rhythms due to its light sensitivity (Emery *et al.*, 1998).

In *cry*0 mutants levels of PER and TIM stay high during the day, and are present in both hyper- and hypophosphorylated forms in a temporally unchanging manner (Stanewsky *et al.*, 1998). The absence of rhythmic expression in LD also highlights the lack of response of TIM to light. Therefore *cry*0 could affect elements of the light entrainment pathway or could affect a protease whose target is TIM. The *cry*0 mutation is a hypomorphic mutation caused by a missense mutation at amino acid 410, which is a conserved residue within a flavin binding site, resulting in the substitution of Asp for Asn. Transcription of *cry* does not cycle in these mutants. The mRNA is expressed at 65-70% of that seen in WT, but this is not enough to
explain the lack of CRY seen in Western analysis. The anti-CRY antibodies were produced using a peptide fragment upstream of the mutation site, suggesting that $cry^b$ may be acting as a null allele. The mutation is within a flavin binding site and perhaps results in rapid degradation of CRY.

Studies into CRY's role in the circadian mechanism have pointed towards an interaction between TIM and CRY to be important in regulation of rhythmicity (Ceriani et al., 1999). The effects of CRY on other clock proteins were studied in S2 cells in culture. CRY was shown to have no effect on CLOCK/CYCLE activation of the per or tim E-box regions. The PER/TIM inhibition was seen to exist on CLOCK/CYCLE in the absence of CRY, but when CRY was added in light conditions the PER/TIM complex could no longer inhibit CLOCK/CYCLE activation of transcription. When CRY$^b$ was used in place of CRY this effect was not seen.

Coimmunoprecipitation (coIP) assays were performed and CRY and TIM were observed to precipitate in the same complex. To test whether this interaction was dependent on light, cells used for these assays were cultured under light or dark conditions. The interaction was found within the dark cultured cells. A yeast two-hybrid assay was also conducted to test for TIM/CRY interaction. TIM was shown to interact with CRY, but only in light. This result is in contrast with the coIP result. This could be attributed to two possibilities. Firstly, in the yeast system the interaction observed occurs in the nucleus, whereas in the coIP all cellular components are studied. Secondly, it is possible that other proteins are present in the S2 cells used for the coIP assays that could contribute to detection of CRY and TIM in the same complex in darkness. CRY$^b$ was also shown to not interact with TIM.

Transfection of a CRY-GFP reporter into cells expressing only TIM shows CRY to remain cytoplasmic. As already stated, it is known that TIM remains in the cytoplasm in the absence of PER. When PER and TIM are present CRY is shown to translocate to the nucleus, inferring that the PER/TIM complex is required to move CRY to the nucleus before it can mediate any regulation.
1.8 The second, interlocked feedback loop

Whilst PER:TIM interaction with CLK:CYC inhibits *per* and *tim* transcription this interaction appears to have the opposite effect on *Clk* transcription, suggesting that CLK:CYC acts to repress *Clk* transcription and PER:TIM interaction relieves this (Allada *et al.*, 1998; Rutila *et al.*, 1998; Glossop *et al.*, 1999). This is supported by the fact that *Clk* mRNA levels are constitutively high in both *Clk*<sup>irk</sup> and *cyc*<sup>o</sup> mutants (Allada *et al.*, 1998; Rutila *et al.*, 1998). It was found that this repression of *Clk* was facilitated by CLK:CYC activation of VRI, which acts to repress *Clk* (Blau and Young, 1999).

VRI, the product of the *vrille* gene, is a basic-zipper transcription factor. It is activated by CLK:CYC via E-box elements within it promoter (Blau and Young, 1999). Overexpression of *vrille* in pacemaker neurons causes reduction in *Clk* mRNA levels leading to reduction or absence of *per* and *tim* and leads to long behavioural rhythms. Conversely flies with reduced VRI levels show short behavioural rhythms, consistent with a role in repression. As expected for a gene under CLK:CYC activation *vri* mRNA cycles in phase with *per* and *tim* mRNA, but unlike PER and TIM there is no temporal delay in formation of the VRI protein (Glossop *et al.*, 2003). Thus VRI cycles in opposite phase to *Clk* mRNA.

Recent data suggests that VRI binds E4BP4 consensus binding sequences within the *Clk* promoter region as a homodimer to facilitate repression (Glossop *et al.*, 2003). These sequences are also found within the *cry* promoter and VRI also acts to repress *cry* transcription. However VRI overexpression resulting in constant high levels of VRI does not full repress *Clk* or *cry* in wild-type flies in either the late evening or early morning. This suggests temporal differences in VRI’s ability to repress transcription that could involve another factor.

Searches for homologues for VRI revealed one other gene that showed cycling of mRNA levels within the fly head, termed *Pdpl* (Cyran *et al.*, 2003). *Pdpl* (*PAR domain protein 1*) and *vrille* both belong to a group of b-ZIP transcription factors with highly conserved DNA binding domains. Like *vrille*, *Pdpl* is directly activated by CLK:CYC via E-box elements although their RNA and proteins accumulate with a different phase (Cyran *et al.*, 2003). Heterozygotes for a *Pdpl* null allele (homozygotes are lethal) show long behaviour rhythms, opposite to that seen for VRI reduction, suggesting that these genes have opposite functions. This is supported by a
synergistic effect of combining VRI over expression with PDP1 reduction. Consequently PDP1 was shown to be able to bind and activate transcription from the Clk promoter.

Six isoforms of Pdpl are present within the fly head but only one, Pdplz, shows cycling at the mRNA level. Peaks in Pdpl mRNA occur 3-6h after that of vri and PDP1 also occur 3h later than VRI. Both VRI and PDP1 bind the same E4BP4 consensus sequences within the Clk promoter, suggesting that they compete for binding sites. VRI over expression in wild-type flies suggests that VRI out competes PDP1 when at high levels, so PDP1 is likely to only be able to activate Clk after VRI levels fall. One possibility for the temporal regulation of VRI levels is a very short half-life of the mRNA.

Both feedback loop are illustrated in Figure 1.4.

![Figure 1.4 Interlocked feedback loops in Drosophila (Adapted from Cyran et al., 2003). CLK:CYC heterodimers activate per, tim vri and Pdpl transcription. PER:TIM heterodimers then feedback to block activation of their own transcription. VRI feeds back to negatively regulate transcription of Clk. Pdpl is also activated by CLK:CYC and activates Clk transcription once competition for binding sites in the promoter are relieved by falling VRI levels.](image-url)
1.9 Outputs from the Clock

Several known components of clock output pathways have now been identified. These genes are controlled by the clock at either transcriptional or translation levels and function to transduce circadian information along output pathways that result in circadian phenotypes such as locomotor activity and eclosion.

LARK is an essential RNA binding protein that functions in the circadian regulation of adult eclosion, as well as having an important role in embryonic development (McNeil et al., 2001). Mutants for lark show normal locomotor activity rhythms but the eclosion rhythm is disrupted and over expression of lark delays the phase of eclosion (Newby and Jackson 1993; McNeil et al., 1998). Although LARK is expressed in many cell types it co-localises with CCAP, a known factor influencing ecdysis (eclosion, Ewer and Truman 1996; Gammie and Truman 1999). In these cells LARK is found within the cytoplasm, rather than in the nucleus as in other cell types (Zhang et al., 2000). Although the RNA is expressed constitutively the LARK protein cycles robustly in a circadian manner, thus although transcription is not circadianly regulated some post-transcriptional modifications are (McNeil et al., 1998, Newby and Jackson 1996). Proof that these post transcriptional modifications are circadianly regulated is seen in per^0 flies, where no cycling of LARK immunoreactivity can be detected in CCAP expressing cells, where it is expressed at constitutively high levels (Zhang et al., 2000).

In the CCAP cells LARK levels reach their minimum at night, several hours prior to eclosion (McNeil et al., 1998). Along with gene dosage analysis (Newby and Jackson 1996) this suggests that LARK acts as a repressor of eclosion. It has been suggested that changes in LARK abundance may regulate a factor that is important for CCAP release (Zhang et al., 2000).

Another output factor is PDF (pigment dispersing factor), which influences locomotor activity. Mutants for PDF shown abnormal behaviour in LD, with no morning peak anticipating the lights on transition and an early evening peak in activity, whilst in DD there is a high incidence of arrhythmicity (Renn et al., 1999). Misexpression of PDF leads to increased activity and arrhythmia (Helfrich-Forster 2000). Normal expression of PDF is found within the ventral lateral neurons of the brain, LNvs, which are known pacemaker cells. There is no oscillation in mRNA levels however PDF cycles in the termini of axons projecting from the small LNs.
Both the Clk\textsuperscript{irk} and cyc\textsuperscript{0} mutants lead to loss of pdf mRNA and protein in larval neurons and the adult sLNvs but CLK:CYC are not required for pdf expression in adult lLNvs (Park et al., 2000). Loss of cycling in the axonal termini in per\textsuperscript{0} and tim\textsuperscript{0} suggests that PER and TIM are required for correct posttranscriptional regulation of PDF (Park et al., 2000).

dFMRF1 is the Drosophila homologue of FMRF1, the mutation of which leads to fragile-X syndrome in humans, which is the most common form of inherited mental retardation (Inoue et al., 2002). In Drosophila loss of function mutants lead to arrhythmic locomotor activity, altered gating of eclosion and normal courtship behaviour (Inoue et al., 2002; Dockendorff et al., 2002). Overexpression of dFMRF1 causes lengthening of the period of locomotor activity (Dockendorff et al., 2002). Loss of function mutants also revealed that normal dFMRF1 activity is required for circadian oscillation of CREB, which is a known clock controlled gene (Belvin et al., 1999). dFMRF may also affect the PDF pathway, as although PDF expression remains normal in the mutants, there are subtle defects to the branching of axonal projections (Dockendorff et al., 2002).

As stated dCREB2 exhibits circadian fluctuation in activity and when mutated shows short period rhythms (Belvin et al., 1999). In these mutants per oscillations are dampened and in per\textsuperscript{0} mutants dCREB2 activity rhythms are eliminated. Another output factor connected to CREB is the cAMP dependent Protein Kinase A (PKA, Majercak et al., 1997). DCO mutants, which are mutants of the major catalytic subunit of PKA show arrhythmic locomotor activity although normal eclosion rhythms persist.

1.10 Location of known clock cells within the Drosophila brain

There are six distinct clusters of neurons within the brain that express both PER and TIM along with many glial cells (Kaneko et al., 2000; Kaneko and Hall 2000). Three of these are located within the dorsal protocerebrum and are known as dorsal neurons (DNs). The other three are found within the anterior lateral cortex, close to the inner margin of the medulla. These are termed lateral neurons (LNs).

The LNs are further divided into three groups (Helfrich-Forster, 1995; Kaneko 1998). One of the clusters is located relatively dorsal to the others and these six
neurons are named dorsal lateral neurons (LNds). The two lateral groups (LNvs) are separated by the size of their somata, with one group having relatively small somata (s-LNvs) and the other having relatively large ones (l-LNvs). The four to five l-LNvs project into the medulla, showing large arborisations over the surface of the medulla and also towards the s-LNvs and into the posterior optic tract (Helfrich-Forster, 1995). The four or five s-LNvs send processes into the superior protocerebrum, arborizing dorso-frontal to the calyces of the mushroom bodies. Here they appear to contact another group of neurons (DN3, see below) that project towards the pars intercerebralis and then via the median bundle towards the oesophageal foramen (Helfrich-Forster, 1995).

In total 55 neurons of the DN class exist in each hemisphere and they are subdivided into three further groups, DN1, DN2 and DN3 (Kaneko and Hall 2000). In the adult the DN1 number 15 neurons located in the dorsalmost cortex which project ventrally towards the region where the sLNvs terminate, before projecting medially to form a V-shaped commisure in the superior protocerebrum. This commisure is located above and anterior to the calyces of the mushroom bodies. The DN2 only number two cells located above the calyces of the mushroom bodies. Within the dorsolateral cortex, lateral to the lateral horn are found the DN3 group. These cells project medially, below the commisure formed by the DN1, and terminate in the pars intercerebralis.

The LNvs also produce the output factor PDF and are known to be required for robust behavioural rhythms (Helfrich-Forster 1995; Kaneko and Hall 2000). Mutants of disconnected are devoid of both LNds and LNvs and whist they entrain to LD cycles they are largely arrhythmic in DD (Dushay et al., 1989; Hardin et al., 1992; Zerr et al., 1990). Those individual flies that are rhythmic were found to have some sNLvs and their neurite projections, suggesting that these neurons are important for behavioural rhythmicity (Helfrich-Forster, 1998). Ablation of the LNvs by other means also generated arrhythmic individuals under DD but also show abnormal behaviour in LD as their evening peaks are advanced by 0.5h (Renn et al., 1999). Those individuals that are rhythmic in DD show short behavioural rhythms. Flies that have their LNvs ablated and are also mutants of disconnected show combined effects of the single deficiencies, showing that the LNds must also have a role in behavioural rhythmicity (Kaneko et al., 2000). As some individuals are still rhythmic whilst lacking all or most of the lateral neurons suggests that other PER/TIM expressing
cells are important. This is supported by the fact that PER expression in some glial cells is sufficient to maintain weak behavioural rhythms (Ewer et al., 1992).

All of the cells discussed in this section are mapped in Figure 1.5.

1.11 The clock in other organisms

The circadian clock is being studied in several model organisms. A brief summary of how the mechanism works in several of these organisms is given in the sections below.

1.11.1 Cyanobacteria

Studies of the circadian clock in cyanobacteria have been carried out on *Synechoccus elongatus*. The three main components are termed KaiA, KaiB and KaiC (Ishiura et al., 1998). The promoter of the *kaiA* gene gives rise to a monocistronic mRNA, whilst transcription of the *kaiBC* promoter produces a dicistronic mRNA. Both transcripts are produced rhythmically (Ishiura et al., 1998) and both the KaiB and KaiC proteins are rhythmically abundant (Xu et al., 2000). As with all other organisms a transcription/translation feedback loop drives the circadian rhythm. KaiA is a positive regulator of the KaiBC promoter, producing KaiB and KaiC proteins. KaiC is rhythmically phosphorylated at serine and threonine residues to produce the clock active PKaiC during the subjective night (Xu et al., 2003). KaiA enhances KaiC phosphorylation whilst KaiB acts to inhibit phosphorylation thus between these two proteins the phosphorylation state of KaiC is controlled via Kai complex formation.

Constitutive expression of KaiC results in low expression levels from the kaiBC promoter, suggesting that KaiC negatively regulates its own transcription (Ishiura et al., 1998). However KaiA mediated activation of the kaiBC promoter has been shown to be KaiC dependant, suggesting that KaiC also plays a positive role in its regulation (Iwasaki et al., 2000). Although KaiA acts to produce KaiB and KaiC rhythmically it has recently been shown that the kaiBC promoter is not required for circadian rhythmicity (Xu et al., 2003). In fact it is the ratio of KaiC:PKaiC and the phosphorylation induced rate of degradation of KaiC that is important in generating...
Figure 1.5 Position of clock neurons within the *Drosophila* brain
A and B) Position of the lateral neurons and their projections (taken from Helfrich-Forster, 1995). C) Position of both the lateral and dorsal neuron subgroups and their projections (Taken from Kaneko and Hall, 2000).
circadian rhythmicity. KaiC is capable of autophosphorylation \textit{in-vitro} but it is likely that other factors influence phosphorylation \textit{in-vivo}. Two such candidates are the histidine kinase SasA, which has been shown to interact with KaiC (Iwasaki \textit{et al.}, 2000), and the bacteriophytochrome CikA that has conserved histidine kinase domains and acts in the light input pathway (Scmitz \textit{et al.}, 2000).

This rhythmmical cycling of protein stability and the presence of auto regulatory feedback loops is a similarity between clocks of different organisms. Another similarity exists in the rhythmic sub-cellular localisation of the clock components in \textit{Synechoccus}. It has been suggested that after synthesis KaiC interacts with KaiA in the cytosol, where KaiC is phosphorylated, peaking in phosphorylation at CT16 (Kitayama \textit{et al.}, 2003). At this time the total cellular levels of KaiB peak but the cytosolic levels of KaiB do not peak for another 4h at CT20. At CT20 the formation of the KaiB:KaiC complex is at its highest and this also corresponds with the decline in KaiC phosphorylation. Thus although the Kai proteins share no homology with clock components of higher organisms similarities in the regulation of the circadian clock can be seen.

Figure 1.6 shows a diagrammatic representation of the clock in Cyanobacteria.

1.11.2 \textit{Neurospora}

The three main components of the \textit{Neurospora} circadian clock are \textit{frequency} (\textit{frq}), \textit{white collar}-1 (\textit{wc}-1) and \textit{white collar}-2 (\textit{wc}-2). Like several components of other clocks WC-1 and WC-2 are PAS proteins that interact to form the WHITE COLLAR COMPLEX (WCC), which activates transcription of \textit{frq} in a rhythmic manner (Loros and Dunlap, 2001). In fact WC-1 is a sequence homologue of BMAL1 and CYC (Loros and Dunlap 2001). FRQ proteins start to accumulate during the early morning and quickly enter the nucleus where it interacts with the WWC through WC-2 to negatively regulate its own transcription (Garceau \textit{et al.}, 1997; Denault \textit{et al.}, 2001). Thus an auto regulatory feedback loop is also present in \textit{Neurospora}.

Another common factor with the \textit{Drosophila} system is the presence of a lag between the mRNA and protein peaks for \textit{frq}, suggesting that post transcriptional regulation is required to generate the rhythm (Aronson \textit{et al.}, 1994; Garceau \textit{et al.},
Figure 1.6 The Cyanobacteria clock
KaiA activates transcription from the kaiB/c promoter and then promotes phosphorylation of the KaiC protein. KaiB works antagonistically to inhibit KaiC phosphorylation and so KaiA and KaiB determine the ratio of KaiC:PKaiC. This ratio determines KaiC’s effect on transcription of both the kaiA and kaiB/c promoters.
Whilst FRQ represses transcription it also promotes WC-1 synthesis from existing mRNA (Lee et al., 2000). As WC-2 is expressed at constantly high levels the WCC is formed with the new WC-1 and sequestered by the FRQ proteins present at this time. FRQ is also subjected to phosphorylation that triggers its turnover (Lui et al., 2000) and at this time FRQ levels begin to decline, releasing WCC to initiate transcription (Lee et al., 2000). Two kinases have been shown to interact with FRQ, these are a calcium/calmodulin-dependent kinase and Casein kinase II (Yang et al., 2001; 2002; 2003). The role of CKII has been studied further and has been shown to be involved in the clock mechanism (Yang et al., 2003).

A novel PAS protein VIVID (VVD) is also involved in the circadian mechanism within the input and output pathways, although it is not part of the central mechanism (Heintzen et al., 2000). Temperature compensation of the Neurospora clock is achieved through two alternate translation initiation sites in frq that are differentially favoured with temperature (Garceau et al., 1997; Lui et al., 1997). An illustration of the Neurospora clock is given in Figure 1.7.

1.11.3 Mammals

The mammalian clock shows a larger degree of similarity to the Drosophila mechanism than in those species discussed previously. However, as expected, it is more complex. Firstly due to genome duplication events (Clayton et al., 2001) here are several copies of some of the clock genes known to function in Drosophila. For instance there are four mPer genes and two mCry’s (Clayton et al., 2001). The first notable difference is that although many elements are conserved between Drosophila and mammals the way in which they interact is very different. Initially there was controversy over the role of mTIM in the mammalian clock (Sangoram et al., 1998; Zylka et al., 1998). More recent studies have revealed that two copies of tim exist in insects and that the second copy is the true homologue of mTIM and has been termed tim2 in Drosophila (Benna et al., 2000). In Drosophila tim2 has no known circadian function. Mammals only have one copy of tim and so it is thought that the insect tim has arisen and acquired a circadian function after divergence of the insect and mammalian lineages (Benna et al, 2000). It is therefore not surprising that the role of DmTIM in Drosophila has been replaced by another protein in mammals.
The WHITE-COLLAR complex (WC), comprising of WC1 and WC2 activates transcription of *frq*. FRQ then feeds back to inhibit WCC activation. At the same time FRQ increases the translation of *wc1* RNA to form WC1, which binds WC2 to form WCC. FRQ is phosphorylated by CKII which leads to its degradation, thus relieving FRQ repression of WCC and allowing activation of transcription once more.

**Figure 1.7** The FRQ/WCC feedback loop in *Neurospora*

The WHITE-COLLAR complex (WC), comprising of WC1 and WC2 activates transcription of *frq*. FRQ then feeds back to inhibit WCC activation. At the same time FRQ increases the translation of *wc1* RNA to form WC1, which binds WC2 to form WCC. FRQ is phosphorylated by CKII which leads to its degradation, thus relieving FRQ repression of WCC and allowing activation of transcription once more.
Figure 1.7 The FRQ/WCC feedback loop in *Neurospora*

The WHITE-COLLAR complex (WC), comprising of WC1 and WC2 activates transcription of *frq*. FRQ then feeds back to inhibit WCC activation. At the same time FRQ increases the translation of wc1 RNA to form WC1, which binds WC2 to form WCC. FRQ is phosphorylated by CKII which leads to its degradation, thus relieving FRQ repression of WCC and allowing activation of transcription once more.
As in *Drosophila* CLK and BMAL1 (the mammalian homologue of CYC) act as positive factors to initiate transcription of the mPer and mCry genes in a rhythmic manner (Gekakis *et al*., 1998; Yamaguchi *et al*., 2000a). In contrast to *Drosophila* it is BMAL1 that cycles in abundance whilst CLK is constitutively expressed (Shearman *et al*., 2000; Tamaru *et al*., 2000; Lee *et al*., 2001). The mCRYs and mPERs cycle in abundance in a similar phase to each other but opposite to that of BMAL1 suggesting, as with *Drosophila*, that a negative feedback loop is operating (Miyamoto and Sancar 1999; Kume *et al*., 1999). In fact mCRY is at its highest levels when there is maximum inhibition of CLK:BMAL1 activation and either mCRY is capable of potently inhibiting CLK:BMAL1 suggesting that it is CRY that mediates the inhibition (Kume *et al*., 1999; Shearman *et al*., 2000). Whilst negatively regulating CLK:BMAL1, mPER2 also exerts a positive effect on BMAL1 production (Shearman *et al*., 2000).

In order to be able to inhibit transcription CRY must first enter the nucleus. Both mCRY proteins interact with all of the mPERs to form various heterodimers (Kume *et al*., 1999). During times of transcriptional repression both mPER1 and mPER2 are found in complex with the mCRYs (Lee *et al*., 2001). It appears that both mPERs and mCRYs rely on the formation of such heterodimers to facilitate their nuclear entry as neither are confined to the cytoplasm in the absence of the other (Kume *et al*., 1999; Lee *et al*., 2001). There are also reports that an mPER1:mPER3 dimer can enter the nucleus without the aid of CRY (Yagita *et al*., 2000). As well as facilitating nuclear entry formation of the mCRY:mPER2 heterodimer is thought to stabilise mPER2, as TIM is suggested to stabilise PER in Drosophila (Shearman *et al*., 2000; Yagita *et al*., 2000; Lee *et al*., 2001).

Both the Casein Kinase 1ε and 1δ isoforms have been shown to be capable of binding and phosphorylating the mPER proteins in-vitro (Keesler *et al*., 2000; Lowrey *et al*., 2000; Vielhaber *et al*., 2000; Akashi *et al*., 2002). This phosphorylation has been shown to destabilise the mPERs leading to degradation via the ubiquitin-proteasome pathway (Akashi *et al*., 2002). Several reports have suggested a link between mPER phosphorylation and subcellular localisation but the results are conflicting, possibly due to the different in-vitro systems used (Vielhaber *et al*., 2000; Takano *et al*., 2000; Akashi *et al*., 2002). Vielhaber and colleagues reported that CK1ε is essential for cytoplasmic retention of both mCRY and mPER1, whilst Takano
and colleagues report that mPER1 is mainly cytoplasmic and that phosphorylation by CKIε facilitates nuclear entry. Both of these experiments were performed in different cell culture systems. However Akashi and co-workers use the same cells as Takano et al and report that neither CKIε or CKIδ have any effect on mPER1 or mPER2 localisation.

Thus the first feedback loop can be seen to be very similar to that observed in Drosophila and as is the case with Drosophila other loops also interact with the first. The PAR bZIP transcription factor DBP is expressed rhythmically under the control of CLK:BMAL1 (Ripperger et al., 2000; Yamaguchi et al., 2000b). It was first though to play a role in clock output as it controls expression of output genes within the liver (Lopez-Molina et al., 1997). It has since been suggested to be important in mPER1 regulation by binding sites within the mPER1 promoter and enhancing CLK:BMAL1 activation (Yamaguchi et al., 2000b). This enhancement is counteracted by E4BP4, another bZIP transcription factor, which out competes DBP for the binding site within the mPER1 promoter and acts as a transcriptional repressor (Mitsui et al., 2001). E4BP4 is also under circadian regulation although apparently opposite to DBP as they are differentially effected in mCRY deficient mice (Mitsui et al., 2001).

The nuclear orphan receptor REV-ERBa has been shown to be a major activator of cyclic Bmal1 transcription (Preitner et al., 2002). It also affects Clk and Cry transcription to a lesser extent. BMAL1:CLK positively regulates Rev-erba and PER and CRY act as repressors. Mutant animals for Rev-erba are however behaviourally rhythmic in constant conditions, suggesting that it is not the sole regulator of Bmal1, although the animals show altered phase shifting ability.

Another loop is formed by two more recently identified components DEC1 and DEC2. Both of these are bHLH transcription factors that cycle in abundance in a circadian fashion and are activated by CLK:BMAL1 (Honma et al., 2002). They also negatively feedback to inhibit CLK:BMAL1. Diagrammatic representation of the loops is given in Figure 1.8.
Figure 1.8 Interlocked feedback loops of the mammalian clock
The CLK:BMAL1 dimer activates transcription of several genes. PER:CRY and DEC1:DEC2 feedback to negatively regulate their own transcription. CLK:BMAL1 also activates transcription of dbp, the product of which enhances transcription of mPerl. At the same time CLK:BMAL1 regulates E4BP4 which out competes DBP for binding sites within the mPerl promoter and acts to inhibit activation of mPerl.
1.12 Aims of the project

Work on *Musca per* (*Mdper*) had begun prior to my arrival in the laboratory (Piccin, 1998). Cloning of the gene and subsequent sequence analysis revealed the same division between conserved and non-conserved regions as seen for the *Drosophila* species (Colot et al., 1988). Despite the similarity in *Dmper* and *Mdper* sequences, regulation of MdPER appears to be greatly altered to that of DmPER (Piccin, 1998). Although *Mdper* mRNA cycles over the circadian period in LD, *MdPER* does not cycle in abundance in either whole head or thorax immunoblots in either LD or DD conditions (Piccin et al., 1998).

The *per* gene of another insect species, the Lepidopteran *Antheraea pernyi*, had also been cloned (Reppert et al., 1994). Expression studies revealed different regulation of PER and TIM to that seen in *Drosophila* (Sauman and Reppert, 1996). Both PER and TIM are seen to cycle in abundance but are only ever observed within the cytoplasm and axons of neurons that co-express both proteins, although nuclear entry is observed within photoreceptor nuclei in the eye (Sauman and Reppert, 1996). This lack of apparent nuclear entry questions the existence of the negative feedback loop described for *Drosophila*.

It can therefore be seen that clock regulation in insects is not completely conserved. *Musca* lies between *Drosophila* and *Antheraea* in evolutionary terms and so our laboratory sought to develop *Musca* as a comparative system to gain insight into which components of the clock were more conserved and which more diverged. This would help to elucidate which components act as core components and which may act to allow species specific fine-tuning of the clock. The primary aim of this project was to further analyse PER regulation in *Musca* and to investigate TIM regulation at both the mRNA and protein levels. It was necessary to look at regulation at the cellular level as western blots could mask cycling in a small number of cells if a larger number of cells showed constitutive expression. As western blots had suggested a stable PER protein I also wished to investigate if this was the case and if so which mechanisms contributed to this.

In addition to this I also continued two lines of research that I had begun during my MRes degree that would further investigate the *Drosophila* clock. The first of these was to analyse a possible role for CRY within the central oscillator. Secondly I had found an interaction between CRY and TGO, a PAS transcription
factor involved in *Drosophila* development. I wished to establish whether this was real and if it had a biological function, possibly to link developmental and circadian timekeeping.
Chapter 2

Materials and Methods
2.1 Bacterial strains

XL1 BLUE MRP’ (Stratagene): Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr)173 endA1 
  supE44 thi-1 recA1 gyrA96 relA1 lac [F’ proAB 
  lacIqZΔM15Tn10 (Tet’)]

BL21(DE3)pLysS (Novagen): F’ ompT hsdSb(rB mB+) gal dcm (DE3) pLysS

Tuner(DE3) (Novagen): F’ ompT hsdSb(rB mB+) gal dcm lacY1 (DE3)

2.2 Plasmids

pBluescript II KS+ (Stratagene) and pBC KS+ (Stratagene) were used for basic 
cloning and RNA probe synthesis. The plasmid pDK101 (Appendix 1) was used to 
clon PCR products by T-A cloning. For yeast-two hybrid assays the vectors pEG202 
and pJG4-5 were employed. pUAST was used in the production of transgenic 
_Drosophila._

pET 14b and pET 23a were used to bacterially express protein fragments. Two 
farther vectors pJDC4 and pJDC7 (Appendix 2) were a gift from J. Clayton and were 
also used for this purpose.

The plasmid EV2 (Appendix 3) was produced in our laboratory by O. Ozkaya and this 
was used for the production of proteins by _in-vitro_ transcription/translation.

2.3 Plasmid DNA preparations

For high quality DNA both maxi and mini scale preparations were performed 
using the alkaline lysis method as described in Feliciello and Chinali (1993).

When high quality DNA was not required, for example to check which colony 
contained the plasmid with the required insert before a maxi prep was performed, a 
“speed” preparation was performed as detailed below.

2.4 Speed preparation of plasmid DNA

1.5ml of bacterial culture was centrifuged to pellet the bacteria at 5000RPM 
for 3 mins. The pellet was then resuspended in 100µl of solution A (50mM Tris-HCl, 
PH8.0, 4% Triton X-100, 2.5M LiCl, 62.5mM EDTA). 100µl of phenol/chloroform
(1:1) was added to this and the mixture vortexed for 10secs. This was then centrifuged at 13000 RPM in a microcentrifuge for 2 mins and the resulting upper aqueous phase collected. 200μl of cold ETOH (100%) was added to this and the contents mixed. The tube was then placed at -20°C for 30 mins or on dry ice for 15 mins. The mixture was then centrifuged at 13000 RPM to pellet the precipitated DNA and the ETOH then removed. The pellet was then washed in 80% ETOH and resuspended in 20μl of TE (10mM Tris-HCl, pH8.0, 1mM EDTA).

2.5 Restriction digest of DNA

To digest DNA with a restriction endonuclease the required amount of DNA was incubated with the enzyme (or enzymes) of choice in a solution containing the appropriate reaction buffer at the optimum temperature for the enzyme for a maximum of 2hrs. A small sample of the reaction was then run out on an appropriate percentage agarose gel in 0.5% TBE buffer to check that the enzyme had cut the DNA. If this was performed to isolate a band of DNA from, for example a plasmid, then the rest of the digest was run out on the gel in a large well.

2.6 Recovery of DNA from an agarose gel

When the required fragment had been separated by electrophoresis the band was cut from the agarose gel. This was transferred to a piece of dialysis tubing (previously boiled in 1mM EDTA and 2% sodium bicarbonate and stored in 100% ETOH at 4°C) and 400μl of 0.5X TBE gel running buffer was added. The tube was clamped at both ends and placed in an electrophoresis tank. A potential difference of 100V was applied for 15mins after which the tube was viewed using a UV transilluminator to ensure that the DNA had moved out of the gel and into the contained buffer. This buffer was then removed and subjected to a phenol/chloroform extraction and finally ethanol precipitation of the DNA. This was performed as follows. To the collected aqueous phase 1/10 volume of 3M NaAc, pH5.2 and 2 volumes 100% ETOH was added and the solution mixed. This was placed on dry ice for 15mins and then centrifuged at 13000 RPM for a further 15mins to pellet the DNA. The pellet was then washed in 80% ETOH and resuspended in an appropriate volume of TE.
2.7 Ligation and transformation of bacteria with plasmid DNA

Vector DNA for cloning was always subjected to dephosphorylation with shrimp alkaline phosphatase (SAP) before use. This strongly reduces the background of re-circularised plasmid.

50ng of vector DNA was placed into the ligation reaction with an amount of insert DNA that corresponded to twice as many molecules of insert as there was vector. The reaction was carried out in a total volume of 15μl using 3U of T4 ligase. This reaction was performed overnight at 4°C and then stopped by the addition of 1.5μl of tRNA, 1.5μl of NaAc (3M, pH5.2) and 50μl of 100% ETOH. This was left on dry ice for 15mins and then centrifuged at full speed for 15mins. The DNA was washed in 80% ETOH and resuspended in 10μl of dH2O. A control reaction was also performed in this manner, whereby the insert was omitted. This indicated the presence of re-circularised vector plasmid.

3μl of DNA was mixed with 40μl of electrocompetent XL1 BLUE cells (see Appendix 4) and this was used for transformation by electroporation. After electroporation the cells were rescued with 1ml of LB and incubated at 37°C for 1hr with aeration. The cells were then plated on LA plates containing the appropriate antibiotic for selection of the transformed cells. Where possible X-Gal and IPTG were added to the media to enable blue/white selection of transformants. The plates were incubated at 37°C overnight.

Single putative transformant colonies were isolated and grown in ~3ml of LB at 37°C with aeration overnight. These cultures were used for speed preparations of the plasmid DNA. This DNA was then digested with appropriate restriction enzymes to allow identification of the plasmids containing the insert. The speed preparation culture was used to set a maxi preparation culture (200ml LB containing the appropriate antibiotic) from a positive clone.

2.8 PCR

For relatively small products PCR reactions were set up as follows: 10mM Tris-HCl pH9.0, 0.1% Triton-X, 50mM KCl, 1.5mM MgCl2, 0.2 mg/ml BSA, 0.2mM dNTPs, 0.3μM of each primer and 0.05U Taq polymerase. Oil was placed on the top
of each reaction and the tubes placed into a Trio-Thermoblock (BIOMETRA) thermal cycler. Details of conditions will be given for each set of primers in the relevant chapters.

The Roche Expand PCR kit (a mixture of Taq and PWO, a thermostable DNA polymerase with proof-reading activity) was used for the amplifications when it was vital that no point mutations were created. Details of the reaction mixture are given below.

Master Mix: 0.2mM dNTP mix
- 10ng template DNA
- 0.3μM of each primer
- 10μl Reaction buffer with MgCl$_2$ (final conc. 1.5mM)
- 0.05U/μl Taq polymerase
- H$_2$O to a final volume of 100μl

The reaction mixtures were then treated as stated above.

Purified PCR product was then ligated into a previously prepared vector that allowed the cloning of PCR products (see Appendix 1) termed PDK101. All cloned PCR fragments were sequenced using the automated sequencing facility at the University of Leicester PNACL.

2.9 Western blotting

Flies were collected at the required time points and snap frozen in liquid nitrogen. Heads were then removed by vortexing and were collected over dry ice using a sieve. Total protein was extracted by homogenising the isolated heads in two volumes of BW buffer (0.1M KCl, 0.01M HEPES pH 7.5, 5% Glycerol, 0.1% Triton X, 10mM EDTA, 1mM DTT, 0.5mM PMSF, 10μg/ml Aprotinin, 5μg/ml Leupeptin, 5μg/ml Pepstatin).

Protein content of the extract was then assayed using a Bradford reaction and extracts from different time points were equalised from this so that the same amount of total protein was loaded in each lane. For the Bradford reaction 200μl of Bradford reagent (Sigma) was added to 800μl of dH$_2$O and then incubated with 1μl of protein
extract (1μl of BW solution to serve as blank) for a minimum of 5mins and a maximum of 30mins. After this time the absorbance value at 595nm was recorded using a spectrophotometer. Extracts were then equalised to the lowest absorbance value of the range for loading. To each sample a half volume of 3X loading buffer (188mM Tris-Cl (pH6.8), 6% (v/v) SDS, 30% (v/v) glycerol, 15% (v/v) β-mercaptoethenol, 0.03% (w/v) bromophenol blue) was added and the sample boiled for 5mins prior to loading. Samples were then loaded onto a 6% SDS-PAGE gel and run alongside a broad range marker (New England Biolabs). Gel running buffer consisted of 2.5mM Tris, 0.25M Glycine, 0.1% (v/v) SDS.

When the gel had run to completion it was blotted to a nitrocellulose membrane for 3hrs at 300mA using semi-dry blotting buffer (40mM Tris, 40mM glycine, 0.0375% (v/v) SDS, 20% MetOH). Membranes were then stored dry before processing further.

Membranes were blocked for 1hr at room temperature in TBST (10mM Tris-Cl (pH7.5), 150mM NaCl, 0.05% (w/v) Tween-20) containing 5% milk. They were then incubated with the primary antibody diluted in TBST containing 5% milk for 2hrs at 4°C with agitation. After this three 5min washes in TBST were carried out at room temperature before incubation with the secondary antibody (carrying a HRP conjugate, Sigma) for 1hr at 4°C. A further three 5min washes were then carried out in TBST. The membranes were then incubated with 0.1M Tris-Cl (pH 8.5), 6.25μM Luminol, 6.38μM p-Coumaric acid, 2.7mM H2O2 for 1min to create a luminescent signal. This signal was then detected on an auto-radiograph film (Fuji).

Developed films were then scanned into a PC and subjected to pixel density analysis using the scion-image software.

2.10 Immunocytochemistry (ICC)

Using paraffin sections of fly brains

Fly heads for both D. melanogaster and M. domestica were partially dissected (by removing the proboscis and all chitinous material at the front of the head) under 1X Drosophila Ringers solution (Ashburner, 1989). Heads were then fixed in a modified BHS (Bouin-Holland Sublimate) solution (Sauman et al, 1996) to which a 1/10th volume of saturated HgCl2 had been added. Fixation was allowed to occur for
6hrs at 4°C. Fixative was then removed by washing with 70% EtOH until no green colouration of the fixative solution remained in the external solution. The heads were then stored at -20°C prior to the dehydration process. When all samples for a time series had been collected the heads were processed together. Dehydration was carried out by subjecting the heads to two 20min washes in each of the following solutions in order; 95% EtOH, 100% EtOH and 100% Chloroform. The heads were then transferred to moulding cases containing molten paraffin at 60°C. These cases were then held in an embedding oven under vacuum overnight. The following morning the cases were removed and the heads positioned for sectioning (face down) with a hot needle before allowing the wax to cool and set.

Sectioning was then performed using an American Optical Company microtome. 6μm sections were cut and ribbons of consecutive sections were bound to a slide coated in chrome-allumen solution (0.5% gelatine, 0.5% chromium (III) potassium sulphate) at 42°C for 48hrs. Slides were stored at 4°C prior to use.

To carry out the immunostaining the slides were first re-hydrated and processed by incubating the slide in the solutions described below.

Once for 10min in 100% Xylene
Once for 5min in 100% EtOH
Once for 5min in 95% EtOH
Once for 5min 70% EtOH
Once for 5min in dH2O
Once for 3min in Lugols Iodine (1% iodine in 2% aqueous potassium iodide)
Once for 5mins in 7.5% Na2S2O3
Once for 5mins in dH2O
Once for 5min in 1XPBS containing 0.3% Tween-20 (PBS-Tw 0.3%)
Once in 10% normal serum diluted in PBS-Tw 0.3%

Note that the normal serum used is from the same animal as that in which the secondary antibody to be used was raised. Slides were then transferred to a humid chamber a 100μl aliquot of the primary antibody, diluted in PBS-Tw 0.3%, was added to each slide. These slides were incubated at 4°C overnight.

Three 10min washes were performed using PBS-Tw 0.3%. The secondary antibody was then diluted in the same buffer and 200μl of this was incubated with each slide for 2hrs at room temperature. When a fluorescent secondary antibody was
used the slides were then mounted in an appropriate medium and sealed. When the secondary antibody used had a HRP conjugate then an enzymatic reaction was used to stain the slides. Slides were incubated in 0.05M Tris-Cl pH 7.5 containing diaminobenzidine tetrahydrochloride (DAB) at a concentration of 0.1mg/ml and 15μl of H₂O₂ until a suitable level of staining was achieved. The slides were then dehydrated through a series of ethanol washed of increasing concentration and then xylene before mounting with DPX (Fisher).

**Using whole-mount preparations**

Whole brains were dissected from flies under *Drosophila* Ringers solution and then fixed in 4% paraformaldehyde in PBS for 6hrs.

*Drosophila* brains were permeabilised by three 20min incubations in PBS-Triton-X 1.0% (PBS-Tx 1.0%). They were then rinsed in PBS and blocked for 1.5hrs at room temperature in 10% goat serum diluted in PBS-Tx 0.5%. Primary antibody(s) was diluted accordingly in the same buffer and incubated with the brains at 4°C for 72hrs. The primary antibody was then removed and the brains subjected to three 5min washes in PBS-Tx 0.5%. Secondary antibody(s) were diluted in PSB-Tx 0.5% and incubated with the brains for 2-3hrs at room temperature. DAPI solution (2μg/ml) was then added for a further 30mins. Three 5min washes were performed with PBS-Tx 0.5% before rinsing the brains in dH₂O and mounting them in antifade (3% n-propylgallate in PBS (pH 8.5), 80% Glycerol).

*Musca* brains were essentially treated the same with the exception of the permeabilisation step. As the *Musca* brain is much bigger than that of *Drosophila* these brains were subjected to four 30min incubations with PBS-Tx 1.0%.

2.11 **In-situ hybridisation**

Dissection of heads and fixation was carried out as described for whole-mount ICC. Heads were then embedded and sectioned as previously described. Sections were annealed to poly-L-lysine slides.

**Preparation of labelled RNA probes**

Segments of suitable DNA regions were cloned into pBluescript (Stratagene). This was then linearised using a suitable restriction site within the MCS of the vector.
to produce either a blunt end or a 5' protrusion. This was carried out twice so that both the T3 and T7 promoters could be used to produce both a sense and antisense probe from the same DNA. Probes were produced containing a digoxygenin (DIG) label as instructed by the manufacturer (Roche). Probes were precipitated with ammonium acetate prior to use to removed unincorporated nucleotides.

Hybridisation

Tissue sections were cleared through xylene and rehydrated through a graded series of ethanol (10min in xylene (twice), 2mins in 100% EtOH, 30secs in 100% EtOH, 30secs in 95% EtOH, 30secs in 70% EtOH, 1min in DEPC treated dH2O (twice)). The tissue was then deproteinated by incubating it in 0.2M HCl for 20mins and then washed for 5mins in PBS containing 0.3% Triton-X. Slides were then equilibrated in 0.1M triethanolamine (TEA) buffer, pH8, and then subjected to acetylation by incubation in 0.25% acetic anhydride in 0.1M TEA buffer, pH8 for 10mins. This was followed by a 5min wash in PBS followed by two 2min washed in SSC before dehydrating the tissue (5mins in 70% EtOH followed by 2mins in 95% EtOH). Slides were then left to air dry.

The probe was then diluted to 5ng/µl in hybridisation solution (50% deionised formamide, 10% dextran sulphate, 2X Denhart's solution (100X Denhart’s: 2% PVP, 2% Ficoll, 2% BSA), 50mM NaPO4 (pH 7), 2X SSC, 0.1% (v/v) SDS, 5mM EDTA, 0.1% sodium pyrophosphate, 500µg/ml salmon sperm DNA, 500µg/ml yeast tRNA) to which 50µl of 1M DTT had been added. This was boiled for 5mins prior to application. 100µl of the mix was added to each slide and the slides were then incubated over night with a cover slip in a humid chamber at 42-58°C, depending on the probe being used, to allow the probe to anneal to its target mRNA. Controls were conducted using the sense probes produced.

Post-hybridisation washes

Slides were washed twice in buffer 1 (2x SSC, 0.05% sodium pyrophosphate, 1mM EDTA) for 20mins at room temperature before subjecting them to RNase A digestion (10mg/ml in 10mM Tris-Cl (pH 8), 0.5M NaCl) at 37°C for 1hr. This was followed by a further 30min wash in buffer 1 at room temperature. Two 30min washes in buffer 2 (Wash buffer 2: 0.1x SSC, 0.05% sodium pyrophosphate, 1mM...
EDTA) were then performed at the same temperature at which the probe was annealed, followed by two 20min washes in the same buffer at room temperature.

**Detection**

The tissue was then washed in PBS-Tw 0.3% for 15mins and then blocked in 10% sheep serum, diluted in PBS-Tw 0.3%, for 30mins at room temperature. 200μl of a sheep anti-DIG (Roche) antibody diluted 1 in 200 in PBS-Tw 0.3% was then added to each slip and the slides were incubated overnight. Three 5min washes were then performed in PBS-Tw 0.3% before equilibrating the slides in detection buffer (100mM Tris-Cl (pH 9.5), 100mM NaCl, 50mM MgCl₂) for 5mins. The slides were then developed in 75ml of detection buffer containing 270μl NBT stock (75mg/ml in 70% dimethyl formamide), 210μl of BCIP stock (50mg/ml in 100% dimethyl formamide) and 15mg of levamisole until a suitable level of staining was achieved. The slides were then dehydrated through a graded series of EtOH and washed in xylene as described for ICC before mounting in DPX (Fisher).

**2.12 RNA Extraction**

Fly heads were collected as described for protein extraction. They were then homogenised in 400μl TRIZOL reagent (Gibco BRL). A further 600μl of TRIZOL was then added and the homogenate was allowed to stand for 5mins. 200μl of chloroform was then added and the mixture shook vigorously for 15secs. It was then left to stand for 2-3mins before being centrifuged in a microfuge for 15mins at 4°C. The upper phase was then carefully collected and removed to another tube. The tube was then filled to the 1.5ml mark with isopropanol and left to stand at room temperature for 5mins. To precipitate the RNA then tube was then centrifuged for 15mins at 4°C. The pellet was washed in 80% EtOH and then resuspended in 50μl of DEPC treated dH₂O.
2.13 cDNA synthesis

cDNA synthesis was performed using the ImProm-II reverse transcriptase (Promega). Template RNA (total RNA) was diluted to 1µg/µl and 1µl was mixed with 1µl of oligo-dT primer (500ng) and 3µl of DEPC treated dH2O. This was incubated at 72°C for 5mins and then chilled on ice for 5mins. To this 4µl of 5X buffer, 2.5µl of 25mM MgCl2, 1µl of dNTP mix (10mM each), 0.5µl of Rnasin and 6µl of DEPC treated dH2O was added. This was mixed before adding 1µl of reverse transcriptase. The reaction was then incubated at 25°C for 5min, followed by 42°C for 1 hour and then the reaction was terminated by heat inactivation at 70°C for 15mins. This was performed on a Trio-Thermoblock (Biometra) thermocycler. This was then used directly for PCR amplification reactions.

2.14 Reverse-Transcription PCR

Primers were designed to amplify a fragment of 150-300 bp and were placed so as to span an intron in the DNA sequence of the target gene, so as to amplify a larger fragment if DNA contamination was present in the RNA extract. Primers were designed accordingly for the RP49 gene, which is expressed constitutively, to give a control for quantification purposes.

The reverse-transcription coupled PCR reaction was performed using the one-step RTPCR kit (Quiagen). Typically a 25µl reaction would contain the following: -

1µl dNTPs
1µl Enzyme mix
2.5µl Primer 1 (6µM)
2.5µl Primer 2 (6µM)
2.5µl Primer 3 (6µM)
2.5µl Primer 4 (6µM)
5µl 5X Reaction buffer
200-400ng RNA
dH2O to a final volume of 25µl.
Reactions were performed in a Trio-Thermoblock (Biometra) thermocycler using the following conditions: 50°C for 30mins, 95°C 15mins followed by 95°C 30sec, annealing temperature 30sec, 72°C 1min for up to 27 cycles. 12μl of the reaction was then run out on a 2% agarose gel.

The primers were first tested to establish the best annealing temperature at which the reaction worked. Once this was ascertained a series of reactions were performed with each reaction being terminated after a consecutive cycle number (reactions were stopped by the addition of gel loading buffer containing EDTA). This was used to determine the range at which the PCR reaction was proceeding in the linear range. Once this was achieved RNA samples were collected from fly heads at different time points over the circadian day. One reaction was then performed for each time point using the same amount of total RNA in each. Again 12μl of the reaction was loaded onto a 2% gel for analysis. Images of the gel were recorded onto a PC and subsequently used for image analysis.

2.15 **Bacterial expression of proteins**

The appropriate constructs were transformed into the appropriate *E.coli* strain using CaCl₂ competent cells. Approximately 1μg of DNA was added to the cells and incubated on ice for 25mins. The cells were then given a 1.5min heat shock at 37°C and then incubated at room temperature for 10mins. 1ml of LB was then added and the cells incubated at 37°C for 1hr with shaking. 100μl aliquots were then plated on LA containing the appropriate antibiotics.

**Test scale expression**

Single colonies were then grown overnight in 10ml of LB or M9 media (0.185mM NH₄Cl, 0.22mM KH₂PO₄, 0.22mM Na₂HPO₄, 0.4% (w/v) casamino acids, 0.4% (w/v) glucose,1mM MgSO₄, 0.3mM CaCl₂) with antibiotics. The following morning 15ml of fresh medium was then inoculated with 750μl of the overnight culture and this was grown at 37°C until the OD₆₀₀ reached between 0.5 and 0.7. A 2ml un-induced sample was then taken and the expression of the protein induced by the addition of IPTG to the remaining culture to a final concentration of 1mM. The culture was left to grow at 37°C and further 2ml samples were collected every hour.
Cells from these samples were pelleted by centrifugation and resuspended to an OD
600
of 10 in PBS-Tx 0.1%. They were then snap frozen in liquid N2 and stored at -80°C. Cells were then thawed on ice and sonicated with six 6sec pulses at high frequency whilst on ice and then centrifuged at high speed for 5mins at 4°C. The supernatant (soluble fraction) was carefully collected and transferred to a fresh tube. The resulting pellet (insoluble fraction) was then resuspended to the original volume of the sample in PBS-Tx 0.1%. Both fractions were then run, along with others from the time course on a 12% SDS-PAGE gel alongside a broad range protein marker (New England Biolabs).

The gel was subsequently stained overnight with Coomassie (45%MetOH, 10%AcAc, 0.25% Brilliant Blue R-250) and then destained with a solution of 45% MetOH, 10% Acetic acid until a suitable level of staining was achieved. Gels were then stored in 12.5% isopropanol, 10% Acetic acid until they were photographed with a digital camera (Coolpix 4500, Nikon).

Large scale production

Overnight cultures were set from single colonies as previously described. This was then diluted 1 in 50 into 50ml of fresh medium and grown to OD
600
of 0.5-0.7 at 37°C and the cells induced as previously described. The length of time that these cultures were grown for depended on the results from the test run performed for that construct. The cells were then pelleted by centrifugation at 3600 rpm and the pellet snap frozen in liquid N2. 1ml of protease inhibitor cocktail set III (Novagen) was added per 20g of cell pellet and the pellet allowed to thaw on ice. Cells were then resuspended in 5mls of lysis buffer (20mM Tris-Cl, 100mM NaCl, pH8.0, with 5mM Imidazol if a HIS-tag fusion was being used) per 5g of cell pellet and Triton-x was added to a final concentration of 0.1% (v/v). If appropriate lysozyme was then added at this stage to a concentration of 0.1mg/ml and the cells incubated for 10min at 30°C. The cells were then sonicated for 30secs three times and then centrifuged at 13000 rpm. The soluble fraction was then collected and the pellet resuspended in the same volume of lysis buffer. Samples from both fractions were checked for target protein content. If the protein was present in the soluble fraction then this was used to purify the protein, if the protein is was insoluble then further processing was first required.
Solubilisation of proteins from inclusion bodies

To attempt to extract target proteins that were carrying a HIS tag from inclusion bodies the resuspended pellet of the insoluble fraction had urea added to a final concentration of 8M and this was then incubated at room temperature for 30mins. This was then centrifuged at 13000 rpm at room temperature and the liquid fraction collected. The pellet was once again resuspended in lysis buffer and ran on a PAGE gel along with a sample from the solubilised fraction to check the efficiency of the reaction. If a significant amount of the protein was now present in the soluble fraction this was then added to a suitable amount of N^NTA and allowed to bind for 15mins at room temperature. The unbound (liquid) fraction was then removed by pelleting the matrix at 3600 rpm at room temperature.

In order to refold the solubilised protein the matrix was washed with lysis buffer containing a decreased concentration of urea each time. First three washes were performed in lysis buffer, 0.1% Tx, 8M urea, then a series of single washes was performed using the same buffer but with 4M urea, 2M urea, 1M urea and 0.5M urea in order. Finally a single wash was performed using lysis buffer only, to which no urea or Tx had been added. The matrix was then transferred to a column support and serial elutions were made and collected by adding 50μl of elution buffer (20mM Tris-Cl (pH 8), 100mM NaCl, 0.5M Imidazole). Protein concentration was then assayed with a bradford reaction and compared to the OD595 values of BSA standards.

2.16 Synthesis of proteins by in-vitro transcription-translation

200ng of plasmid DNA was added to 8μl of Rabbit TNT quick (Promega) coupled in-vitro transcription-translation mix and 0.4μl of ^35S labelled Methionine (Amersham). The reaction was conducted for 90mins at 30°C. In order to check the efficiency of the reaction 1μl of the sample was then mixed with 19μl of Laemli loading buffer (Sambrook et al, 1989). The mixture was then boiled for 3 mins and half the volume loaded onto a 12% PAGE gel. After running the gel was fixed (7% MetOH, 7% Acetic acid, 1% glycerol) for 5mins and dried. This was then exposed to an autoradiograph film (Fugi) overnight.
2.17 Co-immunoprecipitation of proteins

The two proteins of interest were produced by *in-vitro* transcription-translation. One was radiolabelled the other not. They were then mixed together in equal amounts in 300μl of incubation buffer (25mM HEPES (pH7.5), 100mM KCl, 12.5mM MgCl₂, 100μM EDTA, 20% glycerol, 0.1% NP-40, 1mM DTT, 10μg/ml aprotinin, 5μg/ml leupeptin, 5μg/ml pepstatin, 0.5mM PMSF). This was then incubated for 1hr at room temperature on a rotating wheel. 30μl of a 50% agarose-protein G slurry was then added to the sample and this was mixed thoroughly at 4°C for 15mins before being centrifuged at 2000 rpm at 4°C. The supernatant was removed to a new tube and 3μl of an appropriately diluted antibody was added to it. This was then incubated at 4°C for 4hrs. The agarose-protein G slurry was then added to the mixture and incubated at 4°C over night on a rotating wheel. The following morning the sample was centrifuged at 2000 rpm for 2mins at 4°C and the supernatant removed. The pelleted agarose was washed three times with 500μl of incubation buffer at 4°C and then resuspended in 30μl of 2X Laemli loading buffer. 14μl was then loaded onto a 12% PAGE gel. The immunoprecipitated proteins were visualised by autoradiography.

2.18 Yeast-two hybrid assays

Cloning was performed as described in individual results chapters. The assays were performed as described in Golemis and Brent, 1997. Both the bait and prey plasmids for the yeast two-hybrid system (pEG202 and pJG4-5, respectively) were a gift from R. Brent and their sequences are given in Appendix 5. *dcry* cDNA (a gift from J. Hall) was available as a directionally cloned EcoRI – XhoI fragment in pBs SK- (Stratagene). The period fragment used (PER (233-685) was already available in the yeast vectors in our laboratory, as was TIM (337-915). The numbers given in brackets refer to amino acid number. Full-length PER and TIM clones in the yeast vectors were also available. The *tango* gene was already available directionally cloned EcoRI-XhoI in both pEG202 and pJG4-5 (a gift from S. Crews). The Musca (233-685) fragment had also previously been cloned into the pEG202 vector by A Piccin.
2.19 Fly stock maintenance

*D. melanogaster*

Egg laying food and *Drosophila* sugar medium are detailed in Appendix 6. Flies were reared at 25°C in a 12:12 LD regime.

*M. domestica*

Adult flies were fed on sucrose and dried milk and provided regularly with fresh water. Larval medium was prepared for egg laying and larval development. This consists of 50g Bran, 1tsp dried yeast, 200 ml of milk. To this 1.6ml of 20% Nipagine (methyl p-hydroxybenzoate) was added to prevent fungal growth. Flies were kept in transparent cages at 25°C and subjected to a 12:12 LD cycle.

2.20 Transformation of *D. melanogaster*

The strain *w^{1118}* was used for this procedure. These flies carry a large deletion of the white gene (Hazelrigg et al., 1984) and therefore there is no chance of reversion of the white eye phenotype. The pUAS vector used contains the mini-white gene as a selectable marker, conferring red eye colour to transformants.

Flies of approximately 5 days in age were placed into a large open-ended glass tube. One end was closed with cotton wool whilst the other was placed onto the egg laying food. After 30 mins the embryos were discarded as the first laid were usually too old for injection. Collections of embryos were then made every 30mins. These embryos were dechorionated by hand by rolling them over a piece of double-sided adhesive tape. They were then aligned on a piece of double sided tape on a cover slip with the posterior ends of the embryos slightly overhanging the edge of the cover slip. The slide and cover slip were then placed into a desiccator for 10-15 mins and then the embryos were covered in halocarbon oil to prevent any further desiccation.

The plasmid DNA to be injected was resuspended in injection buffer (5mM KCl, 0.1mM Na$_2$HPO$_4$, pH 6.8) to a concentration of 300ng/μl with the Δ2-3 helper plasmid (A gift from J. Dura) to 150ng/μl. This was then loaded into a glass microinjection needle. Only embryos at the early cleavage stage (15 mins to 1hr 20 mins) were injected. The DNA was inserted into the posterior tip of the embryo as
the cells that give rise to the germ line are situated here. Once all of the embryos had been injected the cover slip was removed and placed into sugar media that had been coloured red with food dye in a petri dish. This allowed the resulting larvae to be visualised more easily. The embryos were once again covered in halocarbon oil to prevent desiccation. The petri dishes were incubated in a humid chamber at 18°C.

Each day the petri dishes were examined for larvae and to ensure enough oil was present. Any larvae found were transferred to a vial containing sugar medium with a mounted needle and left to develop at 25°C. Adult flies were collected and crossed individually with two w^{1118} flies of the opposite sex. Each fly was crossed a minimum of three times.

Progeny from these flies (G1) were screened for the presence of red eyes (transformants).

2.21 Locomotor activity analysis of D. melanogaster

The locomotor activity was monitored using an activity event recorder produced by PIXEL Srl. (Padova, Italy). Approximately 20mm of sugar media was placed into each 80 x 3mm cylindrical glass tubes and the tubes sealed at that end with tape to prevent desiccation. Single male flies (using only males prevents interference of any larvae produced by females) were loaded into these tubes and the other end was sealed with cotton wool. A total of 40 flies of each genotype were used for each experiment. These tubes were then placed into the activity monitoring machine, where two diodes of a photocell detect when the fly disrupts the infra-red beam, sending a signal to an attached PC. Infra-red light is used as flies are not sensitive to this wavelength.

Determination of period length

Flies used for the experiments were all raised in 12:12 LD light regimes (lights on at 0900, lights off at 2100) at 25°C.

When the analysis was performed under LD conditions the incubators housing the monitoring machinery were set to the same light regime as the flies had previously been experiencing i.e. light between 0900 and 2100. The machinery was loaded during the light phase. When the analysis was performed under DD conditions the
flies were loaded into the machinery during the light phase and the LD conditions were maintained for three days. At 2100, the normal lights off stage of the fourth day the lights were turned off for the rest of the experiment. For both LD and DD experiments the flies were given 24hrs to adjust to temperature differences and the experiment started the following day. All experiments were run for 5 days of data. The number of events for each fly was recorded in 30min time windows (bins) and stored in an attached computer.

**Light pulse experiments for phase shifting**

Flies were subjected to a 10min light pulse at either ZT15 or ZT21 and then immediately placed into DD conditions. Such light pulses are known to alter the phase of the flies behaviour and results in what is termed a “phase shift”. Light pulses during the early night (ZT15) result in premature degradation of PER and TIM, but there are sufficiently high levels of their RNA so protein levels build up again, taking the molecular mechanism back to the stage it was in at the beginning of the night. This results in a “phase delay”. Light pulses given late in the night (ZT21) again cause premature degradation of PER and TIM but at this time *per* and *tim* mRNA levels are low and so no further protein is produced. Now the molecular mechanism is in the same stage as it would be at the beginning of the day and so the rhythm for that day is shortened, resulting in a “phase advance”. For comparison control flies were also subjected to this light pulse. As a second control both of these sets of flies were also kept in a second incubator and were not given the light pulse but were switched to DD conditions at the same time as the pulsed flies. In order to assess the change in phase of the behavioural rhythm the time of the evening activity peak was measured. This was taken as the time at which the activity had dropped to half of the value of the maximum observed and this procedure was followed for each individual fly. The time of the evening peak was measured two days after the light pulse. For the final analysis the difference in the time of evening peak was calculated between pulsed and un-pulsed flies of the same genotype to give the phase advance/delay in hours. This result was hen compared between genotypes to ascertain genotypic variations.
Data Analysis

The periodicity of the flies was calculated using two separate statistical techniques, autocorrelation and Fourier analysis. Autocorrelation of the data was performed with the SPSS/PC+ version 2 package. Each data point is correlated with the adjacent one (lag = 1), then the procedure is repeated with the data from bins 2, 3, 4 etc, n bins apart. This is then reiterated n/2 times (n is the total number of bins). The correlation coefficient for each starting bin is used to draw the autocorrelation plot. The highest peak in the autocorrelation plot, within the range of 20-80bins was taken as the period of rhythmicity provided that it's value was higher than the 95% confidence limit.

The Fourier analysis was performed with the CLEAN algorithm of Roberts et al. (1987). The data string is transformed into a mathematical function that assumes the form F(x) = f1(x) + f2(x) + ....+fn(x), where f(x) are functions differing for phase, period and amplitude. A frequency curve is then plotted, with the X-axis representing the period and the Y-axis the amplitude. Peaks in this curve correspond to the major spectral components of the data. The confidence limit was then calculated by randomly shuffling the bins and repeating the spectral analysis on these new strings of data. This was repeated 100 times. For every given period the 100 amplitudes produced were ordered and the 95th and 99th in ascending order were plotted to give the 95% and 99% confidence intervals. The period corresponding to the highest peak was taken as the period of rhythmicity of the fly. If none of the specteral peaks were above the 95% confidence interval, the fly was classed as arrhythmic. The period length given by the Fourier (spectral) analysis was used rather than that from the correlation, which is in x of 30mins.
Chapter 3

Expression of *period* and *timeless* in *Musca*
3.1 Introduction

Musca per is capable of restoring robust rhythmicity to per⁰ Drosophila (Piccin et al., 2000), unlike D. pseudooobscura per (Petersen et al., 1988; Peixoto et al., 1998). This is thought to be brought about by the fact that the MdPER PAS domain is actually more similar to that of DmPER than are the PAS domains of the phylogenetically closer D. pseudooobscura and D. virilis PERs. A more similar PAS domain could theoretically lead to a better association of MdPER and DmTIM in these flies, thus leading to a better behavioural rescue.

In Drosophila, per is expressed rhythmically at both the mRNA and protein levels (Hardin et al., 1990; Edery et al., 1994). The peak in mRNA levels occurs between ZT14 and ZT16, while the protein peaks 4-6h later at ZT20-22 (Edery et al., 1994). Not only does PER oscillate in its abundance over the circadian cycle, it also shows temporal regulation in sub-cellular localisation (Saez and Young, 1996). The period gene has also been cloned in another insect species, the silkmoth Antheraea pernyi (Apper, Reppert et al., 1994). In the silkmoth, both PER and TIM are seen to cycle in abundance but are only ever observed within the cytoplasm and axons of neurons that co-express both proteins, although nuclear entry is observed within photoreceptor nuclei in the eye (Sauman and Reppert, 1996). Another Dipteran per has also recently been cloned from the Australian sheep blow-fly, Lucillia cuprina. Analysis of total mRNA and protein levels reveals regulation almost identical to that of its Drosophila counterpart (Warman et al., 2000).

Yet despite the similarity in Dmper and Mdper sequences, regulation of MdPER appears to be greatly altered to that of both DmPER and ApPER (Piccin, 1998). Although Mdper mRNA cycles over the circadian period in LD, MdPER does not cycle in abundance in either whole head or thorax immunoblots in either LD or DD conditions (Piccin et al., 1998). However, as this result was for whole head extracts, any temporal changes only occurring in a small number of cells would be masked. It could therefore be possible that cycling was occurring in clock cells in Musca. The aim of the work presented in this chapter was to investigate this non-cycling further at the cellular level. I employed immunocytochemistry and in-situ hybridisation techniques to map per expression within the Musca brain. I also wished to repeat and confirm the western blot data for MdPER using other antibodies. Whilst doing this I investigated Mdtim regulation at both the mRNA and protein levels. I
employ western blotting and semi-quantitative RT-PCR to investigate protein and mRNA cycling respectively, alongside the microscopy. I aimed to find cells that co-express both PER and TIM within the *Musca* brain so as to identify putative clock cells.

In *Drosophila*, a subset of lateral neurons (LNvs) that are proposed to be circadian pacemaker cells co-express the *Drosophila* homologue (PDF) of the crustacean pigment-dispersing hormone (PDH, Helfrich-Forster, 1995). As both the α-PER antibodies and the available α-PDH (a gift from I. Sauman) were raised in rabbit it was impossible to co-label cells using both of these antibodies. Although I managed to obtain an α-PDF raised in rat (a gift from M. Rosbash) this antibody failed to recognise the *Musca* protein. I therefore performed ICC to detect PDH expression in both *Drosophila* and *Musca* to compare the location of the immunoreactive neurons between the two species.

In order to learn the ICC and *in-situ* hybridisation techniques I spent a six-month period in the lab of Dr. I Sauman at the Czech Academy of Sciences, Ceske Budejovice, Czech Republic.

### 3.2 Materials and Methods

**Western blot analysis of PER and TIM expression in *Musca***

In order to repeat the western blots for MdPER flies were collected every 4h in a 12:12 LD cycle and subsequent protein extracts run on a 6% PAGE gel and blotted as described in Chapter 2. To investigate TIM cycling flies were collected every 2h in both 12:12 LD and DD conditions and proteins extracted. Primary antibodies used to assess PER levels were α-DmPER (a gift from J. Hall), anti-DmPER (a gift from M. Young) and α-MdPER 774 (Developed in our laboratory by S. Racey, Racey 2002). The α-DmTIM antibody used to detect MdTIM was a gift from M. Young. This was the only available antibody that recognises MdTIM as most α-DmTIM antibodies are raised against C-terminal sequences, which shows a high degree of divergence between DmTIM and MdTIM. The autoradiographs produced by the western blot were analysed with the Scion Image software to assess cycling. All data was subjected to ANOVA analyses of variance using the Statistica software package.
Semi-quantitative RT-PCR

Analysis of mRNA cycling was performed using semi-quantitative RT-PCR as described in Chapter 2. Flies were collected every 2h in both 12:12LD and DD conditions and RNA was extracted. In order to verify that this technique gave an accurate insight into mRNA levels I also investigated per cycling as well as tim. Two sets of primers were designed to amplify both per and tim and one set was designed to amplify Mdrp49 to act as a control. All primers for per and tim were designed to span an intron so that any genomic DNA contamination would be identified by the presence of a larger band. The first set of primers for tim were designed to amplify the first 237bp of the coding sequence (forward: atggacttgctattagaaacg and reverse: gaatacccttttcacctcttgcc). The second set was designed further downstream to amplify a 259bp fragment that starts 1498bp into the coding sequence (forward: tcctcgcagtctgaaccttc and reverse: gtggcttgctgcaccag). Appendix 7 shows the available Mdtim sequence supplied by S. Campesan and B. Collins.

For per the first set of primers were designed to amplify a 192bp fragment starting 232bp into the coding sequence (forward: aagttttactcaaccaagcgtac and reverse: tgggccactgaagggacttc) and the second set to amplify a 288bp fragment starting 1861bp into the coding sequence (forward: cttgaacatggccattttgatgctgac and reverse: gattggtatgtgctgttgga). To provide a control for differences in efficiency of the reaction and in RNA equalisation the constitutively expressed rp49 was used (forward: aacgtacccgccatatgctc and reverse: ttggcacgttcaacaatttcc). The sequence used for Mdrp49 was available from Piccin (1998).

Primers were tested prior to the experiment and the second set for both per and tim were found to be the most efficient. Both tim and rp49 primers were added to the same RT-PCR reaction. Test reactions were performed to establish the PCR reaction cycle number at which to conduct the experiment. This was 22 cycles for these primers. Test experiments with the per and Mdrp49 primers showed that the mdrp49 reaction out-competed that for per and so I performed the reactions separately. Products from both reactions were then loaded together onto the agarose gel as this allows greater accuracy in the subsequent quantification. As the reactions were being run independently the primer concentration was reduced for Mdrp49 so that 1.5μM was added to the reaction rather than 6μM. The Mdrp49 reactions were run for
19 cycles, the *per* reactions for 22 cycles. Gel images were recorded directly onto a PC and analysed using the Scion Image software package.

RNA extracts from *Musca* raised in 12:12 LD conditions at both 18°C and 29°C was made available by B. Collins. These samples were used to assess *tim* cycling at different temperatures. The same was not attempted for *per* as this had already been performed by B. Collins and no differences between temperature had been observed.

All data was subjected to ANOVA analyses of variance using the Statistica software package.

**Immunocytochemical analysis of MdPER and MdTIM expression in the Musca brain**

Initial studies were made for PER using 6μm vertical sections of *Musca* heads as described in Chapter 2, using α-DmPER (Young) antibody. Experiments were then repeated using the α-DmPER (Hall) and α-MdPER 774 antibodies. The secondary antibody used was an α-rabbit HRP conjugate (Sigma) and so the reaction was developed enzymatically using DAB. As the results obtained suggested that this was not sensitive enough to detect PER in the nucleus the whole mount technique was using fluorescent secondary antibodies (α-rabbit Cy3 and α-rat Cy2, Jackson). Both the α-DmPER (Hall) and α-MdPER 774 antibodies were used in separate experiments. The α-DmTIM (Young) antibody was used to detect TIM. Pictures of the whole mount brains were taken using a Zeiss confocal microscope.

Studies were also made to look at PDF localisation within the *Musca* brain using an α-PDH antibody that was a gift from I. Sauman.

**Localisation of per and tim mRNA expression in the Musca brain**

A clone of *Mdper* corresponding to 840bp – 2655bp of the coding sequence in the pDK101 vector was already available in the laboratory. This was at first used to produce DIG labelled probes. The plasmid was linearised with *XhoI* followed by transcribing with SP6 RNA polymerase produced the sense (control) probe and with *EcoRV* and transcribed with T7 RNA polymerase to produce the antisense probe. Due to the results obtained, two further probes were created for *per*. One was targeted to
the 5' end and was termed \textit{Nper}, the second was targeted to the C-domain region and was thus termed \textit{CDper}. These regions were amplified by PCR and cloned into pBs to create the templates to synthesise the probe. For \textit{Nper} the 5' primer (cagctgcagtaacagtcaatc) was placed across a \textit{PstI} site and starts 63bp from the beginning of the coding sequence and the 3' primer (caacgataacgccttagcattac) was placed 781bp into the sequence. PCR amplification resulted in a 718bp fragment that was cloned into pDK101. This was then sub-cloned using \textit{PstI} (one site in the 5' primer, one present in the pDK101 vector 3' to the inserted sequence) into pBs. The plasmid was linearised with \textit{XbaI} for the sense probe and \textit{XhoI} to create the antisense probe. Transcription of the probes was conducted with T3 (sense) and T7 (antisense) RNA polymerases.

To create the \textit{CDper} template the corresponding fragment was amplified using a 5' primer (acacgcataaaagaggatattctt) 1390bp into the \textit{per} sequence and a 3' primer (gccccatgttcagattactgcc) 482bp downstream. Again this was cloned into pDK101 and then sub-cloned into pBs using the \textit{ApaI} and \textit{SacI} sites in the pDK101 vector. The resulting plasmid was linearised with \textit{KpnI} (sense) and \textit{SpeI} (antisense) and probes were transcribed using T7 and T3 RNA polymerase, respectively. As \textit{KpnI} digestion results in a 3' protrusion, the DNA was treated with the Klenow fragment of DNA polymerase for 25°C at room temperature to remove this before the transcription reaction took place.

A partial clone of \textit{Mdtim} was also available within the laboratory. Digestion of this with \textit{PstI} (sites at 1636bp and 2192bp) resulted in a 556bp fragment that was cloned into the \textit{PstI} site in pBs. Orientation of the clone was checked by restriction digest with \textit{EcoRV}. It was then linearised with \textit{BamHI} and T3 RNA polymerase used to create the antisense probe and with \textit{HindIII} and T7 RNA polymerase to produce the sense probe.
3.3 Results

Western blot analysis of PER and TIM cycling in Musca heads

As previously shown (Piccin, 1998) PER levels do not cycle over the circadian period (Figure 3.1). This result was obtained with all three anti-PER antibodies used. No obvious shift is seen that may indicate differences in phosphorylation state, as is seen with DmPER (Edery et al., 1994), indicating that any such events are not as dramatic as those for DmPER.

In 12:12 LD conditions TIM shows cycling in abundance with a peak corresponding to ZT22 (Figure 3.2). After peaking late at night levels drop off steadily after lights on reaching a minimum during the early night between ZT 12 and 18. There is a ten-fold difference between the minimum and maximum levels. Under DD conditions TIM continues to cycle but the amplitude is dampened in comparison to LD conditions, being less than two-fold and the peak is earlier, at CT20 (Figure 3.3). By developing blots of LD and DD samples side by side it was possible to compare protein levels (Figure 3.4). At maximum levels were virtually identical (a difference between the values of 1%). However minimum levels in DD corresponded to 70% of the maximum levels, whereas minimum levels in LD are only 10% of the maximum. Therefore the higher amplitude of cycling in LD is largely driven by light induced degradation of TIM.

Semi-quantitative RT-PCR analysis of per and tim cycling in Musca heads

Musca per was included as a positive control for this technique as RPA assays had previously been performed by A. Piccin (Piccin, 1998) in 12:12 LD. This technique revealed per cycling with a maximum at ZT14 and a ten-fold amplitude in the oscillation. Data obtained from RT-PCR analysis is consistent with the RPA data, showing the same amplitude in the oscillation of abundance although the peak is closer to ZT12 (Figure 3.5). Data obtained for tim can therefore be regarded as an accurate account of cycling.

In 12:12 LD tim cycles with a peak in abundance between ZT 12-16 and a 2.5-fold amplitude in the oscillation (Figure 3.6). The same result was obtained when flies were maintained at 18°C (Figure 3.7). However at 29°C the peak was close to
Figure 3.1 PER levels in a 12:12 LD cycle at 25°C. 

a) Western blot analysis of PER levels in 12:12 LD conditions. Males and females were assessed separately to investigate any sex-specific differences. Neither sex shows cycling PER. An aspecific band created by the PER antibody and a cross-reactive band produced by an α-GFP antibody were used to correct for loading inaccuracies. 

b) Quantification of PER levels. Mean levels are standardised and shown ± SEM. One-way analysis of variance revealed no significant differences between values for different time points (n = 3, Appendix 8).
Figure 3.2 TIM levels in 12:12 LD at 25°C.

a) Western blot of TIM with samples taken every two hours in a 12:12 LD cycle. Cycling in abundance can clearly be seen with a peak late at night at ZT22.

b) Quantification of western blot analysis showing mean, standardised values ± SD. On-way analysis of variance reveals a highly significant difference in the values with respect to time (F(11,12) = 481.80; p<0.00001, n = 2, Appendix 8).
Figure 3.3 TIM levels in DD at 25°C.

a) Western blot of TIM cycling in DD. Flies were entrained for 5 days in 12:12LD and samples were taken every two hours over the first day in DD. A molecular weight marker is given on the left. b) Quantification of western blot analysis showing mean, standardised values ± SD. One-way analysis of variance reveals a significant difference in the values with respect to time (F(11,12) = 9.96; p<0.0002, n = 2, Appendix 8). This shows that a rhythm is present albeit of a lower amplitude than that seen in LD.
ZT12 and the amplitude was vastly increased to fifty-fold (Figure 3.7). Two-way analysis of variance revealed a significant difference between the 18°C, 25°C and 25°C data sets, \( F(8,15) = 9.24; p<0.0001, \) although there is no statistical difference between the 18°C and 25°C data. This suggests that \textit{tim} cycling is both time and temperature dependant. This data is the same as that observed for \textit{tim} in \textit{Drosophila}, where highest levels of \textit{tim} transcripts are observed at higher temperatures (Majercak \textit{et al.}, 1999). However, in \textit{Drosophila} the peak in \textit{tim} levels in LD occurs earlier on colder days (18°C), whereas this data suggests that in \textit{Musca} the peaks earlier on warmer days (29°C).

Interestingly in DD neither \textit{per} nor \textit{tim} appear to cycle (Figure 3.8). This cannot be due to light contamination during collection as collections were made at the same time for both RNA and protein extractions. The protein extractions show elevated levels of light-sensitive TIM in DD, which would not be possible if the flies had been inadvertently exposed to light.

\textbf{Figure 3.4 Relative levels of TIM in both LD and DD at 25°C}
Relative levels of TIM under 12:12 LD and DD measured every two hours. Quantifications are given as standardised means ±SD. Two-way analysis of variance reveals a highly significant difference between the two data sets \( F(11,24) = 60.91; p<0.00001, \) n=2, Appendix 8).
Figure 3.5 *per* mRNA cycling in 12:12 LD at 25°C  

a) RT-PCR analysis of *per* cycling in LD, rp49 is used as an internal control for inaccuracies in initial RNA levels. M refers to a φX174-*HaeIII* marker, bands sizes (bp) are given to the right. b) Quantification of gel image analysis. Values are adjusted for *rp49* levels and standardised and shown ± SEM. On-way analysis of variance reveals a highly significant difference in the values with respect to time (F(11,12) = 381.79; p<0.00001, n = 2, Appendix 8).
**Figure 3.6 tim mRNA cycling in 12:12 LD at 25°C**

a) RT-PCR analysis of *tim* cycling in LD, *rp49* is used as an internal control for inaccuracies in initial RNA levels. M refers to an φX174-*HaeIII* marker, bands sizes (bp) are given to the right. 

b) Quantification of gel image analysis. Values are adjusted for *rp49* levels and standardised and shown ± SEM. One-way analysis of variance reveals a highly significant difference in the values with respect to time (F(11,12) = 8.32; p<0.00001, n = 3, Appendix 8).
Figure 3.7 *tim* mRNA cycling at both 18°C and 29°C in a 12:12 LD cycle. 

a) An example of an agarose gel showing RT-PCR products for both conditions. M refers to a φX174-HaeII marker, bands sizes (bp) are given to the right. b) Quantification is shown as mean standardised values ± SD for each time point. One-way analysis of variance reveals significant differences between the time point values at 18°C (F(5,6) = 11.29; p<0.0052, n = 2, Appendix 8) and highly significant differences between the 29°C values (F(4,5) = 72.51; p<0.0001, n = 2 Appendix 8).
Figure 3.8 tim and per mRNA cycling in DD at 25°C

An example of the agarose gel for the RT-PCR is given for both tim (a) and per (b). M refers to a $\Phi$X174-HaeII marker, bands sizes (bp) are given to the right. c) Quantifications are given as mean, standardised values ± SEM. One-way analysis of variance on the tim data reveals a significant difference between the time points ($F(11,12) = 5.61; p<0.003, n = 3$, Appendix 8) although only CTO is actually significantly different to the other values when the data is examined. Omission of this time point returns a $p$ value of 0.208, revealing no significant difference between the other time points. The same is true for the per data ($F(11,12) = 9.10; p<0.0003, n = 3$, Appendix 8) although it is only the CT18 and CT22 time points that show significant differences to the other time points. It can clearly be seen that there is no evidence of cycling for either per or tim.
Immunocytochemical location of PER and TIM expressing cells within the *Musca* brain

Initial studies were conducted with the αDmPER (Young) antibody on 6μm head sections. The most intensely stained cells in this case were located in the pars intercerebralis but control experiments revealed these to also show staining if rabbit normal serum is substituted for the α-PER antibody (Figure 3.9). The experiment was then repeated using the Hall and 774 antibodies. Both stained several groups of cells (Figure 3.9 and 3.10) all of which show cytoplasmic staining and do not show any oscillation in the intensity of staining over the circadian period. Repeating this with the Young antibody at higher concentrations also revealed these cytoplasmically stained cells but slides showed higher levels of background staining. No staining was achieved for TIM using this method.

Although no photoreceptor nuclei staining was ever observed a structure located at the base of the photoreceptors stained very strongly for PER which has been reported previously (Figure 3.9, Racey, 2002)

As the reaction to develop the staining is enzymatic in these experiments the reaction is stopped before saturation is achieved. Therefore it is possible that the intense cytoplasmic staining observed could mask a small amount of nuclear PER. In order to investigate this possibility whole-mount brains were subjected to ICC using fluorescent secondary antibodies. This experiment was conducted with E. Rosato. When this technique was used fewer cells were observed and the pars intercerebralis cells no longer gave a signal (Figure 3.11). With the aid of a confocal microscope co-localisation of PER and TIM was achieved in a small group of cells located between the optic lobe and the central brain at the level of the oesophagus (Figure 3.12). At night (ZT24) both PER and TIM can be seen within the same cells but whilst PER remains within the cytoplasm TIM becomes nuclear. PER also appears to cycle within these cells as no signal is detectable at ZT19. At ZT24 with this technique both PER and TIM co-localise within the photoreceptor nuclei (Figure 3.13). Thus this technique revealed both PER and TIM staining within cells that could not be seen previously.

Whilst the cells on the border with the optic lobe would appear to be in the correct position to be lateral neurons when compared to *Drosophila*, they would not co-localise with PDF from the position of the PDF positive cells in *Musca*. ICC was
Figure 3.9 PER staining on tissue sections of Musca heads

a) General staining pattern obtained with all available antibodies. Several groups of cells can clearly be seen to stain (yellow arrows). These include cells within the pars intercerebralis. Although no staining is observed in photoreceptor nuclei at night a structure at the base of the photoreceptor can be seen to stain strongly at all times (b, red arrow). c) Replacing the α-PER antibody with rabbit normal serum results in staining of pars intercerebralis cells (yellow arrow), suggesting that these are non-specific for PER staining.

Key: pars int, pars intercerebralis; l ho, lateral horn; v l pro, ventral lateral protocerebrum; oes, oesophagus. Me, medulla; la, lamina; ret, retina.
**Figure 3.10** PER staining on tissue sections of *Musca* heads

a) Staining of a neuron showing cytoplasmic PER staining (arrow) using the α-DmPER (Young) antibody. b) PER staining with the α-DmPER (Hall) antibody during both day and night. At both times PER is cytoplasmic.

Key: CB, central brain; Me, medulla; La, lamina.
Figure 3.11 Confocal microscopy of a whole mount *Musca* brain
General staining for PER within the Musca brain using whole mount specimens. Fewer cells (yellow arrows) show staining than when tissue sections are used. Key: OL, optic lobe; CB, central brain.
Figure 3.12 PER and TIM co-localisation within *Musca* neurons at ZT24

(a) and (b) Upper left panel, PER staining; upper right, TIM staining; lower left, merge. For both sets of cells shown PER is restricted to the cytoplasm, whereas TIM is found in the nucleus in both cases as well as in the cytoplasm. More cytoplasmic TIM can be seen in (b). Both cells shown are from within the same group of neurons.
**Figure 3.13** Photoreceptor staining in *Musca*
Upper left panel: PER staining; upper right: TIM staining; lower left: merge. PER and TIM co-localise within the photoreceptor nuclei (yellow arrows). Whilst PER staining can once again be seen in a structure at the base of the photoreceptor (red arrow, see also Figure 3.9), TIM does not give staining although strong staining for TIM can be seen in cells lying underneath this structure (blue arrow).
therefore carried out on both *Drosophila* and *Musca* adult and larval brains to look at the relationship of these neurons between the two species. In larvae and adults the neurons appear to be in similar positions (Figure 3.14). In *Musca* larvae a group of four cells can be detected within each hemisphere which send projections dorsally. This has recently been confirmed by Pyza and colleagues (2003). In both *Drosophila* and *Musca* adults these PDF cells are located within the accessory medulla (also termed anterior base of the medulla), although tissue sections reveal that the cells in *Musca* are in a more anterior position to those in *Drosophila*. Both species show staining patterns as previously reported (Pyza and Meinertzhagen, 1997; Helfrich-Forster, 1995). In *Drosophila* the l-LNv.s project into the medulla, showing large arborisations over the surface of the medulla and also towards the s-LNv.s and into the posterior optic tract (Helfrich-Forster, 1995). The s-LNv.s send processes into the superior protocerebrum, arborizing dorso-frontal to the calyces of the mushroom bodies. Here they appear to contact another group of neurons that project towards the pars intercerebralis and then via the median bundle towards the oesophageal foramen (Helfrich-Forster, 1995). In *Musca* both groups of cells send processes into the medulla and lamina as previously reported (Pyza and Meinertzhagen, 1997). However I also observed projections from the smaller cells into the central brain, similar to that seen in *Drosophila*. One of the two more ventral processes may be the posterior optic tract. These cells also project dorsally toward the region containing the calyces of the mushroom bodies and on towards the pars intercerebralis, as also seen with *Drosophila*. Both ventral and one of the dorsal processes arborize dorsal to the oesophageal foramen. It can therefore be seen that the pattern of projections from the PDF positive cells is slightly different between the two species.

Localisation of *per* and *tim* mRNA within the brain

The pattern of staining obtained with *per* probes was seen to be of a pattern similar to that seen in *Drosophila* (Figure 3.15, Kloss *et al.*, 1998). This is far more widespread than the protein staining, which suggests post-transcriptional regulation plays a role in cell specific expression.

Only the *CDper* probe gave staining for *per*. Whilst a higher intensity of staining was seen during the night, in accordance with the RT-PCR data, technical problems made the data impossible to quantify meaningfully. It was necessary to
Figure 3.14 Confocal microscopy of PDF localisation within *Drosophila* and *Musca* brains.
Staining patterns within the larval brains of both species are similar, (a) shows an example of a *Musca* larvae whilst (b) shows an example of *Drosophila*. Similarities are also seen between the adult *Musca* (c) and *Drosophila* (d) brains.
Key: l, lamina; aMe, accessory medulla; Me, medulla; L, large PDF neurons; S, small PDF neurons; l-LN, s, large ventral lateral neurons; s-LN, s, small ventral lateral neurons; pars int, pars intercerebralis; oes for, oesophageal foramen.
Figure 3.15 In-situ hybridisation of *Mdper*

General staining pattern produced for a) *Dmper* (taken from Kloss et al., 1998) and b) *Mdper* in-situ hybridisation. c) Negative control showing lack of staining produced when the sense probe is used.

Key: OL, optic lobe; CB, central brain.
treat samples from different time points identically so that comparisons between time points would be possible. However the harsh treatments employed in the technique resulted in at least half of the sections being lost by the end of the experiment. Several sets were processed together in the hope that one intact set could be made at the end of the experiment but this proved to be unsuccessful. Fixation steps were also implemented during processing but these resulted in blocking the probe and so no staining was observed.

3.4 Discussion

In *Musca* both per and tim mRNA cycle in abundance in LD, with per peaking at approximately ZT12 and tim at around the same time, between ZT12 and ZT16. Ten-fold amplitude of cycling is seen in both cases. This is very similar to that observed for *Drosophila* (Edery *et al.*, 1994; Sehgal *et al.*, 1994). However in DD conditions the oscillation of both RNA's is either abolished or significantly dampened, more data is required to ascertain which is the case. At the protein level PER does not cycle under any conditions whereas TIM cycles in both LD and DD, peaking at the end of the night. It is interesting to note that TIM levels are significantly raised in DD, thus dampening the amplitude of the oscillation.

Investigations at the cellular level revealed a broad expression pattern for per mRNA, as seen in *Drosophila* (Kloss *et al.*, 1998), whilst the PER protein is seen in far fewer cells, as again is the case with *Drosophila*. Thus in both species a post-transcriptional mechanisms are important in determining PER regulation of expression. Using enzymatic staining, ICC experiments revealed several groups of neurons that stain strongly for PER. In these cells there is no oscillation in staining intensity and the signal remains cytoplasmic throughout the day. More sensitive procedures using fluorescent secondary antibodies and confocal microscopy revealed two groups of neurons positioned dorso-lateral to the oesophagus that stain intensely for PER within the cytoplasm and correspond to cells seen with the enzymatic staining. However a third group positioned ventro-lateral to the oesophagus also stain although the signal is much weaker. Within this group PER appears to cycle, as staining is seen at ZT24 but not at ZT19, although the PER signal is cytoplasmic. TIM is also detected within these cells and again is only visible at ZT24, suggesting
that TIM is cycling in these cells in accordance with the western data. At this time however TIM is nuclear whilst PER is cytoplasmic. The position of these cells supports the idea that the clock cells reside within the central brain of *Musca*, as severance of the optic lobe does not abolish behavioural rhythms (Helfrich *et al.*, 1985).

Both PER and TIM are found in photoreceptor nuclei at ZT24, suggesting that PER can become nuclear in peripheral tissues, although it does not in the central brain. This is also seen for PER in *A. pernyi* (Sauman and Reppert, 1996).

PDF staining is seen in a similar pattern to that of *Drosophila*. Both the small and large group of cells send projections into the medulla and lamina, with the large cells sending projections that arborise over the surface of the medulla as previously reported (Pyza and Meinertzhagen, 1997). I also observed projections from the small cells leading into the central brain both laterally, possibly along the optic tract and dorsally towards the calyces of the mushroom bodies as previously reported for *Drosophila* (Helfrich-Forster, 1995). However these cells do not appear to be the same as any of those expressing TIM and/or PER, although they may be in a position to allow communication with clock cells, thus potentially maintaining PDF’s role as a circadian output factor. PDF does not co-localise with PER in a number of insect species (Zavodska *et al.*, 2003), in fact *Drosophila* so far is the only species in which it does co-localise. However recent data shows that PDF does co-localise with CRY in *Musca*, further supporting a maintained circadian role for PDF (Pyza *et al.*, 2003).

As PER remains cytoplasmic this challenges the negative feedback mechanism described for *Drosophila*. However it is possible that PER is shuttled between the nucleus and cytoplasm as recently described for DmTIM (Ashmore *et al.*, 2003), although dimerisation does not lead to nuclear retention of PER as seen for DmTIM. Alternatively it may be that the roles of TIM and PER are reversed in *Musca* and that PER facilitates TIMs nuclear entry and it is TIM that is the repressor, either directly or indirectly. A role for TIM in repression could help describe the apparent non-cycling of *per* and *tim* mRNAs in DD. In DD TIM remains at relatively high levels, this could be above the threshold level, leading to constant repression. It is also possible that transient nuclear entry of PER is sufficient for negative feedback, as has recently been suggested for *A. pernyi* (Chang *et al.*, 2003).
Chapter 4

Expression of *Musca period* in *Drosophila*
4.1 Introduction

Previous work by A. Piccin in the laboratory had included the production of transgenic *Drosophila* containing the *Musca per* gene in the laboratory, termed *mml*. The transgene consists of the *Drosophila* promoter and 5' UTR with the full coding sequence and 3' UTR of the *Musca* gene. These flies are in a *Drosophila per* background. Thus the only *per* expression is that of *Mdper* but regulation of this expression is as the native gene. The *Mdper* transgene, when carried as a single autosomal copy, successfully rescues locomotor behaviour but the period of the rhythm is shorter than the wild type 24h at approximately 22h in free-running conditions (Piccin et al., 2000).

This reduction in period length is consistent with my data in that it suggests greater stability of MdPER within the cytoplasm. Stable PER would in theory be able to enter the nucleus earlier, thus down regulating its own production earlier, leading to a faster rhythm. Previous western blot analysis of PER in these flies showed two immuno-reactive bands, only one of which showed any degree of cycling (A. Piccin, pers. comm.). The presence of two bands may suggest different phosphorylation states, which together with the fact that only one form cycles supports the theory of increased stability of MdPER related to a change in the PER-DBT relationship.

In order to investigate the regulation of PER in the *mml* flies I performed the following experiments. Firstly I wished to repeat the western blot to confirm the data and to then use β Protein Phosphatase to show that the two bands were the result of phosphorylation status by reducing them to one single band. To further investigate the idea of an altered relationship with DBT I wanted to look at the activity of *mml* flies in the four *ddbt* mutant backgrounds. Initially I produced *mml* that were also heterozygous for the *dbt* mutations *dbt*5, *dbt*1, *dbt*0 and *dbt*ar. Although only one copy of the *dbt* mutation is present *dbt* mutations are semi-dominant (Price et al., 1998; Rothenfluh et al., 2000b) and so I could still expect to see an effect. In order to cross *mml* into a homozygous *dbt* mutant background it was first necessary to move the *mml* transgene to a different chromosome as both lines available have the transgene on the third chromosome, where *dbt* is also located (Price et al., 1998).

As the *doubletime* arrhythmic mutation (*dbt*ar) has been shown to act via the dPER C-domain (Rothenfluh et al., 2000b) I also wished to see what affect the *dbt*ar mutation had on MM12 flies. MM12 flies carry a chimeric *per* transgene that consists
mainly of \textit{Mdper} but with the c-domain swapped for that of \textit{Dmper}. An illustration of this chimera is given in Figure 4.1.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure4.1}
\caption{Diagrammatic representation of the MM12 transgene}
\textbf{Figure 4.1} Diagrammatic representation of the MM12 transgene
The MdPER sequence is given in blue with the numbers representing the amino acid number, whilst the DmPER sequence is shown in red.
\end{figure}

I also wished to investigate the PER-TIM relationship in \textit{mml} as other results (Chapter 5) suggest that the PER-TIM relationship is different in \textit{Musca} as PER is stable in the absence of TIM. It was therefore interesting to assess whether a MdPER-DmTIM dimer is formed in \textit{mml}. To do this I employed the yeast two-hybrid system to look for protein-protein interaction. I also wanted to look at the behaviour of \textit{mml} in a \textit{Drosophila per}^{01};\textit{tim}^{01} background. This would show whether, in \textit{Drosophila} at least, MdPER requires TIM to function.

\section*{4.2 Materials and Methods}

All experiments were performed with male flies carrying one autosomal copy of the \textit{mml} transgene. Behavioural analysis was performed as described in Chapter 2.

\textbf{Western Blot analysis}

Flies were collected every four hours in a 12:12 LD regime and the heads isolated. Protein was then extracted and subjected to western blot analysis using an anti-PER antibody. Both the anti-DmPER (Hall) and anti-MdPER 774 antibodies were used in separate experiments.
Immunocytochemistry

Heads of *mm1* flies were dissected and fixed every four hours over the circadian period. These were used for ICC as detailed in Chapter 2 using 6μm sections and the α-DmPER (Hall) and α-MdPER 774 antibodies.

Yeast two-hybrid analysis of MdPER-DmTIM protein interaction

Both *Musca* and *Drosophila* PER (233-685) clones were used as bait and challenged with both full length *Drosophila* TIM and the empty prey vector (negative control). The *Drosophila* PER-TIM interaction was performed as a positive control for the experiment. Transformed yeast was incubated at 30°C and assessed for the blue precipitate produced by the β-galactosidase reporter.

Creation of per01;tim01;mm1 flies

To obtain this genotype it was first necessary to create per01;tim01 flies, as detailed in Figure 4.2. *mm1* flies were then crossed to a double balancer strain to the second and third chromosomes and the resulting progeny crossed to per01;tim01 flies. Full details are given in Figure 4.3.

Production of per0; mm1/dbtmut flies

Virgin female *mm1* flies were crossed to males carrying the *dbt* mutation. Both *dbt* and *dbt* flies were homozygous for the mutation and so male progeny of this cross were selected as they would inherit per0 and one copy of *mm1* from the mother and the *dbt* mutant allele from the father. In the case of *dbt* and *dbt* mutants the mutation is balanced and so resulting male progeny were selected for the absence of the balancer chromosome. As a control flies carrying a dper transgene in a dper0 background (tg20, 13.2, referred to as per+) were also crossed to the *dbt* mutants in the same manner. This allowed comparison between two transgenic lines rather than between one transgenic and one wild type line. Male *mm1* and per+ flies carrying one copy of their respective transgene were also assessed for locomotor activity. These have wild type *dbt* activity (*dbt*) and so are used to assess the effect of the *dbt* mutation.
Figure 4.2 Cross scheme to create \(per^0\), \(tim^0\) Drosophila

a) It was firstly necessary to balance \(yw\) \(per^0\) flies for the second chromosome. These flies were then crossed to \(tim^0\) flies (b). Progeny from this cross were then crossed to each other. The presence of the second chromosome balancers were utilised so that their absence in the F2 indicated flies homozygous for the \(tim^0\) mutation. In order to select homozygotes for \(per^0\) the yellow (\(y\)) marker was selected, as this is recessive. Only chromosomes one and two are represented.
Figure 4.3 Production of $per^0; tim^0; mml$ flies

a) MM1 flies were crossed to balance the second and third chromosomes. The progeny were then crossed to $per^0; tim^0$ flies (b). Progeny (F1) for this cross were selected as shown and then crossed to others of the same genotype. The F2 were then selected for the absence of $CyO$ to indicate homozygosity for $tim^0$. At this stage it was impossible to tell if the flies were homozygous for $mml$ and so the F2 were crossed to each other as single crosses and only lines that produced no white eyed progeny were kept as these are homozygous for $mml$. 

\[
\begin{align*}
\text{a} & \quad \frac{wper^0}{wper^0} ; \quad \frac{\pm}{\pm} ; \quad \frac{mml}{mml} \quad \frac{X}{\rightarrow} \quad \frac{w}{w} ; \quad \frac{CyO}{CyO} ; \quad \frac{MKRS}{MKRS} \\
\text{b} & \quad \frac{wper^0}{wper^0} ; \quad \frac{CyO}{CyO} ; \quad \frac{mml}{mml} \quad \frac{X}{\rightarrow} \quad \frac{ywper^0}{ywper^0} ; \quad \frac{tim^0}{tim^0} ; \quad \frac{\pm}{\pm} \\
& \quad \frac{ywper^0}{ywper^0} ; \quad \frac{tim^0}{tim^0} ; \quad \frac{mml}{mml} \quad \frac{X}{\rightarrow} \quad \frac{ywper^0}{ywper^0} ; \quad \frac{tim^0}{tim^0} ; \quad \frac{mml}{mml} \\
& \quad \frac{ywper^0}{ywper^0} ; \quad \frac{tim^0}{tim^0} ; \quad \frac{mml}{mml}
\end{align*}
\]
Assessment of the effect of the \textit{dbt}^{ar} mutation on MM12 flies

The MM12 flies were a gift from K Garner. The transgene is located on the second chromosome and is balanced with the \textit{CyO} chromosome. These flies were crossed to \textit{yw} \textit{per}^{0} flies to create flies homozygous for the transgene in a \textit{Dmper}^{0} background. Females were then crossed to \textit{dbt}^{ar} flies and male progeny selected for \textit{Dmper}^{0} background carrying one copy of the \textit{dbt}^{ar} mutation. Locomotor activity was then assessed for these flies, along with male flies carrying one copy of the MM12 transgene in a \textit{Dmper}^{0} background.

Repositioning of the \textit{mml} transgene to the second chromosome

I chose to move the transgene by crossing the \textit{mml} flies to flies carrying a \textit{A2-3} transgene (see Figure 4.4), that expresses the transposase necessary for excision and re-insertion of the P-element that contains the transgene. After this cross male flies (males do not undergo recombination) were crossed to females carrying a third chromosome balancer, which would allow quick identification of flies carrying the insert on the second chromosome. Progeny from the cross to reposition the transgene were screened for a change in eye colouration (to a lighter shade of red or to orange or yellow) and for segregation of \textit{Sb} and \textit{w}. A change in eye colour may indicate a new positional effect of the repositioned transgene and if all \textit{Sb} flies resulting from the cross carry \textit{w} it indicates that the transgene is still on the third chromosome. If the transgene is relocated to chromosome one then all male progeny will be \textit{w} and if it is relocated to the second then flies that carry \textit{Sb} are both white and red eyed and there is no sex linked eye colour (Figure 4.4).

Removal of the \textit{A2-3} transgene was achieved by excluding \textit{Sb} flies from further crosses. Once it had been confirmed that the \textit{mml} transgene was situated on the second chromosome they were crossed to flies carrying both second and third chromosome balancers to confirm the position of the \textit{mml} transgene and to check that only one copy of the transgene was present. This subsequently also allowed the flies to be crossed to homozygosity. They were then crossed into a \textit{per}^{0} background for behavioural experiments. Full details of the crosses are given in Figure 4.4. These flies were then put into heterozygous \textit{dbt} mutant backgrounds in the same manner as for the original \textit{mml} line, except that, like MM12 flies presence of the transgene is detected by absence of the \textit{CyO} balancer chromosome.
First chromosome combinations

Second chromosome combinations

Third chromosome combinations

a

First chromosome combinations

Second chromosome combinations

Third chromosome combinations

b

First chromosome combinations

Second chromosome combinations

Third chromosome combinations

+ or __________ or ±

+ or ________ or __________ or ±

+ or ________ or ________ or ±

+ or ________ or ________ or ±

+ or ________ or ________ or ±
Figure 4.4 Identification the chromosomal location of the relocated *mml* transgene. The possible combinations for each chromosome are given. **a)** If the transgene remains on the third chromosome then all individuals carrying *sb* will have white eyes as they cannot also carry *mml*, which gives a red eye phenotype as it carries *w*⁺ as a marker. **b)** When the transgene has been successfully relocated to the second chromosome then *sb* flies can also have red eyes. There is no difference between sexes. **c)** Although insertion of the transgene on the first (X) chromosome will also produce individuals that can be both *sb* and red eyed, only females will have red eyes. Thus first and second chromosome insertions can be differentiated.
4.3 Results

**Western blot analysis of PER cycling**

Several attempts were made to perform this experiment using both separate set of protein extracts and both available α-PER antibodies. Blots were performed for extracts made both over LD time courses and for protein extract made at ZT23 (when DmPER levels are high). Each time it was impossible to find an MdPER band as all visible bands were also found in the per₀ control lanes. Examples are shown in Figure 4.5. In order to attempt to rectify this problem I repeated the blots whilst adding double the amount of SDS to the extraction buffer and/or running the extracts on a 8M urea SDS-PAGE gel. Both of these measures were to give harsher denaturing conditions as A. Piccin had previously described that MdPER had been seen in a high molecular weight complex (Piccin., 1998), possibly explaining my lack of a specific band. Neither of these measures, nor a combination of the two, proved successful in yielding a specific PER band on the western blot.

**Immunocytochemistry**

Staining was observed in cells that had the correct anatomical position to be lateral neurons. Unfortunately it was not possible to co-label these cells with α-PDF antibody as both available antibodies were raised in rabbit. At all time points staining appeared to be cytoplasmic and did not vary in intensity over the circadian period (Figure 4.6). No obvious staining is observed in photoreceptor nuclei either at night or during the day. Some staining is observed at night but not at a level sufficiently above background levels to be specific. This suggests that MdPER is also stable in *Drosophila*, as seen in *Musca* (Chapters 3 and 5). It is however possible that as these sections were developed with DAB that a high concentration of PER in the cytoplasm would mask any nuclear signal due to the nature of the enzymatic reaction. Had time allowed I would have repeated this experiment using fluorescent antibodies and confocal microscopy.

**Yeast two-hybrid analysis**

A positive result was observed for both DmPER-DmTIM and MdPER-DmTIM protein interactions whilst no interaction was seen between either PER and
Figure 4.5 Western blot of MM1 protein extracts using an anti-PER antibody (Hall). Many cross-reacting bands can be seen, including the aspecific band often seen for anti-PER antibodies running slightly above the 83kDa marker. However, no band is present in any of the MM1 samples that is absent in either of the per<sup>0</sup> control lanes, indicating that MdPER is not detected. There is also no difference in band patterns at any of the time points analysed for MM1. The sizes of the molecular weight markers (lane M) are given to the left.
Figure 4.6 Immunocytochemical staining of putative LNs and photoreceptors in MM1

Immunocytochemistry reveals cells that appear in the correct position to be lateral neurons (LNs, a and b red arrows). a) Shows a horizontal section whist these cells do not show changes in the intensity of staining over the circadian cycle and always give a cytoplasmic signal (doughnut like in appearance, c) day, d) night). e) (day) and f) (night) show sections through the retina. No obvious staining of photoreceptor nuclei is observed although some weak signal is seen at night (red arrow), this is not sufficiently above background levels to be seen to be specific. All staining was performed with the Hall antibody.

Key: r retina, ol optic lobe, cb central brain
the empty prey plasmid (Figure 4.7). This suggests that MdPER is capable of interacting with DmTIM in the \textit{mm} l flies.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure4.7}
\caption{Yeast two-hybrid analysis of MdPER-DmTIM interactions}
\end{figure}

\textit{mml} flies in a \textit{per}^{01};\textit{tim}^{01} double-mutant background

Locomotor activity analysis revealed that both the double mutant and the double mutant carrying the \textit{mml} transgene entrain to a LD cycle and that both are arrhythmic under free running conditions (Figure 4.8). Therefore although MdPER is stable without TIM in \textit{Musca}, and is apparently stable in \textit{mml} flies, it cannot function without DmTIM in \textit{mml}.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
 & \textbf{12:12LD (n)} & \textbf{DD (n)} \\
\hline
\textit{per}^{0};\textit{tim}^{0};+ & 23.62 \pm 0.29 (10) & 100\% Arrhythmic (10) \\
\textit{per}^{0};\textit{tim}^{0};\textit{mml} & 24.24 \pm 0.30 (22) & 100\% Arrhythmic (22) \\
\hline
\end{tabular}
\caption{Locomotor activity of \textit{per}^{01};\textit{tim}^{01};\textit{mml} flies. Average period (\(\tau\)) of locomotor activity is given \pm SEM. As expected \textit{per}^{0};\textit{tim}^{0} flies are arrhythmic under constant conditions. The same is seen when these flies carry the \textit{mml} transgene. The number of flies tested is given (n).}
\end{table}
Behaviour of \textit{mm1} flies carrying a \textit{dbt} mutation

Data was analysed for all flies and is shown in Figure 4.9. The effect of the \textit{dbt} mutation is the difference in \(\tau\) between the \textit{dbt}\(^+\) control and the \textit{dbt} mutation being assessed for each line. It can be seen that the results observed for \textit{per}\(^+\) flies is consistent with that previously shown in Rothenfluh \textit{et al.} (2000b). For both the \textit{dbt}\(^e\) and \textit{dbt}\(^l\) mutations the effect on \(\tau\) is slightly greater in \textit{mm1} flies than in \textit{per}\(^+\). However the effects of \textit{dbt}\(^e\) and \textit{dbt}\(^{ar}\) are considerably greater in the \textit{mm1} flies compared to \textit{per}\(^+\). \textit{dbt}\(^e\), which is thought to effectively halve DBT activity compared to wild type, shows a reduction in \(\tau\) of close to 1h in \textit{mm1} compared to 0.3h in \textit{per}\(^+\). Wild type flies heterozygous for the \textit{dbt}\(^e\) mutation show wild type \(\tau\) in \textit{Drosophila}, as one functional copy of \textit{dbt} appears sufficient to maintain function (Price \textit{et al.}, 1998).

The \textit{dbt}\(^{ar}\) mutation also shows different effects on the different \textit{PER} proteins. As previously reported for wild type flies (Rothenfluh \textit{et al.}, 2000b) \textit{per}\(^+\) flies heterozygous for \textit{dbt}\(^{ar}\) show an increase in \(\tau\), although this is only of 3h in comparison to 4.5h in wild type, whereas \textit{mm1} only have an increase of 0.6h. It has been reported that \textit{dbt}\(^{ar}\) is a reduced function allele rather than a null like \textit{dbt}\(^e\), which results in slower phosphorylation of \textit{PER} and thus a longer rhythm (Rothenfluh \textit{et al.}, 2000b). This can be seen due to the increase in \(\tau\) of the \textit{per}\(^+\) flies. However no increase in \(\tau\) is seen for the \textit{mm1} flies in this case, suggesting that \textit{dbt}\(^{ar}\) does not alter the dynamics of \textit{MdPER} degradation. Rothenfluh \textit{et al.} also reported that the C-domain of \textit{PER} is important in \textit{dbt}\(^{ar}\) activity, as short period mutants that arise from this area show degradation of hyperphosphorylated \textit{PER} unlike their wild-type counterparts in a \textit{dbt}\(^{ar}\) background.

By analysing the locomotor activity of MM12 flies carrying one copy of the \textit{dbt}\(^{ar}\) mutation it was possible to assess the role of the C-domain of \textit{MdPER}. The \(\tau\) of these flies also shows a 3h increase as seen in \textit{per}\(^+\) (Figure 4.9), indicating that by swapping only the C-domain for that of \textit{DmPER} restores the \textit{Drosophila} \textit{dbt}\(^{ar}\) phenotype. This would support the hypothesis of Rothenfluh \textit{et al.} that normally the C-domain of \textit{DmPER} inhibits DBT action. The \textit{MdPER} C-domain does not block DBT action.

It should be noted that the free running rhythm measured for the \textit{mm1} flies is closer to 23h rather than the previously reported 22h (Piccin \textit{et al.}, 2000). The free running period was measured again on different apparatus and found to be 22h (B.
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**b**

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**Figure 4.9 Effects of dbt mutations on MM1 flies**

a) Mean τ ± SEM is given for each genotype and the number of flies tested (n) is also stated. The left hand column corresponds to the dbt mutation being assessed, flies are heterozygous for this mutation. + indicates a wild type dbt background. per⁺ flies were included as a control. The τ of both genotypes is given in both 12:12 LD and DD conditions.  
b) Data for the free-running (DD) τ is given along with the effect of the dbt mutation (h). The effect is calculated as being the difference between the τ of the mutant fly compared to that of the same genotype with a dbt⁺ background.  
c) The effects of the dbtmut mutation on MM12 flies. Again mean τ is stated ± SEM. The effect of the mutation is calculated as the difference in τ between the dbt⁺ and dbtmut flies. ANOVA one-way analysis of variance shows a significant difference between the periods for each genotype in a dbt+ and dbtmut background with the exception of per+; dbt+ and per+; dbtP, where there is no significant difference (Appendix 9).
Collins pers. comm.), suggesting that this discrepancy is caused by different incubator conditions. As all of my behavioural experiments were conducted in the same machinery I used my data for *mm*l to allow a direct comparison between the genotypes tested.

**Repositioning of the *mm*l transgene to the second chromosome**

For the original cross 150 lines were set up, resulting in the selection of three second chromosome lines termed 97.1, 97.1 RED and 98.1. The original line 97 produced two independent second chromosome lines.

Once these flies were balanced for the second and third chromosomes then it was attempted to cross these flies to homozygosity by removal of the second chromosome balancer upon crossing within each line. However this was not possible to achieve, indicating that for all three lines that the insert was homozygous lethal. This was not really a problem as the transgene carried the *dper* promoter. This is an X-linked promoter and therefore in male flies the transgene will undergo dosage compensation (Bashaw and Baker, 1996; Lucchesi, 1996), resulting in normal expression levels. These flies were then crossed into a *per*\(^0\) background for behavioural analysis and then flies in the *per*\(^0\) background were used to make crosses to produce individuals that carried both *mm*l and a single *dbt* mutant allele as there was insufficient time to cross these flies into a homozygous mutant background.

Locomotor activity of the lines revealed that each line produced a different free-running period. This was 24h for 97.1, 22.7h for 97.1 RED and 23.4h for 98.1 (Figure 4.10). Because of this all lines were assessed in the *dbt* mutant backgrounds. As *dbt*\(^p\) and *dbt*\(^ar\) produced the most interesting results for the original *mm*l line, only these mutants were investigated with the repositioned lines (Figure 4.10). The increase in period length caused by *dbt*\(^ar\) in these flies appears to correlate with the free-running period of these flies in a wild type background. The shorter rhythm 97.1RED is the only one to show a 3h increase with the other lines showing closer to 1.5h. Thus the 97.1 line shows wild type DmPER behaviour. The 97.1 line, which shows a wild type free running rhythm shows a reduced increase of 1.5h whilst 98.1, with a free-running \(\tau\) of 23.4h shows a 1.7h increase in the *dbt*\(^ar\) background. Only 97.1RED and 98.1 were assessed in a *dbt*\(^p\) background and both showed the same effect as the original *mm*l with an increase in \(\tau\) of approximately 1h (Figure 4.10).
### Table a

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### Table b

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### Table c

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### Figure 4.10 Locomotor activity of second chromosome MM1 lines in wild type and \textit{dbt} mutant backgrounds

In a and b mean $\tau$ ± SEM is given in both 12:12 LD and DD conditions, (n) is the number of flies tested. a) Locomotor activity of the repositioned mm1 lines is given in a wild type background. b) Repositioned, second chromosome lines were assessed for activity when carrying either the \textit{dbfp} or \textit{dbptr} mutation. c) Data is compiled for each line to show mean $\tau$ in wild-type (+), \textit{dbfp} and \textit{dbptr} backgrounds. The effect of the \textit{dbt} mutation is calculated in the difference between the mean $\tau$ of the line in a wild-type background compared to that in the \textit{dbt} mutant background. ANOVA one-way analysis of variance shows that there is a significant difference in $\tau$ between the \textit{dbt} and the \textit{dbtmut} background for each line with the exception of 97.1 RED; \textit{dbfp} and 97.1 RED; \textit{dbpt} (Appendix 9).
4.4 Discussion

Previous studies had shown that MdPER cycles in abundance in transgenic *Drosophila* but with an apparently altered phosphorylation relationship to that of DmPER (A. Piccin pers. comm.). Although, due to antibody recognition problems, I could not repeat this, the behavioural data presented here indicates an altered relationship between PER and DBT. Yeast two-hybrid analysis of MdPER-DmTIM interaction suggests that, despite the altered kinetics of MdPER, it can still function in *Drosophila* and maintain a circadian function. The short $\tau$ of *mml* and the ICC data suggest that MdPER is more stable than DmPER. It is possible that repeating the ICC using fluorescent antibodies and a confocal microscope could detect nuclear entry of MdPER. It may be that the phosphorylated form of MdPER enters the nucleus whilst the more stable protein is confined to the cytoplasm. This more stable cytoplasmic protein may be what is being detected in the ICC experiment. MdPER is capable of nuclear entry in peripheral tissues as an MM1-GFP fusion protein is detected in the nucleus of cells in the Malphigian tubules of transgenic *Drosophila* (E. Rosato pers. comm., Figure 4.11).

Combining the data of the original *mml* line with those repositioned on the second chromosome suggests that the effects seen in a $dbt^{\text{w}}$ background may be a combination of an altered PER-DBT relationship and a position effect of the transgene insertion. Three out of four of these lines show a reduced effect of $dbt^{\text{w}}$ compared to wild type. Swapping the C-domain of MdPER with that of *Drosophila* results in a wild type $dbt^{\text{w}}$ phenotype, suggesting that the C-domain is responsible for this altered relationship.

In order to investigate why the C-domain could alter the PER-DBT relationship I looked at the predicted protein structures and predicted phosphorylation sites in this region of both DmPER and MdPER. There is no obvious difference in the predicted structure between the two proteins (Figure 4.12) or in the number of phosphorylation sites (Figure 4.13), although in general DmPER has more predicted sites than MdPER.

In general studying the four *mml* lines (the original plus three repositioned lines) show a stronger effect of $dbt^p$ and a reduced effect of $dbt^{\text{w}}$. This could be explained by a different role of DBT both within the cytoplasm and the nucleus. It is known that DBT affects the turnover of PER in both the cytoplasm and the nucleus.
Figure 4.11 PER-GFP staining in Malphigian tubules of MM1-GFP transgenic *Drosophila.*

a) Cytoplasmic detection of the GFP signal during the day as characterised by the doughnut appearance showing the empty nucleus (arrow).  
b) This signal becomes nuclear at night. Pictures supplied by E. Rosato.
Figure 4.12 Predicted protein structure for the C-domain areas of DmPER and MdPER.  

a) DmPER.  

b) MdPER.  
The C-domain is boxed in both cases, this being the section that was removed from MdPER and replaced with that of DmPER in MM12.
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Figure 4.13 Predicted phosphorylation sites within the mdPER and dPER sequences
The number of predicted sites for each kinase is given for both the full-length protein sequence and also for the number found within the C-domain region that was swapped to create the MM12 flies.
(Price et al., 1998) but the complete action of DBT is not fully understood. There is no direct relationship between phosphorylation and degradation. It is also thought that DBT may effect the nuclear gating of PER. The \( dbf^{p} \) mutation leads to lower levels of total DBT and is therefore likely to effect phosphorylation of PER in both cellular compartments. It may be that in \( mmf \) flies that although \( dbf^{p} \) may lead to a slower turnover of PER, it could lead to faster nuclear entry of PER, thus giving rise to a faster rhythm.

The \( per^{s} \) mutation in \( Drosophila \) has been shown to effect the nuclear turnover of PER (Hamblen et al., 1998; Marrus et al., 1996). It has also been shown that short period alleles repress the action of \( dbf^{ar} \) (Rothenfluh et al., 2000b), thus \( dbf^{ar} \) and \( per^{s} \) may effect nuclear stability of PER in opposite directions. As \( per^{s} \) is positioned within the conserved C-domain and replacement of the \( Musca \) C-domain with that of \( Drosophila \) (MM12) rescues wild type \( Drosophila \) behaviour, it is possible that increased nuclear turnover of PER in \( mmf \) counteracts the effects of \( dbf^{ar} \), thus giving a wild-type rhythm.

Although this work has suggested that the PER-DBT relationship in \( Musca \) is altered the exact nature of this is complicated and requires more work to establish exactly what is occurring at the molecular level.
Chapter 5

Investigation of *Musca* PER stability
5.1 Introduction

The degradation of *Drosophila* PER involves the action of at least two kinases. Casein kinase II (that consists of 2α and 2β subunits) may act as a primary kinase, providing the phosphoserine required by DBT to further phosphorylate PER (Lin et al., 2002). After phosphorylation by the Casein kinase Iε homologue, DBT, the F-BOX protein, SLIMB, acts to target phosphorylated PER for degradation via the ubiquitin-proteosome pathway (Ko et al., 2002). Light accelerates the degradation of both PER and TIM. It is thought that the formation of the PER-TIM heterodimer protects phosphorylated PER from degradation and that when light stimulates TIM degradation PER is left unprotected and is thus in turn also degraded (Vosshall et al., 1994; Price et al., 1995).

All casein kinases share certain characteristics. They have a relatively short amino terminus of 9-76aa followed by a highly conserved kinase domain of 284aa (Cegielska et al., 1998). The C-terminus is highly variable with a length of between 24 and over 200aa. This C-terminal has been shown to have autoregulatory properties in both the CKIδ and CKIε isoforms (Graves et al., 1995; Cegielska et al., 1998). Autophosphorylation of the C-terminus results in down-regulation of enzyme activity. Truncations of the C-terminus lead to increase in enzyme activity of a 10 fold level in CKIδ (Greaves et al., 1995) and approximately 4-fold in CKIε (Cegielska et al., 1998).

In previous chapters I have shown *Musca* PER to be apparently stable. It was therefore obvious to investigate whether an altered relationship between PER and DBT could account for this difference. At the time that this work commenced DBT was the only kinase known to phosphorylate PER, thus targeting PER for degradation (Kloss et al., 1998; Price et al. 1998). I wanted to investigate whether MdPER and MdDBT could physically interact and if MdPER could be phosphorylated by MdDBT. I also wanted to see if DmDBT could interact with and phosphorylate MdPER as this would give me an insight into the behaviour of the MM1 transgenic *Drosophila* carrying a copy of the *Mdper* gene. In order to see if changes in either MdPER or MdDBT or in both proteins was responsible for the possible altered relationship between the two proteins in *Musca* I also planned to test whether MdDBT could interact with and phosphorylate DmPER.
Previous studies had not been able to show direct phosphorylation of PER by DBT in Drosophila (Suri et al., 2000). Instead the authors used the yeast homologue of CKIs, HRR25 (DeMaggio et al., 1992) to perform kinase assays using PER as substrate. For this reason I also produced a pET23a-HRR25 construct as described in Suri et al. (2000). This provided a positive control for the kinase assays and could also then be used to test if MdPER is capable of being phosphorylated if the kinase assays performed with DBT failed to yield results.

Clones of DmDBT in pET14b, pJDC4 and pJDC7 were available in the laboratory from J. Clayton. These had been used for bacterial protein expression but all had been found to produce insoluble protein (J. Clayton, pers comm). I first tried to express DmDBT in tuner cells using varying concentrations of IPTG. This again failed to yield any soluble protein and so I attempted to solubilise the protein from the inclusion bodies by addition of urea. This was only attempted for the pET14b-DmDBT construct that produces a HIS tagged protein. The unfolded protein was bound to a Ni\(^{+}\) agarose column and refolded by washing with progressively lower concentrations of urea until the urea had been removed. The protein was then eluted from the column and assayed with Bradford reagent against known concentrations of a BSA standard. Although protein was recovered it formed aggregates in solution. This is probably the result of incorrect refolding of the protein. As it was therefore impossible to produce protein with which to perform protein-protein interaction assays I decided to clone and express several fragments of DmDBT. I also then made the corresponding fragments for MdDBT, along with full-length MdDBT.

I chose to produce two fragments of DBT along with a full-length version containing an engineered S to A mutation at a predicted autophosphorylation site (Zillan et al., 1999), corresponding to amino acid position 396 in DmDBT and position 406 in MdDBT. This position was chosen as it does not alter the predicted secondary structure of the protein. The first fragment is a C-terminal truncation, referred to as ΔC, that removes the last 134aa of the protein. This removes the autoregulatory region and has been shown to increase enzyme activity (Greaves et al., 1995; Cegielska et al., 1999). The second fragment represents the C terminus, consisting of 145aa, starting at position 295 of the full-length protein. This fragment was generated to allow for investigation of any possible role of the C-terminus in binding PER and also to assess whether the autophosphorylation of DBT is caused by
either inter or intra-protein interactions. A visual representation of the fragments generate is given in Figure 5.1.

5.2 Materials and Methods

Western blot analysis of PER and TIM in LL

Both *Musca* and wild-type *Drosophila* were entrained to a LD 12:12 cycle and then moved to constant light at the lights-on transition on day 5. Collections were taken every 6h for the last day in LD and then for 5 consecutive days in LL. Isolated total head protein was then subjected to western blot analysis. Membranes were probed with both anti-PER and anti-TIM antibodies.

Cloning of HRR25

The yeast homologue of *dbt*, hrr25, was amplified from yeast genomic DNA and cloned into the pET23a expression vector as described in Suri *et al.*, 2000.

Cloning of the *Dmdbt* AC and CT constructs

For *Dmdbt* both the AC and C-terminus (CT) constructs were produced by PCR amplification of the required region. In both cases an *NdeI* site was included in the 5’primer and an *XhoI* site in the 3’primer to allow cloning into the expression vectors. For all constructs two 3’ primers were used, one of which contained a stop codon and one of which did not. This was required due to the expression vectors, pET14b and pJDC4 require a stop codon as the tag is situated at the N-terminus, whereas pJDC7 places the tag at the C-terminus and therefore contains a stop codon in the vector after the tag sequence. Primer sequences are given in Figure 5.2. All PCR produced fragments were first cloned into the pDK101 vector and sequenced to check for unwanted PCR generated mutations before cloning the fragment into the relevant expression vector.
Figure 5.1 Diagramatic representation of the expression fragments for dDBT and MdDBT. The kinase domain is given in red. A) Full length sequence. B) C-terminal truncation (ΔC). C) C-terminal fragment (CT). D) Full-length protein containing an autophosphorylation site knock-out (KO) at position 396 in dDBT (blue) and at position 406 in MdDBT (green).
Figure 5.2 Primer sequences for the cloning of Drosophila dbt AC, CT and Musca dbt CT constructs.

a) Dmdbt AC, b) Dmdbt CT, c) Mddbt CT. All enzyme sites are shown in red and stop codons are underlined. Bold type indicates spacer sequence to allow in frame cloning and expression of the C-terminal epitope tag. For each construct there are two different 3' primers, termed A and B. These are required for the cloning of these constructs into different expression vectors. Those labelled A include a stop codon as required for cloning into the pJDC4 vector, which adds an N-terminal tag, whereas those labelled B do not contain a stop as additional sequence supplied by the vector encodes the C-terminal epitope tag.

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<td>TAGCTCGAGCCCTTGCCCTGCCTCCACGCCACC</td>
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<tr>
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<td>CAGCATATGGCTGCCCAGAAGCAGAATCCCTAACAAG</td>
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<tr>
<td>MDB3'</td>
<td>GCTCGAGCCCTTTTGCATTTTCGTATATCGGCTAC</td>
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Cloning of the Dmdbt autophosphorylation site knock-out

To create the autophosphorylation knock out the S to A mutation was engineered by PCR by creation of a single base pair mutation in the third codon position. Two fragments were created by PCR, the first containing the mutation at the 3’ end, the second containing the mutation at the 5’ end. Both fragments were then used as template for a second PCR, which fuses the two previous fragment incorporating the mutation. For the Drosophila construct (Figure 5.3) the 5’ primer used to generate the mutation-containing fragment was designed over an internal XcmI site and the 3’ primer was placed at the end of the coding sequence and contains an XhoI site in the primer tail to allow cloning. The ddbtΔC construct cloned in pBs was digested with Apal and XcmI to produce a fragment containing the missing 5’ end of the coding sequence. Both fragments were then cloned into pBc using the Apal and XhoI sites in the mcs in a 3-way ligation.

Cloning of the Mddbt CT fragment

The Mddbt CT fragment was also generated by PCR amplification of the required region, again incorporating an Ndel site in the 5’ primer and an XhoI site in the 3’ primer. Primer sequences are given in Figure 5.2.

Cloning of the Mddbt ΔC construct

Producing the ΔC fragment was not so simple due to the presence of two Ndel sites within the coding sequence. The first site, 925bp inside the coding region, was removed by PCR engineered single base pair mutation (A926C). This created a sense mutation at the third position of an isoleucine codon. In order to create this mutation primers were designed in both directions over the site and the single base pair mismatch was placed within them. Two separate PCR reactions were then performed to produce one fragment (A) with the mismatch at the 3’ end of the product and one (B) with the mismatch at the 5’ end of the product. Both products were then mixed and used as template for a second PCR reaction, using the 5’ primer used to generate A and the 3’ primer used to generate B. This effectively fuses A and B, incorporating the mutation within the resulting fragment. The 5’ primer in this case was placed at the start of the coding region and incorporates an Ndel site for cloning. The 3’ primer was placed over the second Ndel site (NdeI5’), thus creating an Ndel-Ndel fragment.
**Figure 5.3** Cloning of the *Drosophila dbt* autophosphorylation site knock-out.

**a**) Primer sequences are shown. Restriction sites are given in red, bold type indicates the single base pair miss-match to create the S396A mutation (tcg→gct at bp 1205) and spacer sequence is given in italics. Stop codons are underlined. The creation of the S396A mutation was achieved by PCR mediated mutagenesis as described previously for the *Ndel* site knock-out (Figure 4.5). **b**) Two initial PCRs were performed. The XCM15' primer encompasses the internal *XcmI* site and this was used with SA3' to produce a fragment containing the mutation at the 3' end of the fragment. SA5' was then used with one of the DBT3' primers, depending on the expression vector, to create the second fragment with the mutation at the 5' end. XCM15' and the relevant DBT3' primer were used to produce one fragment from the two initial ones as previously described. **c**) The ΔC construct contains the 5' *dbt* sequence until the *XcmI* site (705bp) and this section of the gene is removed by digestion with *Ndel* and *XcmI*. Both this and the *XcmI*-Xhol autophosphorylation site knock out were then cloned into the relevant expression vector cut *Ndel* and *Xhol* using a 3-way ligation.
To create the ΔC construct the remainder of the coding sequence required was amplified by PCR using a 5' primer placed over the second internal NdeI site and a 3' primer designed at the end of the required sequence and incorporating an XhoI site for cloning. After sequencing both fragments the NdeI-XhoI fragment was first cloned into the same sites of the expression vector. This intermediate construct was then digested NdeI and the NdeI-NdeI fragment added. The orientation of this fragment was checked by restriction digest. A schematic representation of the cloning procedure is given in Figure 5.4.

**Mddbt autophosphorylation knock-out**

The NdeI5' primer was used to create the mutation-containing fragment using a 3' primer designed at the end of the coding sequence that contains an XhoI site (Figure 5.5). After sequencing this was then cloned into the expression vector NdeI-XhoI and the NdeI-NdeI fragment previously described was then added and the correct orientation established by restriction mapping.

**Full-length Mddbt**

The full-length Mddbt was assembled by digesting the autophosphorylation site knock-out with XcmI and XhoI and replacing it with the corresponding fragment from the CT construct. XcmI cuts 1647bp into the coding sequence and so digesting XcmI and XhoI removes the section containing the site mutation (Figure 5.6). Sequencing of the DNA region ensured correct replacement of the autophosphorylation site.

**Cloning of dbt for in-vitro transcription/translation**

*Drosophila dbt* had been cloned into the EV2 vector to allow this by another member of the laboratory and was available for use. It was necessary to clone full length Mddbt into the same vector. A linker was first added to the vector to add an NdeI site (Figure 5.7) Two oligonucleotides were designed to be complementary to one another and so that when annealed overhangs were left at either end to anneal to the EcoRI and XhoI restriction sites within the vector. The EV2 vector was then digested accordingly and the annealed linker ligated. This was then digested with
**Figure 5.4 Cloning of the Musca AC construct.**

a) Primer sequences are given. All enzyme site are given in red, stop codons are underlined and spacer sequence is given in italics. Bold type indicates the engineered base pair miss-matches to destroy the first internal Ndel site. b) PCR was performed separately using the MD5' and NDEK03' and using NDEK05' and NDE3' primers to produce two fragments which are depicted. The first fragment contains an added Ndel site at the 5' end which incorporates the start codon and also contains the base pair miss-match that destroys the first internal Ndel site. The second fragment carries this base pair miss-match at the 5' end and it's 3' end encompasses the second internal Ndel site. c) A second PCR is then performed using the MDA3' and NDE3' primers and using the first two fragments as template. This produces a single product that is effectively a fusion of the first two. Once this was achieved a further PCR was conducted using the NDE5' primer that also encompasses the second internal Ndel site and either of the MDA3' primers which add the remainder of the required sequence for the construct. Choice of the 3' primer depends on the expression vector to be used. The 3' primer also contains an XhoI site for cloning. The Ndel-XhoI fragment was cloned first into the same sites of the expression vector and the Ndel-Ndel fragment added.
**Figure 5.5** Cloning of the *Musca* autophosphorylation site knock-out.

a) Primer sequences are given. Restriction sites are given in red, bold type indicates the single base pair miss-match to create the S406A mutation (tcg→gcg at bp 1205) and spacer sequence is given in italics. Stop codons are underlined. b) The NDE5’ primer encompasses the second (intact) internal Ndel site and used with the MDS A3’ primer produces a product containing the required mutation at the 3’ end. MDSA5’ and either of the two MD3’ primers (depending on the expression vector to be used) produces a fragment containing the mutation at the 5’ end and containing an Xhol site at the 3’ end. This fragment was cloned into the relevant expression vector using the Ndel and Xhol sites. To add the remainder of the sequence the Ndel-Ndel fragment that was produced to make the ΔC construct was cloned then into the Ndel site as shown in e, to create the full length autophosphorylation site knock-out.
Figure 5.6 Construction of the full length *Musca dbt* sequence for expression.
All distances in bp are given as distance from the start of the original coding sequence, not the starting point of that construct. 

**a)** The full-length sequence with the engineered autophosphorylation site knock out is depicted. To create the non-mutated full-length sequence a the region carrying the mutation was replaced with the same region but lacking the mutation. An *XcmI* site present at 927bp from the start of the coding sequence was utilised for this. This construct was digested with *XcmI* and *Xhol* and the 3' fragment of the gene removed. 

**b)** The CT construct also contains the *XcmI* site and the non-mutated 3' sequence of the gene. This construct was then digested with *XcmI* and *Xhol* and the 3' fragment removed. 

**c)** The full-length sequence was then assembled by addition of the *XcmI*-*Xhol* fragment from the CT construct. Successful replacement of this 3' region was checked by sequence analysis.
Ndel and XhoI and the Ndel-XhoI Mddbt fragment added. The Ndel fragment was subsequently added to assemble the full-length sequence.

a

NDELINK5’ AATTCCAACCATATGTCAGTCATCTGAC
NDELINK3’ TCGAGTCAGATGACTGACATATGTTGG

b

G AATTCCAACCATATGTCAGTCATCTGAC TCGAG
CTTAG GGTTGGTATACAGTCAGTAGACTGAGCT C

**Figure 5.7** Addition of a *Ndel* linker to the EV2 vector.

a) Oligo sequences are given. These were annealed and then cloned into EV2 cut with *EcoRI* and *Xhol* sites (blue) as shown in b). The *Ndel* site is shown in red.

Cloning of *Dmper* for *in-vitro* transcription/translation

*Drosophila per* was also cloned into the EV2 vector with a N-terminal HA tag. Addition of the HA tag sequence was achieved by PCR amplification of a 5’ fragment of *Dmper*, adding the tag in the primer tail. An *EcoRI* site was included in the 5’HAPER primer which was designed at the beginning of the coding sequence and the 3’SALI primer was placed over the internal *Sall* site 1812bp into the coding sequence. The full-length *Dmper* sequence was available in the pEG202 yeast vector. This was removed by digestion with *EcoRI* and *Xhol* but it proved impossible to subsequently clone this fragment into pBc. Cloning of *dper* into pBc was achieved by cutting the sequence into two fragments (1812bp and 1862bp) and performing a 3-way ligation. This was done because it was necessary to replace the 5’ 181bp *EcoRI*-*Sall* fragment with the PCR fragment containing the HA tag (See Figure 5.8), which is not significantly different in size to the original fragment. It was therefore necessary to change the antibiotic resistance to enable detection of the correct
construct. The HA-per fragment was cloned into pBs using the EcoRI and SalI sites. Both the full-length sequence and the pBc-HA-per construct were then cut with SalI and XhoI and the SalI-XhoI per fragment ligated into the pBc-HA-per vector. Cutting the pBc-per construct in this way gave rise to two bands, the SalI-XhoI per fragment and pBc containing the first 181bp of per. Both of these bands are of a very similar size (3581bp and 3493bp) and are difficult to separate on an agarose gel. In order to ensure the correct band was cloned into pBs-HA-per resulting colonies were replica plated onto chloramphenicol plates and only colonies that grew on ampicillin but not chloramphenicol were further analysed. Diagrammatic representation of this cloning procedure is given in Figure 5.8.

Cloning of Mdper for in-vitro transcription/translation

I was originally provided with three overlapping clones of Mdper that covered the entire coding sequence. However upon sequencing these only one, corresponding to base 504 to base 2330 contained no mutations. In order to reduce the size of PCR products, and thus the risk of generating unwanted mutations, four fragments were produced and then assembled to make the full-length sequence (Figure 5.9). To do this it was necessary to engineer an EcoRI site by PCR mutagenesis, creating a single base pair sense mutation at base 701. The 5' end of the gene was amplified from cDNA using the FL5' primer which incorporated both a HindIII site prior to the ATG start codon and the 3'EcoRI primer which induced the mutation creating the EcoRI site at base 701. The second fragment was amplified using the 5'EcoRI primer, creating the EcoRI site at base 701 and the NcoI3' primer which was designed slightly down stream of the first internal Ncol site at position 1260. This primer also contained an XhoI site in the primer tail to allow sub-cloning. It was possible to generate the third fragment by restriction digest of the 504-2330 fragment with Ncol. The fourth fragment was again amplified from cDNA using the 5'Ncol primer, which encompassed the second internal Ncol site at position 2053 and the XhoI3' primer designed at the end of the coding region to include the stop codon and incorporate an XhoI site for sub-cloning.

The first two fragments were cloned into pBs HindIII-EcoRI and EcoRI-XhoI fragments in a three way ligation. This was then digested with Ncol and XhoI and the fourth fragment added. Digestion of the resulting construct with Ncol allowed addition of the third fragment, completing the full-length sequence. Once this was
Figure 5.8 Cloning of the HA-dper construct.

a) Primers are shown. Restriction sites are shown in red, the start codon is underlined and bold type represents the HA tag. The 5' primer adds the tag and also an EcoRI site for cloning. The 3' primer encompasses an internal Sail site. b) Amplification with these primers produces a small 5' fragment containing the HA tag in frame with the original per start codon. This is sub-cloned into pBs using the EcoRI and Sail cloning sites. c) dper is removed from the pEG202 vector and cloned into pBc by means of a three-way ligation. This changes the antibiotic resistance background of the clone in order to facilitate easy identification of the correct clone at the final stage, when fragment sizes are too similar to allow identification by restriction mapping. d) The pBs construct containing the 5' fragment with the HA tag is cut Sail and Xhol, as is the pBc construct containing full-length dper. Using pBs-Haper as the vector the rest of the coding sequence cut Sail and Xhol from pBc is ligated in to create the full-length HAdper construct.
a

HINDIII5' ATCAAGCTTCATATGGAAAGTGAATCTACGGAATC
ECORI3' GGAATTCCCGTAGTTATCTGGC
ECORI5' ACGGGAATTCCATTGCCGAG
NCOI3' ATCCCTCGAGCTGAAGACAGCATGTGTGTC
NCOI5' GATAAACCATGGAGTATAGCGG
XHOI3' ATCCCTCGAGTTACAGTCCACCTGACCGCGTGTG

b

HindIII--------- ACGGGAATTCCATTGCC

ACGGGAATTCCATTGCCGAG---------Xhol

c

\[
\begin{array}{c}
\text{HindIII} \\
-6\text{bp}
\end{array} \quad \begin{array}{c}
\text{EcoRI} \\
701\text{bp}
\end{array} \quad \begin{array}{c}
\text{Xhol} \\
1310
\end{array}
\]

\text{Ncol} \\
1260bp

d

\[
\begin{array}{c}
\text{HindIII} \\
-6\text{bp}
\end{array} \quad \begin{array}{c}
\text{EcoRI} \\
701\text{bp}
\end{array} \quad \begin{array}{c}
\text{Ncol} \\
1260\text{bp}
\end{array} \quad \begin{array}{c}
\text{Xhol} \\
3153\text{bp}
\end{array}
\]

\text{Ncol} \\
2053bp
Figure 5.9 Cloning of the full-length *Mdper* sequence.

a) Primer sequences are given. All restriction sites are given in red, with the exception of *Ndel*, which is given in blue, start and stop codons are underlined and the single base pair miss-matches to create the *EcoRI* site are given in bold type. b) The *EcoRI* site is created by PCR mediated mutagenesis. The first two fragments are created using the HINDIII5'-ECORI3' and ECORI5'-NCOI3' primer combinations. The HINDIII5' primer amplifies the start of the coding sequence and adds a *HindIII* site and an *Ndel* site 5' to the start codon. The NcoI3' primer is situated slightly downstream of the first internal *Ncol* site and contains an *Xhol* site in the primer tail. Both of the ECORI primers include a single base miss-match that creates an *EcoRI* site. Unlike previous experiments these fragments are not combined by a further PCR. Both fragments were sub-cloned. c) The first fragment (black) was cut using *HindIII* and *EcoRI*, the second fragment (blue) with *EcoRI* and *Xhol*. Both fragments were cloned in a three-way ligation into pBS using the *HindIII* and *Xhol* sites. d) The resulting construct was then digested with *Ncol* and *Xhol*. The 3' end of the coding sequence was amplified using the NCOI5' primer, that encompasses the second internal *Ncol* site, and the XHOI5' primer that is placed at the very end of the coding sequence and adds an *Xhol* site adjacent to the stop codon. This was digested with *Ncol* and *Xhol* and cloned into the construct containing the first two fragments (red). e) To complete the sequence the Ncol-Ncol fragment was removed from an available cDNA fragment by restriction digest (purple) and cloned into the *Ncol* site of the construct containing the rest of the coding sequence.
assembled in pBs it was then necessary to move the full-length sequence to the EV2 vector. It was considered advantageous to add a HA epitope tag to the PER proteins to allow easier antibody recognition and manipulation of the resulting protein if necessary. This was attempted by adding a linker containing the HA tag and an Ndel site to allow in frame cloning (Figure 5.10). However it proved impossible to ligate this linker into the EcoRI and Xhol sites of EV2 and so the full-length sequence was cloned into the HindIII and Xhol sites without addition of the HA tag.

5.3 Results

As Musca PER is non-cycling in both western blots and ICC of adult brains (Chapter 3) I first wanted to investigate if MdPER would be reduced to low levels in constant light conditions (LL), as is seen with Drosophila PER (Marrus et al., 1996). Western blot analysis was performed as described in Materials and Methods. It can be seen that for Drosophila that both PER and TIM cycle robustly in LD and that this cycle is then dampened in LL, eventually levelling off to a steady level just above minimal LD levels by the third day in LL (Figure 5.11). This is in accordance to previous reports (Marrus et al., 1996). However in the case of Musca, PER remains at a constant level as seen in both LD and DD (Chapter 3) even though TIM is immediately degraded in response to light and then remains at constant low levels during the 3 days in LL. This would suggest that in Musca PER is not reliant on TIM for stability.

In Drosophila TIM protects phosphorylated PER from degradation via formation of the PER/TIM heterodimer (Vosshall et al., 1994; Price et al., 1995). It is not known whether the PER-TIM heterodimer is formed in Musca although MdPER can interact with DmTIM (Chapter 5). Whether this dimer is formed or not, MdPER does not require MdTIM for stability, which would suggest that a step prior to the formation of the dimer is deficient in the Musca system. Therefore it is feasible that MdPER is not sufficiently phosphorylated to target it for early degradation. On a western blot MdPER does not undergo obvious mobility shifts that could be attributed to changes in phosphorylation state, unlike DmPER (Chapter 3).

The Musca doubletime gene was cloned in the laboratory by B. Collins. Analysis of the predicted protein sequence reveals a 440aa protein with 82.7%
a) Oligonucleotide sequences are given. The HA tag is underlined and the *Ndel* site is shown in red. Oligos were designed so that when annealed overhangs were left at the end that would anneal to *EcoRI* and *XhoI* restriction sites, thus enabling them to be cloned into these sites of the EV2 vector (b).

**Figure 5.10** Addition of a linker containing an *Ndel* site and a HA tag to EV2

a) Oligonucleotide sequences are given. The HA tag is underlined and the *Ndel* site is shown in red. Oligos were designed so that when annealed overhangs were left at the end that would anneal to *EcoRI* and *XhoI* restriction sites, thus enabling them to be cloned into these sites of the EV2 vector (b).
Figure 5.11 Western blot analysis of PER and TIM in flies kept in LL. Both Musca and Drosophila were entrained in LD 12:12 and then transferred to LL conditions. Collections were made every 6h for the last day of LD and then for 4 days in LL. Protein extract were used for western blot analysis of PER and TIM levels. In Musca PER remains at constant levels throughout the experiment. Musca TIM cycles robustly in LD and then is degraded in response to light and remains at low levels in LL. Both Drosophila PER and TIM show dampening in the amplitude of oscillation once transferred to LL. This dampening increases the longer the flies are kept in LL.
identity to *Drosophila* DBT. High homology is also seen to other CKIε sequences (Figure 5.12) within the kinase domain (Fish *et al*., 1995), as is expected. All of the sequences show high divergence at the C-terminus. Surprisingly the degree of divergence between the *Drosophila* and *Musca* sequences in this region is higher than that seen between the mammalian sequences. Using the BLAST2 tool human and mouse sequences are 78% identical, whereas the fly sequences share significant identity other than a 27aa region which is shared by both species (Figure 5.12). If the EBI ALIGN tool is used the mammalian sequences are 95.9% identical with 96.7% similarity. The fly sequences are only 52.2% identical and 58.6% similarity. This may be an important factor in the function of DBT in these two species.

Once all of the cloning was complete for the bacterial expression construct small scale expression experiments were conducted to assess protein solubility. All cultures were grown at 37°C in LB. Most of the fragments, if expressed, were found in the insoluble fraction. Most fragments also run at an apparent molecular weight smaller than that predicted, indicating that these fragments may be partially degraded after expression in bacteria. Only the pJDC DmPER autophosphorylation knock-out and the pET14b MdDBT CT fragments yielded any soluble protein (Figures 5.13, 5.14 and 5.15). Surprisingly, although HRR25 was cloned as previously reported (Suri *et al*., 2000) it failed to yield soluble protein. It was therefore expressed at 30°C in M9 media with the hope that the combination of a lower temperature and a media known to aid the production of soluble protein (Novagen technical manual) would result in expressed protein that could be found in the soluble fraction. This failed to yield any protein either (Figure 5.16). Unfortunately I did not have time to make further attempts to express soluble protein for any of the constructs. As previous attempts to solubilise dDBT had failed I did not attempt this either.

Due to time constraints it was not possible to attempt to produce both PER and DBT as S<sup>35</sup> labelled in-vitro transcription/translation products and to perform Co-IP studies using these.
Figure 5.12 Alignment of CKι protein sequences.

Sequences for MdDBT, dDBT, mouse CKιe, human CKι and Saccaromyces HRR25 are compared. The kinase domain is highly conserved between the proteins (shown in red), whereas the C-terminus is diverged. In fact a higher degree of divergence is seen when comparing the Drosophila and Musca sequences than when comparing those of mouse and human.
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**Figure 5.13** Expression of DBT in bacteria.
Results of the trial expression experiments are given as whether the recombinant protein is expressed and if expressed whether found in the soluble or insoluble fraction upon purification.
**Figure 5.14 Expression and extraction of soluble proteins**

Gel photographs of the two constructs producing soluble bacterially expressed protein. Numbers to the left indicate size of the marker band (lane M) in kDa. Lane numbers correspond to time after IPTG induction of expression, 0 being an un-induced sample and M corresponds to the molecular weight marker. A red arrow to the right indicates the band corresponding to the expressed protein.

**a)** Expression of the Drosophila pJDC4 AutoKO results in production of a 48kDa protein that is in part found in the soluble fraction when extracted from bacteria (red arrow).  

**b)** The CT fragment of mdDBT is found almost exclusively in the soluble fraction when expressed in bacteria using the pET14b vector. A very small amount can be seen in the insoluble fraction 4h after induction.
**Figure 5.15** Expression of constructs that produce an insoluble protein. Examples of three *ddh1* constructs that produce insoluble protein on extraction from bacteria. Numbers to the left indicate the size of the molecular weight marker bands (lane M). A red arrow indicates the band corresponding to the expressed protein and the lane numbers correspond to time (h) after IPTG induced induction. All proteins shown are found exclusively in the insoluble fraction. a) pET14b AutoKO. b) pJDC4 CT. c) pJDC7 ΔC
**Figure 5.16** Bacterial expression of the pET23a HRR25 construct

Although expressed as in Suri *et al.*, 2000 no protein band was seen to be induced after addition of IPTG to bacteria containing the pET23a HRR25 construct. The predicted molecular weight of HRR25 (approx 50kDa) is indicated by a red arrow. Sizes of the molecular weight marker (lane M) are given to the left and lane numbers correspond to time (h) after addition of IPTG.
5.4 Discussion

It has not been possible to produce either *Drosophila* or *Musca* DBT as soluble, bacterially expressed recombinant protein. The C-terminal truncation, similar to that used for mammalian CKIε studies (Cegielska *et al.*, 1998) did not facilitate the expression of soluble protein. It was therefore not possible to pursue this experiment further. The production of the constructs for in-vitro transcription/translation was severely slowed down as the *Mdper* constructs that I had been told were available turned out to contain unwanted PCR induced mutations. As a result I had to re-assemble the coding sequence by amplification from cDNA. This in itself proved problematic, as it was very difficult to produce the 3’ *NcoI-XhoI* fragment without mutations due to slippage during the PCR reaction. The length of time taken to produce these constructs meant that there was not sufficient time to use proteins produced in this manner to test for interactions.
Chapter 6

Does a putative NES sequence function in *Musca* PER?
6.1 Introduction

I have previously shown that MdPER is a predominantly cytoplasmic protein (Chapters 3 and 5), with no nuclear localisation ever being detected in central brain cells at any stage of the circadian cycle. In Drosophila it is not only the cycling in abundance of PER but also its sub-cellular localisation that is important for circadian rhythmicity (Saez and Young 1996; Sehgal et al., 1999). Nuclear entry of PER (and TIM) during the late night is required for negative regulation of its own transcription via interaction with the CLK-CYC heterodimer (Glossop et al., 1999). If PER does not enter the nucleus then this form of regulation cannot take place in Musca. The experiments presented in this chapter were performed to investigate the possibility that PER does enter the nucleus but is actively transported back to the cytoplasm by a nuclear export mechanism. It should be noted that this work was initiated before the discovery of nuclear localisation of both PER and TIM in Musca photoreceptor nuclei (Chapter 3).

In mammals PER proteins are critical to clock function (Bae et al., 2001; Shearman et al., 2000) and cyclic nuclear accumulation in SCN cells suggests that PER has a nuclear function (Field et al., 2000; Shearman et al., 2000). In the mouse it has been shown that both mPER1 and mPER2 undergo nuclear export (Vielhaber et al., 2001). Both proteins contain an 11 aa region that shows high homology to the leucine-rich nuclear export sequence (NES, Gerace, 1995). This sequence binds a CRM1/exportin-1 nuclear export receptor (Ullman et al., 1997), thus facilitating export. These putative NES sequences from mPER1 and mPER2 were fused to two copies of YFP (Yellow fluorescent protein) and their natural nuclear localisation signals (Vielhaber et al., 2001). The NES was able to facilitate nuclear export when either transiently transfected into HEK 293 cells or microinjected into Xenopus oocyte nuclei, leading to cytoplasmic accumulation of YFP. Both mutation of the NES sequence and addition of the nuclear export inhibitor leptomycin B blocked this cytoplasmic accumulation.

Alignment of the corresponding sequences from Drosophila and Musca PER to the NES region of the mPERs shows that although the NES sequence is not highly conserved in either Drosophila or Musca there is still a sequence that could possibly function as a NES (Figure 6.1). The Musca sequence may be more likely to function as the Drosophila sequence starts with an I, the consensus sequence can contain other
hydrophobic amino acids in place of L but only in the second to fourth positions (Mowen and David, 2000). The Drosophila sequence also only contains three and not the four hydrophobic amino acids required for the consensus. In order to test whether either of these putative NESs could function I adopted a similar approach to Vielhaber and colleagues. A heterologous protein would be produced by fusing the NLS containing region of DmPER to the putative NES for both DmPER and MdPER and two tandem copies of GFP. Two further constructs would then be made for Musca, each containing mutations of the NES. This would then be cloned to express this construct in both transgenic Drosophila and S2 cells.

Consensus NES = LX$_1$-3LX$_2$-3LXL

L can be substituted with M, I, F or V. X represents any amino acid.

**Figure 6.1** Alignment of known and putative NES signals from PER proteins. Alignments are made for the three mPER proteins with DmPER, MdPER and ApPER (Antherae pernyi). The consensus sequence for a NES is also shown. The L residues can also be substituted with M, I, F or V in the second through to fourth positions. It can be seen that MdPER has a better consensus as four residues are present rather than three in DmPER and ApPER, although the third and fourth residues are not separated. (Adapted from Vielhaber et al., 2001)

6.2 Materials and Methods

Cloning of the NLS-NES-GFP-GFP constructs

The NES sequence, either native or mutated was fused to GFP by adding the NES sequence in the 5' tail of the 5'primer that amplifies the GFP sequence. Primer sequences for the four constructs (DmNES, MdNES, Mdmut1NES and Mdmut2NES) are given in Figure 6.2. A SalI site was added in the 5' primer and a BglII site in the 3' primer to allow sub-cloning. The fragments were then produced by PCR.
amplification, sub-cloned into pDK101 and sequenced to ensure there were no unwanted mutations incorporated by the PCR mechanism.

Digesting a construct consisting of Dmtim2 fused to GFP in pBs using SacI and BglII provided a vector containing one of the GFP sequences required. The NLS in both DmPER and MdPER is situated at the N-terminus. In order to incorporate this sequence the pBS-haper construct from Chapter 4 was digested with SacI and SalI and the 5' haper fragment isolated. A three-way ligation was then performed to assemble the sequences into the final construct as detailed in Figure 6.2.

This construct was then cut SacI and EcoRV to transfer the assembled sequence to pAct and NotI and XhoI to transfer to pUAST. The pUAST constructs were then used to produce transgenic Drosophila. Unfortunately only one construct (mdNES) yielded transformants and time restraints prevented further attempts to be made with the remaining constructs. MdNES flies were then crossed to flies carrying tim-GAL4 in order to produce progeny that would express this construct in "clock" neurons. After the crosses were set the vials were entrained to a 12:12 LD regime. Resulting larvae were removed at the end of the night ZT24 and dissected. This time point was chosen as this is the time when DmPER is found in the nucleus in WT flies. The larval brains were mounted whole and examined on a fluorescent microscope to assess the location of the GFP-NES fusion protein.

6.3 Results

The GFP signal from the MdNES construct was clearly detectable in the nucleus of the lateral neurons (Figure 6.3). If the protein was cytoplasmic in localisation then staining would also be visible in the projections of these neurons (Kaneko 2000). The speckled appearance of the GFP signal is due to protein aggregation.
Figure 6.2 Production of the NES-GFP fusion fragment

a) Primer sequences are given. Enzyme sites are shown in red, all 5’ primers contain a Sall site to allow in frame cloning with the per-nls fragment and the gfp3’ includes a Bglll site to allow in-frame cloning into the pBs-GFP vector. The nes sequence is incorporated in the 5’ primer tail and the primer anneals to the gfp sequence (underlined). PCR amplification thus fuses the nes sequence to gfp. Induced mutations are shown in bold type, these are simply included in oligo synthesis. b) Protein translation of the primer sequences. GFP sequence is underlined and induced mutations are given in bold type.
Figure 6.3 MdNES GFP signal in lateral neurons of the larval brain. The location of GFP within these cells is exclusively nuclear as cytoplasmic staining would label the projections of these neurons (Kaneko 2000).
6.4 Discussion

Although only one of the constructs produced transgenic flies for this experiment it was possible to ascertain that the putative NES signal in MdPER does not appear to function, as all of the GFP fusion protein was located in the nucleus of cells. It is therefore most probable that the cytoplasmic location of MdPER in the central brain of *Musca* is due to the protein not entering the nucleus rather than being due to nuclear export of the protein. Re-creation of the *Drosophila* feedback loop with *Antheraea* components suggests that although nuclear PER cannot be detected in *Antheraea* low level or transient nuclear localisation of PER may take place allowing negative feedback (Chang *et al.*, 2003).
Chapter 7

The role of *cryptochrome* in the *Drosophila* circadian clock
7.1 Introduction

Cryptochromes are blue light photoreceptors that were first discovered in *Arabidopsis* and have since been identified in many other species, including *Drosophila* and mammals (Reviewed in Cashmore *et al.*, 1999). They show high sequence homology with the 6-4 photolyase family but have no role in DNA repair. Sequence alignment of cryptochromes and 6-4 photolyases shows high homology in the two regions responsible for binding of a chromophore (flavin) but differ greatly in their carboxy-termini (Yang *et al.*, 2001). Cryptochromes serve as blue light photoreceptors in plants and flies (Cashmore *et al.*, 1999; Sancar, 2000) and as part of the core circadian machinery in vertebrates (Griffin *et al.*, 1999; Kume *et al.*, 1999; Field *et al.*, 2000).

The *Drosophila cry* gene was identified through the cry*<sup>b</sup>* mutation, a single amino acid mutation resulting in an Asp → Asn substitution at amino acid 410, a highly conserved position on the flavin binding site (Stanewsky *et al.*, 1998; Emery *et al.*, 1998). Behavioural experiments show that these mutants entrain normally to 12:12 LD cycles, have normal 24hr rhythmicity under DD conditions and can re-entrain to a second light regime (Stanewsky *et al.*, 1998). However, unlike wild type flies, cry*<sup>b</sup>* flies do not phase shift in response to a 10min light pulse administered during the subjective night. Flies hemi/heterozygous for the norpAP41 (*no receptor potential A*) mutation are visually blind but entrain to 12:12 LD cycles normally (Stanewsky *et al.*, 1998). Double mutants for both norp*<sup>a</sup>* and cry*<sup>b</sup>* show severely compromised light entrainment, revealing that the cry*<sup>b</sup>* mutation dramatically disrupts the circadian flow of light information via CRY. As the cry*<sup>b</sup>* mutation is located at such a highly conserved position within the flavin (the catalytic chromophore) binding site, it is feasible that the reduced efficiency is due to the reduced ability of CRY*<sup>b</sup>* to activate any hypothetical signalling partner. Finally these results may also indicate that the light input pathways to the clock, both ocular and extra-ocular, may converge on CRY, which is responsible for transducing the signal to the pacemaker through light dependent redox reactions.

In mammals CRY plays a role in the central oscillator mechanism (Griffin *et al.*, 1999; Kume *et al.*, 1999; Field *et al.*, 2000). Both mCRY1 and mCRY2 physically interact with the three mPER proteins and facilitate repression of *per* and *cry*
transcription (Kume et al., 1999; Lee et al., 2001). Moreover, in vitro studies have shown that both mCRYs on their own can strongly inhibit CLK-BMAL1 induced transcription, suggesting that its interaction with the dimer represses its activity (Kume et al., 1999; Shearman et al. 2000). As is generally the case with PER and TIM in Drosophila, in the liver the dimerisation of the mCRYs with mPER1 and mPER2 is required to facilitate their nuclear entry (Kume et al., 1999; Lee et al., 2001. However, this might not be the case in the SCN, where mPER1 can enter the nucleus without the aid of the mCRYs (Yagita et al., 2000). In mCRY deficient mice, mPER2 levels are severely reduced, suggesting a role for them in the stabilisation of mPER2 (Shearman et al., 2000; Lee et al., 2001).

CRY was originally thought to not play a role in the central oscillator in Drosophila, as PER and TIM still cycle in the lateral neurons in cryb flies (Stanewsky et al., 1998). These neurons are the site of the central pacemaker in Drosophila and are responsible for the generation of rhythmic locomotor activity. The fact that PER and TIM still cycle in these neurons in cryb mutants accounts for the rhythmicity of cryb flies in DD. However, cryb flies show abnormal behaviour under LL conditions, where they remain rhythmic, unlike wild type flies (and other organisms) that become arrhythmic in such conditions (Emery et al., 2000). This abnormal behaviour can be at least partially rescued by targeted expression of cry+ in the lateral neurons, suggesting that CRY may play some role in these cells.

Peripheral tissues in Drosophila have been shown to contain autonomous circadian clocks (Giebultowicz et al., 2000). Recent evidence suggests that in the clock containing peripheral tissues, CRY does play a role within the oscillatory machinery and does not function solely as a photoreceptor (Krishnan et al., 2001; Ivanchenko et al., 2001; Levine et al., 2002).

The aim of the work presented in this chapter was to determine whether dCRY could also function within the central oscillator mechanism, in addition to its role in photoreception. Stanewsky and co-workers (1998) had previously analysed the behaviour of perr;cryb double mutant flies. They showed that in 12:12 LD conditions at 25°C these flies predominantly show a 24h period (due to the light regime) but ~40% also have a minor 19h (perr) component. This behaviour is distinctive to the double mutant and is never found in either cryb or perr single mutants, which entrain
to 24h cycles, indicating that the two mutations are epistatic. In turn this suggests that CRY and PER could perhaps physically interact.

Temperature often exerts a rather dramatic effect on clock mutants (see Hall, 1995). This is because one fundamental characteristic of biological clocks is to be temperature compensated, i.e. the period of rhythmic phenotypes is not changed by variation in temperature. Mutations altering the clock often affect this property, as an example *per* and, to a certain extent, *per* and *cry* mutations become more drastic (displaying longer and shorter periods, respectively) at higher temperatures (Konopka et al., 1989; Hamblen et al., 1998). During my MRes I had monitored the adult locomotor activity of *per*, *cry*, and *w* control flies at both 18°C and 28°C, to test whether an increase in temperature has any effect on the *per*;*cry* behaviour due to possible increased *per* light sensitivity (see Marrus et al., 1996). The results obtained from this experiment are shown in (Figure 7.1).

It can be seen that both of the single mutant *per* or *cry* flies individually entrain to the 12:12LD regime at both temperatures, showing a 24h period and normal distribution of activity around the light/dark transitions (Figure 7.2). In DD both genotypes free run with a period of 24h for *cry* and 19h for *per*. Note that *per* shows a moderate temperature dependency as the rhythm is approximately 1h shorter at 28°C than at 18°C. In DD *per*;*cry* flies behave almost identically to *per* mutants at both temperatures (Figure 7.1). However, the behaviour of the double mutant changes under LD conditions in a temperature-dependant manner. At 18°C all of the *per*;*cry* flies show a periodic component of 24h but approximately 60% of the flies also display a 19h (*per*) component. At 28°C only a small minority are able to entrain, the majority of which (79% of rhythmic flies) are behaviourally blind and free run, showing a *per* periodicity of 19h (Figure 7.2).

This breakdown of entrainment at 28°C in the double mutant could reflect a genuine genetic interaction between the *per* and *cry* mutations. Alternatively this could also suggest that the limits of entrainment at 28°C are reduced in *cry* mutants so that *per*;*cry* might entrain to a T cycle of 20hrs at this temperature (closer to the endogenous 19h *per* period), whereas *cry* mutants (with an endogenous period of 24h), might not. In order to test this hypothesis locomotor activity would be monitored for single and double mutant flies in a LD 10:10 regime at 28°C.
The period of locomotor activity was determined by autocorrelation and Fourier (spectral) analysis and the results compared [26]. The period length given by spectral analysis is presented. N = total number of flies examined
(n) = number of rhythmic flies
a = all flies showed also a rhythm of ca 24 h
b = 4 flies showed also a rhythm of ca 18 h, 1 fly showed only the ca 24 h period.

**Table 7.1** Entrained and free-running locomotor rhythmicities for *cry^b*, *per^a* and *per^a; cry^b* flies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>18°C</th>
<th></th>
<th>28°C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Period</td>
<td>N</td>
<td>Period</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>LD (n)</td>
<td></td>
<td>DD (n)</td>
<td></td>
</tr>
<tr>
<td><em>cry^b</em></td>
<td>24.2±0.1 (32)</td>
<td>36</td>
<td>24.3±0.1 (29)</td>
<td>34</td>
</tr>
<tr>
<td><em>per^a</em></td>
<td>23.9±0.1 (19)</td>
<td>19</td>
<td>19.8±0.1 (15)</td>
<td>17</td>
</tr>
<tr>
<td><em>per^a; cry^b</em></td>
<td>24.6±0.1 (32)</td>
<td>32</td>
<td>19.3±0.1 (32)</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>18.8±0.1 (19^a)</td>
<td>32</td>
<td>18.3±0.2 (23)</td>
<td>31</td>
</tr>
</tbody>
</table>

*Figure 7.1* Entrained and free-running locomotor rhythmicities for *cry^b*, *per^a* and *per^a; cry^b* flies

a) Table showing mean ± SEM for both LD and DD conditions at 18°C and 28°C. 
b) Examples of fourier analysis showing *per^a; cry^b* activity at 18°C (left) and 28°C (right). The predominant 24h rhythm alongside a 19h peak can be seen at 18°C, whereas only a 19h peak can be seen at 28°C.
Figure 7.2 Activity profiles of all flies in LD12:12.
Average locomotor activity profiles (see Figure 7.1 for the number of flies averaged for each genotype) for each genotype is shown double plotted for 5 successive days in 12:12 LD. Data is double plotted (day 1–day2; day 2-day 3, etc.). Single mutants entrain to both temperature conditions. The dark-light transitions correspond with the startle responses at 18 °C, but this shifts to the light-dark transitions at 28 °C for all genotypes. This phenomenon is also observed in the wild-type and single mutants. At 18 °C, entrained cycles with ~24 h period are observed (dashed vertical lines) for the double mutant, whereas at 28 °C, a free-running locomotor activity cycle with a ~19 h period is superimposed upon the startle response.
The data also suggested a physical interaction between PER and CRY. In order to further investigate this I wished to perform yeast two-hybrid experiments to test for physical interactions between PER and CRY. I also wished to investigate any role of the C-terminus of CRY in this interaction by engineering a deletion of 20 of the last 22 aa at the C-terminus. The rationale of this experiment is that the C-terminal of CRY molecules, not present in the closely related phytochromes, may play a pivotal role in modulating signalling after light reception. An example in this direction can be seen in Arabidopsis where the C-terminus of CRY in isolation gives a constitutive light response when expressed as a fusion protein with GUS (GUS-CCT, Yang et al., 2000). Plants expressing GUS-CCT show light-grown phenotypes (e.g. short hypocotyl length) even when kept under shade conditions (red light and far-red light) where wild type plants normally show elongated hypocotyls.

At an early stage of this work another group identified a physical interaction between dCRY and dTIM (Ceriani et al., 1999) but failed to find such an interaction between dCRY and dPER.

7.2 Materials and Methods

Cloning of full-length cry into yeast two-hybrid vectors

To create the full length clone of cry for the yeast two-hybrid assay a 5’ fragment was amplified using 5’ GGAATTCATGGCCACGCGAGGGGCGAATG and 3’ GCGGATCCCAAAGCGTGTGTGATA primers (Restriction sites are underlined). The 5’ primer was designed to place an EcoRI site adjacent to the translational start site to allow in frame cloning of the fragment into the yeast vectors. The 3’ primer was placed over a BamHI site located 432bp into the cry sequence. This amplified fragment was then cloned into the yeast vectors with the remainder of the coding sequence, which was removed from the original clone by digestion with BamHI and XhoI, to create the full-length fragment.

Creation and cloning of cryΔ

The cryΔ construct was created by deleting the sequence encoding 20 of the last 22 amino acids from the CRY C-terminus. The same 5’ primer was used as for the full-length construct and the 3’ primer
(GCGCCTCGAGTCAAACCACCGGCGGGGTGATC) was designed to anneal upstream of the region I wished to delete and to contain the sequence for the final two valine residues, the stop codon and an \textit{XhoI} site in the tail (underlined). This was then cloned into the yeast vectors using the \textit{EcoRI} and \textit{XhoI} sites.

7.3 Results

Locomotor activity was monitored for single and double mutant flies in a LD 10:10 regime at 28°C. Both single mutants entrain under these conditions, whereas the double mutant shows some evidence of entrainment during the first two cycles but this rapidly breaks down and the flies free run, with their daytime activity advancing by about 90mins each day (Figure 7.3).

Therefore the entrainment defect at high temperature shown by the double mutant \textit{per$^a$;cry$^b$} flies is the result of a specific interaction between the mutations rather than a defect in the entrainment of \textit{cry$^b$} alone. In \textit{Drosophila} the visual system is involved in the reception of circadian-relevant light information (Stanewsky \textit{et al}, 1998). This system is perfectly functional in the double mutant, as demonstrated by the startle response shown at the lights on transition at both temperatures (see Figure 7.2). Therefore \textit{per$^a$;cry$^b$} flies are able to detect light, but are deficient in the transmission of light information to the clock in a temperature dependant manner.

The genetic interaction seen between \textit{per$^a$} and \textit{cry$^b$} led us to investigate whether or not PER and CRY could physically associate \textit{in-vitro} using the yeast two-hybrid system (Golemis and Brent, 1997). Full-length \textit{cry} was cloned into the pEG202 vector as described to create a direct LexA-CRY fusion. This was then challenged with \textit{PER} (233-685) as prey. This fragment contains the major protein-protein interaction domains described for PER. As a positive control I also challenged \textit{TIM} (377-915) against \textit{PER} (233-685) as this fragment of TIM is known to bind \textit{PER} (Gekakis \textit{et al}, 1995) and contains the relevant regions for PER/TIM dimerisation (Saez and Young 1996). This same TIM fragment was also tested against LexA-CRY. Neither \textit{PER} or \textit{TIM} showed an interaction with LexA-CRY in the dark (Figure 7.5a). Whilst these experiments were being performed it was reported that CRY interacts with full-length TIM, but not with \textit{PER} under constant light conditions (Ceriani \textit{et al}, 1999). However, we found that in light LexA-CRY
Figure 7.3 Activity profiles of per\(^s\), cry\(^b\) and double mutant flies in LD 10:10. Average locomotor activity profiles of 18 flies for each genotype are shown double plotted for 5 successive cycles in 10:10 LD at 28 °C. per\(^s\) and cry\(^b\) mutants show entrained cycles with ~ 20 h period (dashed vertical lines). Double mutant per\(^s\); cry\(^b\) flies show some evidence of entrainment only during the first two LD 10:10 cycles, afterwards the entrainment breaks down and a shorter ~ 18 h free-running period is evident (dashed lines) (Rosato et al., 2001).
binds strongly to PER (233-685) but not to TIM (377-915) (Figure 7.4a). We then challenged LexA-CRY with full-length PER and TIM, both in darkness and constant illumination. No interactions were observed in the dark. A CRY-TIM interaction was observed in light (Figure 7.4a) but no interaction was observed between CRY and PER, in agreement with the observations of Ceriani et al. Thus there was an inconsistency between our results in that full-length PER failed to give an interaction, but the PER(233-685) fragment gave a strong interaction. To investigate this, LexA-TIM was challenged with both full-length PER and PER (233-685) as prey. Although the PER(233-685) fragment gave an interaction, no interaction was observed using full-length PER (Figure 7.4b). Subsequent Western blot analysis revealed that, in our system, full-length PER is poorly expressed and this would account for the lack of interaction seen when using this construct (Figure 7.5).

From these results we can conclude that CRY can interact with PER ex in-vivo and that PER and TIM dimerisations with LexA-CRY are light dependent. This data also suggests that the amino and/or carboxyl terminus of TIM is required for TIM-CRY interaction. The fact that we observed a strong interaction between LexA-CRY and PER (233-685), in contrast to Ceriani et al, could be due to either the different yeast two-hybrid systems employed or that I produced and used a direct LexA-CRY fusion, whereas Ceriani and co-workers placed a linker between LexA and CRY.

To assess whether this PER-CRY interaction could take place in a more functional context, E. Rosato performed a coimmunoprecipitation (coIP) assay in Drosophila S2 cells (Rosato et al, 2001). This is a system where PER is stable in the absence of TIM and could therefore be used to investigate PER-CRY interaction without the possibility of complexes being formed via PER-TIM and TIM-CRY interactions. Immunoprecipitation confirmed the existence of a TIM-independent, PER-CRY complex in darkness (Rosato et al., 2001). This experiment was not performed in light due to the light labile nature of CRY in S2 cells (Ceriani et al, 1999).

As we had shown a meaningful interaction between PER and CRY we wished to assess whether the C-terminus of CRY played a regulatory role. In order to do this I deleted 20 amino acids from the C-terminus of CRY by PCR (see methods), to create CRYΔ. This was then challenged with PER (233-685) and full-length TIM in
Figure 7.4 Yeast two-hybrid analysis of protein interactions.

Triplicate yeast patches from independent transformations were grown on plates containing the β-galactosidase substrate X-gal. A blue precipitate represents cumulative β-galactosidase activity produced by activation of the lacZ reporter by protein-protein interaction. Baits (rows) and prey (columns) were challenged at 30 °C. Assays with pJG4-5 (empty bait vector) represent the negative control to assess for self-activation of the prey. (a) LexA-CRY interacts with PER(233-685) and full length TIM only under constant light conditions. No interactions were ever observed with either full length PER or TIM(377-915). (b) LexA-TIM interacts with PER(233-685) but fails to do so with full length PER both in light and dark. (Rosato et al., 2001)
Figure 7.5 Western blot analysis of PER stability in yeast.
Full length PER and PER(233-685) prey fusions were probed with anti-HA antibodies in cells also expressing LexA-CRY. Western blot analysis shows that PER(233-685) is expressed at high levels whereas the full length PER fusion (expected at the level of the arrow) cannot be detected. The same results were obtained under light and dark conditions and also in LexA-TIM expressing cells. The lower molecular weight bands represent truncation or (most likely) degradation products. (Rosato et al., 2001)
both light and darkness. Interactions were evident between LexA-CRYΔ and both PER (233-685) and TIM, in both conditions (Figure 7.6a). As CRYΔ removes the light dependence of the CRY-PER interaction it can be concluded that the C-terminus of CRY mediates this light-dependant interaction. It had been reported that LexA-CRYb was unable to interact with TIM in yeast cells (Ceriani et al., 1999), possibly because it had lost its photoresponsiveness. E. Rosato challenged both LexA-CRYb and LexA-CRYbΔ (a construct containing the cryb mutation (D[410]N) and carrying the C-terminal deletion) with PER (233-685) and TIM. Although both LexA-CRYb and LexA-CRYbΔ are expressed strongly in yeast (Ceriani et al., 1999 and E. Rosato, pers.comm.) neither is able to interact with either PER (233-685) or TIM (Rosato et al., 2001). Given the light-independence of CRYΔ, this would suggest that the cryb mutation confers a gross structural defect to LexA-CRYb, rather than affecting its photoreceptor ability.

As CRY can interact with both PER and TIM, and PER and TIM form a heterodimer (Saez and Young, 1996) we wished to map the regions of PER involved in the PER-CRY interaction in order to suggest whether it would be feasible for all three proteins to be found in the same complex. To map the sites of interaction I challenged LexA-CRYΔ with several overlapping PER fragments. I observed that LexA-CRYΔ does not associate with PER (233-390) (PAS A domain) or PER (233-485) (PAS A+B region), but does interact with the downstream C-Domain [PER (524-685)] (Figure 7.6a). We confirmed that LexA-TIM (377-915) interacts with the PAS A domain of PER [PER (233-390)] and with PER (233-685) (Huang et al., 1995; Figure 7.6b). As this shows that TIM and CRY may interact with different regions of PER and, since CRY interacts with regions of TIM outside of the (377-915) fragment (Ceriani et al, 1999), we hypothesise that PER, TIM and CRY could be found in the same complex.

7.4 Discussion

In order to assess the regions of CRY involved in the light dependant action of CRY, random Taq-induced mutations were introduced into full-length cry by PCR and LexA-CRY mutants were created by in-vivo gap repair (Muhlrad et al., 1992). These experiments were performed by G. Mazzotta. The mutants were then
Figure 7.6 Yeast two-hybrid analysis of CRYΔ interactins. LexA-CRYΔ interacts in a light-independent manner. (a) LexA-CRYΔ interacts with PER(233-685) and PER(524-685) but not with PER(233-390) or PER(233-485) both under light and dark conditions. LexA-CRYΔ also binds to TIM. (b) LexA-TIM (377-915) interacts with PER (233-685).
challenged with PER (233-685) in the dark. In total, 14 bona-fide light-independent mutations were identified that gave PER-CRY interactions in darkness. All of these proved to carry either a premature stop mutation or a frame-shift mutation in the C-terminus (Rosato et al., 2001), further supporting the view that the C-terminus of CRY mediates the light-dependant PER-CRY and TIM-CRY interactions.

It is possible that the removal of the C-terminus of CRY induced a conformational change to a form that is active in darkness. Alternatively, we hypothesised that there could be a C-terminal bound, light-inhibited repressor of CRY in yeast. By mutagenising yeast cells it was possible to find mutants in which the LexA-CRY:PER (233-685) interaction was light-independent (Rosato et al., 2001). Therefore a trans-acting factor in yeast could be mutated to disinhibit nuclear CRY activity in darkness.

The data presented here demonstrates that CRY can bind PER in both yeast and a Drosophila cell culture system. This interaction is light dependent and the light regulation is conferred by the C-terminus of CRY. Previous studies have reported light-dependent activity in the nucleus of S2 cells (Ceriani et al., 1999), where CRY is suggested to undergo a conformational change after light absorption, allowing it to bind TIM. However, CRY coimmunoprecipitates with TIM and with PER in the cytoplasm of S2 cells in darkness, suggesting that light is not required to alter CRY into its active conformation (Ceriani et al., 1999). We therefore predict that in both yeast and S2 cells a nuclear factor may interact with the CRY C-terminus in darkness, preventing CRY from binding either PER or TIM.
Chapter 8

TANGO – A Possible role in the *Drosophila* circadian clock
8.1 Introduction

The PAS domain was named after the first three proteins in which it was found, PER, ARNT and SIM. It consists of a stretch of 250-300aa in which there is a pair of highly degenerate 50aa subdomains, termed the PAS A and PAS B repeats (Reviewed in Gu et al., 2000). Many proteins that function in signal transduction have been found to belong to the PAS superfamily (Gu et al., 2000). Of these, most are involved in the regulation of transcription although some, such as PER do not have this function. In mammals and flies most PAS proteins are bHLH transcription factors, containing a bHLH motif immediately N-terminal to their PAS domain. These transcription factors act as heterodimers, interacting through their PAS domains. In prokaryotes, which contain no bHLH transcription factors, proteins exist that have homology to PAS proteins (Pellequer et al., 1999; Taylor and Zhulin 1999). They often harbour histidine kinase activity, transducing signals via phosphorylation cascades that activate transcription factors (Agron et al., 1994). A PAS kinase has now been found in mammals which contains two PAS repeats followed by a canonical serine/threonine kinase domain (Rutter et al., 2001a).

The PAS proteins have been divided into three subgroups (Gu et al., 2000). The α-class proteins are described as sensors of environmental signals, such as hypoxia, light and xenobiotics (Reisz-Porszasz et al., 1994; Wang et al. 1995a). β-class proteins are defined as being broad spectrum partners that target the heterodimer to the genomic target and the third class, the γ-class are co-activators.

In mammalian cells the presence of a xenobiotic, such as dioxin, activates the aryl hydrocarbon receptor (AHR). AHR then dimerises with the AHR nuclear translocator (ARNT) and together they bind to target sequences termed xenobiotic response elements (XRE), initiating transcription of genes involved in response mechanisms (Hankinson, 1995). ARNT also binds to HIF1α, and together they activate hypoxia response elements (HRE) in response to oxidative stress (Wang et al., 1995a and 1995b). Both the XRE and HRE sequences contain a GTG at the 3' of the element, which is the binding site for ARNT.

PAS proteins are also found in the circadian pathway. PER, CLOCK and CYCLE all contain PAS domains. CLOCK and CYCLE are both bHLH transcription factors, which act as a dimer to initiate transcription of per and tim by binding a specific sequence termed an E-box (Darlington et al., 1998; Rutila et al., 1998). PER
binds to TIM through its PAS domain, but has no bHLH domain or any other domain to allow it to initiate transcription (Huang et al., 1993). In *Neurospora* the positive factors white-collar proteins WC1 and WC2 are PAS proteins that contain GATA type zinc finger domains (Cheng et al., 2002).

In *Drosophila* PAS proteins are found in several developmental pathways. The TANGO (TGO) protein is the *Drosophila* homologue of ARNT and forms heterodimers with several other PAS bHLH proteins. The TGO-SIM (product of the *single-minded* gene) heterodimer controls the development of glia and neurons that lie on the midline of the central nervous system by binding the CME (central midline element) (Sonnenfeld et al., 1997). The same element is bound by TGO-TRH (TRACHEALESS) to control tracheal development. Another protein, the product of the *spineless-aristapedia* gene (SS) is the closest known homologue of AHR and together with TGO binds an element with the same core as the XRE (Emmons et al., 1999) to regulate distal antennal identity, establishment of the tarsal region of the legs and bristle growth.

My interest in TGO came from a result obtained whilst conducting the yeast two-hybrid assays that identified the PER-CRY interaction reported in Chapter 7. In order to test if the PER-CRY reaction was specific or if this interaction was the result of non-specific interaction caused by the PAS domain of PER, I tested CRY against two other PAS proteins, TGO and SIM. An interaction was seen between CRY and TGO but not between CRY and SIM. This observation suggested a possible role for TGO in the regulation of CRY. Thus I investigated whether this interaction was real, and assessed any biological significance by altering expression of *tgo* in flies to see if this would affect the circadian clock.

To produce flies that either over-expressed or down-regulated *tgo* I used the GAL4/UAS system (Brand and Perrimon, 1993). This allowed me to target the altered regulation of *tgo* to specific cells only, i.e. clock cells, using flies carrying the *S. cerevisiae* GAL4 fused to the *timeless* promoter, thus avoiding the lethal effects of *tgo* (Sonnenfeld et al., 1997).

In order to down-regulate *tgo* RNAi was undertaken. It is known that the presence of double-stranded RNA (dsRNA) can trigger degradation of a cognate gene as the result of a complex mechanism that is referred to as RNA interference (RNAi, Ambros 2001). In order to generate dsRNA I wished to clone two copies of the *tgo* cDNA in opposite orientations. Piccin *et al.* (from our laboratory) had already
reported success in down-regulating yellow using a heterologous spacer between the two cDNA copies (Piccin et al., 2001). The heterologous spacer was used in order to simplify the cloning procedure, as direct inversions are lethal to bacteria (A Jeffrey pers. comm.). It has also been reported that the inclusion of an intron, in place of the spacer, facilitates the formation of a hairpin dsRNA via the splicing mechanism (Smith et al., 2000). Other laboratories have since employed the same technique successfully in Drosophila, also coupling this mechanism with the GAL4/UAS system (Reichhart et al., 2002).

8.2 Materials and Methods

Cloning of tgo-T7

A T7 tag was added by PCR, including the tag in the 3' primer tail (Figure 8.1). The 5' primer was placed over an internal BgII site, producing a PCR product of approximately 900bp. The full-length sequence including the T7 tag was then assembled in pBs by means of a three way ligation using a 5' EcoRI – BgII fragment and the BgII – XhoI PCR fragment.

Bacterial expression of proteins

In order to produce TANGO-T7 as a bacterially expressed GST fusion this PCR fragment was then cloned into the pGEX-4T-1 vector (Pharmacia Biotech) using the EcoRI and XhoI sites. The remaining EcoRI fragment was then generated by restriction digest and cloned into the EcoRI site of pGEX-4T-1 containing the EcoRI-XhoI fragment. This was then checked to have entered in the correct orientation by restriction analysis. CRY was also cloned into the pGEX-4T-1 vector (Ozkaya, 2000). The finished constructs were transformed into both XL1-Blue MRF' and BL21-pLysS cells. Expression of the protein was induced by addition of IPTG to a final concentration of 1mM.

Cloning of ha-cry

In order to allow co-immunoprecipitation (Co-IP) experiments to be performed a HA-tagged full-length cry was cloned by PCR amplification of CRY and the HA tag from the pJG4-5 CRY construct. This would allow the use of anti-HA antibodies to be used to "pull down" the complex. The primers used were 5' hacry
**TGO-T7 5’**

ATGCTGGCATGCAGGCTCCTACG

**TGO-T7 3’**

CCGCTCGAGTTAGCCCATCTGCTGGCGCGCCGGTCATGCTGGCCATCTCGAAGCGCGTGCTGAACAT

**Figure 8.1** Primer sequences for the cloning of tango-T7.
The TGO-T7 5’ spans an internal BgII site (red). The TGO-T7 primer adds the T7 tag in the primer tail (bold) that is immediately followed by a translational STOP codon (underlined). An _XhoI_ site is also included in the primer to allow cloning (red).
GCTCTAGACAACATGTACCTTATGATGTGCGAGTTAT and 3' cry
GCGCCTCGAGTCAAACCACCGGCAGGCGTGTC. An XbaI site is included in the 5' primer and an XhoI was placed site in the 3' primer (both underlined). A kozac recognition sequence was also included in the 5' primer (italics) adjacent to the start of the HA tag. After sequencing to check for PCR induced mutations ha-cry was cloned into pBs using the XbaI and XhoI sites.

Cloning of tgo and cry for in-vitro transcription/translation

Both tango-T7 and ha-cry were also cloned EcoRI-XhoI into the EV2 and pBsMYC vectors produced by O. Ozkaya (Appendix 3) to allow in-vitro transcription/translation. Both constructs for each gene were used for the reaction and the resulting 35S methionine labelled proteins were visualised on a 12% PAGE gel by autoradiography.

Up-regulation of tgo in flies

The tango EcoRI-XhoI fragment was cloned into the pUAST vector using the same restriction sites. This was performed in two stages as an internal EcoRI site exists within the tango coding sequence. The GAL4/uas system was employed to target the over-expression to clock cells only as I feared for the viability of transformants widely over-expressing this gene. Transgenic Drosophila were subsequently produced with this construct, as described in Chapter 2. Transformant flies were mapped for the position of the insert and subsequently crossed to tim-GAL4 flies. Progeny of this cross carry both transgenes and therefore will up-regulate tgo expression in tim-expressing cells. These flies were assessed for locomotor activity and also for the ability to entrain to a second light regime after several days in DD. Flies were entrained to LD 12:12 and then placed in DD for three days. The second light regime (also LD 12:12) was then initiated at CT5. The ability of these flies to phase-shift in response to a light pulse administered during the dark phase was also assessed. Experimental procedures are given in Chapter 2. Flies heterozygous for UAS-tgo but lacking the tim-GAL4 driver were also subjected to this light pulse and serve as a control in order to assess the effect of the up-regulation of tgo. Both genotypes were also kept in a parallel incubator and did not receive the light pulse, but were placed in DD at the same time. Flies of the same genotype were compared, pulsed vs. un-pulsed, to determine the length of the phase shift. Those flies over-
expressing tgo were then compared to the control (driver-less) flies to assess the difference in phase shift conferred by tgo up-regulation.

**Down-regulation of tgo in flies**

To clone the tango RNAi construct (tgo-ogt) the second intron of the single-minded (sim) gene was fused to tango by PCR. A fragment of tango was amplified that stretched from the internal EcoRI site to the end of the coding sequence. A region of 10bp was added to the 3’ primer tail that corresponded in sequence to the 5’ end of the sim intron sequence (Figure 8.2a). The sim intron was also amplified by PCR. The 5’ primer contained a tail with a 10bp stretch corresponding to the last 10bp of the tango coding sequence, whilst the 3’ primer added an XhoI site for cloning (Figure 8.2a). Once these fragments were produced they were mixed and used as a template for a second PCR using the 5’ tango primer and the 3’ sim intron primer. This second PCR fused the two individual fragments by virtue of the 20bp overlap engineered between them (Figure 8.2b). This was then cloned into pUAST using the EcoRI and XhoI sites. The 5’ tango EcoRI-EcoRI fragment was then added. At the same time the tango coding sequence had been cloned into pBluescript (pBs) EcoRI-XhoI. The full-length tango sequence was then excised using the Xbal and XhoI sites of pBs and cloned into the same sites in pUAST, placing this sequence in reverse orientation to the first. This construct was used to produce transgenic Drosophila. Again, transformants were mapped and crossed to tim-GAL4 flies.

**Production of tgo probes for in-situ hybridisation**

The pBs-tango construct was used to produce a single strand DIG-labelled RNA probe for in-situ hybridisation. The 5’ EcoRI-EcoRI fragment was also cloned separately into pBs to produce a second, shorter probe that lacked the bHLH and transcriptionally active domain encoding regions.
**Figure 8.2** Creation of the *tgo-ogt* construct.

a) Primer sequences. An *EcoRI* site (underlined) is included in Tango 5’ and an *XhoI* site (underlined) is in Intron 3’. Regions of complementarity to the opposite fragment are given in red. b) A schematic representation of the creation of *tgo-ogt* by PCR mediated “fusion”. i) Overlapping regions are created by primer tails in the first PCR reaction, shown in red. ii) A second PCR, using the first two products as template, joins the two fragments together. The regions of overlap allow the second fragment to continue to act as template after the first fragment has been amplified.
8.3 Results

For the yeast two-hybrid analysis LexA-CRY was challenged with TGO as prey and a weak but positive interaction was observed (Figure 8.3). It was therefore necessary to test if this interaction was real with further protein-protein interaction assays.

Both CRY and TGO-T7 were expressed in bacteria as GST fusion proteins and expressed protein was extracted for purification. GST-CRY was found to be almost exclusively in the insoluble fraction (Figure 8.4, Ozkaya, 2000) and solubility could not be increased by lowering the incubation temperature to either 30°C or to room temperature. An attempt was also made to express GST-CRY in tuner cells (Novagen, Chapter 4) using varying concentrations of IPTG (1mM, 500μM, 250μM, 125μM and 62.5μM) to increase the amount of soluble protein. This failed to give a higher yield of soluble protein.

GST-TGO-T7 was also expressed under the same conditions. In BL21-pLysS cells this fusion protein expressed well and gave some soluble protein when grown at room temperature and collected three hours after induction (Figure 8.4, Ozkaya 2000). Attempts were made to purify the small amount of soluble protein in each case using glutathione agarose resin but this failed to yield enough protein of a sufficient quality to attempt a GST-pull down against an in-vitro transcription/translation produced protein.

As it was not possible to use bacterially expressed proteins for the Co-IP assay this experiment was instead performed using both proteins produced by in-vitro transcription/translation. Both HA-CRY and TGO-T7 were produced by this method as S\(^{35}\) labelled proteins (Figure 8.5a). PSP64luciferase (Promega) was also produced to serve as a positive control. HA-CRY (non-radiolabelled) and TGO-T7 (radiolabelled) were mixed together to allow interaction to occur. Any resulting complexes were then pulled down using anti-HA antibodies that couple the complex to protein G bound agarose beads. As a negative control equal amounts of HA-CRY and LUCIFERASE (radiolabelled) were mixed and processed in parallel. The resulting complexes were then denatured and ran on a 12% PAGE gel. The presence of CRY and TGO was then assessed by autoradiography (Figure 8.5b). It can be seen that the anti-HA antibodies precipitate HA-CRY. TGO-T7 is also present in the
Figure 8.3 Yeast two-hybrid interaction between TGO and CRY
The blue precipitate indicates a positive interaction between CRY and TGO. This is not a false positive caused by non-specific binding through the TGO PAS domain as CRY does not interact with the PAS protein SIM (product of the single-minded gene). No self activation is seen when LexA-CRY is challenged with the empty pJG4-5 vector.
Figure 8.4 Bacterial expression of pGex4T-1-HACRY and pGex4T-1-TGO-T7.

a) Expression of pGex4T-1-HACRY. Recombinant BL21pLys S cells were induced with IPTG at 37°C and 30°C. The HACRY protein is found almost exclusively in the insoluble fraction (arrow) at both temperatures but some soluble protein is present at T4 in both cases. The apparent MW of the HACRY protein in 70kDa.

b) Expression of pGex4T-1-TGO-T7 in BL21 pLysS at RT. The majority of the protein is found in the insoluble fraction but some soluble protein can be seen at T3. TGO-T7 runs at an apparent MW of 85kDa.
Figure 8.5 Co-immunoprecipitation of CRY and TGO.

a) Autoradiograph of a 12% PAGE gel showing 35S labelled in-vitro transcription/translation products. Lane 1: LUCIFERASE positive control. ha-cry (lane 2) and tgo-t7 (lane 4) expressed in the EV2 vector show higher expression levels than when expressed in pBsMYC (lanes 3 and 5). b) CoIP assay. Proteins were produced as 35S labelled in-vitro transcription/translation products. HA-CRY was then mixed in equal amounts of Tgo-T7 or LUCIFERASE and immunoprecipitated with anti-HA antibodies and protein G. TGO-T7 (lane 2) but not LUCIFERASE (lane 1) co-precipitates with HA-CRY. For comparison one tenth of the total protein used for the CoIP assay is loaded (input, lanes 3-5).
precipitation when the two proteins are mixed prior to the pull down, confirming that
CRY and TGO interact *in-vitro*. This can be seen to be a specific reaction, as
LUCIFERASE is not precipitated in the negative control. This part of the work was
conducted with O. Ozkaya (Ozkaya 2000).

The next step was to see if either up- or down-regulating TGO had an effect
on the circadian behaviour of *Drosophila*, to assess whether this interaction had a
biologically significant role within the circadian mechanism.

Three separate lines of UAS-tgo transformants were created, termed 19.1, 25.1
and 52.1. Line 19.1 carries the transgene on the X chromosome and this line is male
lethal. It has therefore not been used for any of the subsequent analyses. Line 25.1
maps to chromosome 3 and 52.1 maps to the X chromosome and these were used for
the experiments. Flies were crossed to flies carrying the *tim-gal4* transgene and the
resulting male progeny were used for behavioural analysis as described in Chapter 2
and this chapter’s materials and methods.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Period LD (n)</th>
<th>N</th>
<th>Period DD (n)</th>
<th>N</th>
</tr>
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<td>24.52 ± 0.14 (25)</td>
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<td>24.18±0.09 (27)</td>
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<td>24.39 ± 0.09 (23)</td>
<td>25</td>
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<td><em>uas-tgo; +</em></td>
<td>24.18±0.07 (29)</td>
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<td>24.68 ± 0.24 (15)</td>
<td>18</td>
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<tr>
<td><em>uas-tgo; uas-tgo</em></td>
<td>24.25±0.14 (26)</td>
<td>31</td>
<td>23.55 ± 0.29 (15)</td>
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<tr>
<td><em>tim-gal4; tim-gal4</em></td>
<td>23.74±0.31 (18)</td>
<td>31</td>
<td>Arrhythmic</td>
<td>20</td>
</tr>
</tbody>
</table>

*Figure 8.6* Locomotor activity of flies over-expressing *tgo*

The mean period (τ) of locomotor activity ± SEM is given for each genotype monitored at
25 °C. The total number of rhythmic flies (n) is given as well as the total number of flies
tested (N). All flies entrain to a 12:12 LD cycle. All flies show an approximate 24h
period in DD with the exception of the *tim-Gal4* homozygotes, which can be seen to be
arrhythmic. This is likely to be due to a position effect of the *tim-Gal4* transgene.
Overexpression of TGO does not appear to have any effect on the period of locomotor
activity.
The \textit{tim}-GAL4;UAS-\textit{tgo} flies entrained to the LD cycle and showed normal (wild type) rhythmicity under DD (Figure 8.6). This data therefore suggested that the over-expression of \textit{tgo} in clock cells does not affect the circadian clock. As CRY is known to function in photoreception (Emery \textit{et al.}, 2000) I decided to see how well the \textit{tim}-GAL4;UAS-\textit{tgo} flies could entrain to a second light regime after a period in DD. These flies entrained perfectly well to the new light regime (Figure 8.7) as seen by comparing them to wild-type flies, however the flies with increased TGO levels showed a much greater “startle response” to the light being switched on compared to their wild type counterparts (Figure 8.7). This increased masking effect prompted me to check whether these flies may be more sensitive to light input to the circadian clock.

To test this further the flies were subjected to a 10min light pulse at either ZT15 or ZT21. Such light pulses are known to alter the phase of the flies behaviour and results in what is termed a “phase shift”. Light pulses during the early night (ZT15) result in premature degradation of PER and TIM, but there are sufficiently high levels of their RNA so protein levels build up again, taking the molecular mechanism back to the stage it was in at the beginning of the night. This results in a “phase delay”. Light pulses given late in the night (ZT21) again cause premature degradation of PER and TIM but at this time \textit{per} and \textit{tim} mRNA levels are low and so no further protein is produced. Now the molecular mechanism is in the same stage as it would be at the beginning of the day, resulting in a “phase advance”.

Once the average time of the evening peak was calculated the difference between flies that had been subjected to the light pulse and those that had not was determined (Figure 8.8). Activity charts are shown in Figures 8.9, 8.10 and 8.11. It can be seen that the over expression of TGO leads to an extended phase delay when compared to the driver-less control (showing normal \textit{tgo} regulation), showing an increase of 1 hour. This is not observed at ZT21, where only a difference of 0.4hrs is seen in the phase advance, although sample size is small (N<10). However, the data suggests that the over-expression of TGO leads to increased sensitivity to light during the early subjective night.
Figure 8.7 Mean activity profiles of timGAL4; UAS tgo and timGAL4/+ flies at 25°C. Data is double plotted. The flies were entrained in LD12:12 where light was given 0900 to 2100hrs and then subjected to 7 days DD. After this the flies were subjected to a new LD12:12 light regime where the lights on point was shifted forward by 5hrs, light being switched on at 1300hrs. Light is represented by a white bar, darkness by black. Flies entrain to the LD regime (two days are shown) and stay rhythmic during the DD period (the first two days and last day are shown). When the light is turned on to give the new light regime the timGAL4; UAS tgo flies shown a strong startle response (represented by a red arrow). This is not shown by the timGAL4; + control flies (a yellow arrow represents the point of light on).
<table>
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<td>12.91±0.26 (23)</td>
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<td><strong>ZT 21</strong></td>
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<td>uas-tgo;tim-Gal4</td>
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<td>10.43±0.32 (7)</td>
<td>7.92±1.11 (6)</td>
<td>2.51</td>
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</tbody>
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**Figure 8.8** Phase-shift response to ZT15 and ZT21 light pulses.

The time of the evening peak of locomotor activity was calculated for both flies over-expressing tgo (uas-tgo;tim-Gal4) and control flies carrying the transgene but lacking the driver for expression (uas-tgo; +). Results are shown as circadian time (CT) ±SEM. The number of flies tested is given (n). The effect of the light pulse is given as the difference between flies exposed to the light pulse and unpulsed controls of the same genotype. Data is given for both light pulses at ZT15 and ZT21.

The next step was to assess if down-regulating tgo would have an effect on circadian behaviour. In total 5 independent lines were produced for UAS-tgo-ogt, termed 3, 52.1, 1Y, 24.1 and 1R. Of these two lines (3 and 24.1) are unmapped, two (51.2 and 1Y) map to the second chromosome and 1R contains two inserts, one of which is on the second chromosome and one on the third. As tgo-ogt3 was crossed to homozygosity this line was used for subsequent analysis. It was then crossed to produce tim-gal4;uas-tgo-ogt flies and their locomotor activity was analysed. As a control uas-tgo-ogt; + flies were also included in the locomotor activity experiments. The uas-tint flies which down-regulate TGO in clock cells show wild type activity rhythms in both LD and in DD (Figure 8.12). This suggested that either down-regulation of TGO has no behavioural effect or that this RNAi construct was failing to facilitate a down regulation. In order to check that the RNAi approach was effective uas-tint flies were crossed to a stronger ubiquitously expressed driver. For this purpose actin-gal4 was used. As it is known that all tgo mutants are lethal (Sonnenfeld et al., 1997) we expected the progeny of this cross to be non-viable. In fact this was seen to be the case and so I can conclude that TGO is effectively down
Figure 8.9 Phase-shift response to a ZT15 light pulse.
Activity charts of UAS-tgo;tim-GAL4 and tgo;+ (control) flies are shown. Data is double plotted for two days in 12:12 LD and then six days in DD. It can clearly be seen that following the light pulse at ZT15 (red arrow) both genotypes show a phase shift (delay) in their activity (red dotted line).
**Figure 8.10** Phase-shifting in response to a ZT15 light pulse shown against non-pulsed flies. Activity charts are shown for UAS-igo;tim-GAL4 flies. Data is double plotted. After a ZT15 light pulse (red arrow) flies are transferred to DD for the rest of the experiment. The flies clearly show a phase delay in their evening activity peak (red dotted line). Control flies that did not receive the light pulse (time at which the light pulse would have been given is shown by a yellow arrow) do not show a shift in their activity (dotted yellow line). Control flies were transferred to DD at the same time as the flies given the light pulse.
Figure 8.11 Phase-shifting in response to a ZT21 light pulse shown against non-pulsed flies. Activity chart are shown for UAS-tgo;tim-GAL4 flies. Data is double plotted. After administration of a light pulse at ZT21 (red arrow) a clear phase advance can be seen (red dotted line). This cannot be seen in control flies that are not exposed to the light pulse (right, yellow dotted line). The point at which the light pulse would have been given is indicated with a yellow arrow. Both sets of flies were transferred to DD immediately after administration of the light pulse.
regulated in tim expressing clock neurons and that this has no effect on the behaviour of the fly. There was not sufficient time to assess whether these flies had an impaired response to light pulses.

<table>
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<td>tim-Gal4; tim-Gal4</td>
<td>23.74±0.31 (18)</td>
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<td>Arrhythmic</td>
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**Figure 8.12** Locomotor activity of flies down-regulating *tgo*.
The mean period (τ) of locomotor activity ± SEM is given for each genotype. The total number of rhythmic flies (n) is given as well as the total number of flies tested (N). All flies entrain to a 12:12 LD cycle. It can been seen that the effect of down-regulating TGO does not alter the period of activity as the *tim-Gal4; tgo-ogt* shows the same period as the control flies *timGal4;+* and *tgo-ogt;+*. The *tgo-ogt* homozygotes show a slightly shorter period, probably due to a position effect of the insert.

Towards the end of this study an antibody against TGO was made commercially available. This enabled me to visualise the extent of both the up- and down-regulation of TGO on the total TGO levels in the fly head by western blot analysis (Figure 8.13). It can be seen that both *tim-GAL4;UAS-tgo* 25.1 and 52.1 lead to an obvious increase in TGO levels, whereas UAS-*tgo-ogt3* shows only a small decrease in total TGO levels.

In order for the TGO-CRY interaction to have a biologically significant role both proteins must be co-expressed within the same cell. To assess this in-situ hybridisation and ICC was performed on wild-type *Drosophila* brains to identify the pattern of TGO expression within the adult brain. Both in-situ probes gave the same, broad pattern of expression (Figure 8.14). This pattern is very similar to that found for *per* (Figure 8.14) and so it is feasible that *tgo* could be expressed in clock cells.
Figure 8.13 Western blot of total head protein extracts using anti-TANGO antibody.

a) The TGO band is indicated with a red arrow. It can clearly be seen that the uas-tgo flies over express TGO (lanes 5 and 6). The difference between line 52.1 and 25.1 can be explained due to the position of the transgene. Line 52.1 carries the transgene on the x chromosome whereas line 25.1 carries the transgene on an autosome. As all progeny were collected from the cross of female tim-GAL4 to male UAS-tgo then the progeny from 25.1 will overexpress TGO but only half of the progeny (females) of 52.1 will do so. No dramatic down-regulation of TGO can be seen in the tgo-ogt flies (lanes 2-4) compared to the wild-type control (lane 1). b) Quantification of the blot. No statistics were performed as only one blot was available. It can be seen that the tgo-ogt flies (lanes 2-4) do have a small decrease in TGO levels compared to tim-Gal4 (lane 1), however it is impossible to say whether this is a statistically significant decrease.
Figure 8.14 In-situ staining for tgo in Drosophila
a) and b) both show tgo staining in wild-type Drosophila. This pattern is very wide spread and closely resembles that seen for per (Kloss et al., 1998; Figure 3.15, Chapter 3). Intense staining is seen in both in the retina, suggesting that tgo is expressed within the photoreceptors.
Key: CB, central brain; La, lamina; Me, medulla; Re, retina.
However *per* expression is much broader than PER expression, probably due to post-translational mechanisms. To investigate this further it was therefore necessary to perform whole mount ICC when the anti-TGO antibody became available. No staining was achieved using various dilutions of the antibody and so at this stage it is not possible to be certain if TGO is expressed in clock neurons.

8.4 Discussion

I have shown an interaction between CRY and TGO using both ex *in-vivo* and *in-vitro* protein-protein interaction assays. Although both over-expression and down-regulation do not affect the behaviour of flies in either LD or DD, I have shown that increased levels of TGO enhance light sensitivity. In fact *tim-GAL4;UAS-tgo* flies show an augmented startle response (masking) to light and increased phase delay after a light pulse administered at the beginning of the night (ZT15). No effect was seen in the advance zone, when the pulse was given at ZT21. This would perhaps suggest that the TGO:CRY interaction is not critical for the functioning of the central oscillator but might play a role in the light input pathway to the clock. This could be achieved either within clock neurons or within photoreceptor cells. Although the *in-situ* data shows *tgo* RNA expression in eyes and in areas corresponding to the location of the clock neurons, further studies with the α-TGO antibodies need to be performed before any real conclusions can be drawn.
Chapter 9

General Discussion
In *Drosophila* the core of the central oscillator mechanism consists of at least two interlocked feedback loops. The CLK:CYC heterodimer activates transcription of per and tim whose products then feed back to negatively regulate their own transcription by blocking the action of CLK:CYC (Hao *et al.*, 1997; Darlington *et al.*, 1998; Saez and Young, 1996; Gekakis *et al.*, 1995). At the same time CLK:CYC also activates both the activator (PDP1) and repressor (VRI) of *clk* transcription (Blau and Young, 1999; Glossop *et al.*, 2003; Cyran *et al.*, 2003). PER is expressed rhythmically at both the mRNA and protein levels and also cycles in sub-cellular localisation (Edery *et al.*, 1994; Saez and Young 1996). Nuclear entry of PER is essential for its role in the repression of its own transcription (Chang *et al.*, 2003). However studies in other insects have revealed that whilst PER cycles in abundance it is only detected in the cytoplasm of neurons within the central brain (Sauman and Reppert 1996; Zavodska *et al.*, 2003), although nuclear entry is detected within photoreceptors (Sauman and Reppert 1996). This raises questions as to how conserved the *Drosophila* mechanism is within other insects and if and how the negative feedback mechanism works in other species.

In order to investigate this further we have sought to establish the housefly *Musca domestica* as a comparative system. *Musca* is a close relative within the Diptera and so it is possible to use this species to study clock gene regulation so as to establish which are the more conserved factors and which could contribute to species-specific fine-tuning mechanisms. Previous work within the laboratory had shown that in *Musca* PER did not cycle in abundance in whole head protein extracts. Despite this it was capable of rescuing behavioural rhythms in *per*01 *Drosophila* (Piccin *et al.*, 2000). The aims of this project were to further investigate the lack of cycling, especially at the cellular level. It is possible that whilst the majority of PER expressing cells do not show cycling a few that may, and could be the important clock cells within the brain, would therefore be masked. I also studied TIM regulation and its relationship with PER as the PER:TIM dimer has an important role in the *Drosophila* clock (Saez and Young, 1996; Gekakis *et al.*, 1995; Ashmore *et al.*, 2003). Finally I carried out work to further investigate the *Drosophila* clock, so as to provide additional information that would also help in investigating the *Musca* clock.

In *Musca* both *per* and *tim* mRNA cycle in abundance in LD, with *per* peaking at approximately ZT12 and *tim* at around the same time, between ZT12 and ZT16. Ten-fold amplitude of cycling is seen in both cases. This is very similar to that
observed for *Drosophila* (Edery *et al.*, 1994; Sehgal *et al.*, 1994). However in DD conditions the oscillation of both RNA’s is either abolished or significantly dampened, more data is required to ascertain which is the case. At the protein level PER does not cycle under any conditions whereas TIM cycles in both LD and DD, peaking at the end of the night. It is interesting to note that TIM levels are significantly raised in DD, explaining the dampening the amplitude of the oscillation. Also experiments in LL show that exposure to light leads to rapid degradation of TIM and TIM remains at constant minimum levels throughout the rest of the experiment in LL. In *Drosophila* TIM levels are above minimum in LL (Marrus *et al.*, 1996), suggesting that MdTIM is more light labile than DmTIM. As PER levels remain high and constant in LL it can be concluded that PER does not require TIM for stability, unlike in *Drosophila*.

Investigations at the cellular level revealed a broad expression pattern for *per* mRNA, as seen in *Drosophila* (Kloss *et al.*, 1998), whilst the PER protein is seen in far fewer cells, as again is the case with *Drosophila*. Thus in both species post-transcriptional mechanisms are important in determining PER regulation of expression. Using enzymatic staining, ICC experiments revealed several groups of neurons that stain strongly for PER. In these cells there is no oscillation in staining intensity and the signal remains cytoplasmic throughout the day. More sensitive procedures using fluorescent secondary antibodies and confocal microscopy revealed two groups of neurons positioned dorso-lateral to the oesophagus that stain intensely for PER within the cytoplasm and correspond to cells seen with the enzymatic staining. However a third group positioned ventro-lateral to the oesophagus also stain although the signal is much weaker. Within this group PER appears to cycle, as staining is seen at ZT24 but not at ZT19, although the PER signal is cytoplasmic. TIM is also detected within these cells and again is only visible at ZT24, suggesting that TIM is cycling in these cells in accordance with the western data. At this time however TIM is nuclear whilst PER is cytoplasmic. The position of these cells supports the idea that the clock cells reside within the central brain of *Musca*, as severance of the optic lobe does not abolish behavioural rhythms (Helfrich *et al.*, 1985).

Both PER and TIM are found in photoreceptor nuclei at ZT24, suggesting that PER can become nuclear in peripheral tissues, although it does not in the central brain. This is also seen for PER in *A. pernyi* (Sauman and Reppert, 1996).
PDF staining is seen in a similar pattern to that of *Drosophila*. Both the small and large group of cells send projections into the medulla and lamina, with the large cells sending projections that arborise over the surface of the medulla as previously reported (Pyza and Meinertzhagen, 1997). I also observed projections from the small cells leading into the central brain both laterally, possibly along the optic tract and dorsally towards the calyces of the mushroom bodies as previously reported for *Drosophila* (Helfrich-Forster, 1995). However these cells do not appear to be the same as any of those expressing TIM and/or PER, although they may be in a position to allow communication with clock cells, thus potentially maintaining PDF's role as a circadian output factor. This is supported by recent findings that CRY co-localises with PDF in adult Musca (Pyza *et al*., 2003). PDF does not co-localise with PER in a number of insect species (Zavodska *et al*., 2003), in fact *Drosophila* so far is the only species in which co-localisation does occur.

In transgenic *Drosophila* carrying the *Mdper* gene in replacement of *Dmper*, MM1, PER can also seen to have increased stability as PER does not appear to cycle and remains cytoplasmic in pacemaker neurons. However, as *Mdper* restores behavioural rhythmicity to these flies the negative feedback loop must be functional. As is the case in *Musca*, PER can become nuclear in peripheral tissues as seen in the Malpighian tubules using an MdPER-GFP reporter. Crossing these flies into double-time mutants revealed, in *Drosophila*, possible differences in the PER:DBT relationship that might occur in *Musca*. The effects of *dbp* and *dbar* are considerably greater in the *mm1* flies compared to *per*+. *dbp*, which is thought to effectively halve DBT activity compared to wild type, shows a reduction in $\tau$ of close to 1h in *mm1* compared to 0.3h in *per*+. *per* flies heterozygous for *dbar* show an increase in $\tau$ of 3h, whereas *mm1* only have an increase of 0.6h. DBT affects PER phosphorylation in both the nucleus and the cytoplasm and may regulate PER nuclear entry (Price *et al*., 1998; Kloss *et al*., 2001). The *dbp* mutation leads to lower levels of total DBT and is therefore likely to effect phosphorylation of PER in both cellular compartments. It may be that although *dbp* may lead to a slower turnover of PER, it could lead to faster nuclear entry of PER in *mm1*, thus giving rise to a faster rhythm. The *per* mutation in *Drosophila* has been shown to effect the nuclear turnover of PER (Hamblen *et al*., 1998; Marrus *et al*., 1996). It has also been shown that short period alleles repress the action of *dbar* (Rothenfluh *et al*., 2000), thus *dbar* and *per*.
may effect nuclear stability of PER in opposite directions. As \(per^\phi\) is positioned within the conserved C-domain and replacement of the \textit{Musca} C-domain with that of \textit{Drosophila} (MM12) rescues wild type \textit{Drosophila} behaviour, it is possible that increased nuclear turnover of PER in \textit{mml} counteracts the effects of \textit{dbt^c}, thus giving a wild-type rhythm.

In order to further study the apparently altered dynamics of PER stability I attempted to produce MdDBT in bacteria and to investigate PER:DBT interactions and PER phosphorylation. It has been reported that previous attempts to produce active DBT in bacteria have failed (Suri \textit{et al.}, 2000). To overcome this problem I chose to make constructs of DBT, one carrying a C-terminal deletion and another containing an S to A mutation of a predicted autophosphorylation site (Zillan \textit{et al.}, 1999). This C-terminal has been shown to have autoregulatory properties in both the CKI\(\delta\) and CKI\(\epsilon\) isoforms of mammals (Graves \textit{et al.}, 1995; Cegielska \textit{et al.}, 1998). Autophosphorylation of the C-terminus results in down-regulation of enzyme activity. Truncations of the C-terminus lead to increase in enzyme activity of a 10 fold level in CKI\(\delta\) (Greaves \textit{et al.}, 1995) and approximately 4-fold in CKI\(\epsilon\) (Cegielska \textit{et al.}, 1998). It was hoped that a similar result would be achieved with both DmDBT and MdDBT. In case this did not work I also chose to use the yeast homologue of DBT, HRR25, as has been done previously (Suri \textit{et al.}, 2000). Unfortunately very few of the constructs produced soluble protein when expressed in bacteria and so no protein:protein interaction or kinase assays were performed. Although the HRR25 construct was cloned as previously described (Suri \textit{et al.}, 2000) no protein was produced.

Along with investigating PER stability I also wished to understand why it is only detected within the cytoplasm of brain neurons. A plausible explanation is that the protein enters the nucleus and is then actively transported back to the cytoplasm. Investigation of a putative NES signal that is shared with the mPer's showed that coupling this sequence to a NLS-GFP reporter did not lead to cytoplasmic retention of the protein, suggesting that it does not function. This does not however exclude the possibility of nuclear export via other sequences. Alternatively TIM facilitates PER nuclear entry and, whilst in the nucleus, PER acts to repress transcription before being quickly degraded.

Another scenario implies that PER might act to facilitate the nuclear entry of TIM or other clock proteins, such as CRY, that may have taken on PER's role in
repression. In *Drosophila* TIM has recently been shown to shuttle between the cellular compartments until it is held in the nucleus through PER:TIM dimerisation (Ashmore *et al.*, 2003). Although it has now been shown that PER and TIM can enter the nucleus independently they each influence the others nuclear entry (Shafer *et al.*, 2002; Ashmore *et al.* 2003). I have shown that MdPER and MdTIM can dimerise in *ex in-vivo* studies and so it may be that the PER: TIM relationship is reversed in *Musca* and it is PER that is shuttled. However MdPER is not retained in the nucleus through PER:TIM formation. Another supporting factor for a reversal in the roles of PER and TIM is the apparent non-cycling of *per* and *tim* mRNA in DD when compared to the protein cycles. TIM cycles in both LD and DD, but the removal of light induced degradation (TIM is seen to be very light labile in *Musca*, as shown by the LL study) leads to a dampened oscillation due to relatively high levels of TIM throughout the subjective day. If TIM acts in repression in *Musca* and these levels are above a threshold for repression, then this could lead to constant repression and therefore non-cycling of both *per* and *tim* in DD. Finally, if another component has taken PER’s role in *Musca*, CRY would be a prime candidate, as it acts in place of TIM in mammals as PER’s binding partner and also has a role in repression of the CLK:BMAL1 heterodimer (Kume *et al.*, 1999; Shearman *et al.*, 2000).

I was also interested in gaining further understanding of the *Drosophila* circadian mechanism. This involved investigating the role of CRY, a study that had initially started in my Masters degree.

I had previously shown that double mutant *per*:cry flies were unable to entrain to 12:12 LD cycles at 28°C and free ran with a *per* behavioural period. This suggested that these mutants didn’t convey light information to the clock. This could suggest that the limits of entrainments were altered and that these double mutant flies would entrain to a T cycle of 20h (10:10 LD). It also suggested that PER and CRY may physically interact.

Behavioural analysis showed that in 10:10 LD conditions at 28°C either single mutant entrained but the double mutant could not and showed an advance of daytime activity by approximately 90mins each day. Therefore interaction of the two mutations leads to a genuine break down in light perception in a temperature dependant manner. Using the yeast two-hybrid system I have shown that PER and CRY can physically interact in a light dependant manner. This is supported by Co-IP
studies performed by E. Rosato. A deletion construct, CRYΔ, that misses 20 of the last 22aa of CRY suggests that this region mediates the light dependant interaction of CRY with both PER and TIM. Experiments performed by co-workers supported this as both frame shift and premature stop mutations within this region also removed the light dependency of both PER:CRY and TIM:CRY interactions.

We therefore hypothesised that either the removal of the C-terminus lead to a conformational change in CRY or that a nuclear, light-inhibited repressor bound the C-terminus in the dark. Mutagenesis of yeast by G. Mazzotta revealed mutants in which the light dependency of PER:CRY interactions was relieved, giving support to the C-terminal bound repressor theory, at least in yeast. In addition to this recent data from our laboratory suggests that in flies CRYΔ is a constitutively active form of CRY (S. Dissel pers. comm.). My interest for CRY also led my research to another area as I found that CRY also interacts with TGO in both ex in-vivo and in-vitro protein:protein interaction assays.

Homology searches reveal that TGO is very similar in sequence to CYC. In mammals a second BMAL1 protein exists (also termed MOP9) as well as a second homologue of CLK, termed NPAS2 (Reich et al., 2001). NPAS2 acts as a partner for BMAL1 in the forebrain but not in the SCN, suggesting its role in circadian timing in other tissues (Reich et al., 2001). Not only that but this dimer appears to be responsible in transmitting redox state information to the clock (Rutter et al., 2001b). As in mammals different clock genes are expressed in peripheral tissues, could tgo play a role in the peripheral clock of Drosophila? Further analyses using transgenic flies have shown that although neither over expression or down regulation of TGO affect the free-running behaviour rhythm, over expression leads to increased light responses. Such flies show an increased startle responses to light and are also more sensitive to light pulses administered during the early night than wild-type flies. This suggests that whilst TGO probably does not function in the central clock it may function in peripheral tissues such as the eye, where it may affect light transduction. As so far over expression and down regulation was restricted to clock cells and the tim-expressing photoreceptors it is possible that using a stronger, more broadly expressed driver for the eyes, such as rhodopsin, may give a stronger effect.

Other indirect evidence supporting a role of tgo in the clock comes from studies on the output neuropeptide PDF (Park, et al., 2000). In Drosophila PDF
expression is greatly altered by both the cyc⁰ and Clk⁰ mutations. However this is more drastic in Clk⁰ mutants, causing severe developmental defects in the PDF expressing lateral neurons. This suggests that another PAS protein may interact with CLK, maintaining some function of the CLK:CYC dimer. Searches of the Drosophila genome sequence reveals that no homologue of MOP9 exists. Thus TGO may be a prime candidate to act as a CLK binding partner in place of CYC. The fact that TGO is involved in developmental regulation in Drosophila (Sonnenfeld et al., 1997; Emmons et al., 1999) and the development of lateral neurons is affected in Clk⁰ mutants further supports the possibility the TGO can function, at least partially, in place of CYC. Therefore it is possible that a CLK:TGO dimer functions in peripheral tissues and has a function in the circadian mechanism within such tissues.
Appendices
Appendix 1  pDK101

Construction of the pDK101 vector. A is digested with NcoI (B) and the linker inserted to create C. This is then digested with XcmI (D) to produce the T overhang required for the cloning of PCR products produced by Taq polymerase.
Appendix 2 Expression vectors for bacterial protein expression

pJDC4

This vector was produced by adding an amplified GST signal from a pGEX4T-3 vector with a linker containing a thrombin cleavage site and NdeI, XhoI and BamHI sites into the Ncol and BamHI site of pET14b (Novagen). A diagrammatic representation is given below. Cloning the start ATG into the Ndel site allows expression of the inserted sequence.

![Diagram of pJDC4 vector]

pJDC7

This vector was created by adding a linker sequence including a thrombin cleavage site followed by the GST signal into the BamHI and Bpu1102I site of the pET23a vector (Novagen).

![Diagram of pJDC7 vector]
Appendix 3 Expression vectors for *in-vitro* transcription/translation

**EV2 vector**
- pBs EMCV tail: 3.76 kb
- EMCV T7 Promoter
- XhoI (1444)
- EcoRI (1392)
- NcoI
- Kpnl (1474)
- BgII
- BamHI (692)

**pBsMYC vector**
- pBsMYC: 3062 bp
- UTR, KOZAK, MYC
- EcoRI (102)
- EcoRV
- HindIII
- SaII
- XhoI
- Kpnl
- XbaI (0)
- NotI
- SstI
- T7
Appendix 4

Preparation of cells for electroporation

The bacterial cells were grown in 1L of LB media with the appropriate antibiotic to an $\text{OD}_{600}$ of 0.5. They were then pelleted by centrifugation at 4000rpm for 15mins at 4°C.

The cells were then washed in a series of sterile, ice-cold solutions to concentrate them and to remove electrolytes present in the media. Between each wash the cells were pelleted as described above.

1L distilled H2O
0.5L distilled H2O
20ml of 10% glycerol
2ml of 10% glycerol

The final suspension of cells was divided into 40μL aliquots and quick frozen in a dry ice/ alcohol bath. These were then stored at -80°C until required for use.
Appendix 5 pEG202 and pJG4-5

The diagrams show the structures of pEG202 and pJG4-5, including restriction sites and genetic elements. The text provides additional details about the construction of these plasmids, mentioning ADH promoter, pBR backbone, and genetic markers such as AmpR, His3, and TRP1.

Restriction enzymes and their cutting sites are indicated on the diagrams. For example, EcoRI, BamHI, SalI, NcoI, and NotI are shown at various positions. The fusion cassette regions are also highlighted, including GAL and B42 domain elements.

The genetic sequences are presented in the text, including nucleotide sequences for the fusion cassette and other genetic elements. This information is crucial for understanding the construction and function of these plasmids in genetic experiments.
Appendix 6  *Drosophila Media*

**Egg laying media**

15g of agar was boiled in 500ml of H$_2$O and allowed to cool.  
16.5g of sucrose was added to fruit juice and the mixture boiled and allowed to cool.  
These two mixtures were then mixed and poured into small petri dishes.

**Sugar media**

15g agar  
46.3g sucrose  
46.3g dead yeast  
H$_2$O to 1L.

The above were mixed and autoclaved in 400mL aliquots and stored at 4°C.
Appendix 7 Musca tim cDNA partial sequence

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CGTTGGCGCGGAGCCTGAGTGAGTTGAGTTGATAGTGACCTCACGTAAGTGCTTCTGGTAACCTT
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601

112
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a S E D N V H I K M I D H V S -
Appendix 8  Raw data from ANOVA analysis on RNA and Protein cycling data (Chapter 3)

The following pages contain the results of ANOVA one-way analysis of variance with Newman-Keuls post-hoc test.
TIM levels in LD

Newman-Keuls test; TIM (timprotld.sta)
Probabilities for Post Hoc Tests
MAIN EFFECT: TIEMPOIN

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TIM levels in DD

Newman-Keuls test; VALUES (timprotdd.sta)
Probabilities for Post Hoc Tests

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Newman-Keuls test; PER (perld.sta)
Probabilities for Post Hoc Tests
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Summary of all Effects; design: (perld.sta)

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**per RNA levels in DD**

Newman-Keuls test, PER (perrmadd.sta)
Probabilities for Post Hoc Tests

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**tim** RNA levels in DD

Newman-Keuls test; TIM (timdd.sta)  
Probabilities for Post Hoc Tests

**MAIN EFFECT: TIMEPOIN**

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### tim RNA levels in LD at 29°C

Newman-Keuls test; TIM (tim29.sta)
Probabilities for Post Hoc Tests

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Newman-Keuls test; TIM (tim18.sta)
Probabilities for Post Hoc Tests

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Summary of all Effects; design: (tim18.sta)

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125
Appendix 9 Raw data for Chapter 4 ANOVA analysis of activity data

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interaction is essential for maintaining the steady-state level of WC-1 and the function 


