MOLECULAR ANALYSIS OF THE LIGHT REGULATION OF DROSOPHILA CRYPTOCHROME

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Abstract

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Drosophila CRYPTOCHROME (dCRY) is the light receptor in the *Drosophila* central clock. In response to a light signal dCRY interacts with the clock component TIMELESS (TIM), targeting TIM for degradation thus delaying the progress of the molecular oscillations that underlie the clock, until dark-associated repression of dCRY function allows TIM to accumulate. The mechanism by which dCRY is regulated by light is unknown, though recent evidence indicates the important of the unique C-terminal domain. To explore the manner by which the C-terminus regulates dCRY, chimerics were created with the mouse CRYPTOCHROME 1 (mCRY1) whereby the unique C-terminuses were exchanged. mCRY1 is a component of the central clock and has not been found to be a photoreceptor or light regulated. Yeast assays using dCRY/mCRY variants against the clock components *Drosophila* PERIOD (PER) and TIM reveal that the mCRY C-terminal domain interacts with PER light-independently, and that the dCRY photolyase domain can mediate a light signal to regulate this interaction when expressed as a chimeric construct. Extending the study to transgenic fly lines expressing dCRY with a mCRY1 C-terminus, lines demonstrate a behavioral profile and TIM abundance profile similar to that of flies in constant light. This raises further questions over the light regulation of dCRY but provides no definitive answers.

In a concurrent study, putative sites for post translation modification in the C-terminal domain of dCRY were determined using a predictive algorithm and altered through site-directed mutagenesis. The dCRY variants were tested as bait against PER and TIM in yeast assays and in a semi-quantitative yeast assay. The assays revealed that residue position 530 may be important in the light regulation of dCRY.

Finally, an anti-body against dCRY was created by expressing the protein as three fragments that together represented the whole protein in *E. coli*. Two of the fragments representing the first and last third of the dCRY protein were successfully expressed, purified and sent to a commercial company used to successfully generate an antibody.

Chapter 1: Introduction

1.1 The circadian clock: an overview

All eukaryotes and some prokaryotes exhibit biochemical, physiological and behavioural rhythms that cycle periodically and which are synchronised with environmental changes that form the context in which the organism operates. These 'biological rhythms' may have periods that coincide with the seasons (circaannual), with lunar changes (circalunar), or be dependent on more localised environmental factors such as changes in the tide (circatidal). However, perhaps the most prevalent and universal rhythm is that which responds to the daily changes in light and dark, and the associated fluctuations in temperature. These rhythms occur with a period of about 24 hours and are called 'circadian' (*circa diem,* about a day) rhythms.

It is thought that circadian rhythms allow an organisation of behavioural and physiological features such as feeding times or the sleep/wake cycle to both maximise feeding opportunities and minimise the risk from predators, and to generally align the organisms behaviour to the most favourable temporal periods. One example is the circadian feature of *Drosophila melangaster* (the model organism of this study) that earned *Drosophila* its name: that is that they emerge from the pupal case (eclose) at dawn (and *Drosophila* means 'dew loving' in reference to this behaviour. In this case behaviour is linked with circadian timing in order to minimise desiccation. Although these behavioural manifestations of circadian rhythm are perhaps the most obvious, circadian rhythmicity permeates many functions within organisms, an example of which is the link between the cell cycle and the circadian clock (reviewed in Reddy *et al.,* 2005).

The advantage of possessing a circadian clock is perhaps most evident through the ubiquity of such clocks across so many organisms (Dunlap 1999). Yet despite this apparent need for a clock, rigorous tests to accurately assess the relative benefits of possessing a circadian clock on the fitness of the organism are sparse. It has, however, been demonstrated that cyanobacteria that have a period similar to the light/dark cycle of

their environment show an increased relative fitness (Ouyang *et al.,* 1998) and that strains with a functioning clock out-compete those that are clock-defective, but only in cycling environment, and not in constant conditions (Woelfle *et al.,* 2004). It has also been shown that the absence of a functioning clock in *Drosophila melanogaster* results in a reduction in reproductive fitness (Beaver, 2002). A difficulty in assessing the fitness of clock mutants arises from the fact that so many processes are controlled by the clock. Hence, whether the clock confers a specific fitness may well be a secondary factor the pervasiveness of the influence of the clock on the biological processes of an organism. However, enforced social and environmental disruptions to the circadian period of our behaviour are known to cause adverse effects in humans. The most common examples include jet lag, which results from a desynchronisation of the circadian clock with the environment due to travelling across time zones, and problems associated with shift-work, where the workers' activity patterns are frequently changed. Such disruptions lead to tiredness and lethargy during periods when wakefulness is desired, and also to disruption in sleeping patterns (Waterhouse *et al.,* 2002). Also, a link has also been established between the disruption in circadian rhythm and the susceptibility to cancer in humans (Stevens, 2005; Davis *et al.*, 2001)

For an organism to be considered to possess a circadian clock, the circadian rhythms must be endogenous and not simply the reaction of the organism to the environment. Hence, the circadian rhythms must persist in the absence of external cues. This endogenous rhythm does not have to be 24 hours in order to be classified as circadian, merely within the range of 22-25 hours. Circadian clocks must also be able to resynchronise to changes in the environment to allow for the shifting of the phase of the cycle, such as with changing day-length over the course of the year. The clock must also be insensitive to changes in temperature, which fluctuate both with the time of day as well as the time of year. All of these features are required for a circadian clock (reviewed in Young, 1999).

Circadian clocks must consist of an oscillator which drives the underlying circadian period, input pathways to allow resynchronisation, and output pathways to generate the behavioural and physiological manifestations of the circadian clock. Inputs are cues which serve to resynchronise the clock, the most important of which is light, although

other factors such as feeding and temperature have been shown to be able to synchronise the clock. The oscillator itself consists of interlocked negative and positive feedback loops of gene expression, the products of which are self-regulatory transcription factors that serve to regulate the expression of both clock genes (genes directly involved in the central oscillator), and to also regulate the expression of genes known as 'clock controlled genes', which represent the output pathway. This system of input, central oscillator and output represents the general structure of the circadian clock. The specific components and organisation of the clock vary across different organisms, and of particular importance to our understanding of the way circadian rhythms are generated and regulated has been the use of model organisms, such as *Drosophila*. It is the *Drosophila* circadian clock that this investigation is primarily concerned with, and therefore the known components and mechanisms of the *Drosophila* clock will now be discussed.

1.2 The *Drosophila* circadian clock

1.2.1 The *period* gene

The *period* gene was the first clock component to be identified in *Drosophila* through the use of chemical mutagenesis used to generate *Drosophila* mutants with aberrant eclosion rhythms (Konopka and Benzer, 1971). Three mutants were identified: per^S which showed a short period (19 hours), per^L which had a long period (28 hours) and a mutant with no discernible period (arrhythmic) that was designated per^0. All three mutations were mapped to the same locus on the X chromosome. The per^S is a serine to asparagine missense mutation (AGC to AAC) at position 589, the per^L mutation is a valine to asparagine at position 243 and the per^0 mutation produces a premature stop coding, giving a truncated protein (Yu *et al.*, 1987; Baylies *et al.*, 1987).

The *period* gene codes for a 1218 amino acid protein (Citri *et al.*, 1987) which possesses a PAS domain (Crews *et al.*, 1988) (see **fig 1.1**) which functions as a protein dimerization motif essential for regulating its own transcription (Huang, 1993). Other important features are an inhibiting domain of the clock components CLOCK (CLK) and CYCLE

(CYC), the significance of which is discussed later. Within this inhibition domain is a novel nuclear localisation signal (NLS) believed to be more potent than a previously defined NLS in the N-terminal region (Colot *et al.,* 1988) and is essential for PER accumulation in the nucleus (Chang and Reppert, 2003). A region known as the Thr-Gly or 'TG' region, which is polymorphic for the numbers of Thr-Gly pairs present (Kyriacou *et al,* 1996), is important for temperature compensation of the circadian clock (Sawyer *et al,* 1997), and the stabilisation of PER (Peixoto *et al.,* 1998).

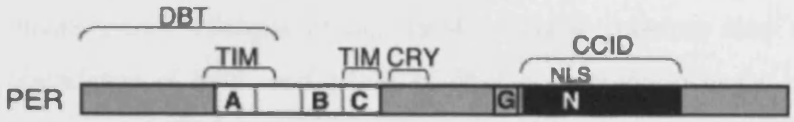


Fig 1.1 The PERIOD protein including important sub-domains. Brackets indicate regions of interactions with other factors. CCID: CLK:CYC interaction domain, indicated as a black box. NLS: nuclear localisation signal within the CCID. TIM=TIMELESS, CRY=CRYPTOCHROME, DBT=DOUBLETIME. G=the glycine-threonine repeat. The white box indicates the PAS domain with A=PAS-A, B=PAS-B and the C=cytoplasmic localisation domain (CLD).

Both *period* mRNA and the PERIOD (PER) protein cycle in abundance in a circadian manner (Hardin *et al.*, 1990; Edery *et al.*, 1994), and the cycling of the *period* mRNA is mainly due to circadian regulation of transcription of the *period* gene (Hardin *et al.*, 1992), and only in part to post-transcriptional controls (Stanewsky *et al.*, 2002). However, the cycling of the mRNA and protein are not synchronised, with the *period* mRNA abundance peaking in the early night, but the protein showing a 4-6 hours delay relative to the mRNA, peaking at late night at a Zeitgeber time (ZT) of 14-16. Zeitgeber (German for "time giver") refers to an external cue that entrains the endogenous circadian clock of organisms; if *Drosophila* are entrained in a 24 hour period of 12 hours of darkness and 12 hours of light (subjective night and subjective day respectively), then Zeitgeber time (ZT) refers to the time in hours during that day. In the context of this study, ZT 0 refers to the start of the subjective day (lights on) and ZT 12 to the start of

the subjective night (lights off). Therefore, period mRNA peaks 2-4 hours into the subjective night and the protein peaks later near the end of the subjective night at ZT 22-20 (see **fig. 1.2**). As well as changes in abundance, PER also undergoes changes in phosphorylation, becoming hyperphosphorylated as it accumulates (Edery, 1994).

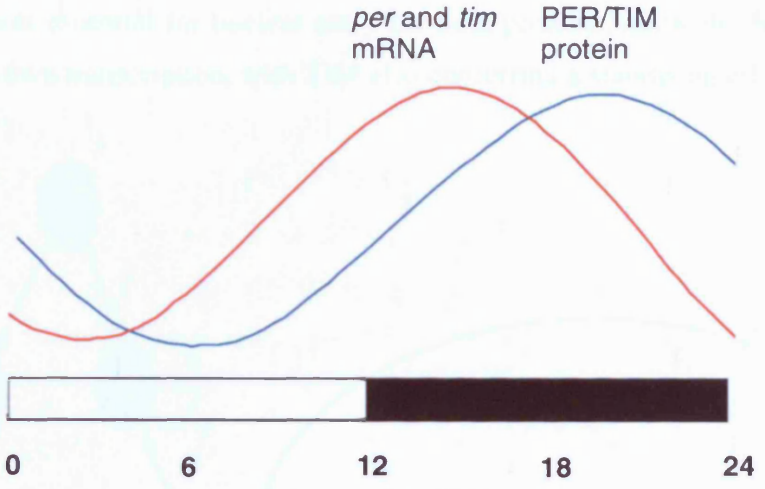
1.2.2 The *timeless* gene

The partner of PER is the product of the *timeless* gene, TIMELESS (TIM), which was first isolated as a mutation that caused an alteration in the eclosion rhythms of *Drosophila melanogaster* (Sehgal *et al.*, 1994). These mutants also showed a decrease in the abundance of PER, and a lack of PER accumulation in the nucleus, indicating that the presence of TIM was required for both PER stability and nuclear localisation. The TIM protein exists in two forms owing to the presence of two possible start sites, one giving rise to a long form (1389 amino acids) which was identified first (Myers *et al.*, 1995) and a short form (1366 amino acids) which was identified later (Rosato *et al.*, 1997).

Both *timeless* mRNA levels and TIM abundance cycle in a circadian manner similar to that of *period* mRNA and PER. Notable differences between PER and TIM are that the peaking of TIM levels precedes that of PER slightly and, importantly, TIM levels fall much more rapidly on exposure to light (Zeng *et al.*, 1996) as compared to PER. In *tim* null mutant (tim^{01}) flies, *per* mRNA doesn't cycle, and the PER protein shows both constantly low levels and hyperphosphorylation. Conversely, in per^{0} flies, *tim* mRNA does not cycle, but TIM levels do in a light:dark cycle, with the decrease in TIM levels coinciding with the onset of light. This cycling of TIM is dependent upon the presence of light, as in constant dark conditions TIM does not cycle, which suggests a mechanism of TIM light-dependent degradation (Zeng *et al.*, 1996) and places TIM as a candidate for the initial target of light entrainment.

Fig 1.2 Cycling profiles of *per* and *tim* mRNA and PER and TIM proteins over the circadian period. *per* and *tim* mRNA cycling is represented by the red line, and the PER and TIM protein levels by the blue line. The subjective time points are given with 0

representing lights on. The white and black bars represent light and dark periods respectively.



1.2.3 The PER/TIM heterodimer

In the tim^{01} mutants the cycling of *tim* mRNA was found to be abolished, combined with the profile of *tim* mRNA and TIM cycling this suggested that TIM was autoregulated in the much the same manner as PER (Sehgal *et al.,* 1995). However, of significance was the finding the in tim^{01} flies, *per* mRNA cycling was also abolished, presenting the possibility that TIM was also involved in the autoregulatory feedback loop of PER (Sehgal *et al.,* 1994). *tim* mRNA cycling was later shown to be dependent on the presence of both PER and TIM, hence it appeared that the negative feedback loops thought to exist for both proteins were interlinked (Sehgal *et al.,* 1995), and this is supported by the findings that PER and TIM dimerize both *in vivo* and *in vitro* (Rutila *et al.* 1996; Saez and Young 1996; Gekakis *et al.,* 1995).

It was later found that TIM appears to have a role in PER localisation in the nucleus (Vosshal *et al.,* 1994; Sehgal *et al.,* 1995), supported by work which found TIM and PER to be codependent for nuclear localisation (Saez and Young 1996; Meyer *et al.*, 2006). Moreover, in tim^{01} flies, PER levels are constitutively low and hyperphosphorylated

(Price *et al.*, 1995) suggesting that TIM is required for the stabilisation of PER, and light-mediated TIM degradation led to a delay in the nuclear accumulation of PER (Lee *et al.*, 1996). Taken together, such evidence led to a model of the clock whereby a PER-TIM dimer was essential for nuclear entry for both proteins, allowing the negative repression of their own transcription, with TIM also conferring a stabilising effect on PER (**fig 1.3**).

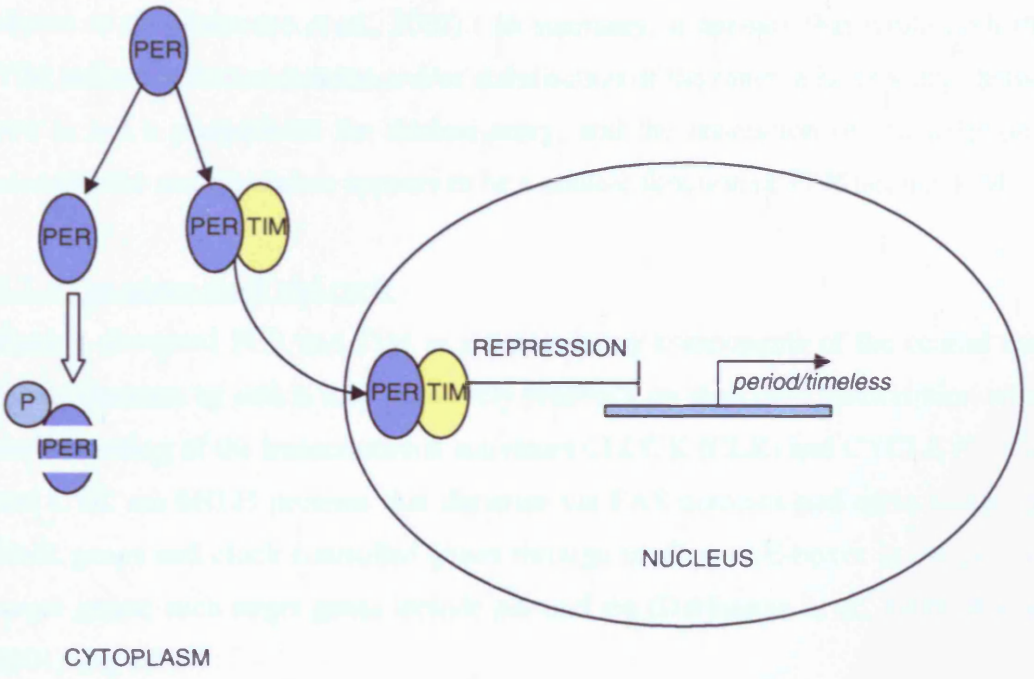


Fig 1.3 PER and TIM associate as a heterodimer to enter the nucleus and repress the transcription of both the *per* and *tim* genes. Phosphorylation of PER in the cytoplasm leads to degradation. However, in the presence of cytoplasmic TIM a PER/TIM heterodimer forms which stabilises PER. The heterodimer enters the nucleus and represses transcription of the *per* and *tim* genes. As PER and TIM levels fall the repression is relieved, and the cycle of negative feedback can resume.

However, it has been subsequently shown that PER nuclear entry precedes TIM nuclear entry and that in certain clock cells this lag is particularly pronounced (Shafer *et al.*, 2002). Moreover PER is able to repress transcriptional activators in the absence of TIM, but such repression can only occur when PER is nuclear (Rothenfluh *et al.*, 2000; Cheng

and Reppert 2003; Nawathean and Rosbash 2004). In addition, while TIM stabilising PER leads to a greater abundance of PER, this does not lead to an increase in PER mediated repression of CLK-CYC in Schneider 2 (S2) cells (Nawathean and Rosbash, 2004). TIM is able to shuttle in and out of the nucleus in a PER dependent manner, with PER being important for the nuclear retention of TIM but not for nuclear entry, but cannot mediate the repression of the *period/timeless* transcriptional activators as PER had shown to do (Ashmore *et al.,* 2003). In summary, it appears that while both PER and TIM influence the localisation and/or stabilisation of the other, a heterodimer between the two is not a prerequisite for nuclear entry, and the repression of transcription that is essential for autoregulation appears to be a nuclear function of PER but not TIM.

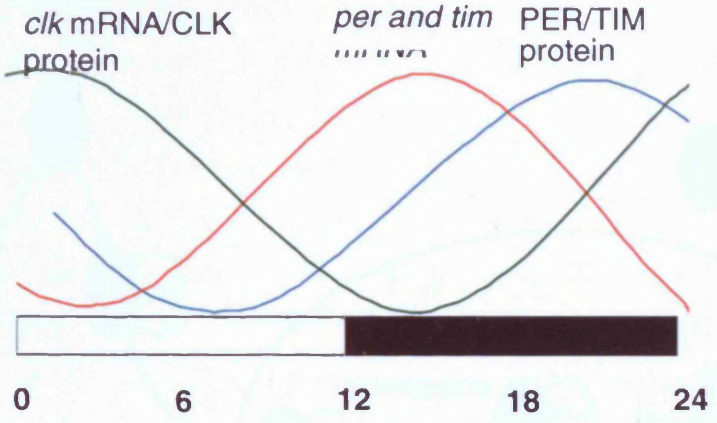
1.2.4 The genes *clock* and *cycle*

Having discussed PER and TIM as autoregulatory components of the central oscillator, the mechanism by which they negatively feedback on their own transcription requires an understanding of the transcriptional activators CLOCK (CLK) and CYCLE (CYC). CLK and CYC are bHLH proteins that dimerise via PAS domains and drive transcription of clock genes and clock controlled genes through binding to E-boxes in the promoters of target genes; such target genes include *per* and *tim* (Darlington *et al.,* 1998; Wang et. al., 2001) (**fig 1.5**).

clock (clk) was first discovered as a mutant that severely disrupted the cycling transcription of *per* and *tim* (Allada *et al.,* 1998). Flies heterozygous for the mutation had alterations in period, while homozygous flies were arrhythmic. Originally known as *jerk* (*jrk*), the gene was renamed *clock* after it was found to be a homologue of the previously identified Mouse *clock* gene (Allada *et al.,* 1998). In keeping with the discovered disruption to *per* and *tim* transcriptional cycling, it was found that both PER and TIM were at constantly low levels in the *clk* mutants. The mutation itself was identified as a truncation that deleted the putative activation domain in the C-terminus (Allada *et al.*, 1998) The levels of *clk* mRNA and CLK protein cycle over the circadian period, which persists in constant darkness (with reduced amplitude), but unlike *per*/PER and *tim*/TIM, *clk* mRNA and CLK protein rise in phase, peaking between ZT 3.5 and ZT

4, in anti-phase with PER and TIM accumulation (Lee *et al.,* 1998; Rutila *et al.,* 1998; Bae *et al.,* 1998). This cycling of *clk* mRNA and CLK protein is mediated through transcriptional activation and repression of the *clock* gene. At first it was thought that the PER-TIM complex positively regulated the transcription of *clock* (Lee *et al.,* 1998; Bae *et al.,* 1998), but it was later shown that this process is regulated by the two genes *vrille* and *pdp1ε*, both of which are activated by the action of the CLK-CYC dimer (Cyran *et al.,* 2003). VRILLE was first identified as a component of the central oscillator in clock cells, transcriptionally activated by CLK-CYC (Blau and Young, 1999), later VRILLE (VRI) was shown to be a transcriptional repressor of clock (Glossop *et al.,* 2003) mRNA and protein accumulation of VRI precedes that of PDP1ε, with VRI acting as a repressor of *clk* transcription. VRI levels are maximal around ZT 15, the early night, when *clk* mRNA and CLK levels are at their nadir. Later on in the night, levels of PDP1ε (which activates *clk* transcription) peak at ZT 18, corresponding to the start of the rise in *clk* levels (Cyran *et al.,* 2003) (**fig 1.4**).

Fig 1.4 The cycling profile of *clk* mRNA and CLK protein compared to *per/tim* and PER/TIM cycling profiles. The profile of *clk* mRNA and CLK protein are synchronised, and represented with a green line. Profiles of *per/tim* are represented with a red line, and profiles of PER/TIM are represented with a blue line. The subjective time points are given with 0 representing lights on. The white and black bars represent light and dark periods respectively. *clk* mRNA and CLK protein cycles almost directly out of phase with *per* and *tim* cycling.



cycle (*cyc*) was first identified by a mutation that resembled the previously discussed *clk* mutant flies: heterozygous mutants were rhythmic, but with altered period, while homozygous mutants were all arrhythmic. Also both *per* and *tim* transcription cycling was reduced (Rutila *et al.*, 1998). Cloning of *cyc* revealed it to be a homologue of the mammalian BMAL1, which was known to partner mammalian CLOCK to form a heterodimer and drive transcription of target genes through the E-boxes (Hogenesch *et al.* 1998), in the same manner that CLK and CYC were later shown to do (Darlington *et al.*, 1998; Hoa *et al.*, 1997). *cycle*, however, was found not to cycle at the mRNA level (Rutila *et al.* 1998).

1.2.5 PER-TIM interaction with CLK-CYC

The method by which PER and TIM negatively feedback on their own transcription is through a direct interaction with the CLK-CYC complex, inhibiting the positive transcriptional activity (Lee *et al.*, 1998, 1999; Bae *et al.*, 2000). This is achieved by prevention of the binding of the CLK-CYC complex (which remains undisrupted) to the target E-boxes (Lee *et al.*, 1999). It is now believed that PER mediates the repressive action on the CLK-CYC complex activity, and that this disruption is maximal when PER has dissociated from the PER-TIM complex in the nucleus (Rothenfluh *et al.*, 2000; Cyran *et al.*, 2005).

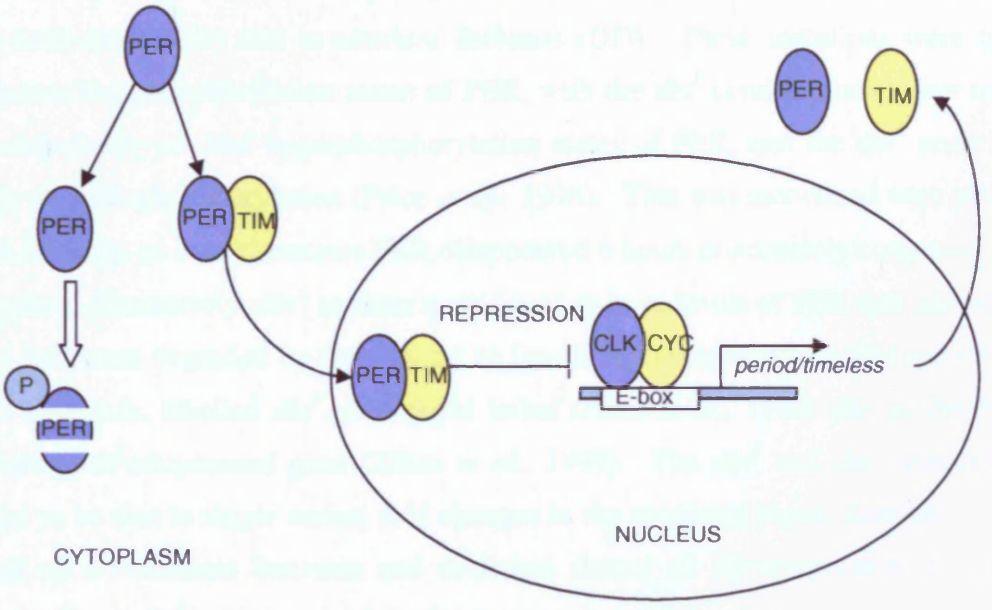


Fig 1.5 Interactions of key clock components in the circadian negative feedback loop in *Drosophila*. CLK and CYC form a heterodimer and drive transcription of *per* and *tim* through binding to the E-boxes in the promoters of the genes. In the absence of TIM, PER is phosphorylated and targeted or degradation. In the presence of TIM, PER is stabilised through the formation of a heterodimer that enters the nucleus and represses CLK:CYC activity.

1.2.6 Post-translational control of the clock components

DOUBLETIME (DBT) is a homologue of the human Casein kinase Iε (Kloss *et al.*, 1998) which was first identified through three mutations. *dbt* mRNA and DBT protein levels do not cycle, but localisation of DBT alters over the circadian period in a manner that corresponds with PER localisation. In per^0 flies, DBT is found to be predominantly nuclear (Kloss *et al.*, 1998, 2001). Two of the mutations gave an altered locomotor and eclosion period; labelled dbt^S and dbt^L, homozygous mutants gave an eclosion period of 20 hours and 27 hours respectively in constant darkness (hence reflecting the endogenous rhythm) and a locomotor period of 18 and 26.8 hours. These mutations also altered the periods of *per* and *tim* mRNA cycling and PER and TIM protein cycling, both in a light and dark cycle (LD) and in constant darkness (DD). These mutations were found to influence the phosphorylation status of PER, with the dbt^S resulting in a faster transition between the hypo- and hyperphosphorylation states of PER, and the dbt^L resulting in a delay in PER phosphorylation (Price *et al.*, 1998). This was associated with changes in PER stability, as in dbt^S mutants PER disappeared 6 hours prematurely compared to wild-type flies. Conversely, dbt^L mutants were found to have levels of PER that persisted after TIM had been degraded by light by up to four hours compared to wild-type flies. The third mutation, labelled dbt^P, was pupal lethal (Price *et al.*, 1998) due to *dbt* being an important developmental gene (Zilian *et al.*, 1999). The dbt^S and dbt^L mutations were found to be due to single amino acid changes in the predicted kinase domain. dbt^P is the result of a P-element insertion and abolishes almost all *dbt* expression (Kloss *et al.*, 1998). Since dbt^P causes pupal lethality it was not possible to examine adult flies, but the effects of the mutation were examined in the brains of third instar larvae. Such studies revealed that the dbt^P mutants lack rhythms of *per* and *tim* expression. Also, PER was found to be both hypophosphorylated and at constitutively high levels in a light/dark cycle, constant darkness and constant light, though this was not found to be due to an increase in *per* transcription as peak levels were comparable to those of wild-type flies. TIM levels were found to cycle in these mutants, though only in an LD cycle, indicating that TIM cycled due to light-mediated degradation, whereas TIM cycling was abolished in DD. Interestingly, although at the time the current hypothesis was that a PER-TIM heterodimer was essential for nuclear translocation of both proteins, nuclear PER was

found in dbt^{P} larval lateral neurons in constant light, a result that was later repeated (Price *et al.*, 1998; Cyran *et al.*, 2005).

Three more DBT mutants have since been isolated: dbt^{s} and dbt^{h}, both of which cause an increase in period length (Suri *et al.*, 2000) and dbt^{ar} which produces an arrhythmic phenotype, and is due to a histidine to tyrosine point mutation in the kinase domain that results in a reduced PER-kinase activity, but with no reduction in the level of PER-DBT interaction (Rothenfluh *et al.*, 2000; Ko *et al.*, 2002). In the dbt^{s} and dbt^{h} mutant flies, PER shows a delay in disappearance in the early morning (as with the dbt^{L} mutant) and the *per* and *tim* mRNA profiles are delayed, but this delay is not seen in the PER and TIM profiles (in that they peak at the same time as in wild type flies) in an LD regime, though the delay does manifest in constant darkness.

Taking all the evidence together, it is believed that DBT functions as a regulator of the circadian clock by interacting directly with PER (but not with TIM) (Kloss *et al.*, 1998, 2001) which leads to PER phosphorylation and degradation (Price *et al.*, 1998; Kloss *et al.*, 1998; Suri *et al.*, 2000; Ko *et al.*, 2002). Also, DBT-phosphorylated PER is found in the nucleus after the removal of TIM indicating that DBT acts upon PER in the cytoplasm and nucleus (Kloss *et al.*, 2001). This function of DBT is blocked by the TIM association with PER, though DBT is still able to associate with the PER-TIM dimer (Price *et al.*, 1998; Kloss *et al.*, 1998, 2001). Once phosphorylated, PER is then targeted for degradation by SLIMB, a member of the F-box/WD40 family (Grima *et al.*, 2002) then targets the hyperphosphorylated PER for rapid degradation by the 26S proteasome (Ko *et al.* 2002).

A further complexity to DBT regulation arose when studies using S2 cells, suggested that the action of DBT was required for PER mediated repression of CLK-CYC activity. Blocking nuclear export of PER was found not to increase the repression of CLK-CYC mediated transcription in the absence of DBT. This led to the proposed model that PER existed in an inactive and active form, the inactive form shuttled between the nucleus and cytoplasm, while the active from was retained in the nucleus. Transition between the

inactive and active form was then partly dependent upon the action of DBT. It was also found that DBT did not influence PER localisation (Nawathean and Rosbash, 2004). However, in more recent studies examining the larval lateral neurons in various dbt^{p}, dbt^{ar} and tim^{01} mutants, it was found that PER can translocate to the nucleus in tim^{01} null mutants but only if DBT kinase activity is inhibited, indicating that DBT also functions to regulate PER subcellular localization (Cyran *et al.*, 2005). The different findings are most likely due to the limitations of the use of S2 cells which are cultured *Drosophila melanogaster* embryonic cells, which do not possess endogenous clocks, and therefore may lack some essential features for such regulation.

In contrast to the action of DBT on PER, PROTEIN PHOSPHATASE 2A (PP2A) is believed to dephosphorylate and therefore stabilise PER. An increase in PP2A activity generally shortens the circadian period, whereas decreasing PP2A lengthens the period. PP2A is a holoenzyme consisting of three units, a main catalytic subunit (*microtubule star – mts*), a regulatory subunit (*twins-tws*) and a beta subunit (*widerborst-wdb*). The level of *wdb* and *tws* mRNA cycle. *tws* may be a target of the CLK/CYC transcription complex, as it has an E-box in the promoter (Sathyanarayanan *et al.*, 2004).

The *shaggy* (*sgg*) gene encodes the SHAGGY (SGG) protein, a homologue of glycogen synthase kinase-3, and like *doubletime* and *vrille* is an essential developmental gene (Siegfried *et al.*, 1992; Bourouis *et al.*, 2002). It has also been shown to influence the circadian period through the phosphorylation of TIM (Martinek *et al.*, 2001). This study conducted a screen for changes in the period of locomoter activity in *Drosophila* following over-expression of genes specifically in pacemaker-cells, and identified a shortening of the locomotor activity due to *sgg* over-expression which produced a shortening of locomotor period by three hours, shown to be due to the premature nuclear entry of the PER-TIM complex, which in turn was associated with SGG-dependent phosphorylation of TIM. Conversely, the study used flies with a *sgg* null mutation that had development rescued via a *sgg* transgene under control from a heat shock promoter in order to investigate the effect in locomotor period in the absence of SGG. Following development, adult flies with the null mutation were maintained at temperatures below

that required to activate the heat shock promoter of the *sgg* transgene, and as such exhibited a lengthening of circadian period and failed to show the characteristic TIM phosphorylation in the subjective late night/early day, when TIM localisation is believed to be nuclear (Martinek *et al.*, 2001). All of this suggests that not only is SGG required for regulating PER-TIM nuclear entry via SGG-dependent phosphorylation of TIM, but that the dissociation of TIM from PER in the nucleus may well be initiated by the action of SGG on TIM. The effects of SGG on the PER-TIM dimer location require the presence of TIM, as PER does not become phosphorylated or nuclear in tim^{01} flies over-expressing SGG (Cyran *et al.*, 2005).

1.2.7 The current model of the central oscillator

Summarising the previously discussed data into an interlinked model gives a central oscillator generated by two interlinked positive and negative feedback loops based upon autoregulation of transcription, and regulation at the translational level through phosphorylation, which serves to both regulate degradation and localisation of certain clock components.

In the negative loop, the transcriptional activators CLK and CYC form a heterodimer (Bae *et al.*, 2000) and drive the expression of the target genes *per* and *tim*, through binding to E-boxes in their promoters (Allada *et al.*, 1998; Rutila et. a., 1998; Blau and Young, 1999). *per* and *tim* mRNA levels peak around ZT 14-16 (the early night) the peak in protein levels occurs around ZT 20-22 (late night) (see **fig 1.4**), 2-4 hours delayed from the mRNA cycle (Hardin *et al.*, 1990; Zeng *et al.*, 1996). At first PER accumulation is impeded by the action of DBT which phosphorylates PER (Price *et al.*, 1998; Kloss *et al.*, 1998, 2000) signalling its degradation via the UBP pathway through association with SLIMB (Grima *et al.*, 2002; Ko *et al.*, 2003). DBT also serves to localise PER to the cytoplasm (Cyran *et al.*, 2005). During the evening PER is dephosphorylated by PP2A which is thought to stabilize PER and perhaps promote nuclear localization (Sathyanarayanan *et al.* 2004). TIM is degraded in response to light during the day (Zeng *et al.*, 1996) but at night, as TIM accumulates, the PER-TIM heterodimer forms which

protects PER from DBT phosphorylation and degradation both in the cytoplasm and the nucleus (Price *et al.*, 1998; Kloss *et al.*, 1998, 2001; Rothenfluh *et al.*, 2000). In the middle of the night, PER and TIM, possibly in a dimer and/or complex with DBT, translocate/s into the nucleus, a process apparently regulated by the actions of SGG which phosphorylates TIM (Martinek *et al.*, 2001; Cyran *et al.*, 2005) and CK2 which phosphorylates PER (Lin *et al.*, 2002; Atken *et al.*, 2003), and which may also enter as a component of the complex. Once in the nucleus, repression of the positive transcription of CLK-CYC takes place (Darlington *et al.*, 1998; Lee *et al.*, 1998, 1999; Bae *et al.*, 2000) most likely through the repressive action of PER either in the dimer and/or as a monomer (Ruthenfluh *et al.*, 2000; Chang and Reppert, 2003; Nawathean and Rosbash, 2004; Cyran *et al.*, 2005). CLK-CYC transcriptional activity has, by this time, already been reduced (So and Rosbash, 1997) most probably due to low levels of *clk* transcription. The PER-TIM heterodimer dissociates with the onset of light, which may be mediated by SGG-dependent phosphorylation of TIM, leading to its degradation (Martinek *et al.*, 2003) at which time PER exerts maximal repression on the positive action of CLK-CYC inhibiting transcriptional activity in the early morning when CLK-CYC levels are actually maximal. PER, having dissociated from TIM, now becomes subject to DBT phosphorylation and associated degradation (Price *et al.*, 1998; Rothenfluh *et al.*, 2000; Kloss *et al.*, 2001). The cycle is thus complete, and transcription driven by CLK-CYC can begin again. A full model of the key components of the molecular cycles that drive the circadian clock can be found in **fig 1.7**

In the second, positive loop, CLK-CYC drives the expression of *vrille* and *pdp1ε* leading to an initial accumulation of VRILLE, which suppresses *clk* expression and then an accumulation of PDP1ε which enhances *clk* expression, which leads to cycling of *clk*, mRNA (and CLK protein) (Cyran *et al.*, 2003). CLK protein and mRNA peak in the early morning and reach their nadir in the early evening (corresponding to maximal amounts of *per* and *tim* mRNA). The lowest point of CLK corresponds to the highest amount of VRILLE, which also peaks in the early evening (around ZT 15) (Cyran *et al.*, 2003). CLK amount begins to rise when PDP1ε reaches its maximal amounts at around ZT 18. In summary, the mRNAs of the CLK-CYC target genes are maximal in early to

mid-night (when CLK is at its lowest). The fact that the mRNAs are not maximal in the early morning (coinciding with the peak of CLK) is due to the lingering presence of PER in the nucleus that inhibits the CLK-CYC action. See **fig 1.6** for a model of the positive loop of the circadian clock.

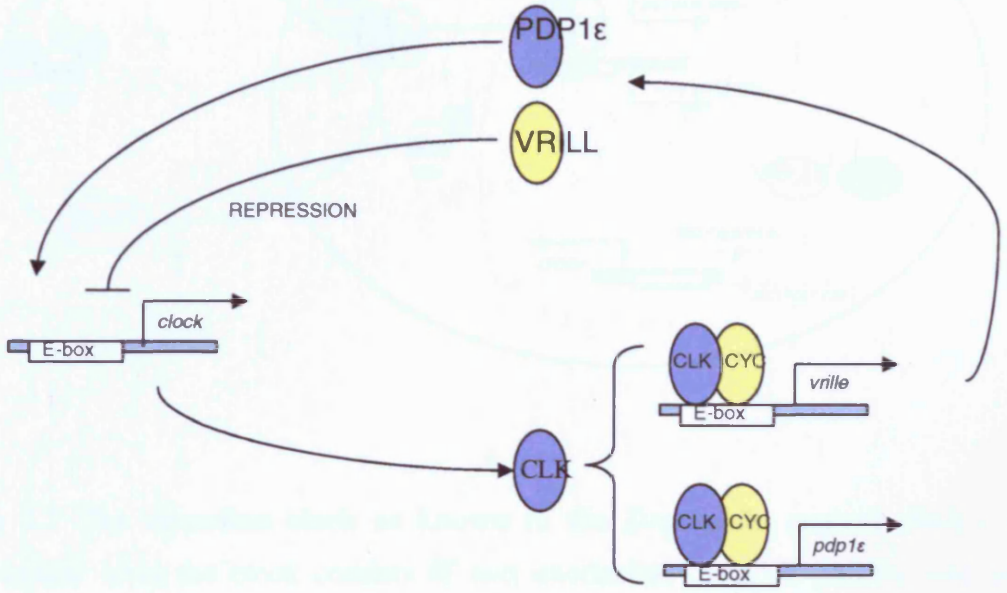


Fig 1.6 The positive loop of the molecular circadian clock cycle as known in *Drosophila melanogaster*. The CLOCK (CLK) and CYCLE (CYC) dimer drives expression of both *vrille* and *pdp1ε* through binding to E-boxes in the promoters of the target genes. Initially, VRILLE represses *clock* transcription with levels of the VRILLE protein peak early in the night; later in the night PDP1ε activates *clock* transcription with levels of the protein peaking approximately 3 hours after VRILLE. This negative and positive feedback loop drives the cycling in abundance of the CLK protein.

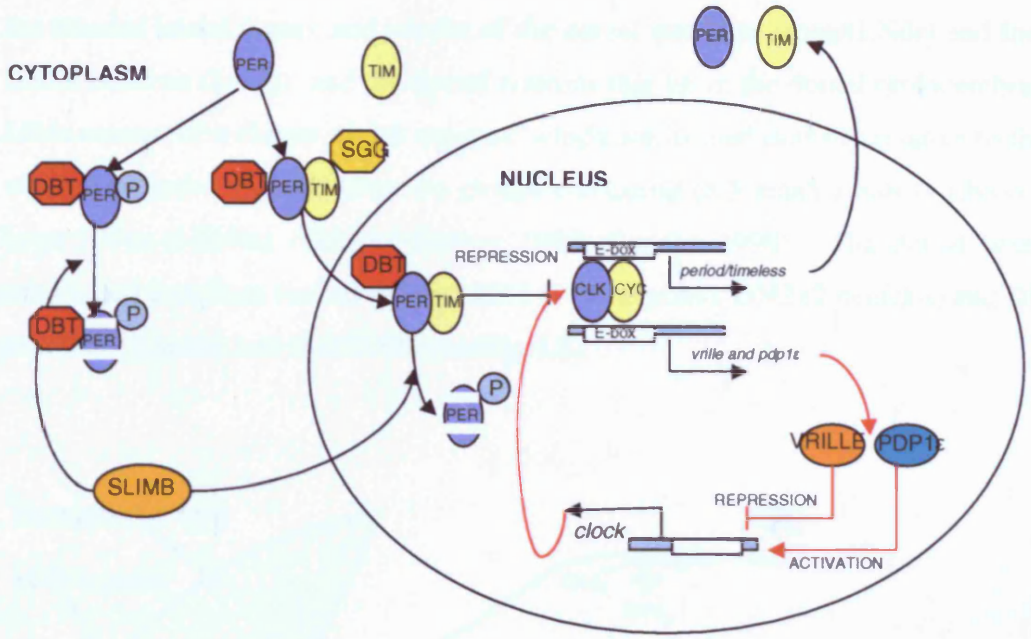


Fig 1.7 The circadian clock as known in the *Drosophila* central clock. At the molecular level the clock consists of two interlocked loops of positive and negative feedback. In the positive loop the CLOCK (CLK) and CYCLE (CYC) dimer drives expression of *period* and *timeless* through binding to E-boxes within the promoters of the genes. Following expression the proteins PERIOD (PER) and TIMELESS (TIM) form a PER:TIM dimer forms and enters the nucleus and represses the positive action of the CLK:CYC dimer. In the absence of TIM, PER is phosphorylated and targeted for degraded through the ubiquitin proteosome via interaction with SLIMB. In the positive loop, CLK:CYC also drive transcription of *vrille* and *pdp1ε,* and the protein VRILLE initially then suppresses *clk* transcription thus lowering CLK abundance. Later, PDP1ε promotes transcription of *clk* increasing CLK amounts.

1.2.8 Location of the central clock cells in *Drosophila*

Several lines of evidence indicate that the central pacemaker responsible for adult locomotor rhythms is located in the *Drosophila* brain (reviewed in Kaneko, 1998). Both *per* and *tim* are expressed in photoreceptor cells, numerous glia cells and in a few neurons. The neurons are divided into two groups: the lateral neurons (LNs) which lie in

the anterial lateral cortex and consist of the dorsal lateral neurons (LNds) and the ventral lateral neurons (LNvs), and the dorsal neurons that lie in the dorsal protocerebrum. The LNds consist of a cluster of 5-8 neurons, which are located dorsally relative to the LNvs, which are further divided into two groups consisting of 5 small LNvs (*s*-LNvs) and 4-6 large LNvs (*l*-LNvs) (Helfrich-Forster 1995; Kaneko 1998). The dorsal neurons are subdivided into three further groups: DN1 (~15 neurons), DN2 (2 neurons) and DN3 (~40 neurons) (Kaneko and Hall 2000) (see **fig 1.8**).

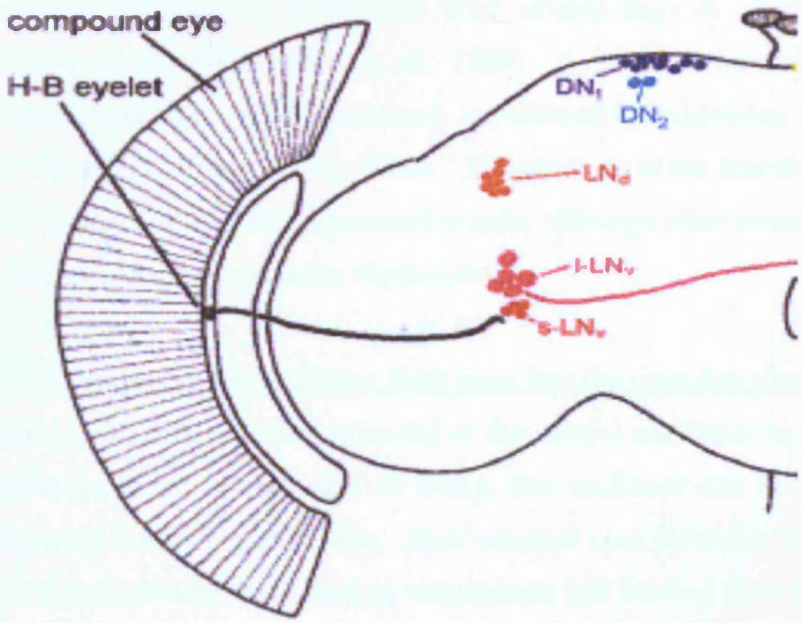


Fig 1.8 A diagram showing the relative positions of the various known clock cells in one side (the arrangement is symmetrical) of the *Drosophila* brain in the dorso-ventral plane. The top of the diagram represents the dorsal and the bottom the ventral side. DN = Dorsal Neurons, LN_d = Dorsal Lateral Neurons, l-LN_v = large Ventral Lateral Neurons, s-LN_v = small Ventral Lateral Neurons

Several lines of evidence indicate the importance of the LNs in generating robust circadian locomotor activity. Photoreceptor cells are not necessary for circadian rhythmicity and entrainment, and, although they contribute to rhythmicity, the glia cells

are not sufficient to maintain rhythmicity in mutants lacking LNs (Ewer *et al.,* 1992), indicating that while these cells contain the *per* and *tim* clock genes, they are unlikely to harbour the central pacemaker. However, in disconnected mutants, which lack LNs, 95% show arrhythmicity (Dushay *et al.,* 1989) and those that do show arrhythmicity were later found to possess at least one LN (Helfrich-Forster *et al.,* 1998). Conversely, flies that have expression of *per* restricted to the LNs show robust circadian rhythmicity, albeit of a longer period and weaker than that of wild-type flies (Frisch *et al.,* 1994). Disruption of the LNvs, either through removing the signalling factor PDF that is expressed only in the LNvs (discussed later) or by inducing specific cell death of these neurons, results in mutants that become arrhythmic after several days in constant conditions (Helfrich-forster *et al.,* 1995; Renn *et al.,* 1999). Mutants for the cry^b mutation, a strong hypomorph allele of *cryptochrome,* are rescued by expression of *cryptochrome* confined to the LNvs (Emery *et al.,* 2000). Therefore, it is the lateral neurons that are the best candidates for the central pacemaker cells, although other neurons may well contribute to various aspects of circadian rhythmicity.

1.2.8 Entraining the oscillator: light input into the circadian clock

Having discussed a current model of the central oscillator in *Drosophila*, consideration must be given to the ways in which this oscillator can be entrained to the external environment by external cues. Such external cues form the 'input' section of the clock. Although external cues such as temperature and feeding cues have been demonstrated to be able to synchronise the *Drosophila* circadian clock, the most potent and best studied entrainment factor is that of light.

It was known for some time that light resets the circadian clock through light-dependent degradation of TIM (Hunter-Ensor *et al.,* 1996; Myers *et al.,* 1996; Zeng *et al.,* 1996; Marrus *et al.*, 1996). In response to light TIM is rapidly phosphorylated on tyrosine residues, ubiquitinated, and degraded via the proteasome (Naidoo *et al.,* 1999). Since PER requires TIM for stability and nuclear entry, light-mediated removal of TIM prevents the accumulation of PER, and PER cannot translocate into the nucleus to mediate repression of the CLK-CYC transcriptional activation, thus delaying the

oscillator until TIM levels can accumulate in darkness. Since TIM itself is not a photoreceptor, it is necessary for there to be at least one photoreceptor to mediate the light input to TIM. *Drosophila* can entrain to light input through several pathways including the visual system, *via* extraretinal structures known as the Haufbaur-Buchner eyelets and throughout direct input to the central oscillator *via* the photopigment *Drosophila* CRYPTOCHROME (dCRY). Elimination of all three of these pathways is necessary to produce mutants that cannot be entrained to light cycles (Helfrich-Forster *et al.*, 2001).

First identified in *Arabidopsis* as proteins involved in blue-light signalling, cryptochromes belong to a family of proteins that show homology to photolyases – a group of proteins important in UV damaged DNA repair - though cryptochromes lack photolyase activity and are characterised by unique C-terminal domains (reviewed in Cashmore, 2003).

Initial experiments indicated the action spectra of both TIM degradation and the resetting of the clock by light was found to correspond to that of blue light indicating the possibility of the photoreceptor being a member of the cryptochrome family (Suri *et al.*, 1998). Two concurrent studies then demonstrated the importance of dCRY in the light entrainment of the *Drosophila* circadian clock. One study identified *dcry* through its homology with the *Drosophila* photolysase6-4 family, and found it was located on the third chromosome (Emery *et al.*, 1998). *dcry* mRNA was shown to cycle in a circadian manner, and that mutants over-expressing *cry* manifested a hypersensitivity to light as determined by measuring the effect on the period of activity following a short light pulse at either ZT 15 or ZT 21. Hypersensitivity was indicated by a greater response to such light pulses. Significantly, dCRY was shown to be degraded in a light-dependent manner (Emery *et al.*, 1998). In a complementary study *dcry* was identified through mapping a mutation which abolished cycling of PER and TIM as determined by bioluminescence of a *per* and *tim-luciferase* fusion gene. The altered gene was identified as *dcry* and the mutation was mapped to a missense mutation at position 410, changing Asp to Asn. This Asp residue is a highly conserved residue in cryptochromes, and is believed to be

important for flavin binding (Stanewsky *et al.*, 1998). This mutant form of dCRY is believed to have no photoreceptor ability (Emery *et al.*, 2000). The abolition of PER and TIM cycling as seen with the original assay does not fit well with the role of dCRY as a photoreceptor. However, cry^b flies showed normal entrainment in LD and PER and TIM cycling was not abolished in the central pacemaker neurons, but only in the peripheral clocks (Stanewsky *et al.*, 1998). cry^b flies also showed poor entrainment to LD regimes in visually blind backgrounds and no phase shift in response to light pulses, indicating that CRY's principal role in the central circadian clock is that of photoreception (Stanewsky *et al.*, 1998). cry^b flies were later shown to retain locomotor rhythmicity in constant light, which produces arrhythmic behaviour in wild type flies (Emery *et al.*, 2000). This indicates that the mutant flies lack the ability to 'see' the constant light, therefore it appears that dCRY is the only dedicated circadian photoreceptor in *Drosophila*. This seems likely, as elimination of the visual and extraocular structures through the $glass^{60j}$ mutations coupled with the cry^b mutation, resulted in a complete lack of entrainment to light-dark cycles (Helfrich-Forster *et al.*, 2001). dCRY is expressed in all clock neurons, and over-expression of dCRY in the ventral lateral neurons increases light sensitivity in wild-type flies, and rescues the reduced light sensitivity in cry^b flies (Emery *et al.*, 2000).

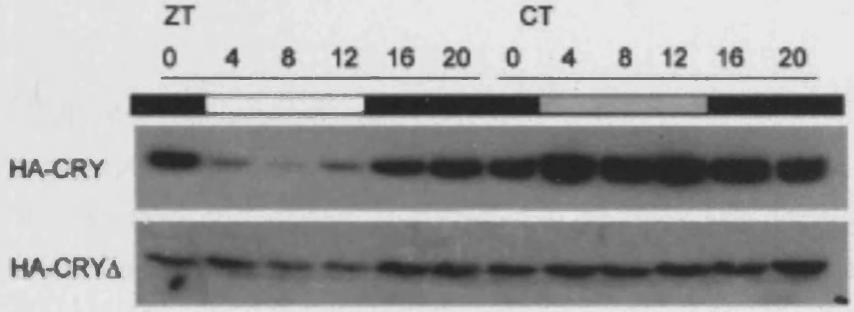
The mechanism by which dCRY mediates the light signal into the circadian clock is through direct interaction with components of the central oscillator. dCRY interacts with both PER and TIM in yeast assays in a light dependent manner (Ceriani *et al.*, 1999; Rosato *et al.*, 2001). CRY^b, in contrast, interacts with neither PER nor TIM in either light or dark (Rosato *et al.*, 2001). Recently, *in vivo* studies using fly extracts have shown a CRY-PER/TIM interaction in wild-type flies and a CRY-TIM interaction in per^0 flies, both of which were light-dependent, although some very weak interaction did occur in the dark. However, a CRY-PER interaction was not found in tim^{01} flies, suggesting that TIM is the primary target for dCRY (Busza *et al.*, 2004). In the same study, it was found that the light-dependent action spectrum for TIM degradation is maximal between 400 and 500 nm, which corresponds the action spectrum for both behavioural resetting and dCRY degradation. TIM is not degraded in response to light in cry^b flies (Lin *et al.*,

2001). Furthermore, it is known that TIM degradation is through phosphorylation and ubiquitination (Naidoo *et al.,* 1999), and that the ubiquitination of TIM has been linked to the action of a light activated dCRY (Lin *et al.,* 2001). dCRY is degraded in response to light, and at first it was thought that dCRY might have a protective role, preventing the degradation of TIM through indirect interaction, perhaps in a complex, until its removal by light allowed TIM to be degraded. However, TIM ubiquitination has been shown to occur before dCRY degradation and also blocking dCRY degradation increases TIM ubiquitination (Lin *et al.,* 2001). Taking into account that dCRY interacts with TIM in a light-dependent manner, it seems more likely that dCRY targets TIM for degradation through interaction. Another study revealed that individually mutating single residues thought to be important for flavin binding in dCRY could abolish both light dependent dCRY mediated relief of PER-TIM repression of CLK-CYC activity and dCRY degradation by light in an assay in S2 cells (Froy *et al.,* 2002). All three mutations that showed loss of light response, also showed constitutively low protein levels. In the same study, they also mutated three tryptophan residues in dCRY which correspond to residues involved in an intramolecular electron transport chain in bacterial photolyases. Light response was removed in two of the mutations which abolished the putative transport chain, which also corresponded to reductions in protein level expression. Significantly, replacement of the tryptophans with different residues that were capable of electron transfer, restored light responsiveness. Both approaches were repeated using mCRY1 to determine the role of the flavin and the intramolecular transport chain in mCRY1 repression, using a similar assay system. The results indicated that mCRY1 activity was not dependent on either flavin binding or the intramolecular pathway (Froy *et al.*, 2002).

Two recent studies have found that deleting parts of the C-terminal domain of dCRY give a truncated form that is both constantly degraded and constitutively active (Dissel *et al.,* 2004; Busza *et al.,* 2004). A truncated form of dCRY, lacking the C-terminus and consisting of residues 1-519 (dCRYΔ) (thus missing 20 amino acids from the C-terminal region) was found to interact with both PER and TIM independently of light in yeast assays, as opposed to the light-dependent interactions of dCRY (Rosato *et al.,* 2001). Interestingly, CRY^b, which does not interact with TIM or PER at all in the same assay,

still does not interact when the same portion of the C-terminus is deleted, suggesting that the lack of function of CRY^b is not due to a disruption in the light-mediated alleviation of repression of dCRY. Over-expressing dCRYΔ via a *tim*-GAL4/UAS-*cry*Δ system in transgenic flies (dCRYΔ flies) causes a lengthening of period in constant darkness, and in constant light such flies stay rhythmic for several days longer than wild type flies (Dissel *et al.*, 2004). At the molecular level flies expressing dCRYΔ show a reduced amplitude of cycling of both PER and TIM, as determined by Western blots on whole head extracts, and there is an apparent absence of the hyperphosphorylated form of TIM (Dissel *et al.*, 2004). dCRYΔ itself is at constantly low levels compared to wild type dCRY, and shows little cycling in response to light dark cycles (**fig 1.9**). This may account for the fact that although dCRYΔ appears to give a constitutive light signal, only a lengthening of period is seen in dCRYΔ flies rather than full arrhythmia (Dissel *et al.*, 2004). The sustained rhythmicity in constant light is hypothesised to be due to the constant removal of the phosphorylated form of TIM which is the primary target for degradation (Naidoo *et al.*, 1999). A similar dCRY mutant that had the last 19 residues removed from the C-terminal domain ($dCRY^M$). This was found by mutagenising flies and screening for rhythmicity in constant light. Hence the $dCRY^M$ fly line did not express endogenous dCRY (Busza *et al.*, 2004). In agreement with the data of Dissel *et al.*, (2004), $dCRY^M$ was found to be at constantly low levels and PER and TIM cycling was found to be reduced in amplitude. Additionally, cry^m flies showed a lengthening of period in low level constant light, unlike cry^b flies, and also exhibited almost normal phase shifts in response to light pulses when entrained in low light conditions (Busza *et al.*, 2004).

Fig 1.9 The circadian profile of dCRY abundance, taken every four hours over a two day period, compared to that of the truncated dCRYΔ which lacks the final 20 amino acid residues, in transgenic *Drosophila*. The black represents darkness and the white bar light. The grey bar represents darkness in the expected light period. dCRYΔ is present at much lower levels, and does not show the same modulation in response to light as full length dCRY. The HA-CRY blot exposure time was 20 seconds; the HA-CRYΔ blot exposure time was five minutes, therefore the intensities are not equivalent (Dissel *et al.*, 2004).



Unlike TIM degradation, dCRY is degraded only through constant light input. Once the light input is removed, dCRY becomes stable again (Busza *et al.*, 2004). This degradation is though to occur through the ubiquitin/proteosome pathway in response to light, and this has been shown to be dependent upon the redox pathway using the flavin, in S2 cells at least (Lin *et al.*, 2001). CRY^M and CRY^b are both degraded via the proteasome independent of light (Busza *et al.*, 2004).

In summary, the current model of dCRY mediated light input to reset the circadian clock, is that a light signal causes a transient association between dCRY and TIM leading to the ubiquitination and degradation of TIM, and, through a separate pathway, the degradation of dCRY. This light-dependent degradation of TIM stops TIM from accumulating and dimerising with PER, which delays nuclear entry and therefore delays the clock. In darkness, dCRY-TIM interaction is repressed, possibly through some function of the C-

terminal domain, allowing TIM to accumulate and dimerise with PER. Removal of the at least 19 residues from the C-terminal domain produces a constitutively active form of dCRY that can interact without a light signal, but which is also constantly degraded. This would suggest that the C-terminal domain both mediates a repression on the function of dCRY in darkness, and also prevents dCRY degradation. However, even in the absence of the C-terminal domain it seems possible for dCRY to mediate a light signal. The fact that blocking the intramolecular pathway in dCRY stops dCRY degradation but enhances TIM ubiquitination (Lin *et al.,* 2001) suggests that the light signal that causes dCRY-TIM interaction may be different to the light input pathway that signal dCRY degradation, because if the flavin-based intramolecular pathway was required for dCRY mediated TIM ubiquitination, then blocking that pathway should block the ubiquitination of TIM.

1.2.9 PDF: an important clock output factor

The neuropeptide Pigment Dispersing Factor (PDF), first implicated in circadian rhythms in crabs (Arechiga *et al.,* 1985), is expressed in the previously discussed LNvs, neurons that are essential for circadian locomotor activity. CLK and CYC are believed to be involved in *pdf* transcription, as Clk^{jrk} and cyc^{0} mutants show no *pdf* mRNA in the *s*-LNvs, although the *l*-LNvs appear unaffected (Park *et al.,* 2000). Although *pdf* mRNA levels do not cycle in abundance (Park and Hall, 1998), accumulation of PDF in the LNvs shows a circadian pattern, with maximal levels occurring at ZT 0-3 and the nadir at ZT 12-15 in a 12:12 hour light:dark cycle. This cycling is abolished in per^{0} and tim^{01} mutants (Park *et al.,* 2000). In a light:dark regime *pdf* null mutants do not show an increase in activity prior to the lights-on period; a feature which is characteristic of *Drosophila melanogaster* activity (referred to as the morning peak). Similarly, the characteristic evening peak in activity appears to be 1 hour earlier as compared with non-mutants, whilst in constant conditions these mutants become arrhythmic after several days (Renn *et al.,* 1999). Furthermore, ectopic expression of PDF leads to increased activity and arrhythmia, though over-expression in the LNvs has no apparent effect on locomotor rhythms (Helfrich-Forster 2000). Taken together, the evidence suggests that PDF is a major molecular output of the central clock.

1.3 The Mammalian Central Clock

The mammalian central oscillator is more complicated than that of the equivalent *Drosophila* circadian clock, which forms the principal focus of study for this project. None-the-less, as an understanding of the mouse circadian oscillator is necessary for the context of certain aspects of this project, particularly the difference in the roles of the cryptochromes (see section 1.4) a brief overview of the mammalian clock, as determined largely through studies in mice, will be discussed.

The mammalian central oscillator is similar to that of the *Drosophila* in that it consists of interlocked feedback loops involving autoregulatory clock factors, many of which are homologues of those involved in the *Drosophila* central clock, although there are differences in functions. As in *Drosophila*, the mammalian clock comprises a heterodimer of mCLK and BMAL1 (the homologue of *Drosophila* CYC) (reviewed by Allada *et al.*, 2001) which drives transcription of target genes through binding to the E-boxes in their promoters (Gekakis *et al.*, 1998; Hogenesch *et al.*, 1998; Bunger *et al.*, 2000). Three *mPer* genes (*mPer1*, *mPer2* and *mPer3*) ad two *mCry* genes (*mCry1* and *mCry2*) are targets of this transcriptional activation. Much like PER and TIM in *Drosophila*, the mPER and mCRY proteins form a complex, translocate into the nucleus and repress the positive transcriptional activity of mCLK and BMAL1 (Griffin *et al.*, 1999; Kume *et al.*, 1999; Shearman *et al.*, 2000; Lee at al 2001). The role of mammalian TIM has yet to be adequately elucidated, but evidence suggests that it is required for stability and may serve to stabilise PER (Barnes *et al.*, 2003). As regards the mCRY-mPER dimmer, since both mCRYs can inhibit CLK-BMAL1 activity *in vitro*, it may be the mCRY association with CLK-BMAL1 that is responsible for repression (Kume *et al.*, 1999; Shearman *et al.*, 2000b). Of the mPER proteins, only mPER1 and mPER2 are thought to be involved in the central oscillator (Bae *et al.*, 2001), and one apparent function may be to localise the mCRYs to the nucleus, as studies in mouse liver cells using mPER1 and mPER2 double-knockouts show the mCRYs to be cytoplasmic (Lee *et al.*, 2001). In turn, the mCRYs have been shown to be important for mPER1 and mPER2

localisation in liver cells, and also stabilise mPER2 in the SCN and liver (Kume *et al.*, 1999; Shearman *et al.*, 2000; Yagita *et al.*, 2000; Lee *et al.*, 2000;).

bmal1 mRNA and protein levels peak directly out of phase with *mcry* and *mper*, whereas CLK levels remain stable (Shearman *et al.*, 2000; Tamaru *et al.*, 2000; Lee *et al.*, 2001). This represents the positive loop of the clock, as mCLK and BMAL1 drive transcription of *rev-erbα*, the product of which represses *bmal1* transcription (Preitner *et al.*, 2002; Ueda *et al.*, 2002). Thus, inhibition of CLK-BMAL1 transcriptional activity by mPER and mCRY prevents *rev-erbα* transcription de-repressing the transcription of *Bmal1*. Interestingly, mPER2, although being part of a complex that inhibits CLK-BMAL1 activity, may also positively activate *bmal1* expression (Bae *et al.*, 2001). Recently another member of the retinoic orphan nuclear receptor family (RORa), has been identified as a positive regulator of *bmal1* transcription, through direct interaction with a RORa element in the *bmal* promoter (Sato *et al.* 2004).

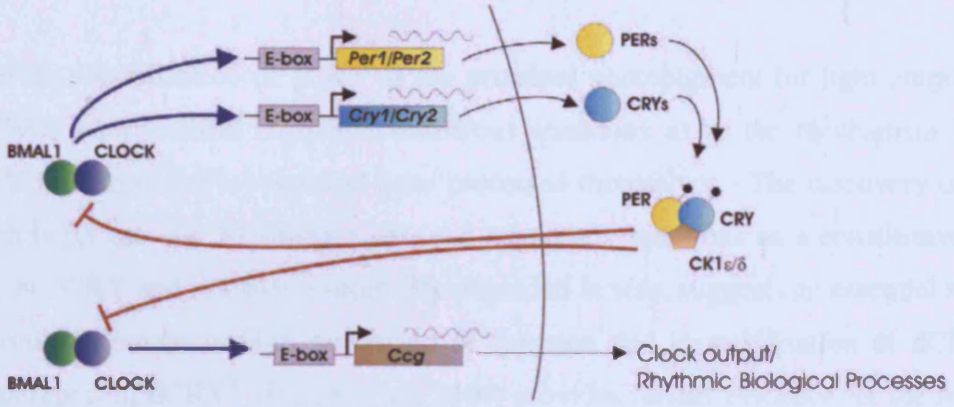
Like the *Drosophila* clock, the mouse central oscillator is regulated by post-translational modifications. Though there is much that is still not known, but mCLK, BMAL1, mPER1 and mPER2 all undergo circadian changes in phosphorylation (Lee *et al.*, 2001). Casein Kinase Iε (the mammalian homologue of DBT) and CASEIN KINASE Iδ have been associated with mPER binding and phosphorylation *in vitro*, which leads to mPER degradation via the ubiquitin pathway and also affects cellular localisation of the proteins (Keesler *et al.*, 2000; Lowrey *et al.*, 2000; Vielhaber *et al.*, 2000; Akashi *et al.*, 2000).

The central pacemaker clock in mammals resides in clock neurons in the region of the anterior hypothalamus known as the suprachiasmatic nucleus (SCN), which entrains to the circadian day then coordinates other peripheral clocks (Reppert and Weaver, 2001). As with *Drosophila*, light is thought to be the most important *Zeitgeber* for entraining the mammalian clock. Unlike *Drosophila*, which can entrain through direct input into the central pacemaker neurons the mammalian central pacemaker is entrained through the retinohypothalamic tract (RHT) (Lucas *et al.*, 2001). Although some studies have found that both opsin-based photoreceptors and the mCRYs are involved in photic entrainment,

(Freedman *et al.*, 1999; Selby *et al.*, 2000; Thompson *et al.*, 2001), melanopsin has been implicated as the most important circadian photopigment in mammals (Provencio *et al.*, 2000, 2002; Hattar *et al.*, 2000).

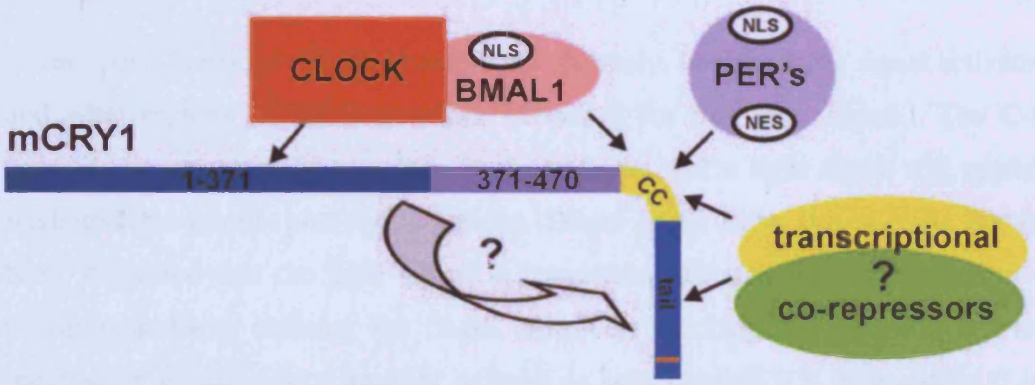
An important difference in the central oscillators of *Drosophila* and mouse is the role of CRY. In *Drosophila* dCRY acts as a photopigment capable of interacting with the clock, while PER and TIM action mediate the negative arm of the feedback loop. However, as discussed, in the mouse oscillator mCRY1 and mCRY2 appear to be components of the central mechanism, and, in the pacemaker neurons, do not possess a photoreceptor function. The repression exerted by mCRY on the transcriptional activity of the transcription factors CLK and BMAL1 is more similar to the role of *Drosophila* PER. Recent evidence suggests that through interaction with mCRY the CLK/BMAL1 complex represses target genes (Kondratov *et al.*, 2006) (see **fig 1.10**). However, there is some evidence that dCRY does act as a component of the central clock in Drosophila peripheral clocks, as cry^b flies entrained to a temperature cycle fail to show circadian rhythms driven by oscillator cells in the antennae (Krishnan *et al.*, 2001). Also, oscillation of PER and TIM are abolished in the Malphigian tubules in cry^b flies in constant darkness (Ivanchenko *et al.*, 2001), and dCRY in conjunction with PER has been found to repress CLK-CYC mediated transcription of certain clock genes in peripheral clock cells (Collins *et al.*, 2006). In fact, as discussed, it was through the abolition in the oscillations of PER and TIM that cry^b was first isolated. Similarly, the mCRYs have been implicated in photoreception, as discussed above. Therefore, while dCRY and the mCRYs show apparent differences in function in the central oscillators of *Drosophila* and mice, it may be that they are more conserved in function than is first apparent, and perhaps it is the context and location of the proteins that defines function.

A



(Taken from Ko and Takahashi 2006)

B



(Taken from Chaves *et al.*, 2006)

Fig 1.10 The molecular interactions that underlie the central circadian clock in mouse A: the negative loop of the mammalian clock whereby CLOCK and BMAL form a heterodimer that drives expression of the target genes *period (per)* and *cryptochrome (cry)* as well as clock controlled genes (ccgs). The products PER and CRY dimerise and repress the transcriptional activity of CLK:CYC. B: the current model for the mechanism of CRY1 action in the mammalian clock. The C-terminal domain is believed to interact with the PERs. The formation of the CRY:PER dimer allows nuclear localisation where CRY interacts with CLOCK and BMAL1. CRY is believed to repress the action of CLOCK and BMAL1 through the recruitment of repressors to the C-terminal domain.

1.4 Aims of the Project

Since the identification of dCRY as the principal photopigment for light-entrainment of the *Drosophila* central oscillator, numerous questions as to the mechanism by which dCRY functions and is regulated have presented themselves. The discovery of dCRYΔ which lacks the last 20 amino acids, and apparently functions as a constitutively active form of dCRY and is also constitutively degraded *in vivo*, suggests an essential role of the C-terminal domain in both repression of function and in stabilisation of dCRY. The recent report of $dCRY^{M}$ (Busza *et al.*, 2004) provides further evidence for the function of the C-terminal domain: that the photolyase domain interacts with relevant clock factors, and the C-terminal domain mediates repression; this being a directly opposite arrangement of function of *Arabidopsis* CRY (Yang *et al.*, 2000).

Several key questions remain unanswered. Namely, how the light signal activates dCRY and what regions of the protein are necessary for this light signal. The C-terminal domain appears to mediate repression in darkness, but a light signal still appears to be mediated through the photolyase domain (Dissel *et al.*, 2004; Busza *et al.*, 2004). It has been suggested that the light signal is transmuted through the intramolecular electron transport pathway utilising the flavin, however, blocking this pathway has led to an increase in ubiquitination in TIM as well as inhibiting dCRY degradation (Lin *et al.*, 2001), suggesting that the light signal required to relieve dCRY repression may be different to that required to signal degradation. It is also unknown as to whether dCRY has an interaction partner which may be involved in light regulation, which has been previously suggested in a yeast study (Rosato *et al.*, 2001). This opens the possibility that dCRY repression may occur through a conformation change, through association with one or more partners, or both. Given the evidence, any such interactions or conformational changes are most likely mediated through the C-terminal domain of dCRY. Finally, although many components of the clock are regulated through such post-translational mechanisms such as phosphorylation, no such regulation has been demonstrated of *Drosophila* CRY, though post-transcriptional modification has been

found to play an important role for other cryptochromes (Shalitin *et al.*, 2003; Eide *et al.*, 2002).

Considering the importance of the C-terminal domain in dCRY regulation, and that dCRY and mCRY functions differ in the respective central osciallators, and given that the dCRY and mCRY proteins show homology in all but the unique C-terminal domains, it is possible that by exchanging the C-terminal domain there may be an associated exchange of function. If the C-terminal domain of dCRY contains a discrete repressor function, by replacing this onto a mCRY photolyase domain this might then confer light regulation onto an otherwise non-light regulated protein.

To assess whether this is the case, a series of dCRY/mCRY chimerics will be created by swapping the apparent unique C-terminal domains to elucidate the intrinsic function of the C-terminal domain of dCRY (and reciprocally, mCRY). The chimerics and also an mCRY will be tested against the *Drosophila* clock components PER and TIM to test for interaction and whether light regulation of these interactions is evident in yeast assays. Since dCRY interacts with PER and TIM only in the presence of light, whereas dCRYΔ interacts independent of the light conditions, identifying chimerics that interact in a light-dependent manner will indicate the domains necessary for light regulation to occur. The yeast studies will also determine if mCRY can interact with the components of the *Drosophila* circadian clock.

Following the yeast studies, transgenic fly lines will be created to generate *Drosophila melanogaster* expressing mCRY and the chimeric dCRY/mCRY constructs; depending on the outcome of the yeast studies priority will be given to the chimeric proteins that have shown features of interest. The transgenic fly lines will be subjected to locomotor activity experiments and phase-shift analysis to reveal any alterations in circadian behaviour and light sensitivity. At the molecular level, Western blots against the clock components TIM and the CRY transgenes used, to determine the abundance of TIM and of the CRY construct over the circadian period. This will determine how the circadian clock is affected at the molecular level. Since it is known that both TIM and dCRY are

degraded in light, and that TIM degradation is dependent on a light-regulated dCRY, then alterations to the putative light-regulation domain in dCRY should have effects on the patterns of TIM and dCRY abundance.

Since a dCRY antibody is not currently available for Western blot analysis and also for immunocytochemistry work, an antibody will be created by expressing dCRY in three overlapping fragments in E.*coli* which will then be extracted and sent to a commercial service to produce an antibody.

Finally, putative sites for post-translation modification, such as phosphorylation, will be altered following an exercise to predict potential motifs. These sites will be altered by site-directed mutagenesis and the effect of the mutations on the interactions of dCRY with PER and TIM and the light-regulation of these interactions determined through yeast assays. The study will be confined to looking at potential sites in the C-terminal domain due to the previously indicated importance of this domain and the necessity to narrow the scope of the study due to time constraints.

Chapter 2: Materials and Methods

Chapter 2: Materials and Methods

2.1 Basic cloning

2.1.1 Plasmid DNA recovery

To obtain large amounts of high quality DNA from transformed bacteria, I used the alkaline lysis method as described in Feliciello and Chinali (1993). In order to obtain small amounts of low quality DNA from transformed bacteria for testing for the presence of an insert/plasmid, the following 'speed prep' was used:

Speed prep.

Solution A:

Tris-HCL pH 8	50mM
Triton X-100	4%
LiCl	2.5mM
EDTA	62.4mM

Protocol

1) A single bacterial colony was inoculated into approximately 3ml of selective Luria Broth and incubated overnight at 37°C with shaking.
2) Approximately 1.5 ml of the overnight bacterial culture was then microfuged at 13,000 rpm for 20 min.
3) Supernatant was removed and the cells resuspended 100µl solution A.
4) 100µl Tris-HCL-buffered phenol/chloroform was added and vortexed for 5 seconds.
5) This was then microcentrifuged at 13,000 rpm for 2 min and the upper layer removed to a new tube.
6) The DNA was precipitated using 100% ethanol and washed with cold 80% ethanol and dried..

The pellet was resuspended in 10µl TE (TRIS-HCL 10mM, EDTA 1µM, pH 8).

This method was used to extract relatively small amounts of plasmid DNA for testing with a restriction digest, and was used to identify bacterial colonies that contained the target DNA.

2.1.2 Quantification

DNA concentrations were determined by running a sample with a known amount of marker on a 0.5-1% agarose gel. Ethidium bromide was used to visualise the DNA, and a comparison between the marker and sample band made by eye to estimate the quantity of DNA.

2.1.3 Restriction digests

Digests of DNA were carried out by incubating the DNA with the appropriate enzyme and buffer provided by the manufacturer in a 37°C water bath. The number of units of enzyme added depended on the purpose of the digest, but the volume added never exceeded 10% of the total reaction volume so as to prevent the presence of excess glycerol interfering with the digest. To test for the presence of an insert in a plasmid, excess restriction enzyme was added: typically 5-10 units for 1-2μg of DNA in a reaction mixture of 20μl, incubated for one hour. To extract an insert from a plasmid for future cloning, or to prepare a plasmid for cloning, the manufacturers guidelines on the specific enzyme use were followed to ensure the correct amount of units were added to sufficiently digest the DNA, while avoiding cutting at non-specific sites. Therefore, the amount of enzyme added and the incubation time varied with the enzyme used. A sample of the restriction digest was then loaded onto an agarose gel and visualised with ethidium bromide. If a large amount of DNA was required for cloning, then the entire restriction digest reaction was loaded into a large well and subsequently extracted and purified.

2.1.4 DNA recovery and purification

DNA from agarose gels was recovered by visualising the DNA using ethidium bromide and a UV transilluminator, then cutting out the relevant section of gel then using the following method:

- The section of gel containing the DNA was placed in a length of dialysis tubing, and sufficient 0.5X TBE buffer (TRIS-HCL 0.445M, EDTA 0.001M, Boric acid 0.0445M pH 8) was added to cover the gel entirely.
- The tubing was sealed at both ends with dialysis clips and placed in an electrophoresis tank and run at 100V until the DNA had migrated completely into the buffer.
- The buffer containing the DNA was removed and an equal volume of phenol/chloroform added and shaken vigorously before being centrifuged at 13,000 rpm for two minutes. The top aqueous layer was then carefully removed and an ethanol precipitation used to recover the DNA.
- The DNA was washed once in 80% cold ethanol before being resuspended in TE.

2.1.5 Ligations

- 50µg of the cut vector was incubated with insert at a vector:insert ratio in mols of 1:3, hence the quantity in ug of insert varied according to its size relative to the vector.
- Ligations were carried out using T4 ligase and the appropriate buffer in a reaction volume of 10µl at 4°C over night, or at room temperature for 4 hours.
- The ligation reaction was stopped with the addition of 3µl NaAc (3M pH 5.2) and 50µl cold ethanol (100%). 3µl of 10 mg/ml Yeast tRNA was added as a carrier to make the DNA pellet visible.
- This was then incubated at -80°C for 10 min then centrifuged at 13000 rpm for 20 min to pellet the DNA.
- The DNA was resuspended in 10µl dH_2O and this solution was used to transform bacteria.
- If it was necessary to dephosphorylate the digested vector or insert before ligation, then the DNA was incubated with 1 unit of shrimp alkaline phosphatase (SAP) per µg of plasmid for one hour at 37°C.
- The enzyme was then denatured by incubation at 65°C for 15 min before proceeding to the ligation reaction.

2.1.6 Bacterial Transformation

For transforming the product of a vector:insert ligation reaction into bacteria, the electroporation method was used.

- 3µl of the ligation product was mixed with 40µl *E. coli* cells in a 0.1 mm Biorad electroporation cuvette.
- The mix was given a pulse using the following settings: 1.5 kV, 1000 Ω and 25 µF.
- Immediately afterwards 1ml of Luria-Bertani (LUB) broth (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) was added, and the cells plus LB transferred to a sterile, glass vial and shaken for 1 hour at 37°C.
- After 1 hour 100µl of the culture was removed and plated onto an LUB agar plate (LUB Broth with the addition of 1.5% bacto-agar) plus the appropriate antibiotic.
- When applicable, 0.96 mg of X-Gal and 0.8 mg of IPTG were added to 25ml of media plate to allow for a blue/white screen for positive colonies.

For each transformation two dilutions of 10X and 100X of the culture were also plated on identical plates in order to ensure that at least one plate would result in a reasonable number of single, non-confluent colonies.

The transformation of supercoiled plasmid DNA was carried out in $CaCl_2$ chemical competent *E. coli*, as follows:

- 100 µl of competent cells, stored at – 80 °C, were thawed on ice for 15 min before adding 1 ng of plasmid DNA with gentle mixing.
- The bacteria-DNA mix was left on ice for 15 min and then plated onto a selective LUB agar plate as previously described.

2.2 Bacterial and Yeast Strains

Bacterial strains

XL1 BLUE MRF' (Stratagene): Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB $lacI^qZ\Delta M15Tn10$ (Tet^r)]

BL21(DE3)pLysS (Novagen): F^- *ompT* $hsdS_B(r_B^-m_B^-)$ *gal dcm* (DE3) pLysS

Yeast strain

Saccharomyces cerevisiae EGY48

2.3 Vectors

pET-14b Expression vector 4.67 Kb (Novagen) used for expressing proteins in *E. coli.*

pBluescript II KS (+) Cloning vector 2.96 Kb (Stratagene). Used for general cloning.

PDK101 T-vector 3.0 Kb (Kovalic *et. al.*, 1991). This vector is a modification of pGEM *5zf* (+) (Promega) by the insertion of a linker. The linker harbours a double *Xcm* I restriction site, that when cut generates sticky ends by a single overhanging T. All initial PCR products were ligated into this vector for amplification.

pEG202 Yeast expression vector, a Lex-A fusion plasmid for expressing bait fusion protein in a yeast two-hybrid assay (Ruden *et. al.*, 1991).

pJG4-5 Yeast expression vector, expresses cloned sequences as a fusion with the acid blob B42 to be used as prey in a yeast two-hybrid assay (Gyuris et. al., 1993).

pUAST P-element Drosophila transformation vector (Brand and Perrimon, 1993).

pUCksΠΔ2-3 Helper plasmid which contains a source of transposase required for P-element injection. It is based on the pUC18 cloning vector (Mullins, *et al.*, 1989).

2.4 PCR for cloning

PCR amplification for cloning was carried out using the proof reading Expand Kit (Roche). The reactions were performed on a Biometra TRIO-thermoblock. PCR reactions to produce sequences for cloning were undertaken as follows: two reaction mixes were made separately

Reaction mix 1	**Reaction mix 2**
1 µl DNA template (10ng/µl)	10 µl 10X buffer*
3 µl 5' primer (10µM)	1µl Taq DNA polymerase (5 units)
3 µl 3' primer (10µM)	39µl H2O
2 µl dNTPs (10mM)	
41 µl H2O	

*10X Buffer: 10mM Tris-HCL, 50mM KCL, 1.5mM $MgCl_2$.

The two reaction mixes were then added together, mixed thoroughly by pipetting, and distributed evenly in three PCR reaction tubes, as all first time reactions were performed in triplicate using either 55°C, 60°C or 65°C in the annealing step to determine the optimum annealing temperature.

The cycle used was as follows:

- 95°C 1 minute,
- 55°C/60°C/65°C for 1 minute,
- 72°C for 1 minute, for ten cycles,
- Then the same cycle again but with the addition of 20 seconds at 72°C per cycle for 15 cycles
- Finally 72°C 10 minutes.

PCR reactions were then loaded into an agarose gel, and the product visualised, extracted, purified and quantified as previously described.

2.5 Protein expression in *E. coli*

The pET expression system (Novagen) was used to express proteins for purification in *E. coli*. Coding sequences for expression were cloned into a pET-14b translation vector which carries an N-terminal 6xHis tag to facilitate purification, an ampicillin selectable marker and a T7 promoter to drive expression of the target gene. Once the coding sequence had been cloned into the pET-14b vector, this was transformed into *E. coli* for expression. Expression from the T7 promoter can only occur in the presence of a T7 polymerase. This allows the plasmid to be amplified without possible toxic effects that the protein may have on the host bacteria which do not produce T7 polymerase. In order for the protein to be produced, the pET-14b (Novagen) vector was transformed into an expression host that carries a chromosomal copy of the T7 RNA polymerase under *lacUV5* control, which gives an additional level of control. Expression is only then induced with the addition of IPTG to the growth media.

Initially, a small scale protein expression was used to determine the optimum conditions for protein expression. This was then scaled up to produce usable amounts of protein.

2.5.1. Culture growth

1) A single colony of transformed bacteria was inoculated into 10 ml LB + antibiotics and incubated overnight at 37°C with shaking.
2) 0.5ml of the overnight culture was then inoculated into 15ml LB + antibiotics and incubated at 37°C with shaking until OD600 reached 0.5.

[At this stage a sample for analysis was taken. The amount taken was sufficient to give an OD600 of 10 when resuspended in final volume of 100µl, and the cells pelleted and resuspended in 100µl PBS + Triton X and kept on ice for immediate use or were stored at –20°C]

3) Expression of target protein was induced by adding IPTG to a final concentration of 1mM to the culture.

4) Incubation was continued at 37°C with shaking and take samples every hour for four hours, starting with one hour post induction. The volume of sample taken should be equal to the volume required to give an OD600 of 10 when resuspended in a final volume of 100µl. Pellet cells and resuspend in 100µl PBS + Triton X and keep on ice for immediate use or store at –20°C.

2.5.2 Preliminary expression

PBS:

NaCl	1.3M
Na_2HPO_4	70mM
NaH_2PO_4	30mM

Adjust to PH 7.5

1) Samples were thawed and keep on ice.
2) These were then sonicated 5 times for 5 seconds at 16 microns amplitude. Throughout, samples were kept on ice and sonication was performed in 4°C constant temperature room.
3) The sample was then centrifuged at 4°C at 13000 rpm for ten minutes. The supernatant was removed to a new eppendorf and kept on ice. This sample represented the soluble fraction of the protein.
4) The pellet was resuspended in 100µl PBS and kept on ice. This represented the insoluble fraction of the protein.

The amount of protein in each fraction was quantified as described in section **2.5.5**.

2.5.3 Large scale expression

Following the intermediate step, the process was scaled up to produce usable quantities of protein. The method was identical to that used for the intermediate stage, with the following modifications:

i) The volume of LB (with antibiotics) inoculated with overnight culture corresponded to the volume required to obtain the desired amount of protein. The LB culture was inoculated 1:50 with the noninduced overnight culture.

ii) A 1ml sample of the culture was taken just before induction, and another taken just before harvesting the cells; these were the non-induced and induced control respectively. Samples were collected to give an OD600=10 when resuspended in 100µl PBS as previously described..

iii) Following centrifugation, the protein was extracted and purified as described in sections **2.5.4**.

2.5.4 Preparation of E.Coli lysates and protein purification under denaturing conditions

Buffers for purification under denaturing conditions

Lysis buffer

Buffer B (1 liter):

100 mM NaH2PO4

10 mM Tris·Cl

8 M urea

Adjust pH to 8.0 using NaOH.

Wash buffer

Buffer C (1 liter):

100 mM NaH2PO4

10 mM Tris·Cl

8 M urea

Adjust pH to 6.3 using HCl.

Elution buffers

Buffer D (1 liter):

100 mM NaH2PO4

10 mM Tris·Cl

8 M urea

Adjust pH to 5.9 using HCl.

Buffer E (1 liter):

100 mM NaH2PO4

10 mM Tris·Cl

8 M urea

Adjust pH to 4.5 using HCl.

Due to the dissociation of urea, the pH of Buffers B, C, D, and E should be adjusted immediately prior to use. Do not autoclave.

Method:

1) The pellet was thawed on ice for 15 minutes and then resuspended in buffer B at 5 ml per gram of the wet weight of the pellet.
2) The cells were vortexed to lyse them, while taking care to avoid foaming which would indicate denaturing of the protein.
3) The lysate was then centrifuged at 10,000x g for 30 min at room temperature and a sample taken for analysis.
4) 1 ml of nickel nitrilo-triacetic Acid (nickel-NTA) agarose resin (Qiagen) was added to 4 ml lysate and mixed gently by shaking on a rotary shaker for 60 min at room temperature.
5) The cleared lysate was carefully removed leaving the slurry at the bottom of the tube. A sample of the cleared lysate was kept for analysis.
6) The resin was washed twice with buffer C by adding the buffer and shaking on rotary shaker for 15 min, then decanting the buffer gently to avoid loss of resin.

7) After washing, the protein was eluted in eight instalments by adding buffer D four times then buffer E four times. This was done by adding the buffer and shaking on the rotary shaker for 15 min then collecting the eight fractions separately.

To assess the protein expression, the following samples were analysed by SDS-PAGE: The cleared lysate sample (from step 3), the cleared lysate sample following addition of the Ni-NTA slurry (from step 5), each of the two wash samples (step 6), and samples from each of the eight elutions. Samples of the non-induced and induced control (see section 2.5.4 (iii)) were also run (see section 2.5.5 for details of analysis).

2.5.5 Replacing the elution buffer with PBS using dialysis

The protein was required to be in PBS. In order to remove the protein from the elution buffer and into PBS, the fractions of elution buffer that contained the protein were placed into a length of 10000 molecular weight cut-off dialysis tubing, and placed in 1L of PBS at 4°C overnight, with stirring. The next day the PBS was removed and replaced with another 1L of fresh PBS, and left at 4°C with stirring for a further two hours.

2.5.6 Quantification of protein

In order to quantify the amount of protein in a given sample, 10-15µl of the sample was run on 12% SDS-PAGE (see section **2.13**). When it was necessary to quantify the amount of protein, known quantities of BSA were run in adjacent lanes for comparison. To visualise the protein, a Coomassie (45%MetOH, 10%AcAc, 0.25% Brilliant Blue R-250) staining was used on the gel, then the gel was washed with a solution of 45% MetOH, 10% Acetic acid 3x 15 min such that the individual bands of protein were visible. The gels were stored in 12.5% isopropanol, 10% Acetic acid. To provide a durable record of the results, the gels were dried onto Wattman paper using a vacuum gel drier.

2.6 Yeast assays

2.6.1 Yeast growth and storage

All yeast two-hybrid assays were performed using the yeast strain EGY48 (*MAT alpha, his3, trp1, ura3, 6LexAop-LEU2*)

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YP media for growing yeast

Yeast extract	10g/L
Peptone	20g/L
Glucose	20g/L
Agar	20g/L (omit for liquid media)

Selective growth media

YNB (without amino acids) 6.7g/L

Glucose 2%

Agar 2% (omit for liquid media)

For selective media add the amino acid/s required by the yeast strain

Tryptophan (8mg/ml)	2.5ml/L
Histidine (8mg/ml)	2.5ml/L
Uracile (2mg/ml)	10ml/L
Leucine (12mg/ml)	3.25ml/L
Adenine (2mg/ml)	10ml/L

For growing and maintaining untransformed yeast stocks, the yeast was grown on YPD plates or in YPD liquid media. Following transformation with a plasmid with a selective marker, the yeast strains were grown on selective media containing the appropriate amino acids for selection of positive transformants.

For long-term storage of a yeast strain, a sample of the yeast line to be stored was grown in the appropriate liquid selection media overnight. 160µl of 80% glycerol was added to 840µl of the overnight culture, mixed thoroughly, and then stored at –80°C. To recover

frozen stocks, a small amount was streaked onto a selective media plate taking care not to thaw the remaining stock, as this reduces viability.

2.6.2 Yeast transformation

Yeast were transformed using the following protocol:

1) 5ml of liquid media was inoculated with a single yeast colony and grown overnight at 30°C with shaking.
2) Following overnight growth, 1 ml of the cell culture was transferred to an Eppendorf tube and centrifuged at 13000 rpm for 3 minutes.
3) The supernatant was then removed and the cells resuspended in 100µl of 0.1M LiAc, and then incubated at 30°C for 5 minutes.
4) The cells were then centrifuged at 13000 rpm for 30 seconds and the supernatant discarded.

The following was added in this specific order

240µl (50%) PEG 3350
36µl 1M LiAc
5µl 10 mg/ml Salmon Sperm DNA
1-2µg of each plasmid to be transformed
65µl dH_2O

5) The samples were then vortexed until fully resuspended and incubated at 42°C for at least 20 minutes.
6) The cells were then centrifuged at 13000 rpm for 1 minute and the supernatant removed thoroughly.
7) The cells were then resuspended in 200µl of sterile dH_2O and plated onto the appropriate selective agar.
8) The plates were incubated at 30°C for 3-4 days until large, single colonies are seen.

If the plates were confluent or the colonies too small, then colonies were re-streaked onto a new plate and allow to grow a further 3-4 days at 30°C.

2.6.3 Yeast Two-Hybrid System

The LexA yeast two-hybrid system uses the DNA binding domain from *E. coli* LexA, while the activation domain is the B42 acid blob (Gyuris *et al.*, 1993). The coding sequence for the bait protein was cloned into pEG202 lexA-fusion plasmid, which uses an *ADH* promoter to drive expression of the target protein fused to the LexA domain, and contains the *HIS3* selectable marker. The coding sequence for the protein to be used as prey was cloned into the pJG4-5 vector, which expresses the second target protein fused to the SV40 nuclear localisation signal, the acid blob B42 and the haemaglutinin (HA) tag, and also contains the selectable marker TRP1. Expression in pJG4-5 is driven from the *Gal1* promoter, which is induced in the presence of galactose and inhibited by the presence of glucose (Johnston et al. 1994). The inducible nature of the expression of the fusion protein reduces any toxic effects the protein may have on the yeast host following transformation but prior to the assay. In order to perform the assay, the pEG202 and pJG4-5 vectors with the target genes, were transformed into yeast cells along with the reporter plasmid pSH18-43. pSH18-34 contains a *lacZ* reporter gene with eight *lexA* operons located upstream. The high number of *lexA* operons enhances the sensitivity of the system, allowing detection of even weakly interacting proteins. PSH18-34 also contains the selectable marker *URA3*.

2.6.4 X-GAL (5-bromo-4-chloro-3-indolyl-b-D-galactoside) Plate assay

X-Gal assay plates:

1. YPD/Agar media

YPD (without amino acids)	8.38g/L
Agar	2.5%

2. Galactose/Raffinose solution

Galactose	20%
Rafinose	10%

3. Bu Buffer

Na2HPO4	70g/L
NaH2PO4	30g/L

Adjust to pH 7
Add dH2O up to 1 L

4% X-Gal 2ml/L

The assay plates were made in three separate components. To make an X-Gal plate the YPD/agar, the galactose/raffinose and the BU buffer were added together in a 8:1:1 ratio. The X-Gal was then added only when the temperature of the media was below 50°C, to prevent precipitation. Plates were stored in darkness to prevent X-Gal degradation.

X-Gal plates use galactose and raffinose in place of glucose to induce expression from the *Gal1* promoter in the PJG4-5 vector, allowing expression of the 'prey' protein. Positive interaction between the bait and prey proteins is evident by the yeast colonies having a blue colour.

To perform the assay, streak at least three yeast colonies for each assay onto a selective media plate containing glucose and allow to grow for 1-2 days. This plate is known as the 'master plate' and the purpose is to provide a good quantity of yeast for streaking onto the X-Gal plates or 'assay plates'. Since the assay plates are not left to grow for long, insufficient yeast can produce poor results. Once grown, streak samples from the 'master plate' into an X-Gal plate and grow overnight. A blue colour indicating interaction should develop within 12 hours. The 'master plate' can be stored at 4°C for up to a month to provide yeast for more plate assays or for liquid assays.

To asses whether the interactions between the bait and prey proteins were light-dependent, two identical plates were prepared for each particular assay, using yeast from the same colonies from the same master plate. The assay was conducted at 37°C, and a light source was placed within the incubator. One plate was left exposed directly under the light source, while the other was placed next to it, but wrapped in tin-foil to prevent exposure to light. If any of the interactions were light-dependent, then the colonies would only turn blue on the assay plate exposed to light, and not on the one in darkness.

2.6.5 Semi quantitative plate assay

As a variation to the plate assay described previously a semi-quantitative method was used, whereby identical amounts of yeast were grown so as to give an indication as to the relative strength of the interactions. The premise being that if an identical amount of yeast was grown, then any difference in the intensity of the blue colour indicating protein interaction, would be due to the relative strength of that interaction.

Firstly for each yeast strain a plate assay as described was carried out. Then samples were taken from the Master plate and grown in 5ml selective liquid media at 37°C with shaking, overnight. When the OD600 of the samples reached 0.5-1, they were removed from the incubator and the absorbance at OD600 measured. A volume equivalent to 200µl of an OD600=0.5 culture was taken for each sample, placed in an eppendorf and centrifuged at 13000 rpm for 2 min. The supernatant was removed, and the yeast cells resuspended in 10µl sterile dH_2O. This was then transferred via pipette and spotted onto an X-Gal assay plate, then incubated at 30°C overnight.

In order to provide some quantitative data on the relative strength of the interactions, the results were scanned and analysed with Scion software, which measured the average pixel density in a specified area which included the colony. To enable comparison, the area measured for pixel density was kept the same for each yeast spot; the reason being, that although the same volume and same number of yeast cells had been spotted onto the plates, the diameter of the spots had some slight variation. Spots with smaller diameters might show greater pigment density than larger ones just because of the greater density of the yeast growth, but since the area measured for each colony was kept the same, the mean density should be unaffected by this.

If the plate assays had shown that the interactions were light-dependent, then during growth the yeast would be exposed to the same light source. Semi-quantitative assays and liquid assays were not used to test the light dependence of interactions.

2.6.6 Yeast western blot analysis

In order to determine whether a negative result from a yeast two-hybrid assay was due to lack of interaction between the bait and prey proteins, or whether it was due to a lack of expression of one of the constructs, a Western blot analysis was used. This could be used check for the expression of the bait or prey protein. A plate assay was performed using all yeast strains that gave a negative result, along with a positive control. For each strain that gave a negative result, and for the positive control, a sample of yeast was taken from the master plate used for the assay plate (see section 2.6.4 for details) and the yeast grown overnight in appropriate liquid media. The overnight culture was then used to inoculate 5ml of liquid media to an OD600=0.1. This was grown until the OD600 was between 0.5-1.0. The actual OD600 of each culture was taken, and a sufficient volume taken to give an OD600=20 when the yeast cells were resuspended in 10µl dH_20. This volume was spun down at maximum speed in a microcentrifuge for 5 minutes, and the supernatant carefully removed. The yeast cells were allowed to air-dry for 5 minutes before being resuspended in 10µl dH_20. These samples were then used directly in an SDS-PAGE and a Western blot analysis, and chemilumiscence with an appropriate antibody (see sections 2.13-2.15).

2.7 Drosophila Stocks

Fly stocks were kept in glass vials containing approximately 10ml yeast/sucrose medium (46 g/l heat-inactivated dried yeast, 46 g/l sucrose, 10 g/l agar) which provided a food source. Flies were kept in temperature controlled room with a 12:12 hour light:dark cycle, with lights-on (beginning of the subjective day) occurring at 08:00, adjusted for British Summer time as necessary.

2.7.1 Fly lines

***w*^{1118}** A null mutant for the *white* gene, thus has a white eye colour phenotype. The genotype is referred to as *w*^{1118} throughout.

tim-GAL4 *yw;tim-GAL4*
A *tim* promoter drives the expression of (*Saccharomyces cerevisiae*) Scer/GAL4. Referred to as *tim-GAL4*.

2.8 Injecting *Drosophila* embryos to generate transgenic fly lines

2.8.1 DNA Preparation

1x Injection Buffer

Solution 1	Solution 2
0.1mM Na_2HPO_4	0.1mM NaH_2PO_4
5mM KCl	5mM KCl

Solution 1 was gradually added to 2 until the a pH of 6.8 was reached.
The filter sterilised and stored at -20°C.

The vectors pUAST (containing the gene for transformation) and pUCksΠΔ2-3 were resuspended in injection buffer to give a final concentration of 300ng/µl and 150ng/µl respectively. This was then used to inject the *Drosophila* embryos. All DNA to be injected was first purified using a Qiagen column from a Qiagen miniprep kit.

2.8.2 Collecting and preparing embryos for injection

Approximately 300 hundred *w*^{1118} flies, between 3-7 days old, were placed into an open-ended glass cylinder. One end was blocked while the other was placed against a cell culture dish containing egg-laying media (330 ml/L mango-apple juice, 15% Agar, 2% Nipagin) upon which the flies were induced to lay. Flies were initially allowed to lay for a forty minute period after which all embryos were discarded so as to remove any embryos that were too old i.e. past the pole cell formation stage. Flies were then allowed

to lay again and embryos were collected every 20 minutes and placed on a strip of scotch tape that was attached to a glass slide. As many embryos as possible (approx 50) had the chorion removed by hand by rolling them across the tape with the tip of a mounting needle, within a 15 minute time limit. Using the mounting needle, the dechorinated embryos were picked up and placed on the edge of the scotch tape so that the posterior region (where the pole cells develop) protruded slightly over the edge. The embryos were placed in a desiccator containing silica gel crystals for 5 minutes, and then covered with mineral oil (Voltalef grade 10s) and transferred to an inverted microscope at 100x magnification. All dechorination and injection procedures were performed at 18°C.

2.8.3 Injection of the embryos

Needles used for the injection of DNA into the Drosophila embryos were pulled on a flaming Brown needle puller with the following settings:

1. H 830 P 30 V 50 T 120
2. H 960 P100 V 220 T 55

The tips of the needles were broken with a razor blade to give an open, sharp point through which the DNA could be injected. DNA was loaded via the back of the needle by warming in a Bunsen flame then drawing the DNA/buffer in as the needle cooled. The DNA/buffer was injected directly into the posterior region of the embryo where the pole cells form. If it was evident that any embryo was at or past the stage of pole cell formation, the embryo was killed. Once all embryos had been injected, the scotch-tape was removed and placed within a 100ml glass jar half full with yeast/agar fly food, with the embryos facing upwards, on the surface of the food. An amount of mineral oil (Voltalef grade 10s) sufficient to cover the embryos was then added, to prevent desiccation and allow embryo development in the absence of the chorion.

Once emerged, the male G0 flies were crossed to 3-5 virgin w^{1118} females and the female G0 flies were crossed to 2-3 w^{1118} males no more than a week old. The crosses were placed into sugar food vials, and the G1 flies were monitored for the red-eye phenotype indicating successful transformation. The transformants were crossed to w^{1118}; *CyO/Sco*; *MKRS/TM6B* to determine which chromosome the gene had inserted into. Once the

position of the insert had been determined, the transformants were crossed to obtain homozygosity for the insert. Inverse PCR was employed to determine the exact location of the insert.

Locomotor activity experiments

2.9.1 Analysis of Drosophila locomotor activity

Cylindrical glass tubes (8cm x 0.3cm) were filled approximately 1cm deep with sugar/agar medium at one end, which was then sealed with wax. Male flies were placed individually into each tube and the open end sealed with cotton wool. The tubes were then loaded into the locomotor activity monitor *Drosophix* (Pixel, srl., Padova, Italy). Each tube was loaded into an individual slot containing an infra-red beam that passed through the glass tube to a detector. Every time the fly passed through the beam, this was recorded as an activity event. No more than one activity event could occur within a 5 second period, and all data was recorded and stored in distinct, consecutive 30 minute periods (bins).

All incubators housing the activity recording apparatus, were set-up to give a 12 hour light:12 hour dark cycle, with lights-on occurring at 08:00. Recording of all fly locomotor activity began at 20:00 of the day that the flies were loaded into the recording incubators regardless of what time they were loaded. The flies were given a minimum of four and a maximum of 6 days in this light:dark cycle (the entrainment period) before light conditions were changed. For constant condition experiments, following the last day of the light:dark cycle, the incubators would be switched either to constant darkness or constant light. The flies were then left for 4-6 days and their activity recorded. For pulse experiments flies were maintained in the light:dark cycle as described, but on the final day were given a ten minute light pulse at a certain point in the subjective night, then maintained in constant darkness for a minimum of four days.

Following the period of locomotor activity recording, the data was collected and used for analysis. The data was analysed using both Excel and Spectral analysis. Using Excel,

flies of the same genotype that had been subjected to the same conditions were grouped together, and an average activity for each bin obtained. Using the averaged values a histogram was generated using the Excel graph function. Data for flies that had died was removed prior to generating the averages. Activity under both the light:dark and the constant condition was analysed. To analyse the periodicity of activity for each individual fly, Spectral analysis was used. Spectral analysis uses the CLEAN algorithm (Kyriacou and Hall 1989; Roberts *et al.* 1987). The Spectral analysis finds sine and cosine waves that most closely match the data, which are then displayed as a spectrogram. 95% and 99% confidence limits for each fly are generated by Monte Carlo simulation, randomising the data 1000 times while performing CLEAN analysis. The circadian period of the fly was taken to be highest peak over the 99% confidence limit, excepting where the highest peak was sometimes the 12 hour period, owing to the bimodal nature of *Drosophila* activity. When this occurred the circadian period was taken to be double the value of the highest peak.

2.9.2 Light pulse locomotor analysis

Drosophila were loaded into the activity recording machines as described in 2.10.1 for recording of locomotor activity. Following the entrainment period of a four to five day 12:12 hour light:dark cycle, a five minute 'light pulse' was given at a specific Zeitgeber time (ZT) during the subjective night by manually turning the lights of the activity machine on for a timed five minute period. The lights were then manually turned off and the Drosophila left in constant dark conditions, and the activity recorded and analysed as described in section 2.10.1.

2.10 Computer analysis

The ANOVA and Newman Keuls *post hoc* comparisons were performed using the SPSS for Windows version 11.0 (LEAD technologies, Inc).

2.11 Fly collections and protein extraction

2.11.1 Collections

Drosophila were kept at 25°C in glass vials in a light:dark regime and collected at set time points. Flies were kept a minimum of three days in the required light regimen to allow for entrainment before samples were collected. Collections were made by taking the vial, anaesthetising the flies and dispensing them into an Eppendorf, whereupon they were immediately transferred into liquid nitrogen to snap freeze them before being stored at – 80°C, preventing any further degradation of proteins from the point of collection. The vial was then discarded and not reused, hence one vial could only represent one time point. At least three separate vials were used for each time point to give three independent samples so as to duplicate results.

2.11.2 Protein extraction

Solutions:

BW Buffer

0.1 HEMG	1915µl
10% Triton X100	20µl
0.5mM EDTA	40µl
1M DTT	2µl
50mM PMSF	20µl
10mg/ml Aprotinin	2µl
10mg/ml Leupeptin	1µl
10mg/ml Pepstatin	1µl

2 ml total.

HEMG

1M KCL	4ml
0.1 HEPES	8ml
0.2 Glycerin	2ml
ddH2O	26ml

40 ml total

Method:

Collected fly samples were transferred into liquid nitrogen and vortexed at maximum speed 5x 3 sec returning the samples in the liquid nitrogen for 5 sec between vortexing bouts. The effect was to dislodge the heads from the body. The fly samples were then sieved through a metal grid that allowed the heads but not the bodies to pass through, and the heads were then collected separately in a new Eppendorf. All of this was done on dry ice to ensure the heads remained frozen throughout. Once the heads had been removed, a volume of BW buffer approximately double that of the heads, estimated by eye, was added and the heads ground thoroughly within the Eppendorf, on ice, using a micropestle (Kontes). The heads and buffer were then centrifuged at 13000 rpm for ten minutes at 4 °C and the buffer removed to a new tube taking care not to transfer any solid fly-matter. The head extracts were then stored at –80°C until use.

The head extracts were analysed by SDS-PAGE and Western Blotting. To ensure even loading into the gel, a Bradford reaction was used to quantify the relative amount of protein in each extract. 1 µl of protein extract was mixed with 800 µl of Bradford reagent (Sigma) and 200 µl of water and thoroughly mixed. After 5 min, but before 30 min, the OD_{595} was measured, reflecting the amount of protein in each sample. Prior to loading on the gel, the samples were equalized.

2.12 SDS-PAGE Analysis

Stock solutions

10X SDS Running Buffer

Tris Base 30.2g

Glycine 188g

to 900ml H2O

Add 100ml SDS just before use.

3X Protein Loading Dye

Tris pH 6.8 188µM

Mercaptoethanol	15%
Glycerol	30%
SDS	6%
Bromophenol	0.03%

Resolving Gel (12%)

H2O	7.7ml
Tris 2M pH 8.8	4ml
SDS 10%	200µl
Acrylimide 30%	8ml
APS 25%	80µl
TEMED	12µl
20ml total	

Gels were poured into Biorad gel assembly apparatus and covered with isopropanol until ready for use.

Stacking Gel (4.5%)

H2O	7.3ml
Tris 1M pH 6.8	1ml
Acrylamide 30%	1.5ml
SDS 10%	100µl
APS 25%	20µl
TEMED	10µl
10ml total	

The stacking gel was poured directly onto the resolving gel and the comb inserted immediately.

To each sample to be loaded 50% volume of 3X protein loading dye was added, then this was incubated at 95°C for 3 min to denature the proteins. Samples were then centrifuged at 13000 rpm for 30 sec, then mixed thoroughly using a pipette prior to loading into the gel wells.

Samples were run at 15 mA per gel through the stacking and at 30mA per gel through the resolving gel. The protein was then transferred to a nitrocellulose membrane.

2.13 Western Blotting

Transfer buffer

5.28g Tris
2.93g Glycine
3.75ml 10% SDS
200ml MetOH
to 1L H2O

The gel was immersed in transfer buffer, overlaid with a nitrocellulose membrane (Protran BA 85 nitrocellulose membrane, Schleicher & Schuell) and sandwiched between sheets of Whatmann 3M paper. The sandwich was loaded in a Western Blotting apparatus and the proteins were transferred for three hours at 400 mA. The nitrocellulose membrane was either used immediately for immunodetection or stored dry at room temperature.

2.14 Immunodetection

TBST

5M NaCl	28ml
2M Tris pH7.5	5ml
10% Tween20	5ml

To 1L H2O

The nitrocellulose membrane was placed in approximately 100ml milk blocking solution (5% powdered semi-skimmed milk in TBST) and left to shake gently on a shaking platform for 1 hour. The milk blocking solution was decanted and the membrane was placed into a plastic envelope where the primary antibody (diluted in 5% milk TBST) was added in a sufficient quantity just to cover the membrane. The envelope was sealed ensuring there were no air bubbles to prevent contact between the antibody and the membrane. The antibody and membrane were left to shake on a shaking platform overnight at 4°C. The primary antibody was decanted and frozen to store for future use. The membrane was washed 3 times in 100ml TBST and placed in a new plastic envelope. The secondary antibody was added as before and left to shake for 2 hours at 4°C. The membrane was removed and washed 3 times in 100 ml TBST as before. The membrane was then stored in TBST until ready for chemiluminescence.

2.14.1 Chemiluminescence

Reaction solution

dH_2O	9ml
1M Tris-HCl pH 8.5	1ml
Luminol Stock (Sigma. Stock – 250mM in DMSO)	50µl
p-coumaric acid (Sigma. Stock 90mM in DMSO)	22µl

Add 3µl H_2O_2 then use immediately.

The nitrocellulose membrane was placed protein side up, on a piece of Clingfilm and the reaction solution poured so as to completely cover the up-turned surface. This was left for 1 min then the solution was poured off and the membrane placed face down onto a piece of Clingfilm, wrapped thoroughly, and transferred to a film cassette. A sheet of auto-radiograph film (Fuji) was transferred to the cassette and left for 1 minute then developed. If necessary this was repeated, varying the exposure time of the film depending on the intensity of the bands. The films were then scanned into a PC and pixel density was measured with Scion Image analysis software (Scion Inc.) to determine the relative amounts of protein.

Chapter 3: Cloning and expression of dCRY in overlapping fragments for generating antibodies

3.1 Introduction

3.1.1 The role of specific antibodies in dissecting the circadian clock

The circadian clock is based upon the cycling of key clock components. Therefore, essential to understanding the mechanisms by which the molecular clock regulates time keeping, is an understanding of how the levels of these key clock components change in abundance over the circadian period, and how such changes are affected by input such as light. Of particular use in this regard has been the application of specific antibodies raised against a particular component of the clock. Western blot analysis of samples taken from organisms at intervals over the circadian period can be analysed with such antibodies, and a quantitative assessment made of the changes in abundance of the clock component of interest. Of equal importance to understanding the circadian clock is to understand the cellular distribution of clock components, and related changes throughout the circadian period. A suitable antibody can be used in immunohistochemical (IHC) studies to analyse just such distribution patterns of clock components.

The use of specific antibodies to visualise clock components following a Western blot has elucidated many important features of the *Drosophila* circadian clock. Some key examples of the use of antibodies in elucidating the time profiles of clock components are highlighted here. All these examples are discussed in greater detail in chapter 1. Period (PER) was shown to cycle over a 24 hour period, the peak in protein level occurring at night, at ZT 20-22 (Edery *et al.*, 1994). TIM protein cycling was shown to be very similar to that of PER, aside from TIM very slightly anticipating the increase in PER and the much more rapid decline of TIM levels, that also precedes the fall in PER levels (Zeng *et al.*, 1996). It was also shown that TIM levels decline rapidly with light input (Hunter-Ensor *et al.*, 1996). CLOCK (CLK) shows temporal changes in both abundance and phosphorylation pattern, like PER and TIM (Lee *et al.*, 1998), whereas CYCLE

(CYC) does not cycle in abundance (Rutila *et al.*, 1998). dCRY has been shown to cycle in a light-dependent manner (Emery *et al.*, 1998).

Investigations into the role of dCRY in the circadian clock undertaken by this research group have shown how mutations in dCRY affect the components of the circadian clock. *Drosophila* lines transgenic for the truncated version of dCRY (dCRYΔ, discussed in chapter 1 and chapter 4 section 1), which behaviourally show a constitutive low-level light response, have an attenuated amplitude for both PER and TIM cycling (Dissel et al., 2004). Importantly, dCRYΔ was also shown to be at constant low levels, indicating constant degradation, which demonstrates the importance of the dCRY C-terminal domain in dCRY stability. Since a principal objective of many of the investigations of this research group is to understand the regulation of dCRY and the way in which dCRY interacts with other components of the *Drosophila* clock, an antibody specific for dCRY is essential. This could be used to generate circadian profiles of dCRY and related constructs for transgenic fly lines, particularly where dCRY has been altered in some way, or in which a component believed to affect dCRY is altered. Also, a suitable antibody could be used in coimmunoprecipitation and immunohistochemcial studies where the antibodies could be used against tissue sections to identify and localise the protein within its native environment. This could be used to identify the spatiotemporal localisation of dCRY and the related mutants in the different clock neurons, thereby highlighting any differences in distribution in clock neurons, which would in turn shed light upon the functions of the different types of clock neuron.

3.1.2 Aims and objectives

In order to produce an effective antibody for dCRY, it was decided that the entire sequence of dCRY would be used to generate the antibody. The first consideration was that, based on previous experience, it was known that full-length dCRY aggregates upon expression in the *E. coli* expression system (data not published). To overcome this problem, it was decided to express dCRY as three fragments, and that each fragment would represent approximately one third of the full-length dCRY, and therefore together would comprise the whole sequence.

To this end, *dcry* was cloned as three fragments representing the first, middle and end thirds of the gene (and therefore the first, middle and end thirds of the dCRY protein), and these regions were expressed in *E. coli* to give dCRY fragments which were then extracted and purified.

The second consideration was whether to try to isolate the dCRY fragments as a soluble or insoluble form. A soluble form of dCRY produced in *E. coli* may be identical, or similar enough, to the native form of dCRY as expressed *in vivo* in *Drosophila*, in which case the antibody generated using this protein might be more usable in IHC studies as compared with antibodies raised against the dCRY protein extracted in an insoluble form. However, this idea would not be assured as it was also probable that due to the fact that expression would be undertaken in *E. coli*, not *Drosophila*, and that the dCRY protein would be expressed in fragments rather than as a whole, that the resulting proteins would be sufficiently different to native dCRY so as to be unable to raise antibodies that would work in IHC. Finally, it was considered quite probable that antibodies raised against insoluble dCRY may well be usable in IHC work and coimmunoprecipitation studies.

Taking into account all considerations as discussed, it was decided to try to extract and purify the protein in the native form initially, in the hope that the antibody generated might be more useful in IHC and coimmunoprecipitation studies, but since there was no assurance of this it was also decided that if the protein fragments proved difficult to express in a soluble form then the insoluble form would be used.

3.2 Materials and Methods

3.2.1 Cloning

All cloning was carried out as described in section 2.1-2.4.

3.2.2 Protein expression and purification

Protein expression and purification were carried out as detailed in section 2.5.

3.3 Results

3.3.1 Cloning *dcry* in three fragments

Using PCR, the *Drosophila cryptochrome (dcry)* gene was amplified as three overlapping fragments. The fragments consisted of the following intervals of *dcry*, expressed in base-pairs, taking the first nucleotide of the coding sequence of *dcry* as position 1: fragment 1 consisted of 1-600 (600 bp) called dCRYf1, fragment two was 540-1296 (756 bp) called dCRYf2, fragment three was 999-1626 (627 bp) called dCRYf3. This was achieved using primers designed specifically to amplify the region to be cloned (**fig. 3.1**)

Fragment 1:

5' primer GGCATATGGCCACGCGAGGGGCGAATG

3' primer GGGGATCCTTACTCGAACAACTTAAGACTTCGGCAG

Fragment 2:

5' primer CGCATATGGAAGACGCCACCTTTGTCGAGC

3' primer GGGGATCCTTACAGCAGCCTTTCAAACGCCGAG

Fragment 3:

5' primer GGCATATGAATGACATCTGCCTGAGCATCCCG

3' primer GGGGATCCTTAAACCACCACGTCGGCCAGCCAG

Fig 3.1 The 5' and 3' primers used to clone *Drosophila cryptochrome* (*dcry*) in 3 overlapping fragments. All primer sequences are written 5' to 3'. The fragments consisted of the following intervals of *dcry*, expressed in base-pairs, taking the first

nucleotide of the coding sequence of *cry* as position 1: fragment 1 consisted of 1-600 (600 bp), fragment two was 540-1296 (756 bp), and fragment three was 999-1626 (627 bp). An *Nde*I restriction site was added to the 5' primers of each, highlighted in blue. To the 3' primer of each an *Xho*I restriction sire was added, highlighted in red, as well as a stop codon when necessary, underlined.

To each of the 5' primers an *Nde*I restriction site was added, and to each of the 3' primers an *Xho*I restriction site was added preceded by a stop codon (**fig 3.1**). The sequences were amplified *via* PCR using the method detailed in section 2.4. These were then ligated into the vector pDK101 and the sequence checked for unwanted mutations. The correct sequence was then excised with an *Nde*I—*Xho*I digest and ligated into the expression vector pET14b (Novagen), which had been cut with the corresponding enzymes. Each fragment in pET14b was first cloned in the bacterial strain XL1-Blue MRF' (Stratagene), from which purified plasmid DNA was extracted. Plasmids were then transformed into the expression host BL21(DE3)pLysS (Novagene) for expression and purification of proteins.

3.3.2 Expression and purification

For each fragment a small scale expression and analysis was done (section 2.5.2). Post induction, the incubation temperature was either 37°C or 28°C. Samples of the culture were taken for analysis at 1 hour intervals for four hours (section 2.5.2), although a greater time period was used when appropriate. The soluble and insoluble protein fractions were then separated and analysed (section 2.5.3). This was used to determine whether the expressed protein was soluble or insoluble, and whether the solubility was affected by the temperature and duration of incubation, following induction.

Once the small scale expression analysis had been used to determine whether the protein was in a soluble or insoluble form, and what the best conditions were for obtaining maximal amounts of protein were, the process was scaled up to give a usable quantity of protein (section 2.5.4). If the protein had been determined to be in a soluble form, then it would be extracted and purified under native conditions i.e. using buffers and solutions

designed to keep the protein in its native, soluble form. If, however, the protein proved to be insoluble, then extraction and purification would be done under denaturing conditions, in which the protein would be solubilised. Based upon the results of the small scale expression analysis, all fragments were extracted and purified under denaturing conditions (see 2.5.4).

3.3.3 Testing the antibody

Once the antibody had been generated, it was tested using a Western blot analysis. Samples known to contain dCRY were subjected to SDS-PAGE analysis and Western blotting (see section 2.13 and 2.14 for details of methodology). The antibody was then used in an immunodetection and chemiluminescence to visualise the dCRY protein (see section 2.15 for details on immunodetection and chemiluminescence).

3.3.4 Secondary structure prediction of CRY

The secondary structure prediction programme (Rost *et al.*, 2004) was used to generate a prediction of secondary structure of the *Drosophila* CRY (dCRY). To decide how *dcry* was to be cloned to give three fragments of dCRY, firstly the protein was divided into roughly equal-sized beginning, middle and end. Then using the secondary structure prediction, and corresponding sequence of *dcry*, the position of the primers was decided such that, where possible, the beginning and end of the expressed fragments would be in regions corresponding to no predicted secondary structure. This was to minimise the disruption to secondary structure motifs present in dCRY that might be antigenic and therefore useful in generating antibodies, as well as attempting to avoid disrupting secondary structure that might cause misfolding of the fragments. Rather than have distinct fragments, some overlap was included between the first and second, and second and third, fragments.

3.3.5 Small scale expression analysis

Initially, host bacteria expressing dCRYf1 were grown at an incubation temperature of 37°C, and samples taken ever hour for 4 hours post IPTG induction. The results of this show that dCRYf1 is present almost entirely in the insoluble form. While some protein is

present in the soluble form, it is present at a much lower level. Optimal amounts of soluble protein appear to be obtained at a two hour incubation period following induction (**fig 3.2**), but even this maximal amount is still much less than the amount of insoluble dCRYf1 at all time points. A similar result is seen with dCRYf1 expressed at 28°C (**fig 3.2**). The protein is present almost entirely in the insoluble form. Maximal amounts of soluble protein are seen three hours post induction, but as with the expression at 37°C, this is still a very small quantity relative to the insoluble form at all time points. As far as can be assessed, the quantity of insoluble form is not affected by the temperature or period of incubation, and is constantly present in large quantities.

The dCRYf2 proved to be toxic to the host bacteria, and cultures did not reach the required OD600 for induction with IPTG initially. To try to overcome this problem 1% w/v glucose was added to the liquid media used for the overnight culture, and also to the growth media used for expression which has the effect to reduce the expression. Also, the IPTG concentration used for induction was lowered to 0.1mM which would also have the effect of reducing expression. The rationale being that if the expression was reduced then the toxicity to the *E. coli* would be reduced allowing accumulation of the soluble form and greater growth of the bacteria. Using these modifications, a time series at 28°C was possible, taking only three time points following induction (**fig. 3.3**). Since the culture was growing slowly, indicating toxicity of the fragment, it was thought unlikely that a long incubation period would be conducive to the accumulation of dCRYf2 protein. It was thought more likely that with an increase in time the build of dCRYf2 would adversely affect the growth of the culture. Therefore the time course was shortened. It can be seen that in none of the samples is dCRYf2 seen in the soluble fraction (see **fig 3.3**), as would be expected for a protein that is toxic to the host.

For dCFRYf3, the first small scale expression was undertaken using an incubation temperature of 37°C, and samples were initially taken every hour for four hours following induction (see section 2.5.2). Analysis suggested that the amount of protein in the soluble fraction increased with time, so the time series was extended a further two hours, giving time points 5 and 6 (see **fig 3.3**). It can be seen from the results that maximal

amounts of protein in the soluble fraction occur at 5 and 6 hours following induction (**fig 3.3**). Analysis of the small scale expression time series incubated at 28°C following induction, shows the maximal amount of soluble protein occurs at 3 hours post induction (**fig 3.3**). Time points at 5 and 6 hours were also taken for dCRYf3 grown at 28°C, as it was thought that since soluble dCRYf3 appeared to show an increase with time at 37°C, this might be the case at 28°C (**fig 3.4**). This was not the case. At both temperatures and at all time points, the majority of the protein is found in the insoluble form. As with dCRYf1, the quantity of insoluble form appears not to be affected by the temperature or period of incubation, being present in large quantities after the first hour of induction onward.

With all three fragments the small scale expression indicates that the great majority of the protein is always in the insoluble fraction, regardless of conditions. Small amounts of soluble protein are present, but in much lower quantities. Altering the growth temperature and incubation time has a small affect on the amount of protein in the soluble form, but at every time point and under all conditions the vast majority of all three protein fragments is in the insoluble form. It was therefore decided at this stage, that it would be more feasible to express and purify the insoluble form of each fragment.

3.3.6 Full scale protein expression

Since the conditions as determined by the small scale protein expression time series did not indicate that the quantity of insoluble protein was greatly affected by the conditions tested, cultures expressing any one of the fragments were grown at 28°C for three hours following induction. The dCRYf2 expression was induced with IPTG to a final concentration of 0.1mM, and 1% w/v glucose was added to the growth media as before in an effort to reduce expression of the protein to a level where it was less toxic to the *E. coli*.

dCRY fragment 1

The protein was extracted and purified as described in section 2.5.6. Most of the protein eluted in fractions 7-10, so these were pooled together and the elution buffer replaced

with PBS as described in section 2.5.7 (see **fig 3.5**). Since the protein was insoluble, on replacement of the elution buffer with PBS, dCRY fragment 1 precipitated out of solution. The PBS was carefully removed and the solid precipitate mixed with 100ul PBS and moved to a 1.5 ml microfuge tube.

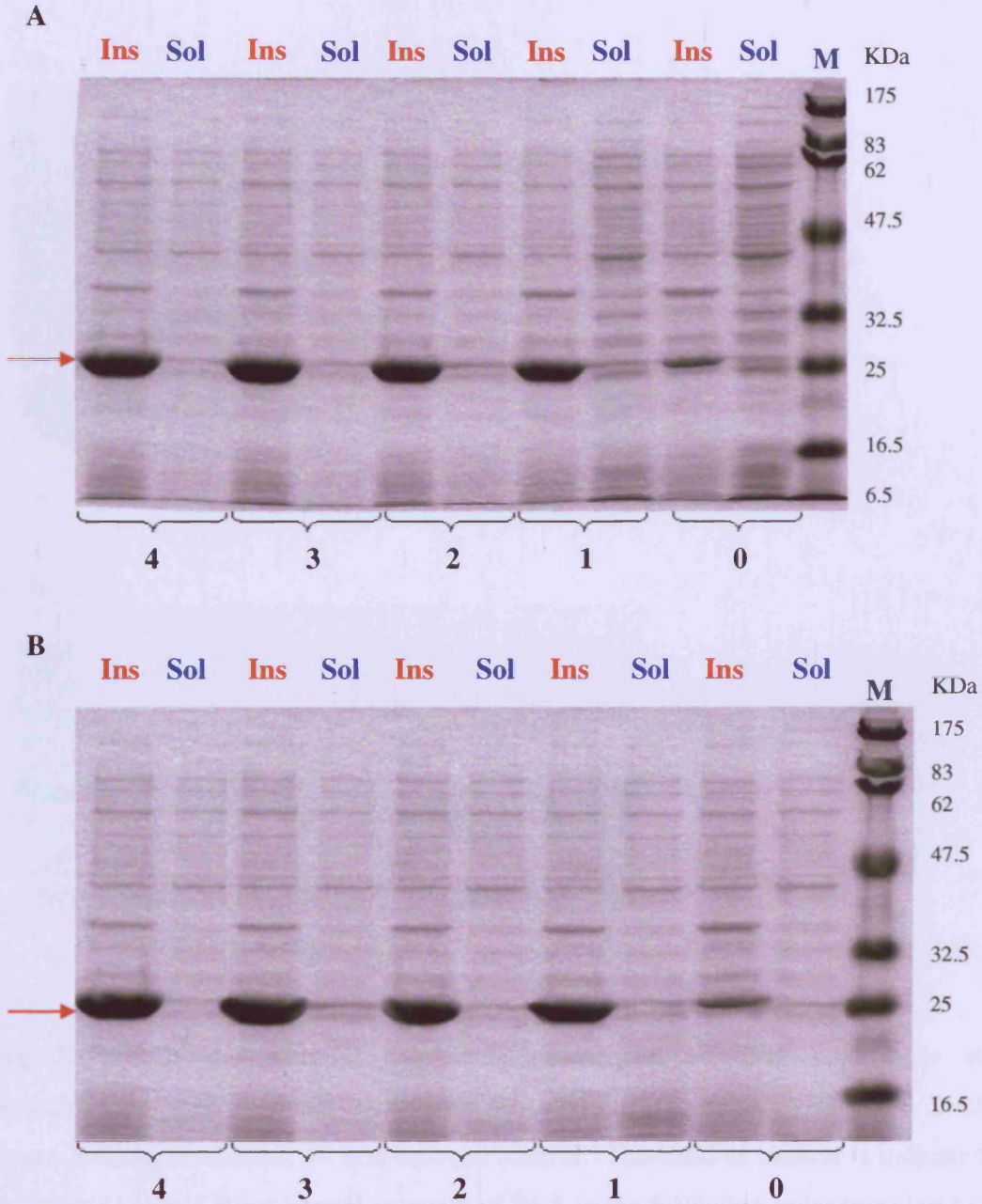


Fig 3.2 dCRY expression in *E. coli*. Coomassie stained polyacrylamide gel showing dCRY fragment 1 (dCRYf1) following expression and extraction from *E. coli* grown at A=37°C and B=28°C. The lane numbers represent the time in hours after induction of expression by IPTG. M is the marker (Broadrange, Biorad) band sizes are listed sequentially in KDa. Ins=the insoluble protein and Sol=soluble protein, at each time point. The red arrow indicates the position of the bands of interest. It is clear that in all conditions the protein is present in the insoluble form.

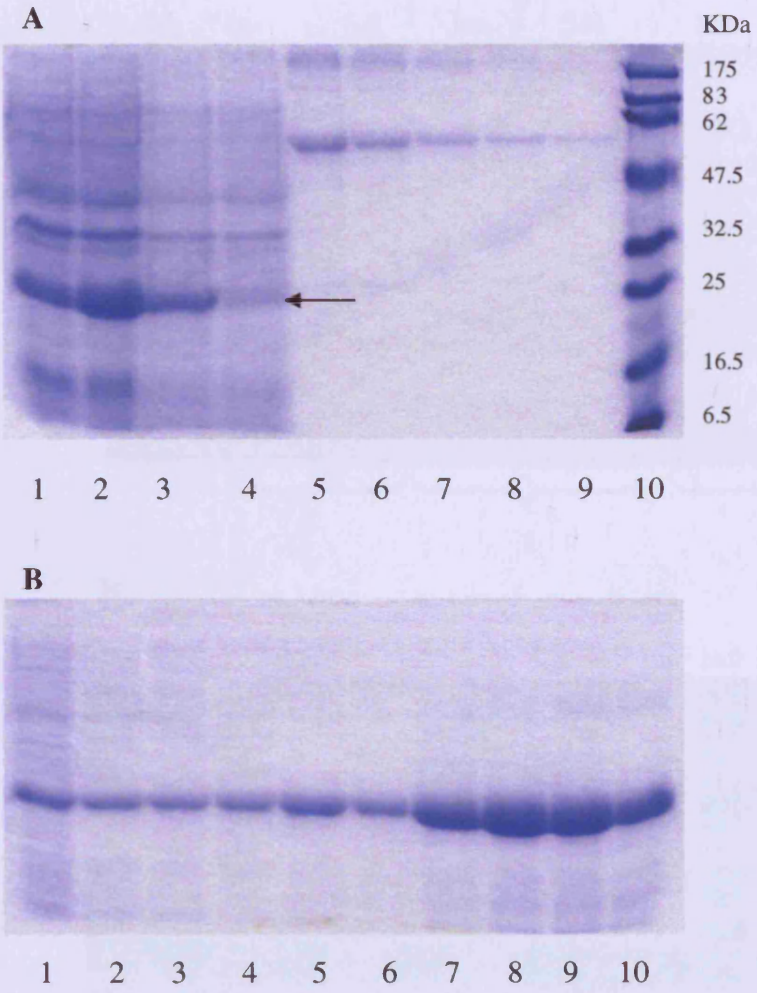


Fig 3.5 dCRY fragment 1 full scale expression analysis. Coomassie stained polyacrilamide gel. **A**. lanes correspond to the following 1= flow through, 2=cleared lysate, 3=induced control, 4= non-induced control. The band of interest is indicated with an arrow. Lanes 5-9 are known amounts of BSA in the following order from left to right: 4µg, 2µg, 1µg, 0.5µg, 0.25µg. M is marker, band sizes are listed sequentially in KDa. (Broadrange, Biorad). **B**. lanes correspond to the following: 1-2 wash buffer fractions, 3-6 elution buffer D fractions, 7-10 elution buffer E fractions. All fractions are presented from left to right in the order they were collected. It can been seen that following purification, most of dCRYf1 is found in elution fractions 7-10.

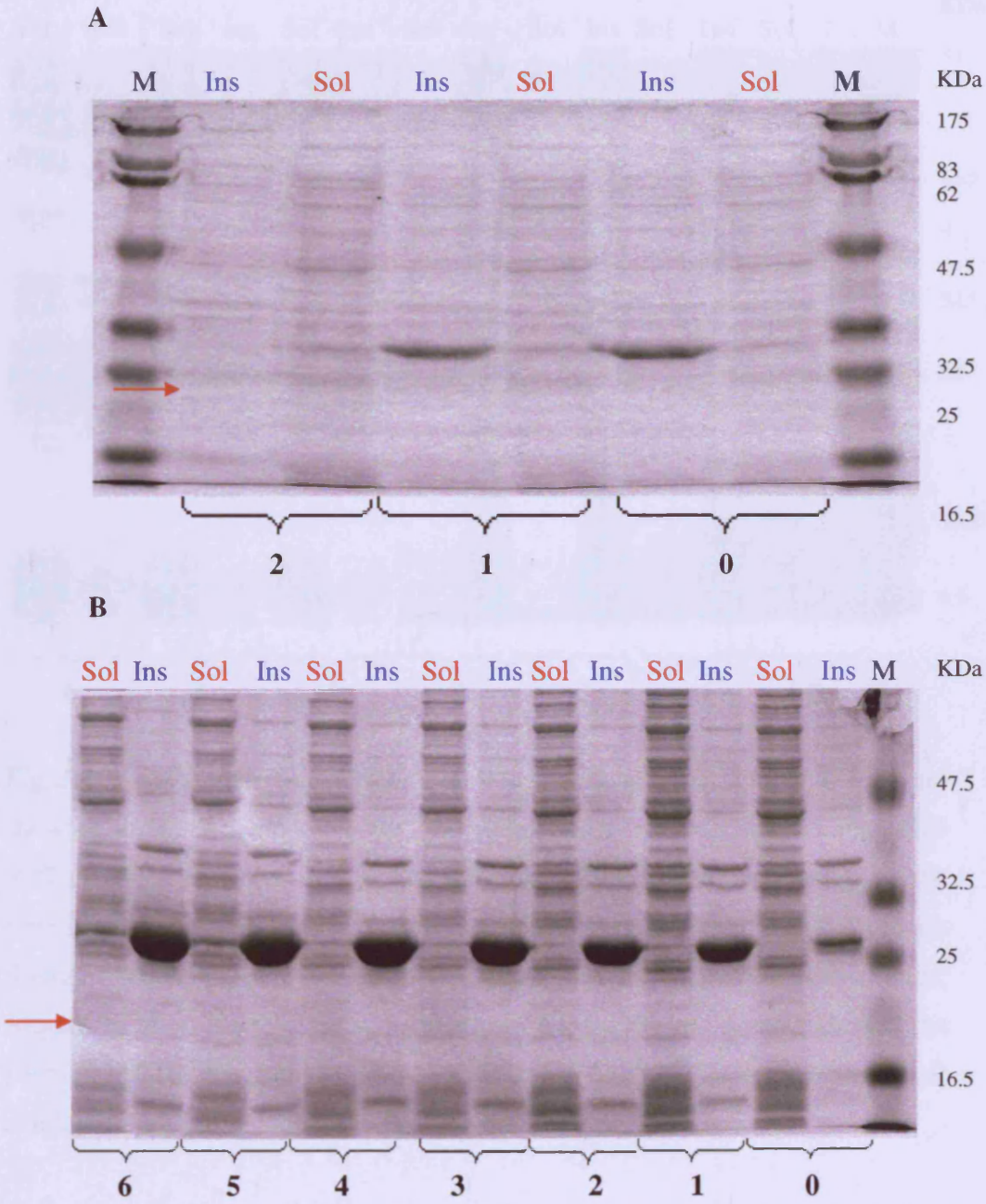


Fig 3.3 dCRY expression in *E. coli*. Coomassie stained polyacrylamide gel showing dCRY fragments following expression and extraction from *E. coli*. **A:** dCRY fragment 2 (region 540-1296) incubated at 28°C. **B:** dCRY fragment 3 (region 999-1626) grown at 37°C. The lane numbers represent the time in hours after induction of expression by IPTG. M is the marker (Broadrange, Biorad) band sizes are listed sequentially in KDa. The red arrow indicates the position of the bands of interest. It is clear that all fragments, under all conditions, are present almost entirely in the insoluble form

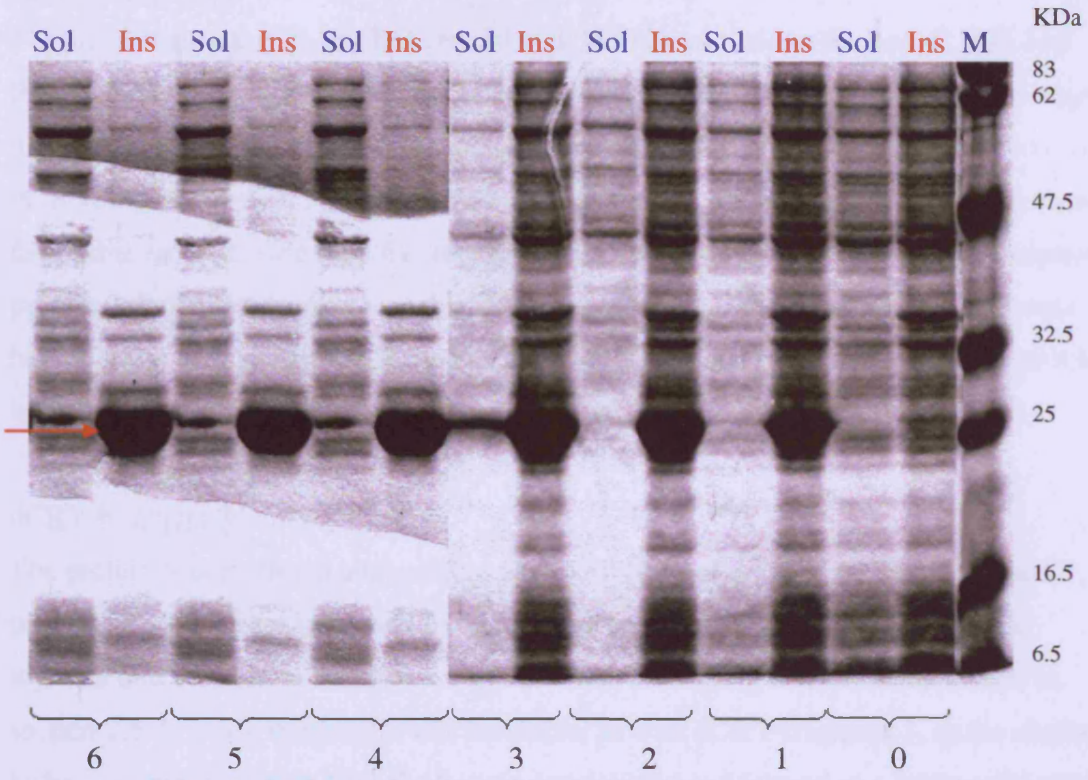


Fig 3.4 dCRY expression in *E. coli*. Coomassie stained polyacrylamide gel showing dCRY fragment 3 (dCRYf3) consisting of the nucleotides 999-1626 (627 bp) expressed in *E. coli* grown at 28°C. The lane numbers represent the time in hours after induction of expression by IPTG. Ins=the insoluble protein Sol=the soluble protein, for each time point. M is the marker (Broadrange, Biorad) band sizes are listed sequentially in KDa. The red arrow indicates the position of the band of interest. It is clear that all fragments, under all conditions, are present almost entirely in the insoluble form.

dCRY fragment 2:

The small scale analysis had indicated that dCRYf2 was toxic to the host *E. coli*, and therefore expression levels, even of insoluble protein, were relatively low when compared to dCRYf1 and dCRYf2. None-the-less, an attempt was made to extract usable amount of dCRYf2. However, following expression and purification, dCRYf2 was not present in detectable amounts (see **fig 3.6**). It may have been possible to produce useful amounts of protein, but the culture may have had to be impractically large, and this in turn would have resulted in a very low concentration of dCRYf2 when extracted, leading to problems in purification. Therefore, it was decided not to pursue dCRYf2 any further.

dCRY fragment 3:

The protein was extracted and purified as described in section 2.5.6. The fractions deemed to have the most protein in them, those fractions being 7-10, were pooled together and the elution buffer was replaced with PBS using dialysis as described in section 2.5.7. Since the protein was insoluble, as with dCRY fragment 1, as the elution buffer was replaced with PBS the protein precipitated and formed as a white solid matter within the dialysis tubing. The PBS was carefully removed and the precipitate mixed with 100ul of new PBS and moved to a 1.5 ml microfuge tube.

3.3.7 Preparation of dCRY fragments

For generating antibodies, the fragments were required to be at a final concentration of 0.4mg/ml, and at least 5ml total was required (hence 2.5ml of dCRYf1 (0.4mg/ml) was added to 2.5ml dCRYf3 (0.4mg.ml). The large scale expression was repeated for each fragment, as described, until enough of each protein fragment had been obtained. dCRY fragment 1 and 3 in PBS were quantified using SDS-PAGE (see section 2.5.8). Based on this quantification, the volume of PBS in each case was adjusted to give a final concentration of 0.4 mg/ml. In all cases this involved adding more PBS. The two volumes were then added together and sent to NeoMPS SA, as instructed.

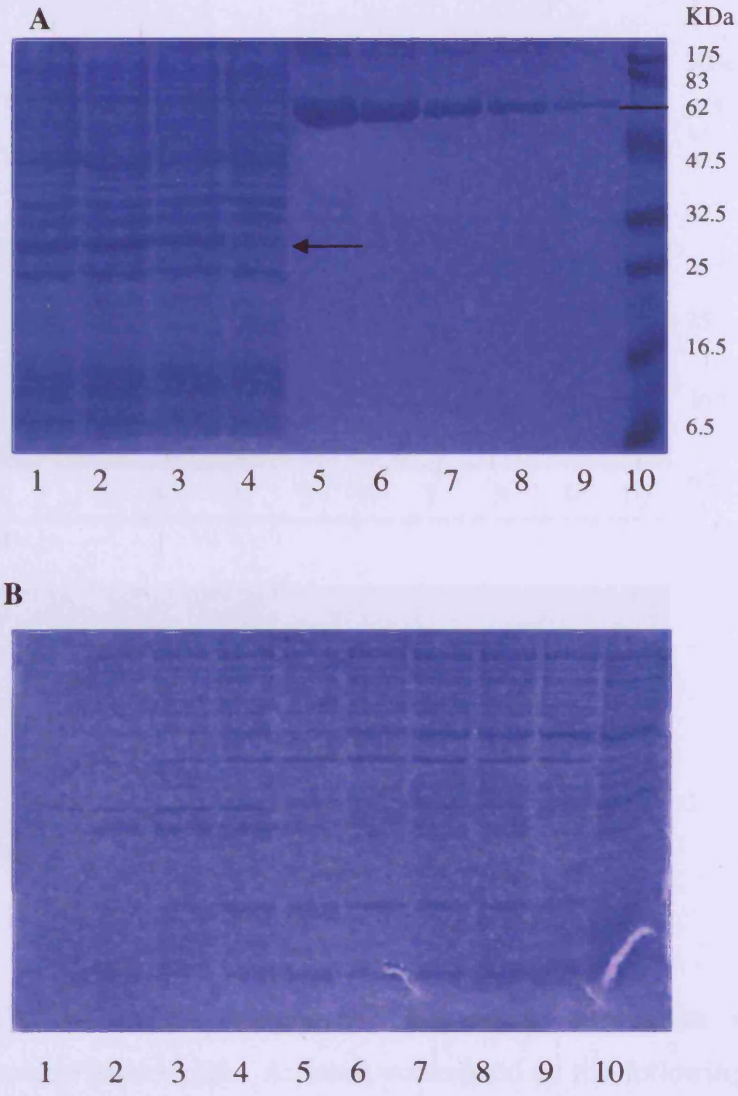


Fig 3.6 dCRYf2 full scale expression analysis: Coomassie stained polyacrilamide gel. **A**. lanes correspond to the following 1= flow through, 2=cleared lysate, 3=induced control, 4= non-induced control. The band of interest is indicated with an arrow. Lanes 5-9 are known amounts of BSA in the following order from left to right: 4µg, 2µg, 1µg, 0.5µg, 0.25µg. M is a marker, band sizes are listed sequentially in KDa. **B**. lanes correspond to the following: 1-2 wash buffer fractions, 3-6 elution buffer D fractions, 6-10 elution buffer E fractions. All fractions are presented from left to right in the order they were collected. While there is some dCRYf2 present in the flow through, cleared lysate and induced control fractions, very little, if any, dCRYf2 is present in the elution fractions.

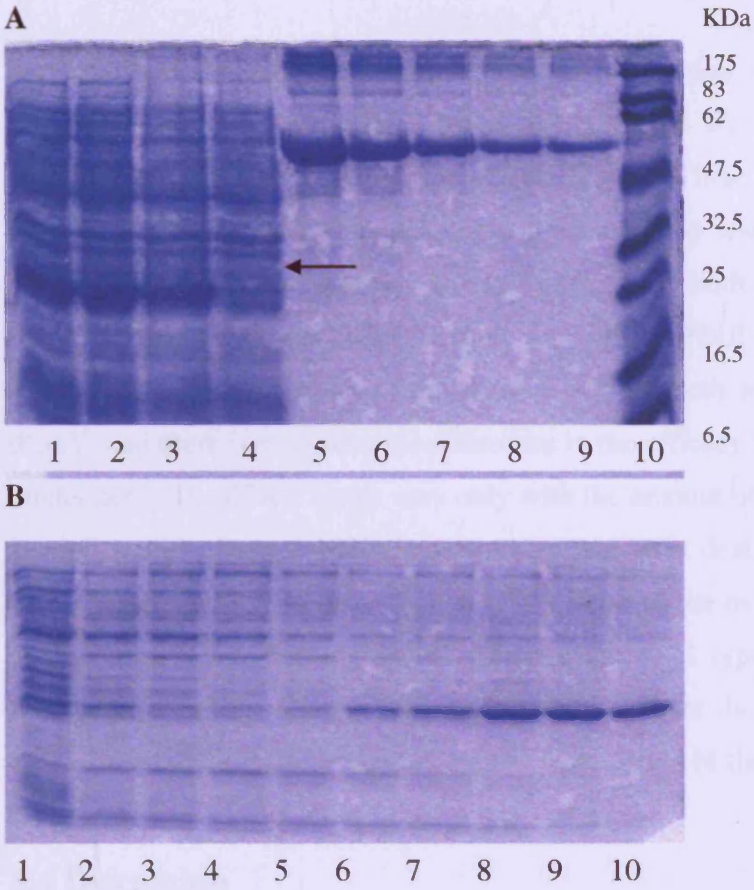


Fig 3.7 dCRY fragment 3 full scale expression analysis. Coomassie stained polyacrilamide gel. **A**: lanes correspond to the following 1= flow through, 2=cleared lysate, 3=induced control, 4= non-induced control. The band of interest is indicated with an arrow. Lanes 5-9 are known amounts of BSA in the following order from left to right: 4µg, 2µg, 1µg, 0.5µg, 0.25µg. M is a marker, band sizes are listed sequentially in KDa **B**: lanes correspond to the following: 1-2 wash buffer fractions, 3-6 elution buffer D fractions, 6-10 elution buffer E fractions. All fractions are presented from left to right in the order they were collected. Following purification, most of dCRYf3 is present in elution fractions 7-10.

3.3.8 Testing of the antibody

The dCRY antibodies were tested as described in section 3.2. The samples used were a *Drosophila* fly line over-expressing dCRY taken at ZT 20 and two samples from a wildtype *Drosophila* line taken at ZT 12 and ZT 20. These samples had been collected as detailed in section 2.12. The dilutions of the antibody tested were 1:1000 and 1:10000. Two sets of antibodies from two different animals (in both cases a rat) were tested in this manner. The secondary antibody used was anti-rat HRP conjugated (Sigma, 1: 8000). See **fig. 3.8** for the results. The antibodies from both animals successfully bound to dCRY, and there is no detectable difference in the efficacy between the two. The relative intensities of the dCRY bands vary only with the amount of dCRY expected to be present in each sample. At a 1:1000 dilution, there is a great deal of cross-reaction, making the dCRY band difficult to see. At a 1:10000 dilution the over-expressing sample, in both cases, gives a very strong band, whereas the wild type gives a less intense band, particularly with the sample collected at ZT 12 where the band is quite faint, owing to low levels of dCRY due to light-triggered degradation of the protein.

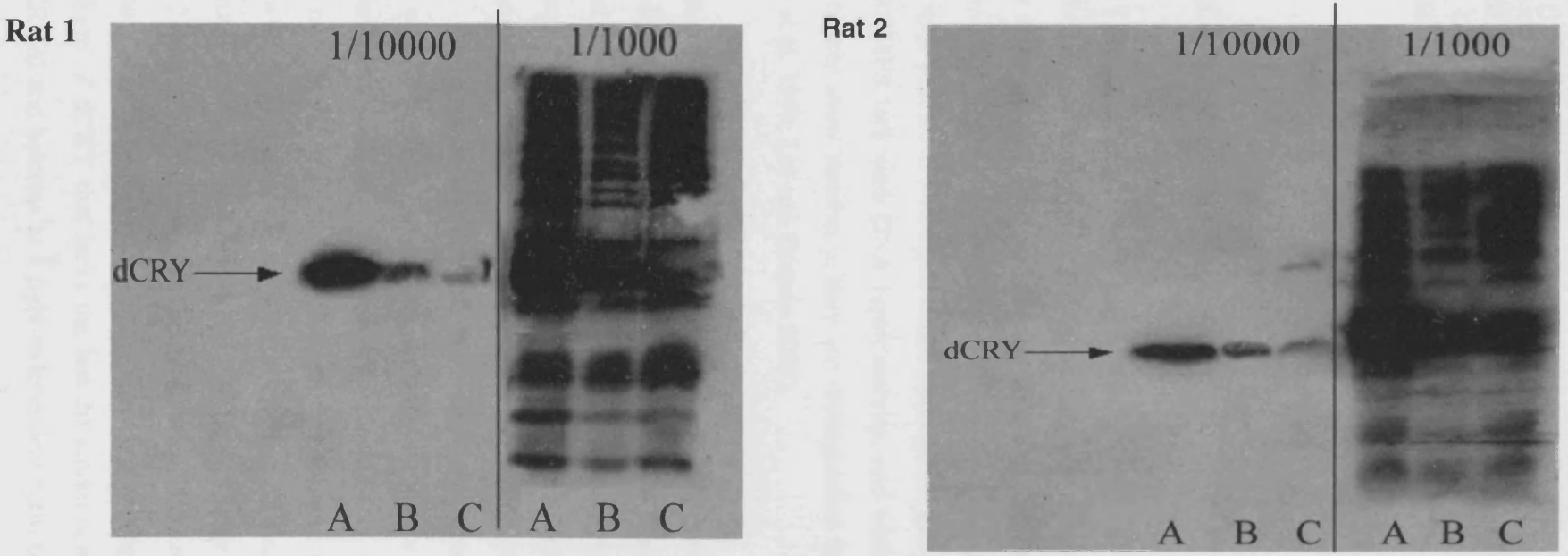
3.4 Discussion

It was not possible to express the dCRY fragments in their soluble form. It may be possible to do so by altering certain conditions, or by attempting to refold the protein following purification. However, it was decided to express and purify all the fragments in their insoluble form due to time constraints, and because of the amount of protein required.

Expressing the protein fragments in the insoluble form proved successful for all fragments apart from dCRYf2, which was toxic to the host bacteria, and difficult to obtain in large enough quantities. Once again, it may have been possible to express dCRYf2 in useful amounts by alteration of the conditions of culture growth, but time constraints meant that pursuing what could be a long process of trial and error was deemed impractical.

Using the insoluble forms of dCRYf1 and dCRYf3 an antibody has been produced that binds to dCRY in Western blot analysis (**fig 3.8**). Since the binding of the antibody to dCRY from extracts not over-expressing were weak (**fig 3.8**), it was decided to use the antibody at a dilution of 1/8000 as standard guideline, subject to modification as required.

Fig 3.8 Results of the dCRY antibody tests: dCRY antibodies from two different rats (labelled Rat 1 and Rat 2) were tested for efficacy against three samples of fly head protein extracts, using two dilutions of antibody. The dilution factor of the antibody solution is written above the relevant columns. The samples tested, as indicated by the letters below each lane, were as follows: A represents a fly line over-expressing dCRY, the sample was taken at ZT 20. B and C represent a wild type fly line, with the samples taken at ZT 20 and 12 respectively. It can be seen that at a dilution of 1/10,000 the antibody effectively binds to dCRY in all three samples, the differences in intensity being due to the differences in the amount of dCRY present. At the dilution 1/1000, cross-reactions make the dCRY band difficult to discern.



Chapter 4: Dissecting the role of the *Drosophila* CRY C-terminus using chimerical constructs

4.1 Introduction

4.1.1 The role of CRYPTOCHROME in the *Drosophila* central clock

Drosophila CRYPTOCHROME (dCRY) is a member of the CRYPTOCHROME family of proteins, which show homology to the DNA repair proteins the photolysases (reviewed in Cashmore 2003). Photolyases are flavoproteins that mediate the repair of UV-B-damaged DNA by removing the resulting pyrimidine dimers (Sancar, 2003). Such repair activity is dependent upon irradiation with blue or UV-A light, hence photolyases are a form of photoreceptor. CRYPTOCHROMES lack such DNA repair activity, and while members of the CRYPTOCHROME family show similarity, they are distinguished by unique C-terminal domains (Cashmore et al. 1999; Lin and Shalitin 2003).

Drosophila CRYPTOCHROME was first identified as an important photoreceptor in two studies (Emery *et al.*, 1998; Stanewsky *et al.*, 1998), and is thought to be the only photoreceptor in the *Drosophila* central clock neurons capable of directly entraining the central oscillator via light input (Emery *et al.*, 2000); flies lacking a functional dCRY coupled with loss of visual and extra-ocular structures are unable to entrain to a light:dark regime (Helfrich-Forster *et al.*, 2001). Although certain lines of evidence suggest that dCRY may act as a component of the central clock in peripheral tissues (Krishna *et al.*, 2001; Ivanchenko *et al.*, 2001), in the central oscillator the evidence indicates that it functions as a photoreceptor. dCRY is believed to operate in this capacity through a light-dependent transient interaction with TIM that leads to TIM ubiquitination and degradation (Naidoo *et al.*, 1999; Ceriani *et al.*, 1999; Rosato *et al.*, 2001; Lin *et al.*, 2001; Dissel *et al.*, 2004; Busza *et al.*, 2004). Thus delaying the accumulation of TIM in light and preventing the negative feedback loop of the central oscillator from proceeding. The recent discovery of a truncated form of dCRY that lacks the last 20 amino acid residues of the C-terminal domain (dCRYΔ) and behaves as a light-independent form of

dCRY that is constitutively active in transgenic flies (Dissel *et al.*, 2004), has led to the idea of the C-terminal domain as an essential region for repression of dCRY activity, and for dCRY stability in darkness. A similar but reversed situation to this has been shown to exist in *Arabidopsis*, where expression of the C-terminal domains of both *Arabidopsis* CRY1 and CRY2 as fusion proteins causes a constitutive photomorphogenic (COP) phenotype in transgenic plants expressing the construct (Yang *et al.*, 2000). It seems that both dCRY and *Arabidopsis* CRY1 and CRY2 consist of a functional domain that is regulated by a distinct and *separate* region of the protein; in the case of dCRY, light regulation is conferred by the C-terminal domain upon the rest of the protein, whereas with *Arabidopsis* CRY1 and CRY2 the opposite is true. This is indicative of the apparent situation that the variable C-terminal domains of CRYs from different organsisms seem to be utilised in different ways. Aside from the differences between *Drosophila* and *Arabidopsis*, in *Xenopus* the C-terminal domain of the endogenous CRY has been shown to be essential for nuclear localisation which, in turn, is required for the repressive function of *Xenopus* CRY (Zhu *et al.*, 2003).

Mouse has two versions of CRY: mouse CRYPTOCHROME 1 (mCRY1) and mouse CRYPTOCHROME 2 (mCRY2). In the central clock these mCRYs function as components of the central oscillator (Griffin *et al.*, 1999; Kume *et al.*, 1999; Shearman *et al.*, 2000; Lee *et al.*, 2001) as dCRY is thought to do in peripheral clocks. It has been shown not to be light regulated in its interactions (Griffin *et al.*, 1999), certainly within the central clock.

4.1.2 Aims and objectives

In order to further understand the mechanism by which the C-terminal domain of dCRY regulates its interactions with components of the central clock in *Drosophila*, chimeric proteins were created by exchanging the dissimilar C-terminal domains of dCRY and mCRY1 to produce constructs that possess the photolyase region of dCRY with the C-terminal domain of mCRY1, or vice-versa. The constructs were tested for interaction against the clock components *Drosophila* PER and TIM in a yeast two-hybrid assay, and some of the constructs were used to make transgenic fly lines expressing the constructs, ultimately under the control of the *tim* promoter. mCRY1 was also cloned and tested against *Drosophila* PER and TIM to look for any interaction, and a transgenic fly line

expressing mCRY1 in the pacemaker clock neurons was generated to test if mCRY could function as an equivalent photoreceptor to dCRY. Transgenic fly lines were analysed at the behavioural level using locomotor activity analysis to determine changes in circadian period and light-entrainment and phase-shift analysis to test for changes in light sensitivity, whereby the change in circadian period following a five minute light pulse at night was assessed. At the molecular level Western blot analysis of TIM and CRY-construct levels was used to determine the abundance and phosphorylation status of the proteins over the circadian period by performing the analysis on fly protein extracts taken across the circadian period.

4.2 Materials and Methods

4.2.1 Cloning
All cloning was carried out as described in section 2.1.

4.2.2 Yeast-two hybrid assays

All plate assays and semi-quantitative assays were carried out as described in section 2.6.

4.2.3 Yeast Western blot analysis

Yeast western blot analyses were carried out as detailed in section 2.6.7. However, since it has previously been determined that PER233-685 and TIM are expressed in pJG4-5 as prey in this yeast strain (Rosato et al., 2001), the analysis was conducted only to determine if the bait constructs in pEG202 were expressed.

4.2.4 Generation of transgenic *Drosophila* stocks

Microinjection of *Drosophila* embryos to create transgenic fly lines was carried out as described in section 2.8, using the embryos w^{1118} (see section 2.7). The micro-injection of the two transgenes, *mcryl* and *dcry-mct,* both in pUAST, was performed using the helper plasmid pUCksΠΔ2-3 (Mullins *et al.* 1989).

4.2.4.1 Maintenance and crossing of *Drosophila* stocks

Stocks [w^{1118}], [*yw; tim-GAL4*], [*w; tim-GAL4; cryb*] (called *tim-GAL4* cry^b thereafter), [cry^b] and [*w; UAS-dcry*] (see section 2.7 for details of the fly lines) were already available in the laboratory. These fly lines were crossed against the transgenic lines generated as required for locomotor experiments (see next section for details). All fly stocks were maintained as described in section 2.7, at 18°C.

4.2.4.2 Crossing schemes for locomotor experiments

All locomotor experiments, including pulse experiments, for assessing the effect of over-expression of a transgene in a wild type background, were performed using a line over-expressing the transgene and a non-over-expressing wild type control, heterozygous for the transgene. The over-expressing lines were generated by crossing homozygous *UAS-*

transgene flies to homozygous *tim-GAL4* flies. The control line was generated by crossing homozygous *UAS-transgene* flies with w^{1118}. Also included in each locomotor activity experiment was a control consisting of *tim-GAL4* crossed to w^{1118}, to control for any effects of the *tim-GAL4* transgene on locomotor activity.

All locomotor experiments for assessing the effect of over-expression of a transgene in a cry^b background were performed as described above, but the over-expressing line was generated by crossing the UAS-*transgene* line to cry^b. The non-over-expressing control was generated by crossing homozygous *UAS-transgene* flies with cry^b flies. Also included in each locomotor activity experiment was a control consisting of *tim-GAL4*; cry^b crossed to cry^b, to control for any effects of the *tim-GAL4* transgene on locomotor activity.

Locomotor experiments, using the lines described above, were carried out as described in section 2.10.

4.2.4.3 Analysis of locomotor experiments

Analysis of locomotor activity was carried out as described in section 2.11. The locomotor activities on transgenic fly lines over-expressing the transgene were compared to the controls; hence any changes in locomotor activity in lines over-expressing the transgene are relative to the non-over-expressing controls.

4.2.4.4 Fly collections and protein extractions

Crosses of transgenic fly lines to give over-expressing and control lines were set up as described in section 4.3.3.2, but instead of performing a locomotor experiment, the flies were maintained and collected as described in section 2.12. Collections were taken every four hours starting at ZT 0. When collections were to be made at time points in the subjective night, the vials were wrapped in foil ten minutes before lights-off, so that the flies were not exposed to light during collection.

These collections were analysed by SDS-PAGE analysis and Western blotting, as described in sections 2.13-2.15.

4.3 Results

4.3.1 Cloning

Cloning of all constructs, including PCR and all basic cloning techniques, were performed as described in sections 2.1-2.4. Additional details for creating the chimeric constructs are detailed below. All references to regions of genes are expressed in nucleotide positions relative to the first nucleotide of the coding sequence, which is position 1. All primers are written 5' to 3'.

dcry in pBS was already available. As well as containing the full *dcry* sequence, this construct also contained a Kozak sequence (Kozak 1984) and an N-terminal hemagglutinin tag (HA tag) and will be referred to in subsequent sections as pBS-*HAdcry*. A map of this vector and insert, with restriction sites relevant to subsequent cloning, is shown in **fig. 4.1**. This construct was used extensively in all subsequent cloning. As can be seen from **fig 4.1**, the *dcry* sequence was flanked directly by an *Eco*RI site at the 5' end and an *Xho*I site at the 3' end. An *Eco*RI-*Xho*I digest removed the entire *dcry* coding sequence, while leaving the Kozak sequence (Kozak 1987) and HA tag. Any sequence ligated into the *Eco*RI-*Xho*I sites would therefore have the Kozak sequence and HA tag in the 5' position. Digestion with *Xba*I-*Xho*I removed the *dcry* sequence, HA tag and Kozak sequence.

Cloning *dcry-mct*

The chimeric sequence *dcry-mct* consists of *dcry* 1-1476 with the region of *mcry* 1402-1818, defined here as the C-terminal domain. It was created using three PCR reactions. In the first PCR reactions the *dcry* region 688-1476 was amplified using the 5' primer CCT TAC TTC TGT TGG ATG AGC GTC (*dcry*PD5) and the 3' primer CAC CAT CGG TTT CGG ATA ATG GAC TCC GAT CAG GC (*dcry*PD3). This region of *dcry* contains a unique *Cla*I restriction site at nucleotide position 793. An additional 12 nucleotides were added to the 5' end of the 3' primer. These 12 nucleotides correspond to the last 12 nucleotides of the 5' end of the non-coding strand of the *mcryl* 1402-1818 region, and are underlined here. The *mcryl* 1402-1818 region was amplified using the 5' primer GTC CAT TAT CCG AAA CCG ATG GTG AAC CAT GCT GAG (*mcry*CT5) and the 3' primer GGC TCG AGT TAG TTA CTG CTC TGC CGC TGG AC (*mcry*CT3)

amplifying the region of *mcryl* 1402-1818. 12 nucleotides corresponding to the last 12 nucleotides of the 3' end of the coding strand of the *dcry* region were added to the 5' end of the 5' primer, shown here underlined. An *Xho*I site was added to the 5' end of the 3' primer, shown here in red. Once these two regions had been amplified, they were extracted and purified. The third PCR reaction used the products from the first two amplifications as template, and the primers *dcry*PD5 and *mcry*CT3. The additional nucleotides added to the 3' end of the *dcry* region and the 5' end of the *mcryl* region allowed the two strands of the template sequences to anneal. Amplification could then take place, giving a chimeric, but not yet complete, sequence *dcry-mct*(f). This was then ligated into PDK101, screened for mutations by sequencing, and extracted with a *Cla*I-*Xho*I digest. PBS-*HAdcry* was prepared with a *Cla*I-*Xho*I digest, and the vector separated from the excised portion of *dcry* and purified. The *dcry-mct*(f) sequence was then ligated into this vector giving the full *dcry-mct* chimeric gene. This cloning is illustrated in **fig 4.2.**

Cloning *mcryl*

mcryl was cloned in two separate sections using PCR. Two versions of the first section consisting of the *mcryl* region 1-1066 were amplified. One version (*mcryl*1A) used the 5' primer GGG AAT TCA TGG GGG TGA ACG CCG TGC ACT G with an added *Eco*RI site shown here in red, and the other (*mcryl*1B), used the 5' primer GGT CTA GACAACA TGG GGG TGA ACG CCG TGC ACT G; identical to the first, except that the added restriction site is an *Xba*I site, shown here in red, and the addition of a Kozak sequence, shown here underlined. In both cases the 3' primer used was AAT GGT GGA TCC AGC CCT CCT GAC which includes a *Bam*HI site, shown here in blue. Following PCR both versions of the *mcryl* sequence were cloned into PDK and the sequence screened for mutations. The *mcryl* sequences were then excised with either an *Xba*I-*Bam*HI or an *Eco*RI-*Bam*HI digest as appropriate, and purified.

The second section of *mcry*1, called *mcryl*2, consisted of nucleotides 1012-1818 and was amplified using the 5' primer CGT GGA TTG ACG CCA TCA TGA C and the 3' primer GGC TCG AGT TAG TTA CTG CTC TGC CGC TGG AC. An *Xho*I restriction site was added to the 5' end of the 3' primer, shown here in red. Following the PCR reaction the product was ligated into PDK101 and screened for mutations. The insert was then

extracted with a *Bam*HI-*Xho*I digest. Using a three-way ligation, this was then ligated along with either the excised *mcry1*1A or *mcry1*1B previously described, into pBS-*HAdcry* cut with *Eco*RI-*Xho*I or *Xba*I-*Xho*I respectively (see **fig 4.1**), thus joining the two sections of *mcry1* together to make the entire coding sequence of *mcry1*. In this way, *mcry1* was cloned in pBS with and without an HA tag (see **fig. 4.3**).

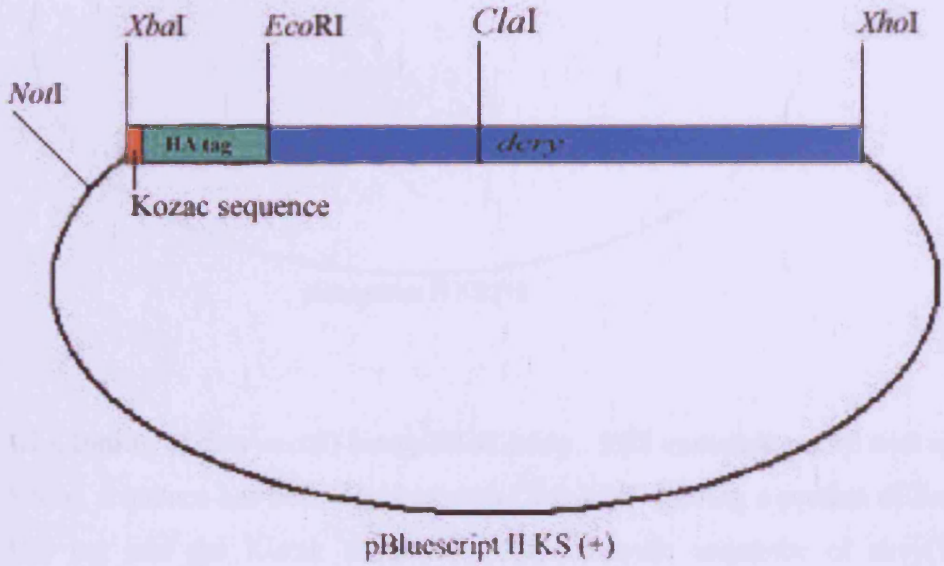


Fig 4.1 Representation of pBS-HA*dcry*: *dcry* in pBluescript II KS (+). The HA tag is labelled and shown here in green. The Kozak sequence is represented in red. Also labelled are the relative positions of several important restriction sites. This map only represents the relative order of the labelled components.

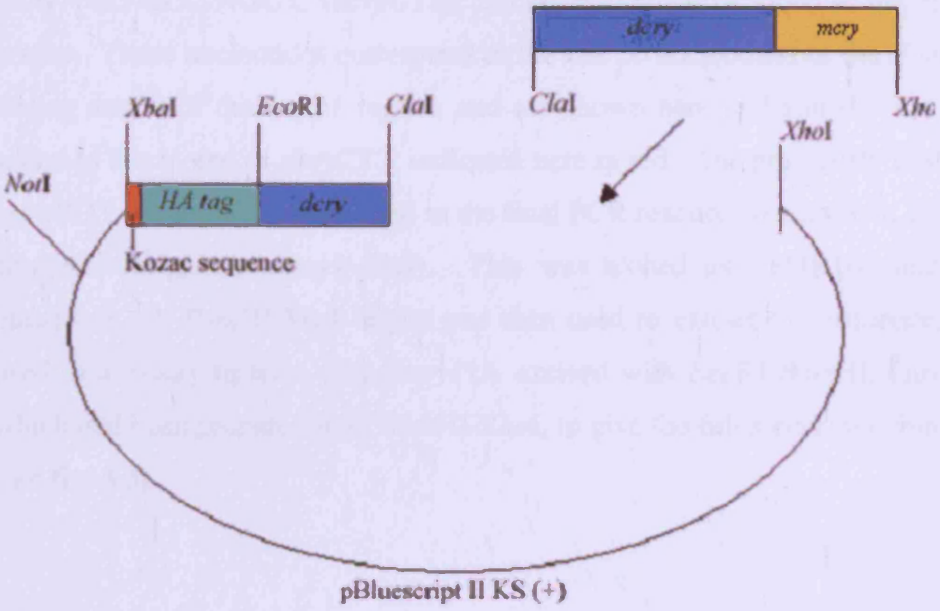


Fig 4.2 Cloning of *dcry-mct*(f) into pBS-*HAdcry*: PBS containing *dcry* with a 5' HA tag and Kozak sequence has been digested with *Cla*I-*Xho*I, leaving a portion of *dcry* (1-793), the HA tag and the Kozak sequence. The chimeric sequence of *dcry*(794-1625)-*mcry1*(1402-1818) which had been excised with a *Cla*I-*Xho*I digestion, was then ligated in via the compatible restriction sites. Important restriction sites are indicated on the diagram. This diagram shows the relative positions of the labelled features.

Cloning *mcry-dct*

mcry-dct is a chimeric sequence consisting of the *mcry1* region 1-1401 region and the *dcry* 1476-1626 region. It was cloned using the same method as *dcry-mct*. The *mcry1* region 1012-1401 was amplified using the 5' primer CGT GGA TTG ACG CCA TCA TGA C (*mcry*PD5) and the 3' primer CAA TGA TCC GCT CGG GGT AAT TAC CTC CTA TCA AAC AC (*mcry*PD3). 13 nucleotides were added to the 5' end of the 3' primer. These nucleotides correspond to the last 13 nucleotides of the 5' end of the coding strand of the *dcry* region of the chimeric sequence, and are shown here underlined. This *mcry1* sequence contains a unique *Bam*HI restriction site at position 1057 (relative to the full sequence). The *dcry* 1476-1626 region of the chimeric sequence was amplified using the 5' primer GGA GTT AAT TAC CCC GAG CGG ATC ATT GAT TTG TCC ATG (*dcry*CT5) and the 3' primer GGC TCG AGT TAA ACC ACC

ACG TCG GCC AGC C (*dcry*CT3). 20 nucleotides were added to the 5' end of the 5' primer. These nucleotides correspond to the last 20 nucleotides of the 3' end of the non-coding strand of the *mcryl* region, and are shown here underlined. An *Xho*I site was added to the 5' end of *dcry*CT3, indicated here in red. The previously described primers *mcry*PD5 and *dcry*CT3 were used in the final PCR reaction to amplify a chimeric, but not complete, sequence, *mcryl-dct*(f). This was cloned into PDK101 and screened for mutations. A *Bam*HI-*Xho*I digest was then used to extract the sequence, and this was used in a 3-way ligation with *mcryl*1A excised with *Eco*RI-*Bam*HI, into pBS-*HAdcry*, which had been prepared with *Bam*HI-*Xho*I, to give the full *mcryl-dct* chimeric sequence (see **fig. 4.3**).

Cloning *mCRY1Δ*

*mcryl*Δ is a truncated form of the *mcryl* gene, consisting of the *mcryl* region 1-1401. PCR was used to amplify the *mcryl* sequence 1012-1401 using the 5' primer CGT GGA TTG ACG CCA TCA TGA C and the 3' primer GGC TCG AGT TAG GGG TAA TTA ACT CCT ATC AAA CA. An *Xho*I site was added to the 3' primer shown here in red. This amplified sequence contains a *Bam*HI site at position 1057. The PCR product was cloned into PDK101 cut with *Xcm*I and screened for unwanted mutations. The correct sequence was then excised with a *Bam*HI-*Xho*I digest giving the *mcryl* fragment *mcryl*Δ(f). This was then ligated in a three-way ligation with *mcryl*1A (*Eco*RI-*Bam*HI excised) into the pBS-*mcryl*1A construct prepared with an *Eco*RI-*Xho*I digest. This gave the complete *mcryl*Δ sequence. See **fig 4.3**.

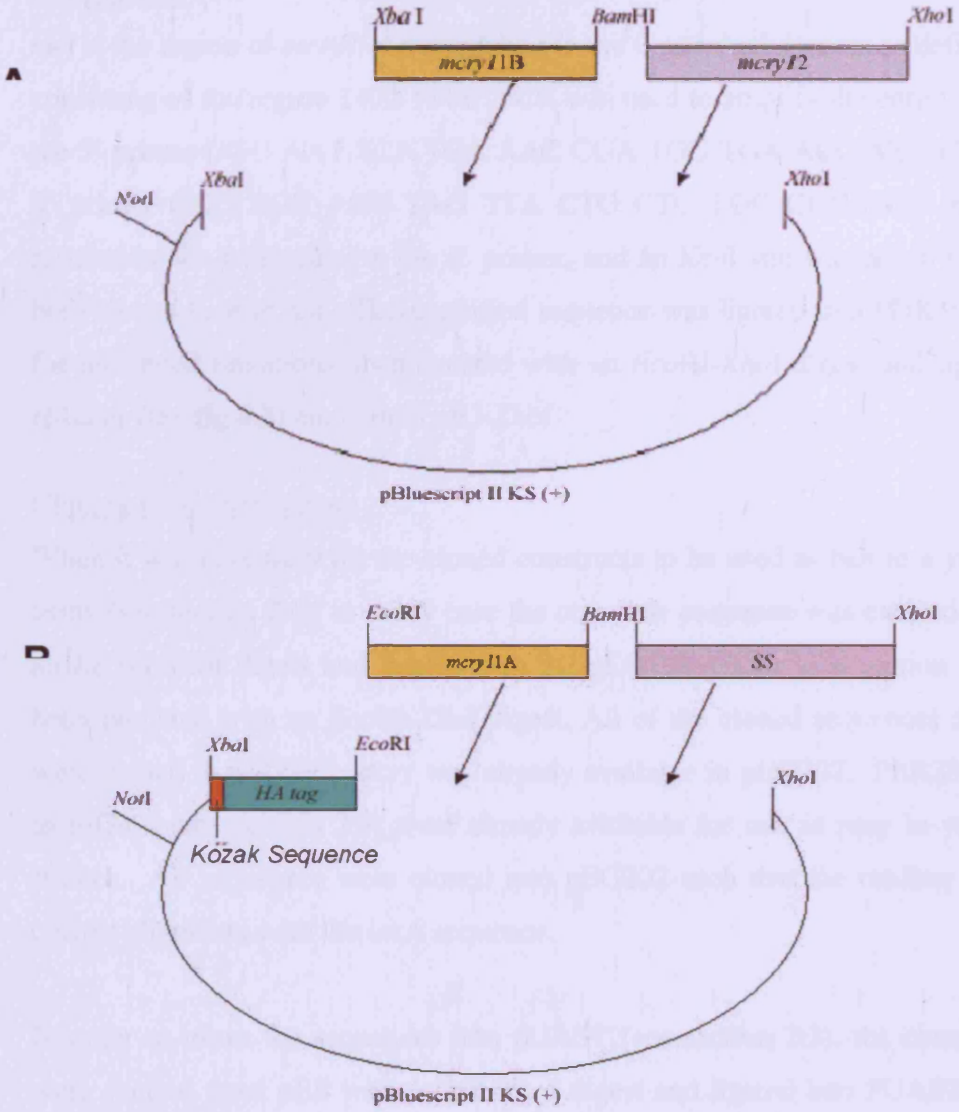


Fig 4.3 Cloning of *mcry1*-based constructs into pBS: A. pBS was prepared with *Xba*I-*Xho*I. *mcry1* 1-1057 (*mcry1*1B) excised with *Xba*I-*Bam*HI and *mcry1* 1058-1818 (*mcry1*2) excised with *Bam*HI-*Xho*I were ligated into the vector in a three-way ligation, giving the full *mcry1* sequence. **B**. pBS-*HAdcry* was prepared with *Eco*RI-*Xho*I, excising the dcry sequence, but leaving a Kozak sequence and HA tag. *mcry* 1-1057 (*mcry1*1A) excised with *Eco*RI-*Bam*HI was ligated in a three-way ligation with a second-sequence excised with *Bam*HI-*Xho*I. This second sequence (*SS*) was either *mcry1*2, *mcry1-dct*(f) or *mcry1*Δ(f). All relevant features and restriction sites are labelled. The diagrams show only the relative positions of the labelled features.

Cloning *mct*

mct is the region of *mcry1* corresponding to the C-terminal domain as defined previously, consisting of the region 1402-1818. PCR was used to amplify the entire sequence, using the 5' primer GGG AAT TCA TGA AAC CGA TGG TGA ACC ATG CTG AG and the 3' primer GGC TCG AGT TAG TTA CTG CTC TGC CGC TGG AC. An *Eco*RI restriction site was added to the 5' primer, and an *Xho*I site was added to the 3' primer, both shown here in red. The amplified sequence was ligated into PDK101 and screened for unwanted mutations, then excised with an *Eco*RI-*Xho*I digest and ligated into pBS-*HAdcry* (see **fig 4.1**) cut with *Eco*RI-*Xho*I

Cloning into other vectors

When it was necessary for the cloned constructs to be used as bait in a yeast two-hybrid assay (see section 2.6), in every case the complete sequence was excised from pBS with an *Eco*RI-*Xho*I digest and ligated into the pEG202 vector (see section 2.3) which had been prepared with an *Eco*RI-*Xho*I digest. All of the cloned sequences described above were cloned in pEG202. *dcry* was already available in pEG202. PER233-685 and TIM in pJG4-5 (see section 2.3) were already available for use as prey in yeast-two hybrid studies. All sequences were cloned into pEG202 such that the reading frames were in correct alignment with the *lex*A sequence.

In order to move the sequences into pUAST (see section 2.3), the complete sequences were excised from pBS with a *Not*I-*Xho*I digest and ligated into PUAST cut with *Not*I-*Xho*I. This put the sequence in the correct orientation for transcription. Complete *mcry1*, *HAmcry1* and *dcry-mct* were ligated into PUAST.

4.3.2 Analysis and comparison of dCRY and mCRY1/mCRY2 sequences

In order to first determine which of the two mCRY proteins would become the chimeric partner of dCRY, a comparison using the protein-protein Blast comparison tool (Tatiana and Madden, 1999) was run for both mCRY1/2 primary sequences against the dCRY primary sequence. This revealed that mCRY1 bears slightly greater homology to dCRY than mCRY2 (see appendix 1). The Blast comparison between dCRY and mCRY1 revealed that all homology occurs within the region dCRY 1-516 and mCRY1 1-491. Since the regions of dCRY 517-542 and mCRY1 492–606 showed no homology, these

were designated the C-terminal domains for the purposes of this project, referring to the regions that are unique between both proteins. For dCRY, this C-terminal domain contains the region believed to be important for light repression (see section 4.1 for discussion), and therefore light regulation of dCRY, as revealed by experiments using dCRYΔ (as discussed in section 4.1). It was therefore decided to exchange the C-terminal domains as defined by the Blast comparison.

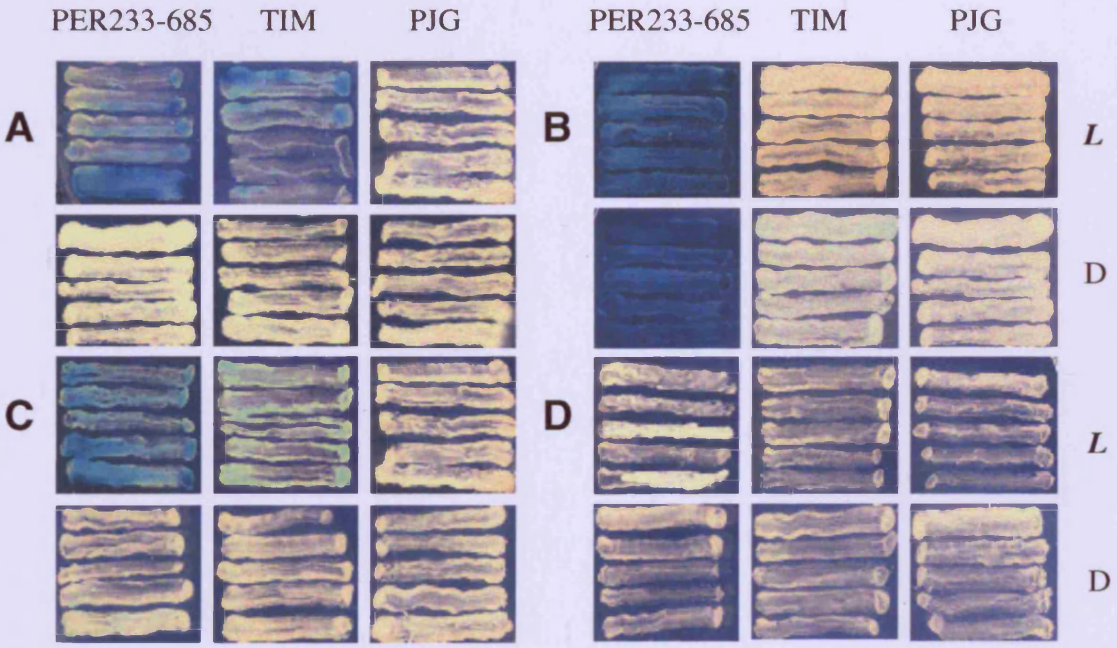
In order to determine the point of exchange of the C-terminal domains, a secondary structure prediction was run on both dCRY and mCRY1 using a secondary structure prediction programme (Rost *et al.*, 2004) which revealed that the N-terminal end of the defined dCRY and mCRY1 C-terminal domains occur in a region of predicted secondary structure in both cases. In order to minimise disruption to the protein structures, the site of the exchange was moved further towards the N-terminal, into the photolyase domain. However, the site of exchange was selected such that the site was the equivalent point in both proteins, as determined by the Blast alignment. This point was amino acid position 492/493 in dCRY and position 467/468 in mCRY1. Therefore, for the ease of reference, the C-terminal domain was defined as the region of dCRY 493-542 (dCT) and the region of mCRY1 468-606 (mCT), even though these regions show similarity between the first 24 amino acid residues. The primers were then designed to clone the chimeric sequences to give proteins with these regions exchanged between dCRY and mCRY1.

4.3.2 Yeast assays

Initially, the following constructs mCRY1, mCRY-dCT and dCRY-mCT were tested in an X-Gal plate assay as bait against PER233-685 and TIM in pJG4-5 as prey, using empty pJG4-5 as a control (as described in section 2.6).

The results show that dCRY interacts with both PER233-685 and TIM, but only when exposed to light during the assay. Hence it can be said that these interactions are light-dependent. mCRY1 interacts with PER233-685 in a light-independent manner, and also light-independently with TIM, but in a very weak manner that is only just detectable. mCRY-dCT does not interact with either PER233-685 or with TIM in either light or darkness. dCRY-mCT interacts with PER233-685 and TIM in a light-dependent manner, in a similar manner to dCRY (see **fig 4.4**).

Fig 4.4 Yeast plate assay results for dCRY/mCRY1 constructs tested against PER233-685 and TIM: A= dCRY, **B=** mCRY1, **C=**dCRY-mCT, **D=**mCRY-dCT. All constructs were tested as bait against PER233-685 and TIM, using empty pJG4-5 as a control, in light and dark conditions. Protein interaction is indicated by a blue colour. The L and D at the far left of each row signify the light conditions the assays were grown in: L=light, D=dark. All yeast assays were repeated using independently transformed yeast strains.



In order to determine whether it was the dCRY 1-492 or the mCRY-CT region that was interacting with PER233-685 and TIM, dCRY 1-492 (dCRYΔII), mCRY 1-467 (mCRY1ΔII) and mCRY1 468-606 (mCT) were all tested in an X-Gal plate assay as bait against PER233-685 and TIM. mCRY1ΔII failed to interact with either PER233-685 or TIM in either light or dark conditions, and the same was true for dCRYΔII. mCT interacted with both PER and TIM in a light-independent manner (see **fig 4.5**).

Semi-quantitative plate assays:

A semi-quantitative yeast assay was performed using the following constructs as bait: dCRY, dCRY-mCT, mCT. PER233-685 was used as prey. Since mCRY-dCT, mCRYΔII and dCRYΔII had failed to interact in the initial plate assay, it was not considered useful to repeat them for this semi-quantitative assay. The results are shown in **fig 4.6** and the relative pixel densities are given in **table 4.1**. These results indicate that dCRY-mCT has a stronger interaction with PER than dCRY which in turn has a stronger interaction than mCT (all difference are statistically significant by ANOVA).

Fig 4.6. Semi-quantitative yeast assay results for all dCRY and mCRY1 constructs: dCRY, dCRY-mCT and mCT were tested as bait against PER233-685 and TIM. **A.** The results for the constructs challenged with PER233-685. **B**. The results for the constructs challenged with TIM. In both cases: 1=dCRY, 2=dCRY-mCT, 3=mCT. The results were performed at least 3 times for each test, using independently grown liquid yeast cultures, and all assays were repeated once more using independently transformed yeast cultures. All assay plates were exposed to constant light during growth.

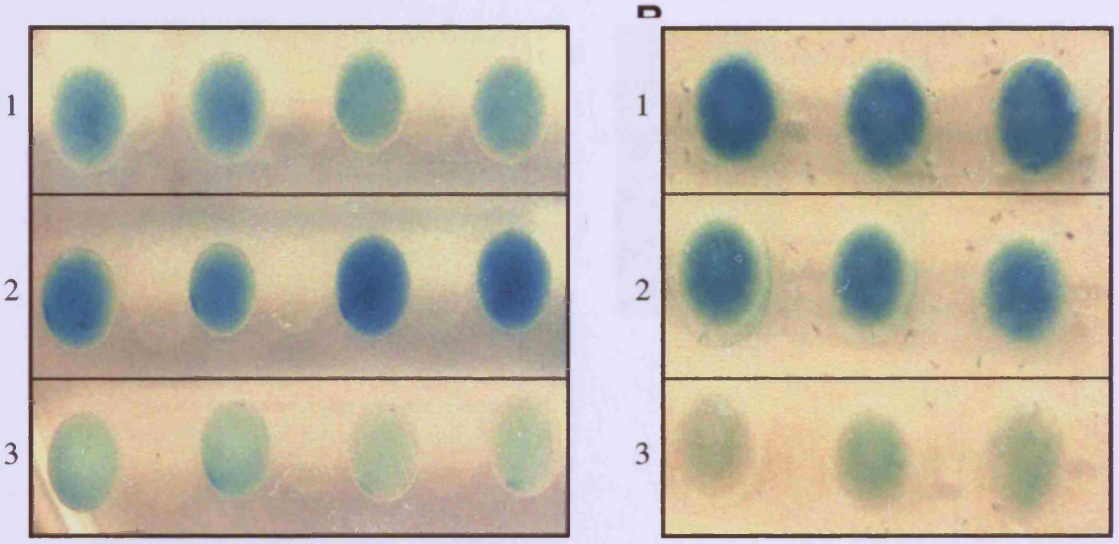


Fig 4.5 Yeast plate assay results for truncated mCRY1 constructs tested against PER(233-685) and TIM: A=mCT, B=mCRY1, C= dCRYΔII D=dCRY and E=mCRYΔII were tested as bait against PER233-685 and TIM, using empty pJG4-5 as a control, in both light and dark conditions. Protein interaction is indicated by a blue colour. The L and D at the far left of each row signify the light conditions the assays were grown in: L=light, D=dark. All yeast assays were repeated using independently transformed yeast strains.

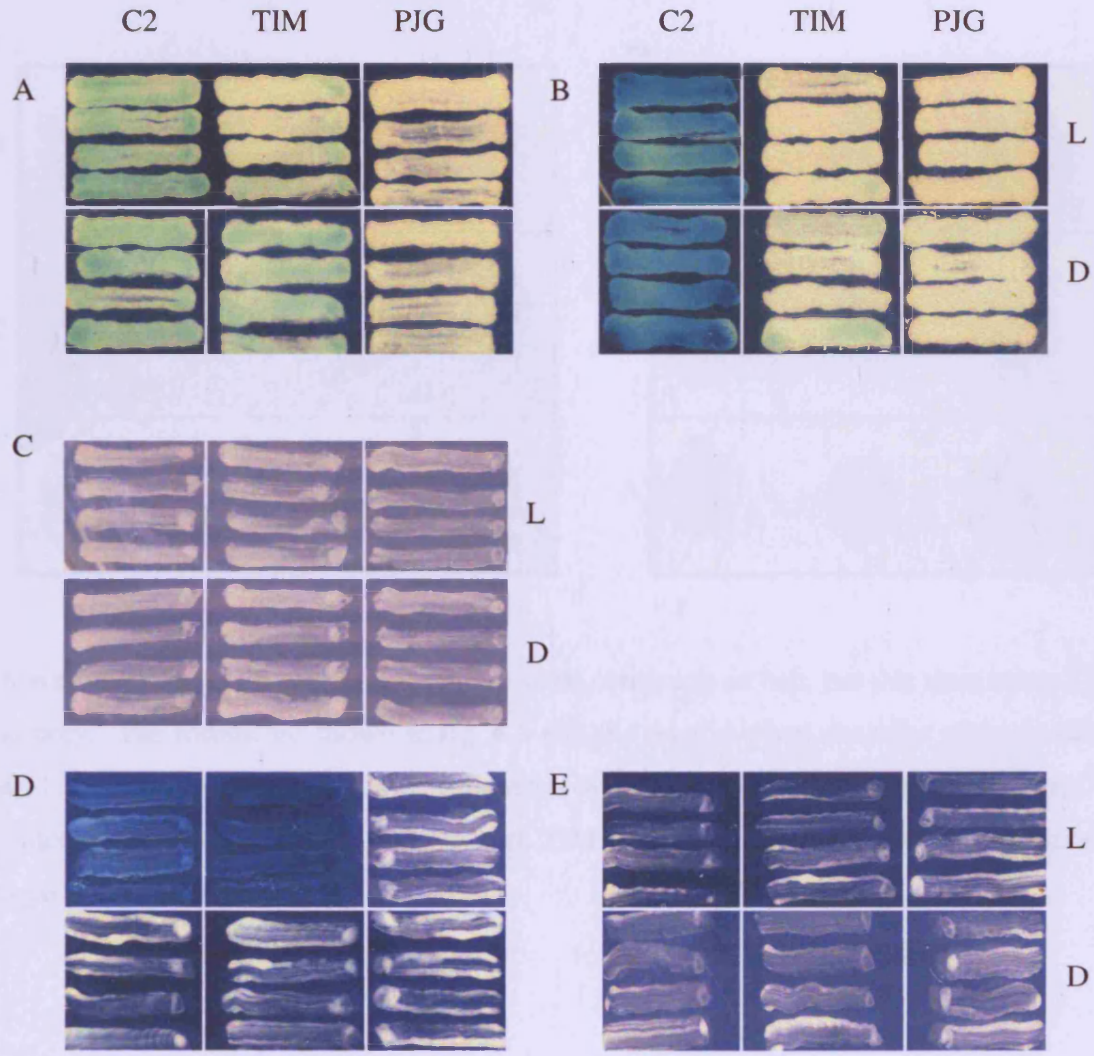
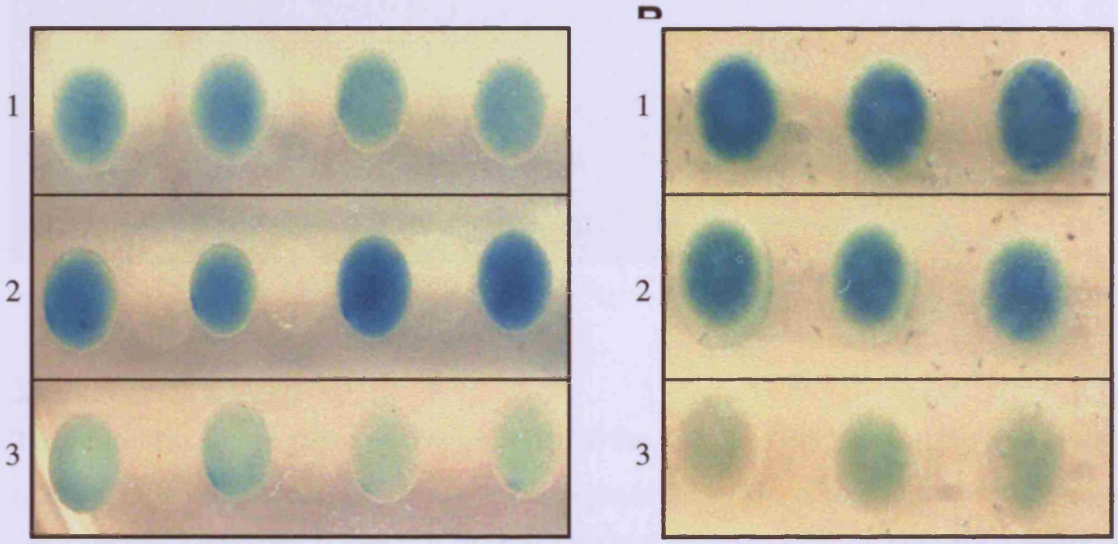


Fig 4.6 Semi-quantitative yeast assay results for all dCRY and mCRY1 constructs: dCRY, dCRY-mCT and mCT were tested as bait against PER233-685 and TIM. **A.** The results for the constructs challenged with PER233-685. **B**. The results for the constructs challenged with TIM. In both cases: 1=dCRY, 2=dCRY-mCT, 3=mCT. The results were performed at least 3 times for each test, using independently grown liquid yeast cultures, and all assays were repeated once more using independently transformed yeast cultures. All assay plates were exposed to constant light during growth.



A similar assay was carried out using the same constructs as bait, but this time using TIM as prey. The results are shown in **fig. 4.6** and the relative pixel densities given in **table 4.1**. These results show that dCRY interacts with TIM more strongly that dCRY-mCT, which in turn interacts more strongly with TIM than mCT (all difference are statistically significant by ANOVA).

Table 4.1: Pixel density analysis of yeast-two hybrid semi-quantitative assays: these values are the relative pixel densities for each colony of the semi-quantitative assays. Values for all constructs versus PER233-685 can be compared, as can all values for the constructs against TIM, but the two sets cannot be compared with each other as they originate from different assay plates and different images.

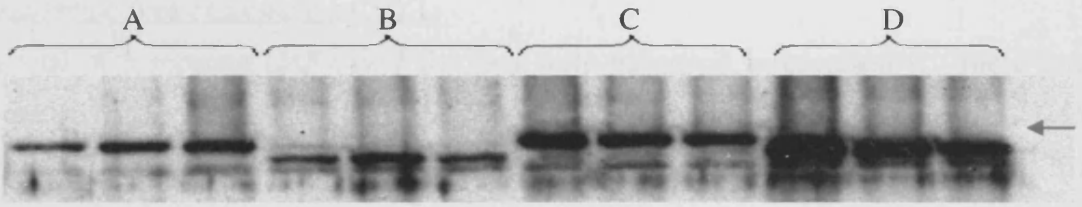
	1	2	3	4	Average±Sem
dCRY vs PER233-685	83.34	84.37	74.22	73.41	**78.8±5.82**
dCRY-mCT vs PER233-685	122.06	98.85	140.05	183.33	**136.07±35.73**
mCT vs PER233-685	63.80	63.52	59.79	58.87	**61.5±2.53**
dCRY vs TIM	156.80	153.36	155.37	155.35	**155.22±1.41**
dCRY-mCT vs TIM	117.25	119.91	118.5	112.21	**116.97±3.35**
mCT vs TIM	64.04	63.70	52.22	66.62	**61.65±6.41**

Yeast Western analysis

To ensure that any negative results were due to the failure of the bait and prey proteins to interact, rather than a lack of expression or degradation of the constructs, a yeast Western analysis was conducted for mCRY-dCT and mCRY1ΔII (see section 2.6.7 for details). In each case, three independent samples were taken from independently grown cultures, each expressing the construct in pEG202 and also containing PER233-685 in pJG4-5 and the reporter vector pSH18-34. Therefore, the Western analysis was performed in triplicate on yeast cultures identical to those used in the assays previously described. Anti-*lex*A (Upstate, 1: 5000) was used as the primary antibody. The results, shown in **fig. 4.7**, indicate that all proteins are expressed in yeast to a level approximately equal to or greater than the level of the positive control, in this case dCRY, particularly mCRY1ΔII, which is present in relatively large quantities; although there is some variation. Therefore, the negative results obtained for the interactions of both proteins are most likely due to a lack of interaction with the prey proteins.

Figure 4.7 Western analysis of constructs expressed in yeast: the results of a Western analysis performed on yeast cultures expressing constructs in pEG202, as fusions with lexA. The constructs were as follows: A=dCRY, B=dCRYΔII, C=mCRY-dCT,

D=mCRY1ΔII. The primary antibody used in each case was anti-lexA. For each construct three independent samples, each from an independently grown culture, were analysed. The red arrow indicated the position of the marker band corresponding to 91KD.



Summary

The combined results of the plate and semi-quantitative assays indicate that dCRY interacts with PER and TIM in a light dependent manner, as discussed in section 4.1. mCRY also interacts strongly with PER233-685 , but very weakly with TIM. Since mCT but not mCRYΔII interacts with PER233-685 (and TIM), this suggests the mCRY1 interaction domain is located in the region defined as mCT, and therefore the region of dCRY-mCT responsible for interacting with TIM and PER is the mCT part of the chimeric protein. Since mCT interacts with TIM and PER light-independently, this would suggest that the dCRY region of the chimeric, mediates the light regulation. The fact that dCRYΔII does not interact with either PER233-685 or TIM is not surprising, as this truncation removes part of the putative flavin binding domain, and flavin binding has been shown to be essential for dCRY function (Froy *et al.*, 2002). None-the-less, it was thought it would be worth testing this construct in a yeast assay, but the lack of interaction may be due to several factors including loss of flavin binding, and not necessarily due to the absence of a binding domain.

4.3.3 Activity analysis of transgenic fly lines

Activity studies

Two sets of transgenic fly lines were created using the method described in section 2.8: one set with the insertion *UAS-mcryl* and the other with *UAS-dcry-mct.*

Transgenic flies expressing mCRY1

A total of 5 separate *UAS-mcryl* fly lines were generated independently. These lines were given the designation *UAS-HAmcryl.1*, *UAS-HAmcryl.2*, *UAS-HAmcryl.3*, *UAS-HAmcryl.4*, *UAS-HAmcryl.5* (the numbers are arbitrary to distinguish the lines). The insert was mapped to the second chromosome for the lines *UAS-HAmcryl.1, UAS-HAmcryl.2*, *UAS-HAmcryl.3*, *UAS-HAmcryl.5* and the third chromosome for the line *UAS-HAmcryl.4.* Inverse PCR was attempted to map the inserts further, but was not successful, hence the position within the chromosomes is not known.

Effect of over-expressing *mCRY1* on the circadian period

The *UAS-mcryl* fly lines were crossed to give over-expressing lines as described in section 4.2.3.2 and the activity cycles monitored and measured as described in 2.10 All six over-expressing fly lines entrained normally to a light:dark regime, giving a bimodal pattern of activity, with a morning peak of increased activity following lights-on and an evening peak of increased activity anticipating lights off. This pattern was seen with all the fly-lines, the data for *UAS-HAmcryl.1* is shown in **fig 4.8** as a representation. It can be seen from the figure that the flies over-expressing mCRY1 (*yw;tim-GAL4/UAS-HAmcryl.1*) appear to show a faster decline in activity following the morning peak at lights-on, as compared to the control w^{1118};*UAS-HAmcryl.1/+*;, as well as an earlier anticipation of lights-off (the activity prior to the evening peak). However, the *tim-GAL4* crossed with w^{1118} control also has these features; hence it is reasonable to say that they are a feature of the *tim-GAL4* line and not of over-expressing mCRY1.

Similar analysis reveals that all the lines are rhythmic in constant darkness, as are the controls (see **fig 4.9**), but with no difference in the activity pattern. In constant light, all lines become arrhythmic (see **fig 4.10**). Interestingly, the fly lines over-expressing mCRY1 appear to become arrhythmic faster than the controls, including the *tim-GAL4*

crossed with w^{1118} line, which shows distinguishable peaks of activity 1-2 days into the constant light period. These may indicate a greater photosensitivity in these fly lines. Since this occurs in all over-expressing lines tested, but not in the controls, it is most likely due to the expression of mCRY1.

Spectral analysis to determine circadian period

Spectral analysis results for all lines UAS-*mcry1* are given in **table 4.2**. From this it can be seen that in a 12:12 light:dark regime, all fly lines entrain to an approximate 24 hour period. However, in constant darkness, all fly lines over-expressing mCRY1 show an increase in period relative to the controls. Spectral analysis of flies from a constant light regime simply showed the flies to be arrhythmic, as indicated by the activity plots.

Table 4.2 The circadian periods of all *UAS-mcry1* over-expressing and control lines in a light:dark regime: The numbers represent the average periods of the UAS-*mcry1* lines crossed with either *tim-GAL4* to give over-expressing lines, or with w^{1118} to give control lines. All lines were entrained in a 12:12 light:dark regime, and the period determined by spectral analysis of the activity. *tim-GAL4* crossed with w^{1118} represents a control line. The number of flies (N) used in each case is given under each value.

Genotype	n	Period ±SEM
yw;tim-GAL4/UAS-HAmcry1.1	33	24.04±0.12
yw;tim-GAL4/UAS-HAmcry1.2	19	23.98±0.09
yw;tim-GAL4/UAS-HAmcry1.3	14	24.04±0.12
yw;tim-GAL4/+;UAS-HAmcry1.4/+	31	23.96±0.08
yw;tim-GAL4/UAS-HAmcry1.5	18	24.07±0.11
w^{1118};+/*UAS-HAmcry1.1*	39	23.96±0.11
w^{1118};+/*UAS-HAmcry1.2*	22	23.98±0.13
w^{1118};+/*UAS-HAmcry1.3*	11	23.95±0.07
w^{1118};+/+;*UAS-HAmcry1.4/+*	20	23.96±0.12
w^{1118};+/*UAS-HAmcry1.5*	10	24.00±0.12
yw;tim-GAL4/+;+/+	22	24.07±0.10

[Raw data in appendix 2]

Table 4.3 The circadian periods of all UAS-*mcry1* lines in constant darkness: The numbers represent the average periods of the UAS-*mcry1* lines crossed with either *tim-GAL4* to give over-expressing lines, or with w^{1118} to give control lines. All lines were entrained in a 12:12 light:dark regime then left in constant darkness, and the period determined by spectral analysis of the activity. *tim-GAL4* crossed with w^{1118} represents a control line. The number of flies (n) used in each case is given under each value.

Genotype	n	Period ±SEM
yw;tim-GAL4/UAS-HAmcry1.1	51	24.65±0.61
yw;tim-GAL4/UAS-HAmcry1.2	20	24.26±0.54
yw;tim-GAL4/UAS-HAmcry1.3	28	24.64±0.41
yw;tim-GAL4/+;UAS-HAmcry1.4/+	28	24.56±0.53
yw;tim-GAL4/UAS-HAmcry1.5	35	24.51±0.5
w^{1118};+/*UAS-HAmcry1.1*	42	24.11±0.49
w^{1118};+/*UAS-HAmcry1.2*	27	24.16±0.49
w^{1118};+/*UAS-HAmcry1.3*	28	24.05±0.37
w^{1118};+/+;*UAS-HAmcry1.4/+*	28	24.2±0.51
w^{1118};+/*UAS-HAmcry1.5*	27	24.12±0.42
yw;tim-GAL4/+	29	24.35±0.49

[Raw data in appendix 3]

Statistical analysis of spectral data

Initially a statistical analysis was performed using the spectral data for each individual fly, comparing all the over-expressing lines with each other to determine if there were any significant differences due to line effects. The analysis showed no significance difference in period between the over-expressing lines although the result was border line ($F_{4,157}$=2.18; P=0.073). However, the *tim-GAL4/UAS-HAmcry1.2* over-expressing line shows a shorter period than the other four over-expressing lines (**table 4.3**), and although this period is longer than the w^{1118}/*UAS-HAmcry1.2* control line, the difference is less than the equivalent for all the other lines, and it is shorter than the period of the *tim-*GAL4 crossed with w^{1118} control. Comparing this line with the *tim-GAL4* crossed with

w^{1118} control reveals no significant difference between the two sets of data ($F_{1,47}$=0.345; p=0.560). Therefore, it is likely that this line shows a reduced over-expression of the *UAS-mcryl* construct due to a position effect and the decision was made to exclude this fly line from further analysis. With the removal of the *UAS-HAmcryl.2* data the comparison of the remaining over-expressing lines was repeated ($F_{4,137}$=1.755; P=0.142). A similar comparison was done between the control lines, including *tim-GAL4* crossed w^{1118}; this revealed no significant differences between the different lines used as controls ($F_{45,175}$=1.381; P=0.233). The over-expressing lines were then grouped together, excluding *UAS-HAmcryl*.2 data, to form one group of data representing flies that over-express mCRY1, and the same was done for the control lines. These two groups were then compared and found to be significantly different ($F_{1,321}$=61.125; P<<0.001), indicating that the observed lengthening of period is a result of the expression of mCRY1.

Since the 4 out of the 5 fly lines tested all show a similar increase in circadian period, and *UAS-HAmcryl*.2 does not, the most probable explanation is that this is due to a line effect of *UAS-HAmcryl.2*, likely due to the position of the insertion. Since this data is apparently different, it was omitted when calculating the average period increase. Using the spectral data of all the over-expressing lines (i.e. not the averages of the individual lines) (n=142) the period length is 24.60 hours, compared with the mean period length of the controls (n=181), which is 24.16. This gives a total increase of 25.8 minutes.

In summary, over-expressing mCRY1 appears to cause an increase of approximately 26 minutes in flies kept in constant darkness, as compared to flies not over-expressing mCRY1.

Light pulse locomotor activity experiments

Due to time constraints, only two *UAS-mcryl* lines were tested, these were *UAS-HAmcryl.1* and *UAS-HAmcryl.3*, chosen because they had shown the greatest increases in period, hence were most likely to demonstrate any effect of over-expressing mCRY1. The data, summarised in table 4.4, reveals a phase delay in all flies pulsed at ZT 15. However, both overexpressing and control lines show this phase shift, indicating that there is no effect due to the expression of mCRY1. This was tested using a univariate ANOVA. Firstly the data for the lines over-expressing were compared with each other,

revealing no significant difference ($F_{1,45}$=3.238; P=0.079). The pulsed control lines, the control lines, were then compared with each other and were not significantly different ($F_{2,62}$=1.009; p=0.370). The over-expressing lines, as one group, were then compared to the control lines, as one group, and this revealed a significant difference ($F_{1,110}$=26.570; P<<0.01), indicating that the increase in delay is due to the over-expression of mCRY1.

For the flies pulsed at ZT 21, there is a distinct phase advance for the *UAS-HAmcry1.1* and *UAS-HAmcry1.3* control lines, that is not seen in the equivalent over-expressing lines, with *UAS-HAmcry1.1* over-expressing showing a much smaller phase advance, and *UAS-HAmcry1.3* over-expressing showing an apparent phase delay. *tim-GAL4* crossed with w^{1118} control line also shows a phase advance, but this reduced compared to the other control lines. The differences between the groups were tested as before. The data for the control groups for *UAS-HAmcry1.1* and *UAS-HAmcry1.3* showed no significant difference ($F_{1,40}$=2.825; p=0.101), however, when this data was pooled as one group and compared to the *tim-GAL4* crossed with w^{1118} control line data, there was a significant difference between the two sets ($F_{1,49}$=7.155; P=0.010), indicating that the *tim-GAL4* line has an effect on the phase shift. Comparing the two over-expressing lines separately with *tim-GAL4* crossed with w^{1118}, *UAS-HAmcry1.1* over-expressing was found to not be significantly different ($F_{1,31}$=0.997; P=0.326) whereas *UAS-HAmcry1.3* over-expressing was found to be significantly different ($F_{1,24}$=7.477; P=0.012). A comparison of the lines *UAS-HAmcry1.1* over-expressing and *UAS-HAmcry1.3* over-expressing also revealed a significant difference between the two sets of data.

Table 4.4: Phase shifts resulting from a 5 minute light-pulse on transgenic fly lines expressing mCRY1: *UAS-HAmcry1.1* and *UAS-HAmcry1.3* represent two transgenic fly lines containing mCRY1. Crosses with *tim-GAL4* represent lines over-expressing mCRY1 while crosses with w^{1118} represent non-over-expressing controls. The final column represents the control that lacks the mCRY1 transgene. ZT 15 = light pulse 3 hours after lights off. Unpulsed = no light pulse given. ZT 21 = light pulse given 3 hours before lights on. n = numbers of flies analysed.

	yw;tim-GAL4/UAS-mcry1.1	*yw;tim-GAL4/UAS-mcry1.3*	*w^{1118};UAS-mcry1.1/+*	*w^{1118};UAS-mcry1.3/+*	*yw;tim-GAL4*
ZT 15	396.04	397.37	393.29	393.24	396.64
N	28	24	19	24	11
Unpulsed	388.63	389.21	386.21	387.56	388.89
N	19	19	19	16	16
Shift/hours	-7.41	-8.16	-7.08	-5.68	-7.75
ZT 21	388.08	390.56	384.1	385.36	387.33
N	24	20	16	22	9
Unpulsed	388.63	389.21	386.21	387.56	388.89
N	19	19	19	16	16
Shift/hours	0.55	-1.35	2.11	2.2	1.56

The pulse experiments indicate that mCRY1 over-expression has an effect on the phase response of the flies. A pulse at ZT 15 causes a delay in the phase, which is enhanced in the *UAS-mcry*1 over-expressing lines. A pulse at ZT 21, causes a phase advance in pulsed flies, but this advance is either attenuated or becomes a delay in flies over-expressing mCRY1.

Transgenic flies expressing mCRY1 in a *cry*^{b} background

Since *dcry* is found on the third chromosome, to get fly lines that are homozygous *mcry1*, and homozygous *cry*^{b}, fly lines that had the UAS-*mcry1* inserted on the second chromosome were used, thus a recombination event was not be required. Since there were three lines with *mcry1* on the second chromosome, it was felt that there were sufficient fly lines to test the effects of expressing mCRY1 in a *cry*^{b} background, and that if there were line effects, it was very unlikely they would be the same for all three lines. Hence *UAS-HAmcry1.1*, *UAS-HAmcry1.3* and *UAS-HAmcry1.4* were crossed into a *cry*^{b} background (*UAS-HAmcry*;*cry*^{b}).

Effect of expressing mCRY1 on the circadian period in *cry*^{b} flies

UAS-mcry1;*cry*^{b} lines were crossed to give over-expressing lines as describe in section 4.2.3.2. Locomotor activity experiments reveal that the *UAS-mcry1* transgenic lines in a *cry*^{b} background show a normal pattern of activity in a 12:12 hour light:dark regimen, with a morning peak of activity following lights on, and an evening peak of activity anticipating lights off (see **fig 4.11**). In constant darkness they show a 24 hour activity rhythm, although the separate morning and evening peaks of activity are lost and replaced with a single peak of activity (see **fig 4.12**). Over-expressing dCRY in a *cry*^{b} background results in arrhythmicity in constant light, however this does not occur for the lines over-expressing mCRY1, indicating that mCRY1 cannot rescue dCRY behaviour (see **fig 4.13**).

Table 4.5 The circadian periods of all *UAS-mcry1* lines in a cry^b background constant darkness: The numbers represent the average periods of the *UAS-mcry1* lines crossed with either *tim-GAL4* to give over-expressing lines, or with w^{1118} to give control lines. All lines were entrained in a 12:12 light:dark regime then left in constant darkness, and the period determined by spectral analysis of the activity. *tim-GAL4* crossed with w^{1118} represents a control line. The number of flies (n) used in each case is given under each value. cry^b /cry^b

Genotype	N	Period ±SEM
yw;tim-GAL4/UAS-HAmcry1.1;cry^b /cry^b	23	24.39±0.28
yw;tim-GAL4/UAS-HAmcry1.3;cry^b/cry^b	24	24.44±0.25
yw;tim-GAL4/UAS-HAmcry1.4;cry^b/cry^b	22	24.39±0.25
yw;tim-GAL4/UAS-dcry;cry^b/cry^b	16	24.36±0.36
w;UAS-HAmcry1.1/+;cry^b/cry^b	31	24.30±0.42
w;UAS-HAmcry1.3+;cry^b/cry^b	13	24.19±0.37
w;UAS-HAmcry1.4/+; cry^b/cry^b	12	24.26±0.33
yw;tim-GAL4/+; cry^b /cry^b	14	24.34±0.28

[Raw data in Appendix 4]

Table 4.6 The circadian periods of all *UAS–mcry1* lines in a cry^b background in a constant light regime: The numbers represent the average periods of the *UAS-mcry1* lines crossed with either *TG4*; cry^b to give over-expressing lines, or with w^{1118} to give control lines. *Arrh*=arrhythmic. All lines were entrained in a 12:12 light:dark regime, and the period determined by spectral analysis of the activity. *TG4* crossed with w^{1118} represents a control line. The number of flies (n) used in each case is given under each value.

Genotype	N	Period ±SEM
yw;tim-GAL4/UAS-HAmcry1.1;cry^b/cry^b	28	24.64±0.72
yw;tim-GAL4/UAS-HAmcry1.3;cry^b/cry^b	35	24.66±0.79
yw;tim-GAL4/UAS-HAmcry1.4;cry^b/cry^b	22	24.13±0.32
yw;tim-GAL4/UAS-dcry;cry^b/cry^b	20	Arrhythmic
W;+/UAS-HAmcry1.1;cry^b/cry^b	44	24.84±0.66
w;+/UAS-HAmcry1.3;cry^b/cry^b	28	24.92±0.84
w;+/UAS-HAmcry1.4;cry^b/cry^b	26	24.40±0.63
yw;tim-GAL4/+;cry^b/cry^b	13	24.12±0.26

[Raw Data in Appendix 5]

Transgenic flies expressing dCRY-mCT

A total of 7 independent fly lines with the transgene UAS-*dcry-mct* were generated. These were designated *UAS-HAdcry-mct1*, *UAS-HAdcry-mct2*, *UAS-HAdcry-mct3*, *UAS-HAdcry-mct4*, *UAS-HAdcry-mct5*, *UAS-HAdcry-mct6*, *UAS-HAdcry-mct7*. *UAS-HAdcry-mct2* and *UAS-HAdcry-mct6* were mapped to the second chromosome, *UAS-HAdcry-mct1* and *UAS-HAdcry-mct7* were mapped to the third chromosome, and lines *UAS-HAdcry-mct3* and *UAS-HAdcry-mct5* had the insert on the sex chromosome.

Effect of expressing dCRY-mCT on the circadian period

UAS-*dcry-mct* flies were crossed to give over-expressing lines as described in section 4.2.3.2. Over-expressing and control fly lines *UAS-HAdcry-mct2*, *UAS-HAdcry-mct6* and *UAS-HAdcry-mct7* were entrained in a 12:12 hour light:dark regime and then subjected to constant light or dark. Analysis of the activity data shows that all the lines tested entrain normally to a light:dark regimen with a bimodal activity pattern, with one peak of activity occurring at lights on, and another anticipating lights off. Plotting the activity data as graphs reveals no apparent differences between the over-expressing lines and the controls (see **fig 4.14**).

In constant darkness both the over-expressing lines appear rhythmic for the first 3 days. The activity graphs also indicate that the over-expressing lines show a lengthening of period, which the control flies do not (**fig 4.15**).

In constant light, the control flies become arrhythmic quickly, within the first two days. However, the over-expressing lines can be seen to stay rhythmic for up to three days into the constant light period. This suggests that the flies have a decreased ability to perceive light (**fig 4.16**).

Spectral analysis to determine circadian period

The spectral analysis data (see table 4.4) reveals that in all lines tested, the UAS-*dcry-mct* over-expressing and control flies entrain normally in a light:dark regime, with a period of ≈24 hours.

Table 4.7 The circadian periods of all UAS-*dcry-mct* lines in a light:dark regime: The numbers represent the average periods of the UAS-*dcry-mct* lines crossed with either *tim-GAL4* to give over-expressing lines, or with w^{1118} to give control lines. All lines were entrained in a 12:12 light:dark regime, and the period determined by spectral analysis of the activity. *tim-GAL4* crossed with w^{1118} represents a control line. The number of flies (n) used in each case is given under each value.

Genotype	n	Period ±SEM
yw;tim-GAL4/UAS-HAdcry-mct2	15	24.00±0.11
yw;tim-GAL4/UAS-HAdcry-mct6	20	24.15±0.16
yw;tim-GAL4/+;UAS-HAdcry-mct7/+	17	24.02±0.16
w^{1118}*;UAS-HAdcry-mct2/+*	15	24.03±0.11
w^{1118}*;UAS-HAdcry-mct6/+*	20	23.94±0.09
w^{1118} *+/+;UAS-HAdcry-mct7/+*	17	24.04±0.22
yw;tim-GAL4/+	23	24.05±0.14

[Raw data in Appendix 6]

Table 4.8 The circadian periods of all UAS-*dcry-mct* lines in constant darkness: The numbers represent the average periods of the UAS-*dcry-mct* lines crossed with either *tim-GAL4* to give over-expressing lines, or with w^{1118} to give control lines. All lines were entrained in a 12:12 light:dark regime then left in constant darkness, and the period determined by spectral analysis of the activity. *tim-GAL4* crossed with w^{1118} represents a control line. The number of flies (n) used in each case is given under each value.

Genotype	n	Period ±SEM
yw;tim-GAL4/UAS-HAdcry-mct2	42	26.83±1.00
yw;tim-GAL4/UAS-HAdcry-mct6	15	26.27±0.62
yw;tim-GAL4/+;UAS-HAdcry-mct7/+	36	25.84±0.56
w^{1118}*;UAS-HAdcry-mct2/+*	34	23.95±0.34
w^{1118}*;UAS-HAdcry-mct6/+*	45	23.93±0.37
w^{1118}*;+/+;UAS-HAdcry-mct7/+*	26	23.86±0.38
yw;tim-GAL4/+	23	24.56±0.58

[Raw data in Appendix 7]

Western blot analysis of *dCRY-mCT* expression

In order to assess the circadian profile of the abundance of dCRY-mCT protein extracts from heads were produced from dCRY-mCT overexpressing flies and analysed, as described in section 2.12-2.15. The primary antibody used was anti-HA (Sigma, 1:10000), since the dCRY-mCT construct was tagged with an HA sequence on its N-terminus (see section 4.2.1). **Fig 4.17** shows that dCRY-mCT is constitutively expressed under light-dark conditions. Using the detection of HSP70 as a loading marker shows that the samples were sufficiently equalised. Table 4.6 shows the relative "weight" of each band, after adjusting relative to the HSP70 data, for three independent Western blots, and the average for each time point.

Table 4.9: Analysis of relative pixel density of Western blot analysis of extracts from a *UAS-dcry-mct* transgenic fly line: The pixel density of the results of the Western blot analyses were taken for both the dCRY-mCT bands, and the HSP70 bands. The values for the dCRY-mCT was divided by the HSP70 band, and expressed as a percentage of the highest value. The final relative values are shown in the table for each time point for three independent Western blots.

ZT	Western blot 1	Western blot 2	Western blot 3	Standard deviation	Average
0	98.45	98.90	96.67	1.18	98.01
4	96.95	100.00	94.74	2.64	97.23
8	96.67	93.89	96.33	1.52	95.63
12	93.36	83.25	98.87	7.93	91.83
16	100.00	89.70	100.00	5.95	96.57
20	97.85	85.91	100.00	7.59	94.59

The Western blot of TIM over the circadian period in the *UAS-dcry-mct2* fly line shows several interesting features (**fig 4.18 A**). There is no cycling of TIM levels, indicating that TIM is not degraded under light conditions. There is also a distinct absence of the hyperphosphorylated form of TIM, indicated by the lack of the upper band seen at time points 0, 16 and 20 of the control. The quantification (**fig 4.18 B**) shows that TIM is at intermediate levels relative to the control throughout the circadian period, in that TIM is neither at maximal nor minimal levels as defined by the control results.

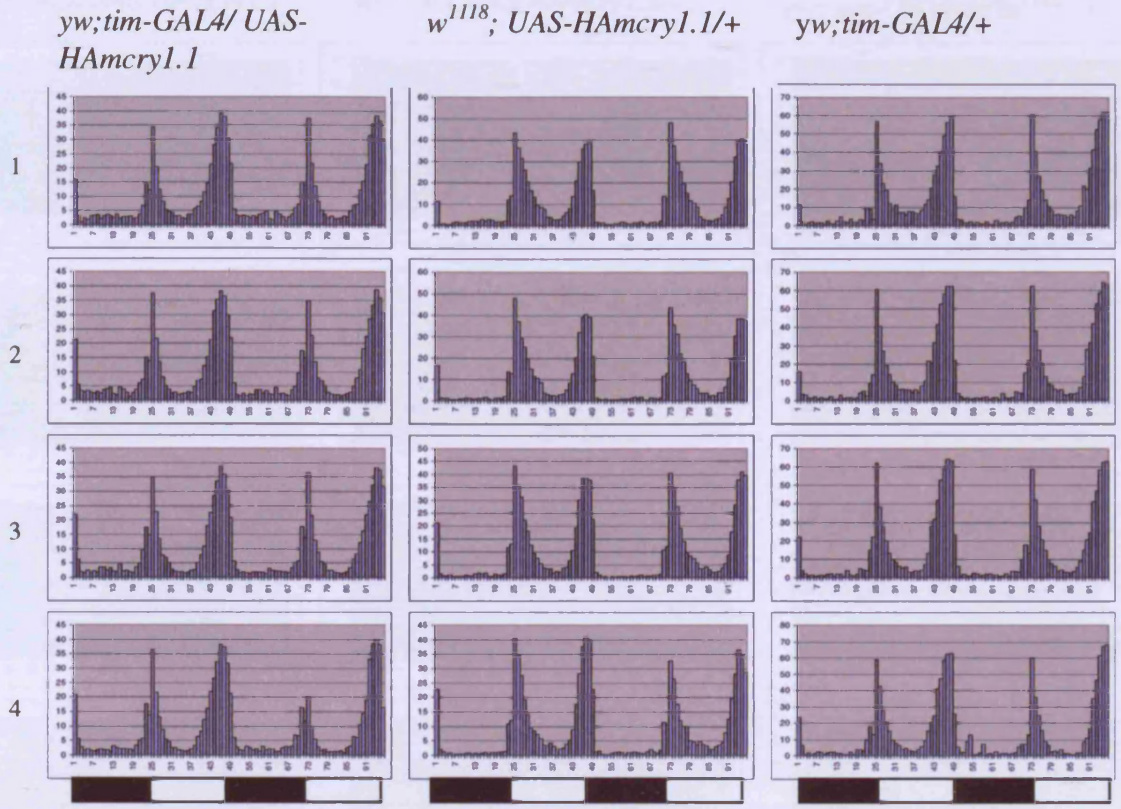


Fig 4.8 The activity pattern of *UAS-mcry1.1* over-expressing lines and control lines in 12:12 light:dark regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. The results show that transgenic fly lines over-expressing mouse CRYPTOCHROME 1 entrain to a 24 hour period in a 12 hour light:12 hour dark cycle no differently from the control lines.

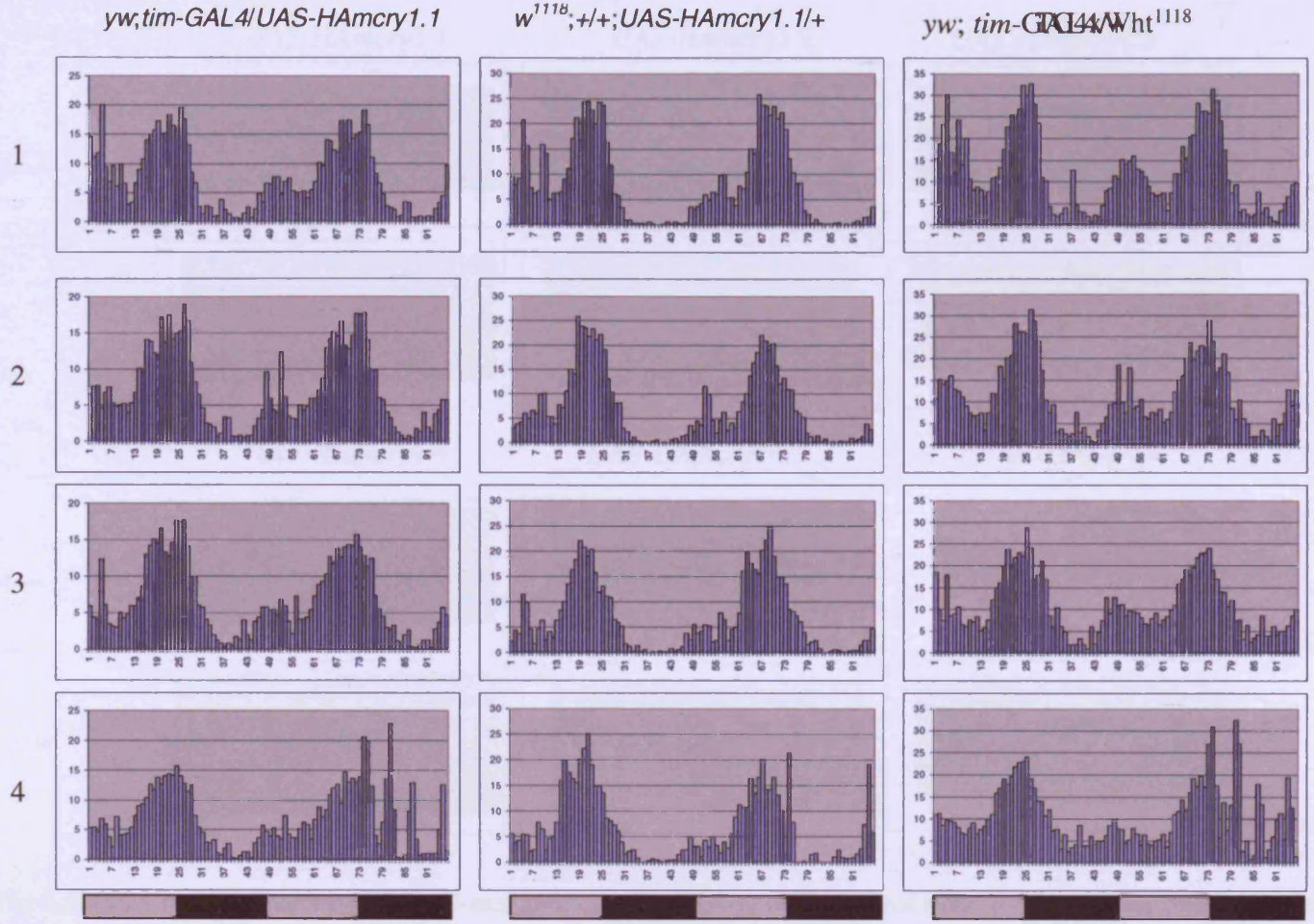


Fig 4.9 The activity pattern of *UAS-mcry1.1* over-expressing and control lines in a constant dark regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. Differences between the control and over-expressing lines are not immediately apparent without further analysis.

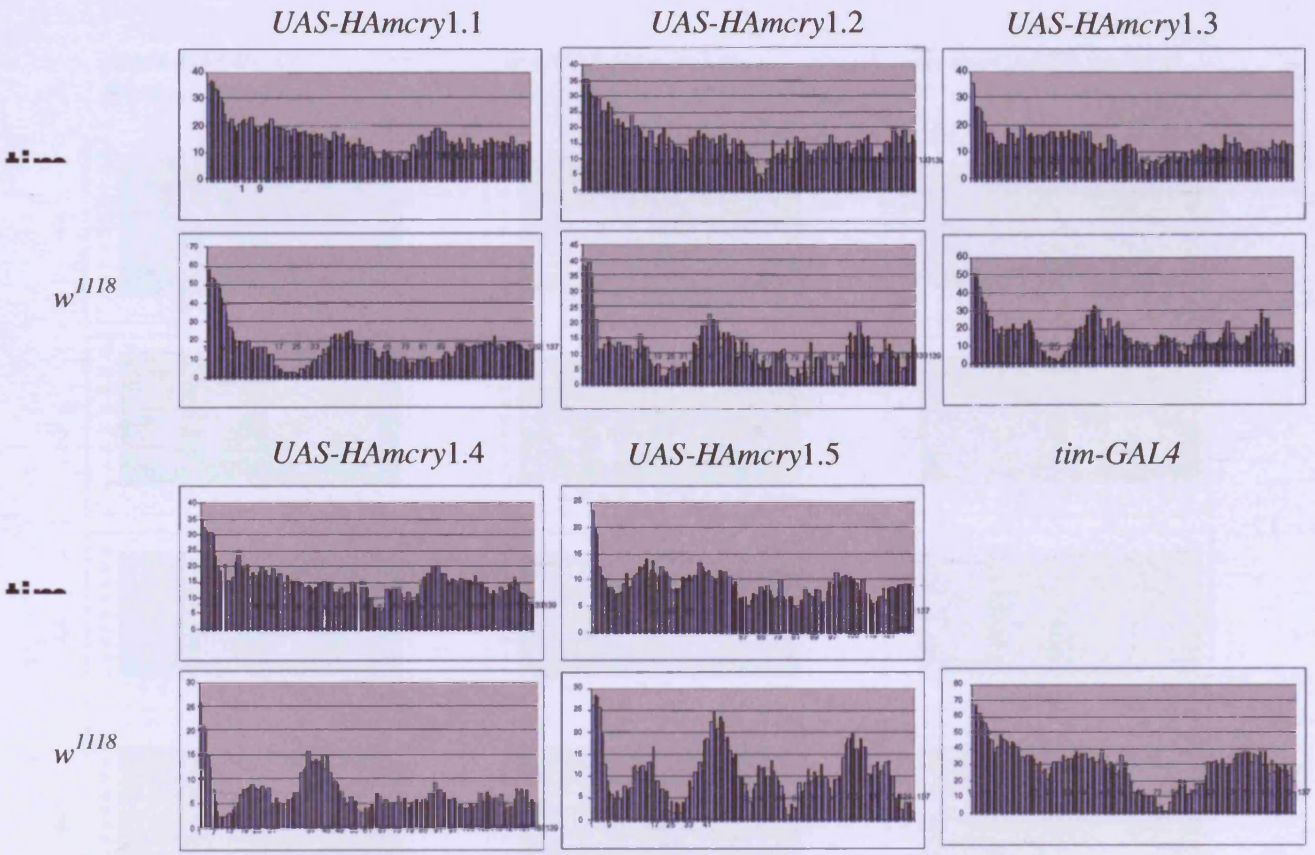


Fig 4.10 The activity pattern of UAS-*mcry1* over-expressing and control lines in a constant light regime: The first 3 days of the constant light period are shown here for each fly line tested. *tim-GAL4* rows represent the over-expressing line (*yw;UAS-HAmcryt/tim-GAL4*), and the w^{1118} rows represent the control (w^{1118}; *UAS-HAmcry/+).* The *tim-GAL4* / w^{1118} line is a further control line. All lines become arrhythmic, however those over-expressing mCRY1 appear to become arrhythmic faster.

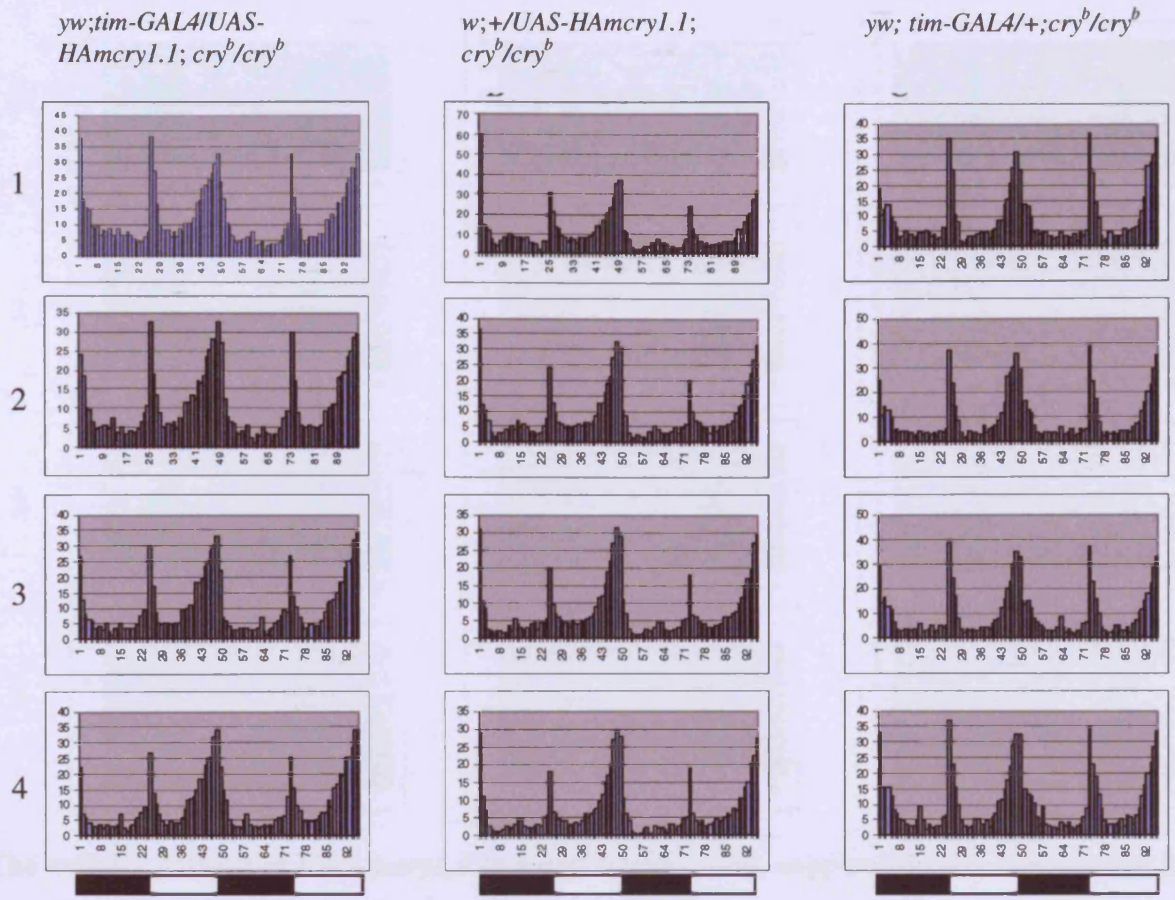


Fig 4.11. The activity pattern of *UAS-mcry1.1* in a *cry*^{b} background, over-expressing and control lines in a 12:12 light:dark regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. All lines entrain and show similar activity patterns.

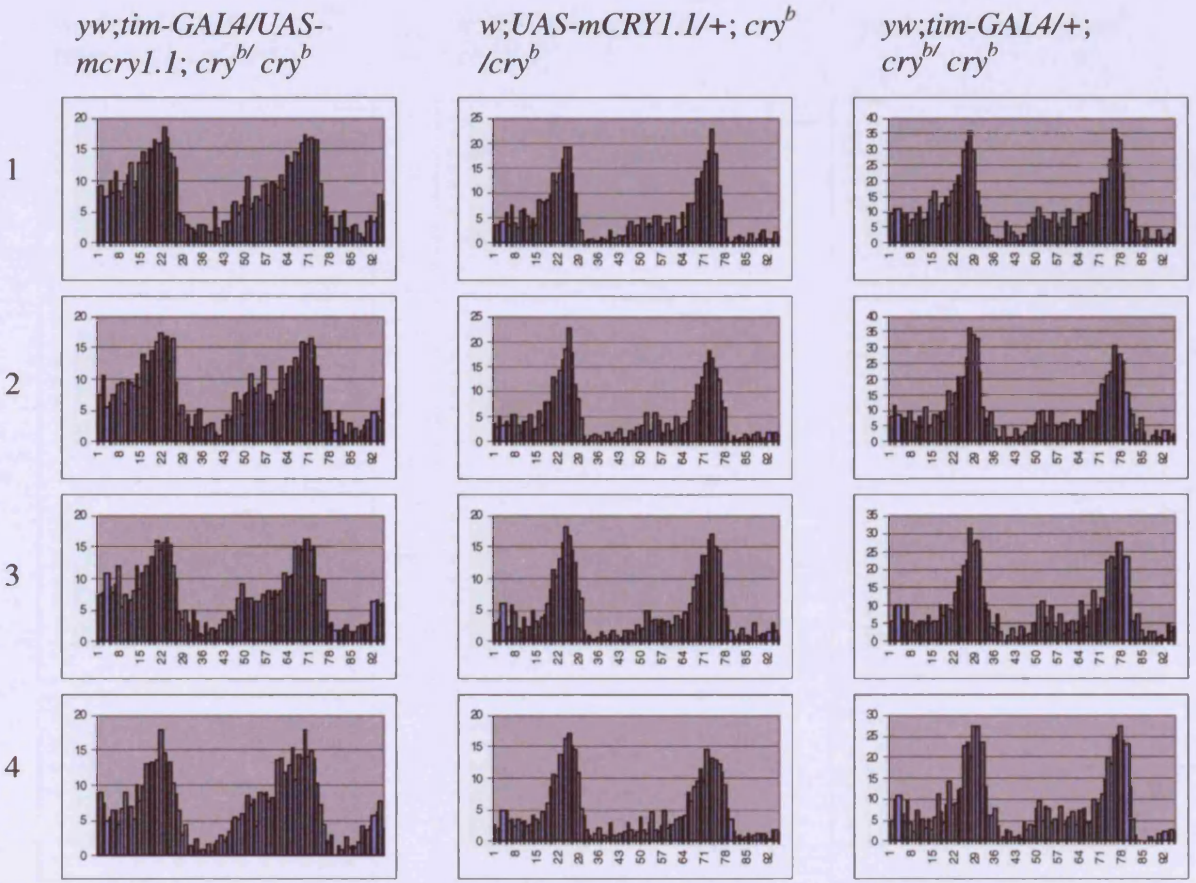


Fig 4.12 The activity pattern of *UAS-mcry1.1* in a *cry*^{b} background, over-expressing and control lines in a constant dark regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. All lines entrain, with the over-expressing lines showing less distinct peaks of activity as compared to the controls.

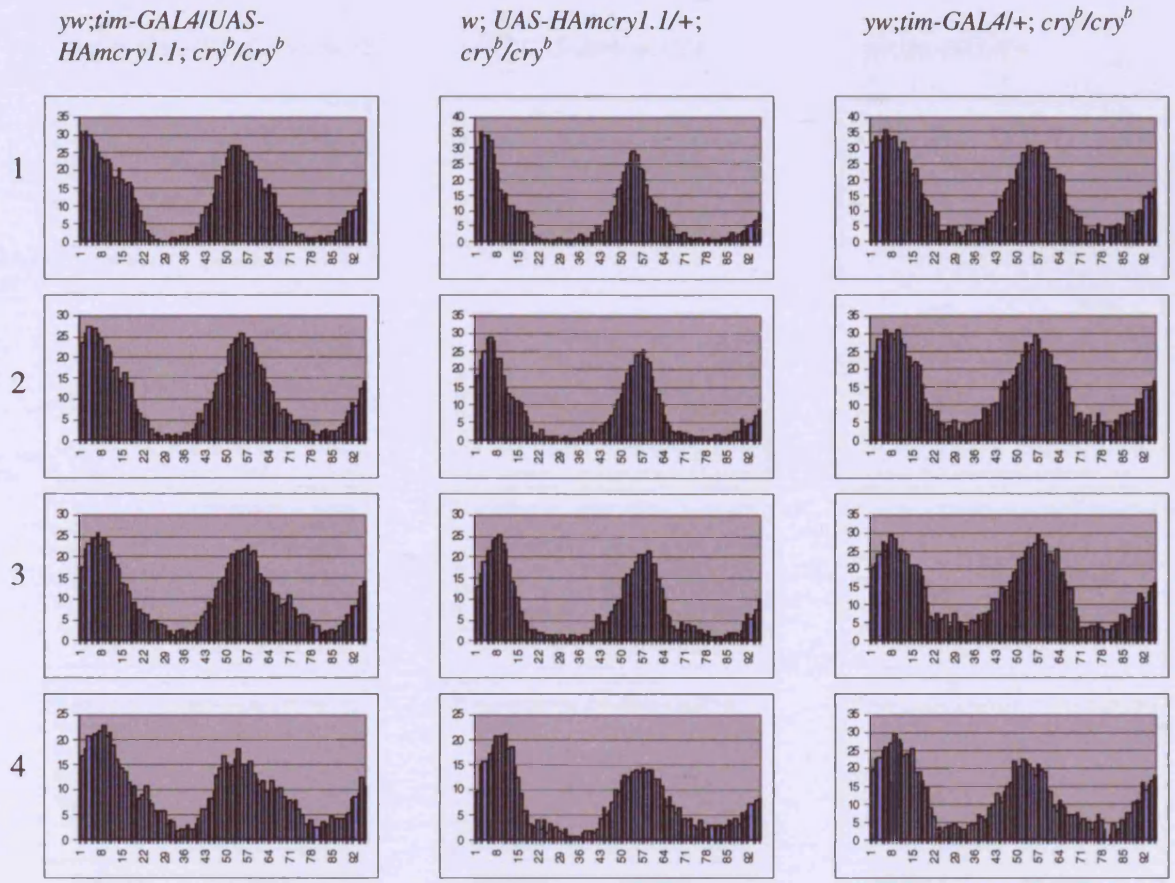


Fig 4.13 The activity pattern of *UAS-mcry1.1* in a *cry*^{b} background, over-expressing and control lines in a constant light regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. All the lines stay rhythmic indicating no rescue of dCRY function.

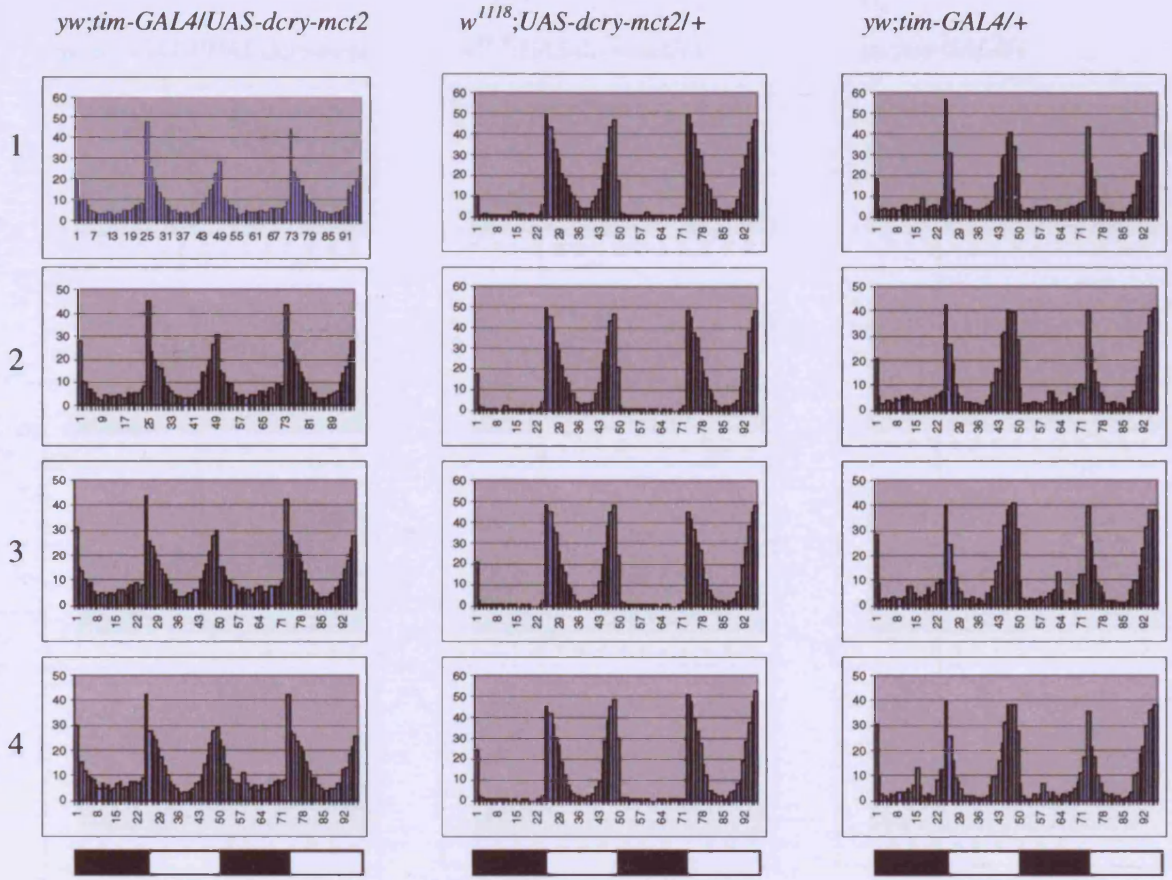


Fig 4.14 The activity pattern of UAS-*dcry-mct2* over-expressing and control lines in a 12:12 light:dark regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. All lines entrain, however the over-expressing lines show less distinct peaks of activity and more consistent activity over the recorded period.

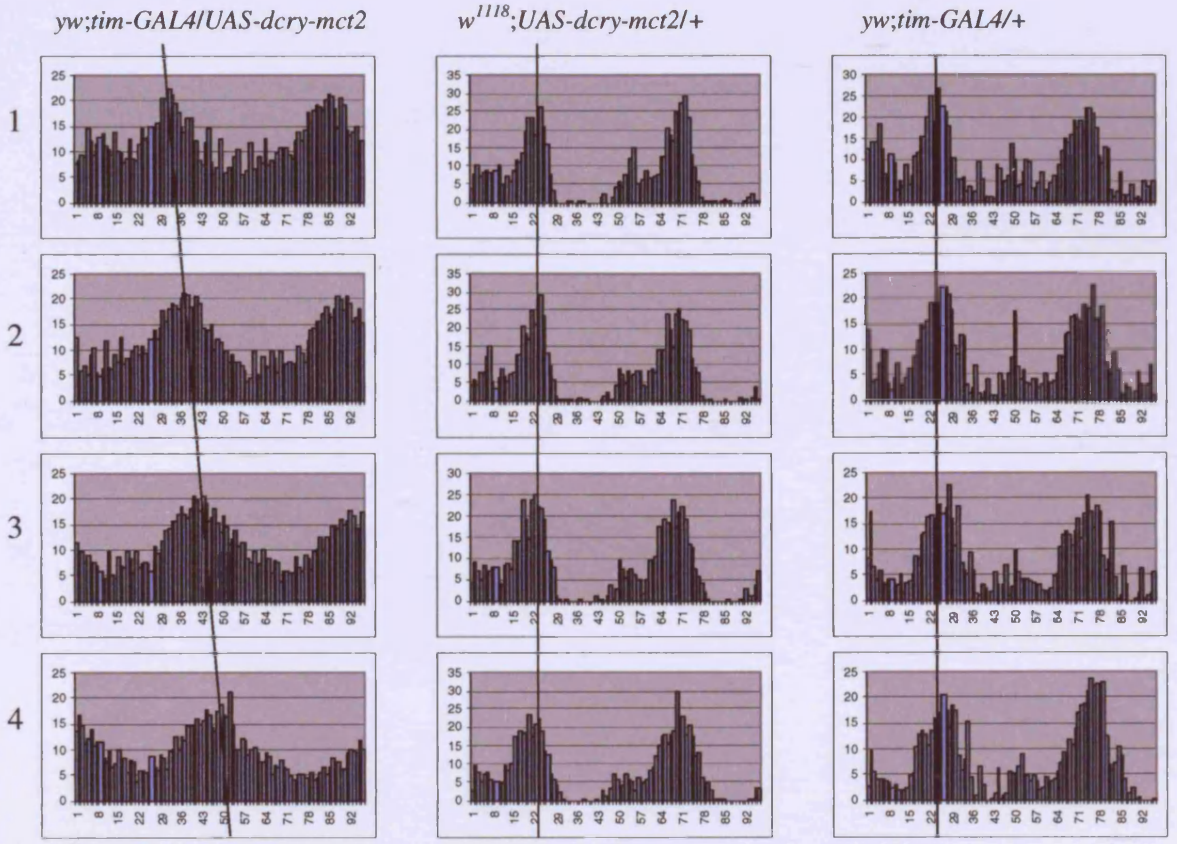


Fig 4.15 The activity pattern of UAS-*dry-mct* over-expressing and control lines in a constant dark regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. Over-expressing lines show an increase in period (represented by the vertical line) that the control lines do not exhibit. Therefore the construct dCRY-mCT lengthens the circadian period when expressed.

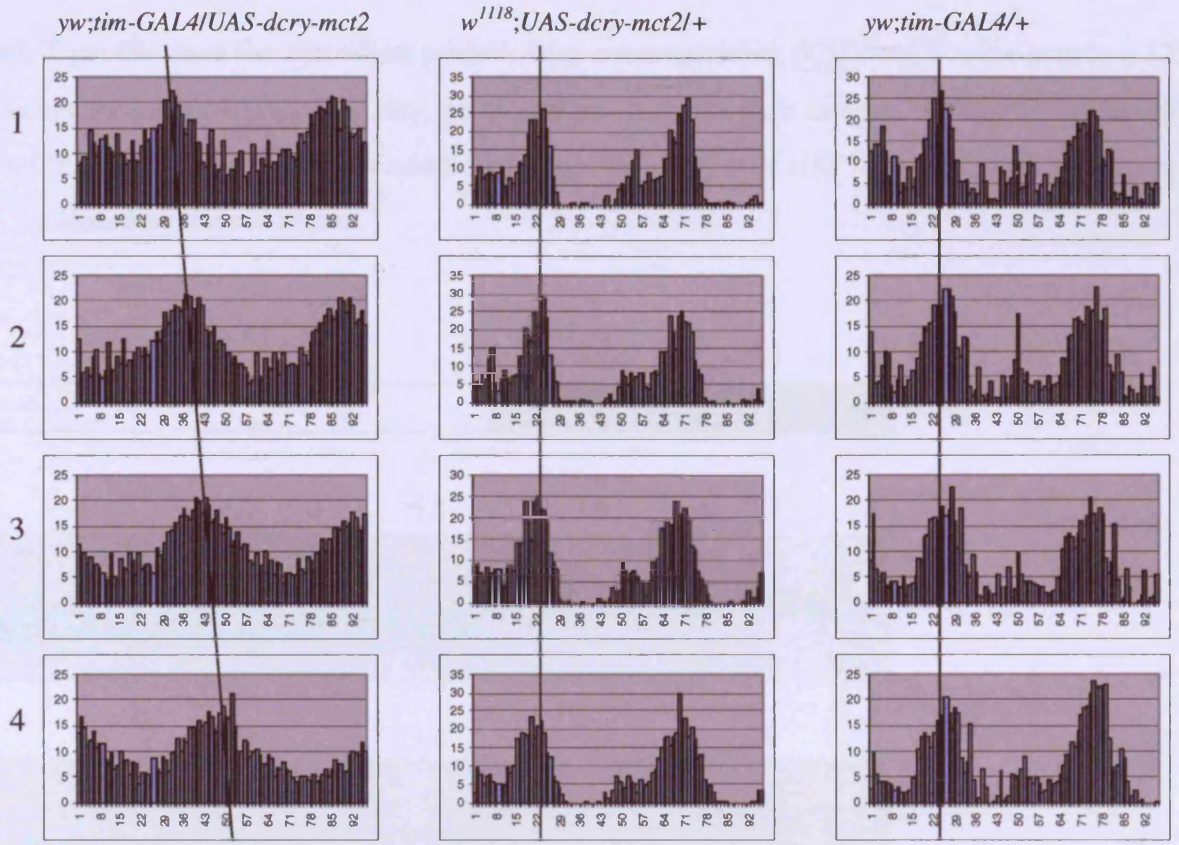
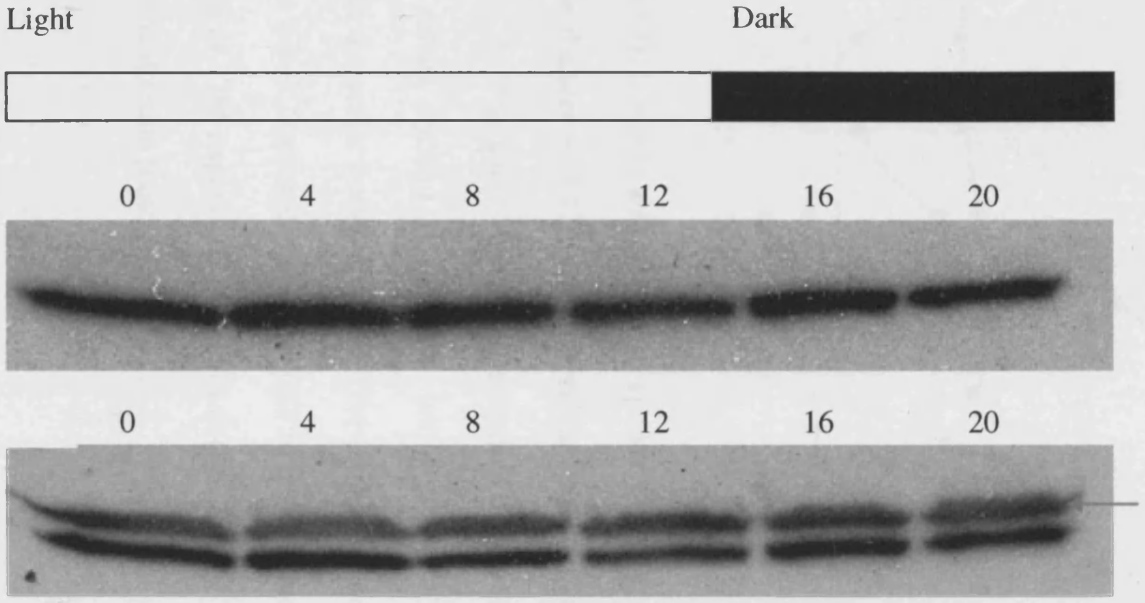


Fig 4.15 The activity pattern of UAS-*dry-mct* over-expressing and control lines in a constant dark regime: In each case row 1=day 1-day 2, 2=day 2-day 3, 3=day 3-day 4, 5=day 4-day 5, hence all activity profiles for each day are double plotted for ease of comparison. Over-expressing lines show an increase in period (represented by the vertical line) that the control lines do not exhibit. Therefore the construct dCRY-mCT lengthens the circadian period when expressed.

Fig 4.17 dCRY-mCT profile over the circadian period: flies overexpressing dCRY-mCT were kept in a 12:12 light:dark regime, and samples collected every 4 hours starting at ZT=0. The time points are given above each sample. A= Immunodetrection with α-HA antibodies, the band represents dCRY-mCT. B= The same blot after additional immunodetection of HSP70. The bottom band represent dCRY-mCT and the top band represents HSP70, indicated by the red arrow.



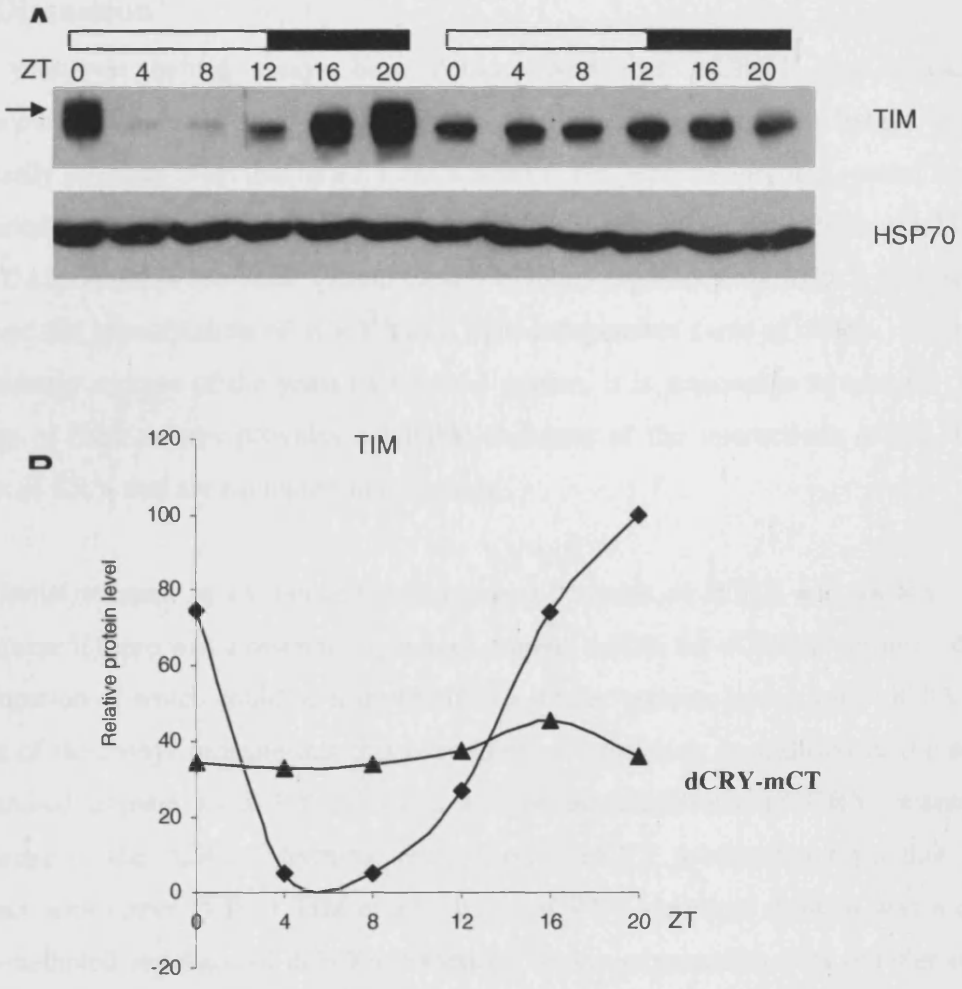


Fig 4.18 TIM cycling in LD. Controls and flies overexpressing dCRY-mCT (line *UAS-dcry-mct2.2*) were entrained under a 12:12 light:dark regime and sampled every 4 hours (ZT 0, 4, 8, 12, 16, 20). **A.** Top panel. Immunodetection of TIM using an anti-TIM antibody: TIM shows robust cycling in controls but not in dCRY-mCT flies. Hyperphosphorylated high molecular weight forms of TIM (arrow) do not accumulate in dCRY-mCT flies. Bottom panel. Immunodetection of HSP70 in the same blot as loading control **B**. Quantification of TIM expression in control and dCRY-mCT flies. HSP70 expression was used as normalisation standard.

4.4 Discussion

The yeast-two hybrid assays have demonstrated that mCRY1 can interact with *Drosophila* PER but not TIM. Whilst the context of the yeast-two hybrid system is markedly different from that of a fly clock neuron, the yeast two-hybrid system has so far provided a remarkably predictive assay for dCRY interactions with PER and TIM, and dCRY expressed in the yeast system clearly exhibits regulation by light; a feature which allowed the identification of dCRYΔ as a light-independent form of dCRY. Based upon the historic success of the yeast two-hybrid system, it is reasonable to assume that the results of these assays provides a reliable indicator of the interactions of the different forms of CRY that are examined in this study..

The initial concept of swapping the C-terminal domains of dCRY and mCRY1 was to determine if there was a discrete regulatory domain within the dCRY C-terminal domain, the function of which could be transferred to a similar protein, in this case mCRY1. The result of the assays indicate that this is certainly not the case, as addition of the mCRY1 C-terminal domain to dCRY produces a light-regulated form of CRY, whereas the exchange of the dCRY C-terminal domain onto mCRY produces a form that fails to interact with either PER or TIM at all. If the dCRY C-terminal domain was a discreet light-mediated regulator of dCRY interactions, it was expected that its transfer onto the photolyase domain of mCRY1 would produce a light regulated construct (assuming the construct functioned at all). The light-dependent nature of the dCRY-mCT construct provides further confusion, as this appears to indicate the dCRY photolyase domain confers light-regulation, which is supported by the evidence that the mCRY C-terminal interacts light-independently with PER and to a lesser extent with TIM in the absence of the dCRY photolyase domain. It seems that the notion of the C-terminal of dCRY as a light-regulator is an oversimplification of a more complex process.

Several interesting aspects have emerged from these studies. The initial yeast assays revealed that mCRY1 interacts with *Drosophila* PER in a light-independent manner, which is not surprising considering that mCRY1 is believed to partner mammalian PER in the endogenous circadian clock, including the SCN, which is located deep in the brain, hence far away from direct illumination. Conversely, mCRY1 does not interact with

Drosophila TIM as would be expected from the fact mCRY1 does not partner the TIM homologue in the mammalian clock. Based upon this data, it was predicted that mCRY1 may cause a lengthening of period when over-expressed in transgenic flies, as its interactions with PER would remove (or sequester) PER, delaying the formation of the heterodimer with TIM. However, studies using the mCRY1 expressing transgenic fly lines supported this hypothesis, with the fly lines showing a subtle, although statistically significant, increase of approximately 26 minutes of the locomotor activity period. Moreover, the effect on period requires the presence of functional endogenous dCRY, as period lengthening is not seen in a cry^b (a strong hypomorph mutant) background. All together, these results suggest that the behavioural effect elicited by mCRY1 overexpression might be caused by interference with the normal function of dCRY rather than being the product of an autonomous effect of mCRY1 on PER. What has certainly been shown is that mCRY1 cannot function as a circadian photoreceptor in *Drosophila*, as its expression fails to rescue light responses in fly without a functioning dCRY. The divergence in function between dCRY and mCRY1 is apparently due to intrinsic differences to the proteins, as well as or instead of the difference in contexts.

Further insights into the interactions of mCRY1 can be gleaned from the results that the mCRY1 photolyase region fails to interact with PER or TIM, whereas the mCRY1 C-terminal domain interacts light-independently with *Drosophila* PER and TIM. This suggests that the necessary interaction domain for mCRY1 lies in the region defined here as the C-terminal domain (see section 4.3.1 for definition of this region). Since the C-terminal domains of both dCRY and mCRY1 vary greatly, it is a sensible hypothesis that the region of interaction is the area of homology that the mCRY1 C-terminal domain shares with dCRY that was retained to minimise disruption of the secondary structure (see section 4.3.1). The data does not exclude that it is the non-homologous region of the mCRY1 C-terminal domain that is responsible for the interactions, but this seems unlikely. The question then arises as to whether this holds true for dCRY. The construct dCRYΔII did not interact with either PER or TIM, even though it expressed to levels equivalent to dCRY in yeast (see fig 4.7). Certain evidence (discussed in depth in section 5.1) indicates that if the dCRY protein is truncated before residue 519 it will not show any interactions with PER or TIM. It may be that the interaction domain is dependent on the inclusion of the amino acid residues immediately before residue 519. This is far

from conclusive evidence as to the interaction domain of dCRY, as truncation before this point may produce a gross structural change and is not site specific, but it does provide an intriguing suggestion as to the whereabouts of the dCRY PER/TIM interaction domain, which can be explored further (see future work below).

Perhaps the most interesting result came from the construct dCRY-mCT, which interacted with PER and TIM in an unexpected manner. Since removal of the final 20 amino acid residues from dCRY produces a constitutively active form, it was thought that removal of those same residues, plus additional residues, would produce a constitutively active form or a form that did not interact at all. The addition of the mCRY1 C-terminal domain was not expected to have an effect on the light sensitivity of the construct. The yeast assay, however, revealed the construct to interact with both PER and TIM, and to be light-regulated. Since the light regulation domain was thought to lie within the C-terminal domain of dCRY, suggested by the behaviour of dCRYΔ, by removing the entire C-terminal domain, including those residues absent from dCRYΔ, it was not thought that the remaining protein fragment would be regulated by light. An explanation could have been that the mCRY1 C-terminal domain was now mediating a light signal. However, this idea can be dismissed for two important reasons: firstly, mCRY1 is not light regulated in its interactions with PER and TIM in the yeast assay, therefore any ability to mediate a light signal via the mCRY1 C-terminal domain must be a unique feature of its attachment to the dCRY photolyase domain, which, while possible, is not probable. Secondly, the testing of dCRYΔII and mCT as separate entities against PER and TIM revealed that mCT interacted in a light-independent manner, whereas dCRYΔII did not interact at all. The explanation that this data most credibly lends itself to, is that the mCT region contains the PER/TIM interaction domain (as discussed above) and that the light signal is mediated by the dCRYΔII photolyase domain. This runs contrary to the evidence generated from studies with dCRYΔ, which is the dCRY photolyase domain and which appears to be free from repression in the absence of light. However, as discussed in Chapter 1, dCRYΔ, while being constitutively active, does still exhibit some regulation by light, as over-expression of dCRYΔ in a cry^b background gives flies that show a lengthening of period in constant light, as opposed to the stable period of control cry^b flies. Perhaps the divergence in the functional domains between dCRY and *Arabidopsis* CRY is not as great as first thought, as *Arabidopsis* CRY show light

regulation through the photolyase domain, and interaction through the C-terminal domain.

Expression of dCRY-mCT in transgenic fly lines produced another surprise: the flies behaved as if in constant low-level light. In fact, they showed the same behavioural patterns as dCRYΔ expressing flies. The lengthening of the locomotor activity period in darkness and the suppression of TIM cycling under LD conditions, suggests that the construct behaves as a constitutively active dCRY. In constant light the flies exhibit periodicity for at least four days into the regimen, whereas control fly lines become arrhythmic. In anything, the features of dCRYΔ are not only manifested in these fly lines, but are even more distinct. At the molecular level, dCRY-mCT does not show any cycling in a light dark cycle, and is present at constantly high levels. This differs from dCRYΔ which is present in constitutively low levels, owing to its constant degradation. It seems that the addition of the mCRY1 C-terminal domain to dCRY confers stability in light to this construct, even though it is active. In wild type flies, light activation of dCRY results in degradation of dCRY and TIM. In dCRY-mCT overexpressing flies dCRY-mCT is stable but TIM is degraded, showing for the first time that CRY activation and degradation are not mechanistically linked and confirming that CRY and TIM degradation unfold along separate pathways (Busza *et al.*, 2004). Thus the phenotypic effects we see are most probably due to a constitutive CRY activity of dCRY-mCT but with a novel molecular function.

These transgenic fly results appear to run contrary to the yeast results for dCRY-mCT, which predicted a light regulated CRY, not a constitutively active form. However the possibility exists that although constitutively active, this form of CRY is also light activated. Unfortunately, due to time constraints, it was not possible to test the effect of dCRY-mCT overexpression under LL and in light pulse experiments neither in wild type or in a cry^b background, which would have demonstrated whether dCRY-mCT retained any light regulation.

Taking all the data into account, it may be that both *m1.1* and *m1.3* over-expressing lines do not show the phase advance seen with the equivalent controls. The *tim-GAL4* crossed

with w^{1118} line also shows a decreased phase advance as compared to these controls. The most probably explanation is that the over-expression of mCRY1 has an effect on the phase advance, but that there is also a similar effect associated with the *tim-GAL4* line. In this case, the effect of over-expressing mCRY1 in the m1.3 line is sufficient to distinguish it from the *tim-GAL4* line effect. Statistically, however, the *UAS-HAmcry1.1* over-expressing line appears to not be different than the *tim-GAL4* crossed with w^{1118} line, which would, if only taking into account the statistical test, suggest that the effect we see is due only to the presence of the *tim-GAL4*. However, the mean of the *UAS-HAmcry1.1* over-expressing line is clearly less than that of the *tim-GAL4* crossed with w^{1118} line by approximately 30 min, and also this control line has fewer data sets which means a greater variance. In summary, I would suggest that the over-expression of mCRY1 has an effect on phase shifting after a ZT 21 pulse, but that for the line *UAS-HAmcry1.1* this is partially masked by the effect of the *tim-GAL4* line and the small number of data points. Further work on these lines should repeat these pulse experiments to gain sufficient data for statistical analysis.

4.5 Future work

Firstly, a possibly region of interaction with PER233-685 and perhaps TIM has been identified for mCRY1, which suggests that the interaction domain for dCRY may be the homologous region to that identified in mCRY1. Regardless of whether this is the case, there is a strong case for mapping the interaction domain of dCRY with PER and TIM, as these interactions form the basis for the function of dCRY and for the yeast assay used, therefore any further understanding of the interactions of dCRY may provide insights into its regulation.

Regarding the mCRY1 fly lines, and the effect of the lengthening of period, the hypothesis was formulated and borne out that mCRY1 would interact with PER delaying the circadian cycle, which was seen in the locomotor experiments. However, ICC work would be useful in revealing whether this was the case, which may become apparent through identification of delayed entry of PER and/or TIM into the nucleus. Due to time constraints it was not possible to undertake Western blot analysis of the mCRY1 fly lines. This would also be a future objective, to perform Western blot on a time series of samples

to look for cycling of mCRY, PER and TIM. It may be expected that TIM would show a slight delay in peaking (though it is questionable whether the Western blot technique would be sensitive enough to detect this). ICC work to detect the location of the construct for comparison with dCRY would also be useful, as mCRY1 may have different localisation patterns.

Work with dCRY-mCT could involve expressing the construct in a cry^b background. This could demonstrate whether dCRY-mCT still functions as a photoreceptor, as any lengthening of period or arrhythmicity in a constant light regimen would be due to dCRY-mCT effects. Conversely, no lengthening of period would suggest that dCRY-mCT has lost all regulation by light, which run contrary to the yeast results. This construct has demonstrated that the initial model of light regulation of dCRY was over-simplified, now there is the long task of unravelling the mechanism by which light regulates dCRY, and the role of the photolyase domain in the apparent repression of the mCT interactions as seen in the yeast studies.

Chapter 5: Site Directed Mutagenesis of Potential Regulatory Sites in the dCRY C-terminal domain

5.1 Introduction

As discussed in both Chapter 1 and Chapter 4, the C-terminal domain of dCRY has been shown to be essential for repression of dCRY interactions in darkness, and for dCRY stability in darkness (Dissel *et al.*, 2004; Busza *et al.*, 2004). Truncation of the C-terminal domain of dCRY by 20 amino acid residues (dCRYΔ) gives a form of dCRY that interacts with *Drosophila* PER and TIM independent of light, in contrast to wild type dCRY that requires light in order to interact in the same yeast assay (Rosato et al., 2001). dCRYΔ expressed in the clock neurons in transgenic *Drosophila* in a wild-type dCRY background behaviourally show a low level light response indicated by a lengthening of period in constant darkness eventually becoming arrhythmic, and a failure to become arrhythmic in constant light in the first few days (Dissel *et al.*, 2004). At the molecular level, TIM and PER cycle with reduced amplitudes, and there is an absence of the phosphorylated forms of both proteins, particularly noticeable with TIM. dCRYΔ levels are also constitutively low, which has been shown to be due to constant light-independent degradation (Dissel *et al.*, 2004; Busza *et al.*, 2004).

In order to find an interaction partner, a strategy of introducing mutations into the C-terminal domain has been undertaken. The rationale for this lies partly with research undertaken where random mutations were induced in dCRY and a screen for light-independent interactions with TIM carried out in a yeast assay (Rosato *et al.*, 2001). All mutations of dCRY that produced a positive result were found to generate stop codons or frame-shift mutations that gave truncated forms of dCRY similar to dCRYΔ (**fig 5.1**). Having re-examined the data recently it was noticed that all these mutations fell between residues 519 and 533, which led to the following conclusions: firstly, that truncating the protein before position 519 led to a non-interacting protein, as seen with the chimeric protein studies. Secondly, there were no mutations past position 533 that gave a positive

result. Since it seems unlikely that the addition of more of the C-terminal domain somehow produces a non-functioning protein, it is more likely that truncating dCRY downstream of position 533 gives a light regulated form of dCRY, which would not be revealed by an assay for light independent mutants. This then posses the question as to what is important about the region prior to residue 533 in dCRY.

Fig 5.1 Random PCR-induced, light-independent mutations. Random mutagenesis of the dCRY gene followed by a yeast assay revealed the following mutations that produced light-independent interactions with TIM (taken from Rosato *et al.*, 2001).

C-terminal mutation	Other mutation(s)
V[489]FRAME-SHIFT + STOP	N[253]T
P[519]FRAME-SHIFT	V[297]M
P[520]FRAME-SHIFT	—
P[520]FRAME-SHIFT	T[149]A
P[520]FRAME-SHIFT	R[390]H
C[523]STOP	—
R[524]STOP	—
R[524]STOP	—
R[524]STOP	—
E[529]STOP	—
E[530]STOP	E[200]A, T[303]P
Q[533]STOP	T[438]A, N[514]S
Q[533]STOP	K[445]R, I[167]N
Q[533]STOP	—

An analysis of the region about position 533 using a predictive algorhithm (Puntervoll *et al.*, 2003) reveals several possible binding motifs for protein factors in the region of dCRY N-terminal to the 533 residue. Since light regulation is seen in yeast assays, any interaction partner that serves to modify dCRY must be present in both yeast and dCRY, and therefore be highly conserved. Though it has not been determined whether yeast have circadian clocks, they do possess photolyases, and many components of the *Drosophila* clock have been found to be generic proteins that serve numerous functions, and are found conserved throughout many organisms, DOUBLETIME and SHAGGY being two examples. The analysis was conducted to find possible

binding sites in the C-terminal domain in a *Drosophila* and yeast background, then only domains for protein factors found in both were considered (**fig 5.2**). The results indicated a possible site for phosphorylation via a Protein Kinase A motif at residues 524-526 and a class III PDZ motif. PDZ motifs occur in a number of proteins allowing binding to a diverse range of signalling, cell adhesion and cytoskeletal proteins (reviewed in Schillace and Scott 1999). Further more, prediction of secondary structure of dCRY reveals alpha helical structure in the putative region of light regulation.

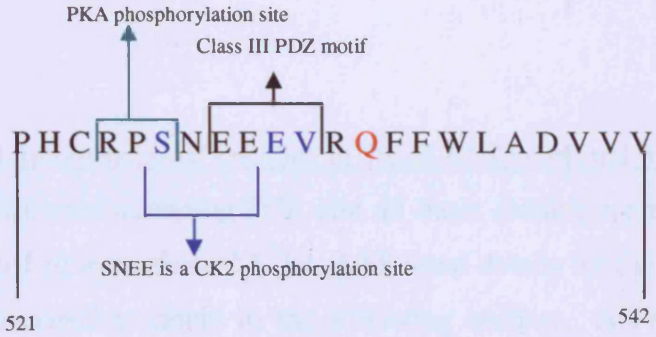


Fig 5.2 Previous results of a random mutagenesis of dCRY revealed that the region N-terminal to residue 533 may be important for light regulation. In order to determine whether any trans-acting factor might interact with this domain, a search for protein motifs was undertaken for this specific region. The analysis revealed three possible binding domains. The residues highlighted in blue represent sites that were subjected to site-directed mutagenesis to alter the putative domains.

To determine the importance of each of these sites, the following mutations have been introduced into the C-terminal domain of dCRY: S to D (526) (dCRY(S526D)) which is a possible phosphorylation site, E to P (530) (dCRY(E530P)) which eliminates all α-helical structure in this region, and V to K (531) (dCRY(V531K)) which lies in a possible class III PDZ domain (**fig 5.2**).

5.2 Materials and Methods

5.2.1 Cloning

All cloning was carried as described in section 2.1.

5.2.2 Yeast-two hybrid assays

All plate assays and semi-quantitative assays were carried out as described in section 2.6

5.3 Results

5.3.1 Site directed mutagenesis of residues in the *dCRY* C-terminal domain

Cloning of all constructs, including PCR and all basic cloning techniques, were performed as described in sections 2.1-2.4. Additional details for the site directed mutagenesis is described in detail in the following section. All references to regions of genes are expressed in nucleotide positions relative to the first nucleotide of the coding sequence, which is position 1. All references to amino acid positions are relative to the first methionine which is position 1. All primers are written 5' to 3'.

Specific mutations were induced in the C-terminal domain of *Drosophila* cryptochrome (*dcry*) using PCR described in section 2.4. Two constructs were already available in the lab. The first consisted of *dcry* in pBS. As well as containing the full *dcry* sequence, this construct also contained a Kozak sequence and an HA tag and will be referred to in subsequently as pBS-HA*dcry* (see **fig 5.3**). As can be seen from **fig 5.3**, the *dcry* sequence was flanked directly by an *Eco* RI site at the 5' end and an *Xho*I site at the 3' end. An *Eco*RI-*Xho*I digest removed the entire *dcry* coding sequence, while leaving the Kozak sequence and HA tag. Any sequence ligated into the *Eco*RI-*Xho*I sites would therefore have the Kozak sequence and HA tag in the 5' position. The second construct that was used extensively in cloning the *dcry* constructs was a sub-clone of *dcry* in pBS (see **fig 5.4**). This vector contains the *dcry* sequence 422-1625 flanked by a *Bam*HI site at the 5' end and an *Xho*I site at the 3'end. The aforementioned

*Bam*HI site is an endogenous site to *dcry*, therefore a digest using *Bam*HI will not give any additional sequence at the 5' end. The necessity for this sub-clone will be discussed in the specific descriptions of the cloning given below.

Fig 5.3 Representation of pBS-HA*dcry*: *dcry* in pBluescript II KS (+). The HA tag is labelled and shown here in green. The Kozak sequence is represented in red. Also labelled are the relative positions of several important restriction sites. This map only represents the relative order of the labelled components.

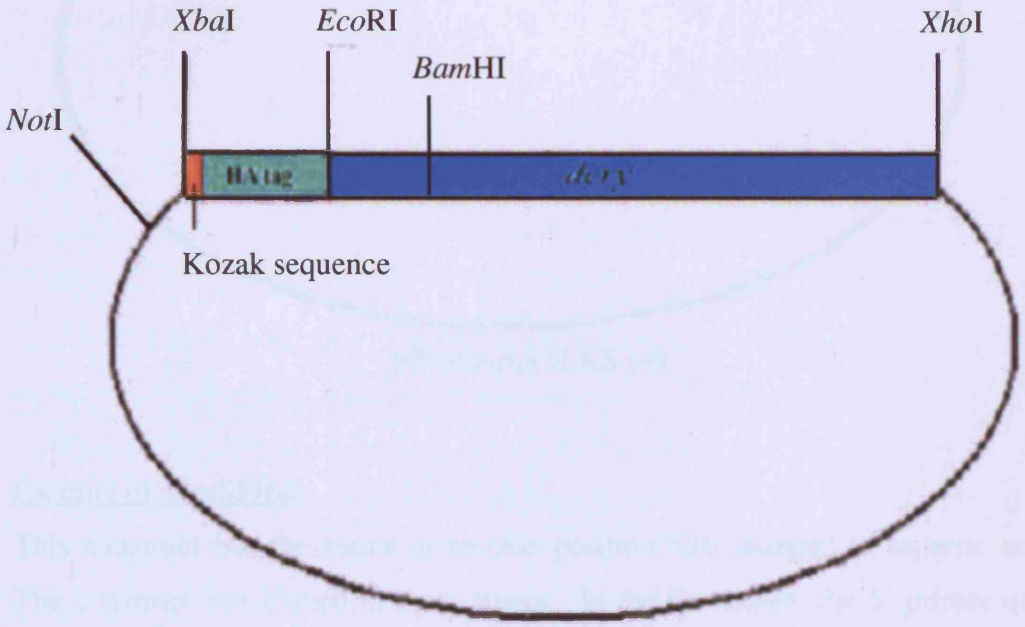
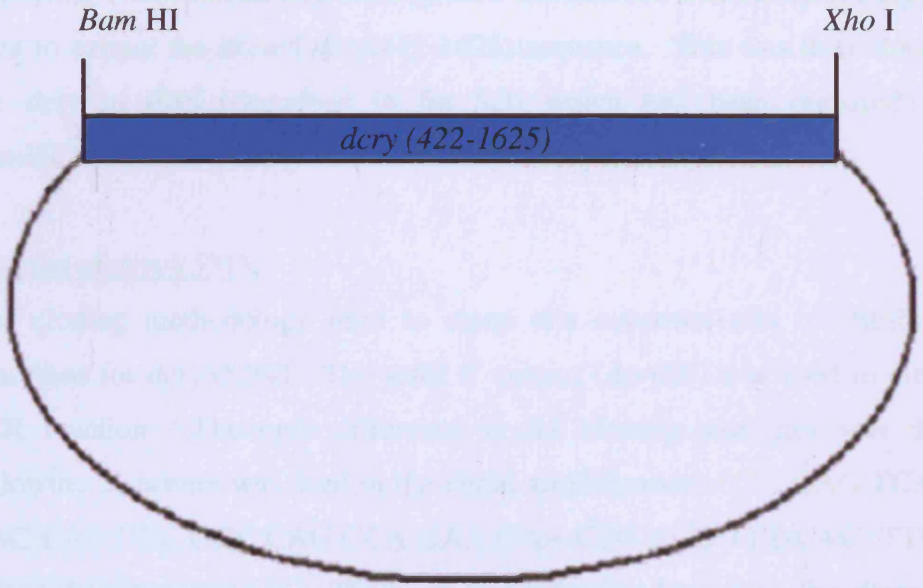


Fig 5.4 Representation of pBS-HA*dcry* sub-clone: *dcry(422-1625)* in pBluescript II KS (+). This map only represents the relative order of the labelled components.



Cloning of *dcry*S526D

This construct had the serine at residue position 526 changed to aspartic acid. The construct was cloned in three stages. In the first stage, the 5' primer used was TGC CTG ATC GGA GTC CAT TAT CCG, referred to in subsequent sections as *dcry*5E and the 3' primer used was CTC GAG TCA AAC CAC CAC GTC GGC CAG CCA GAA GAA CTG ACG CAC TTC CTC CTC GTT GTC(GA) TGG TC. The bases highlighted in red indicate the added *Xho*I restriction site and the bases highlighted in blue indicate the substitution, with the original bases shown in brackets. The alteration of the bases changed the coding such that the altered codon coded for aspartic acid rather than serine. This PCR reaction amplified the section of *dcry* 1450-1625 (region expressed in base pairs starting with the first base pair of the coding region as position 1). *dcry* contains an *Nco*I site at position 1495, therefore this amplified region contained this restriction site. Once this section had been amplified by PCR as described, it was cloned directly into PDK and sequenced to check that the sequence corresponded to the altered base-pairs. When the correct sequence had been identified, it was

extracted from PDK with an *Nco*I/*Xho*I digest. This was then cloned into the sub-clone of *dcry* in PBS (described in **fig 5.4**) which had been prepared with an *Nco*I/*Xho*I digest. The necessity for this additional step was that *dcry* contains another *Nco*I site 5' to the *Bam*HI site. This construct was then sequenced. Following confirmation that the sequence was correct, a *Bam*HI/*Xho*I digest was used to extract the altered *dcry*(422-1625) sequence. This was then cloned into the *dcry* in PBS (described in **fig 5.3**) which had been prepared with a *Bam*HI/*Xho*I digest; this gave the full length *dcry*S526D in PBS.

Cloning of *dcry*V531K

The cloning methodology used to clone this construct was identical to that described for *dcry*S526D. The same 5' primer (*dcry*5E) was used in the initial PCR reaction. The only difference in the cloning strategies was that the following 3' primer was used in the initial amplification: CTC GAG TCA AAC CAC CAC GTC GGC CAG CCA GAA GAA CTG ACG TTT(CAC) TTC CTC CTC GTT GGA TGG TC. The bases highlighted in blue show the altered bases in the primer, while the bases in brackets denotes the codon as it appears in wild-type *dcry*. The alteration causes a substitution of valine for lysine at position 531 of the coded protein. All other cloning steps are as described above for *dcry*S526D.

Cloning of *dcry*E530P

The cloning methodology used to clone this construct was identical to that described for *dcry*S526D and *dcry*V531K. The same 5' primer (*dcry*5E) was used in the initial PCR reaction. The only difference in the cloning strategies was that the following 3' primer was used in the initial amplification: CTC GAG TCA AAC CAC CAC GTC GGC CAG CCA GAA GAA CTG ACG CAC TGG(TC) CTC CTC GTT GGA TGG TC. The bases highlighted in blue show the altered bases in the primer, while the bases in brackets denotes the codon as it appears in wildtype *dcry*. The alteration causes a substitution of glutamic acid for proline. All other cloning steps are as described for *dcry*S526D.

Cloning of *dcry* variants into pEG202 for yeast-two hybrid assays

When it was necessary for the cloned constructs to be used as bait in a yeast two-hybrid assay (see section 2.6), in every case the complete sequence was excised from pBS with an *Eco*RI-*Xho*I digest and ligated into the pEG202 vector (see section 2.3) which had been prepared with an *Eco*RI-*Xho*I digest. All of the cloned sequences described above were cloned into pEG202. *dcry* was already available in pEG202. PER233-685 and TIM in pJG4-5 (see section 2.3) were already available for use as prey in yeast-two hybrid studies. All sequences were cloned into pEG202 such that the reading frames were in correct alignment with the *lex*A sequence.

Cloning of *dcry* variants into pUAST

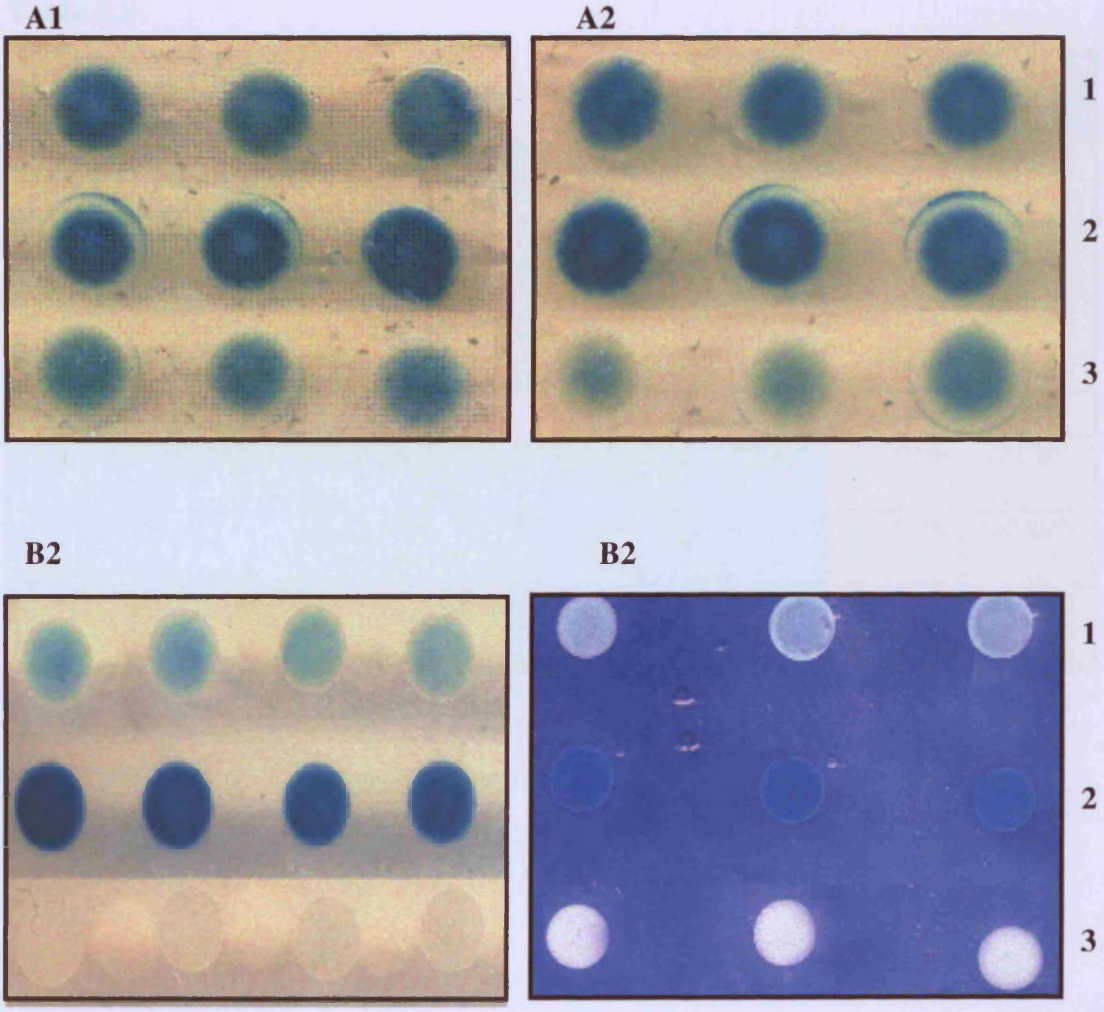
In order to move the sequences into PUAST (see section 2.3), the complete sequences were excised from pBS with a *Not*I-*Xho*I digest and ligated into PUAST cut with *Not*I-*Xho*I. This put the sequence in the correct orientation for transcription. All *dcry* variants described in this chapter were ligated into PUAST for future generation of transgenic fly lines.

5.3.2 Yeast plate assays

The yeast plate assays revealed that dCRYS526D and dCRYV530K interacted with *Drosophila* PER and TIM in a manner indistinguishable from dCRY. Therefore, both mutated forms of dCRY interacted with both PER and TIM in a light dependent manner, with no interaction occurring in darkness (see fig **5.5**). There was no distinguishable difference in the intensity of the interaction (as assessed by any variations in the intensity of the blue colour) when comparing the interactions of the mutated forms of dCRY with PER and TIM with the interactions of unaltered dCRY with PER and TIM. However, the yeast plate assay is not designed to be a quantitative assay, therefore unless the difference in interactions intensity was very great, it would be unlikely to be evident in a plate assay. dCRYE530P does not interact with PER or TIM in either light or dark conditions. This is not surprising as the mutation completely disrupts the α-helical structure that is the likely binding surface for PER and TIM.

However, the semi-quantitative assay reveals that dCRYS526D interacts more strongly with both PER and TIM as compared to dCRY, whereas dCRYV531K shows reduced interaction (see **fig 5.6**). The Scion analysis for the blots gives quantitative data about the relative interactions (see **table 5.1**). As discussed in Chapter 2, the scion analysis merely shows the relative difference between the interactions for the assays, and provides quantitative data to support the plate assays. From table 5.1, it can be seen that the interaction between dCRYS526D and TIM is more than twice the values for dCRY. This does not indicate the dCRYS526D interacts twice as strong with TIM as compare to dCRY, as the assay is not that precise, however, it does indicate that the interaction differences are significant, and that the point mutation Serine(526) to Aspartic acid increases the affinity between dCRY and TIM relative to wild-type dCRY. Conversely, the mutation of V to K at position 530 has significantly reduced the interaction between dCRYV530K and TIM, relative to both dCRY and dCRYS526D. Clearly, the point mutations have had opposite effects from each other.

Fig 5.6 Semi-quantitative yeast assay results for point mutations of dCRY tested against PER(233-685) and TIM: dCRY, dCRYS526D and dCRYV531K were tested as bait against PER233-685 and TIM. **A1 and A2:** Two independent assays, both representing the results for the constructs challenged with TIM. **B1 and B2**: Two independent assays, both representing the results for the constructs challenged with PER233-685. In all cases: 1=dCRY, 2=dCRYS526D, 3=dCRYV531K. The results were performed at least 3 times for each test, using independently grown liquid yeast cultures, and all assays were repeated once more using independently transformed yeast cultures. All assay plates were exposed to constant light during growth.



Tables 5.1 and 5.2: Pixel density analysis of yeast-two hybrid semi-quantitative assays: these values are the relative pixel densities for each colony of the semi-quantitative assays. Table 5.1 shows the results for the dCRY variants challenged with TIM, with A1 and A2 representing independent assays. Table 5.2 shows the results for the dCRY variants challenged with PER233-685, with B1 and B2 representing independent assays. The column headed 'relative to control' shows the value of the averages as percentages of the values for the dCRY assays; this has been calculated separately for each assay. The column headed 'relative to highest' shows the value of the averages as percentages of the highest value; this has been calculated separately for each assay.

Table 5.1

A1	1	2	3	Average	Relative to control	Relative to highest
dCRY vs TIM	134.75	131.33	133.44	**133.17**	100.00%	81.37%
dCRYS526D vs TIM	171.38	170.32	149.32	**163.67**	122.91%	100.00%
dCRYV530K vs TIM	90.4	85.2	103.51	**93.04**	69.86%	56.84%
A2						
dCRY vs TIM	130.18	125.76	130.71	**128.88**	100.00%	77.03%
dCRYS526D vs TIM	162.07	177.99	161.86	**167.31**	129.82%	100.00%
dCRYV530K vs TIM	104	98.93	100.68	**101.20**	78.53%	60.49%

Table 5.2

B1	1	2	3	4	Average	Relative to control	Relative to highest
dCRY vs PER233-685	71.93	70.7	65.29	61.42	**67.34**	100.00%	37.81%
dCRYS526D vs PER233-685	199.76	188.26	161.91	162.47	**178.10**	264.50%	100.00%
dCRYV530K vs PER233-685	40.78	32.12	31.98	29.04	**33.48**	49.72%	18.80%
B2							
dCRY vs PER233-685	101.68	90.34	94.07		**95.36**	100.00%	47.68%
dCRYS526D vs PER233-685	200.08	198.95	201		**200.01**	209.74%	100.00%
dCRYV530K vs PER233-685	59.57	54.08	59.29		**57.65**	60.45%	28.82%

5.4 Discussion

As discussed in section 5.1, the region around the C-terminal of dCRY from 519 to 533 may be significant in regulating dCRY interactions. The search for protein motifs around this region revealed three possible binding sites, which were altered using site-directed mutagenesis to produce point mutations that altered the residues at key sites. The change of Serine to Aspartic acid at position 526 was originally intended to place a negatively charged residue at a possible phosphorylation site, being an essential residue for the binding of CK2 and PKA. The presence of the negative charge at this site was thought to confer the same effect as being phosphorylated; hence the point mutation might give a form of dCRY constantly pseudo-phosphorylated at this site. Since phosphorylation has been shown to be important for the activity of CRY in other organisms, as discussed in Chapter 1, it is likely that phosphorylation may also play a role in the regulation of dCRY activity, and taking the other evidence into account, this site was a likely candidate for investigation. While these assays are crude and can only be used to compare like with like, the analysis clearly shows that the Serine to Aspartic acid substitution gives a variant of dCRY that interacts

with both TIM and PER233-685 more strongly that wild-type dCRY. The second apparent feature is that this increase in strength of interaction appears to be more pronounced for PER233-685 as compared with TIM. Therefore it can be concluded that this mutation produced a form of dCRY (dCRYS526D) that interacted with both PER233-685 and TIM with a significantly greater affinity than wild-type dCRY.

The point mutation V to K at position 531 removes the class III PDZ domain binding site which was identified in the same process as described above, seems to reduce the affinity between dCRY with the mutation (dCRYV531K) and PER233-685 and TIM, but to a lesser degree with TIM (see table 5.1), or rather the mutation produces a more pronounced effect on the interactions with PER233-685. In fact, in the conditions of the semi-quantitative assay it is not possible to say whether dCRYV531K interacts with PER233-685 at all, as no blue colour is detectable. Certainly the difference in the strength of interactions is more pronounced with the interactions with PER233-685 as compared to those with TIM.

The dCRY point mutation that changes E to P at position 530 produced a form of dCRY that interacted with neither PER233-685 nor TIM under either of the light conditions used. The mutation was designed to remove the secondary structure of the region that had been predicted. The result is not unexpected, as the predicted alteration generated by this mutation to this region is substantial.

In conclusion, it would appear that the region identified as being important to dCRY interactions with PER233-685 and TIM does have a function in these interactions, as the affinity between dCRY and PER233-685 and TIM can be altered through producing substitution mutations in possible protein binding sites. Whether this is due to altered interactions with some trans-partner of dCRY, or whether the mutations have an inherent effect on the affinity of dCRY with PER233-685 and TIM is not clear from these experiments. Further work is required, particularly in identifying whether the predicted possible trans-acting factors do serve to modify dCRY, and if so, in what way. Phosphorylation is a likely modification, and a likely candidate site at residue position 526 has been

identified, the alteration of which increased dCRY affinity with TIM, but to a greater extent with PER233-685. Exactly why the effect should be to modify the affinity between dCRY and PER233-685 and TIM is unknown. The region that the mutations were induced in is unique to dCRY, and therefore were the interaction domain to lie in this region, this would run contrary to the previously discussed notion that the interaction domain lies in a region homologous between dCRY and mCRY1 (see Chapter 4). It is possible that this region from 519-530 is necessary for light regulation, but that the way that it prevents dCRY interacting with PER233-685 and TIM is altered by the mutations, which lead to effects seen in light.

It is difficult to suggest a mechanism behind the results at this stage, and more work is required to dissect the mechanism of light repression of dCRY. The first step would be to map the interaction domain/s of dCRY with PER233-685 and TIM, then to define the region essential for light regulation, and how these two domains might interact. The identification of any proteins that interact and modify the C-terminal of dCRY would be another important focus of any further investigations.

The next step for the mutated version of dCRY already generated would be the creation of transgenic flies, as the yeast assay, while providing a good basis for assessing dCRY interactions as a first step, needs to be supporting by analysis of the expression of the mutations in Drosophila at the behavioural and molecular level. In summary, this work has identified a possible region of importance for light regulation and possible regions for protein modification, but has only begun the work that is required to gain a full understanding of the regulation of dCRY.

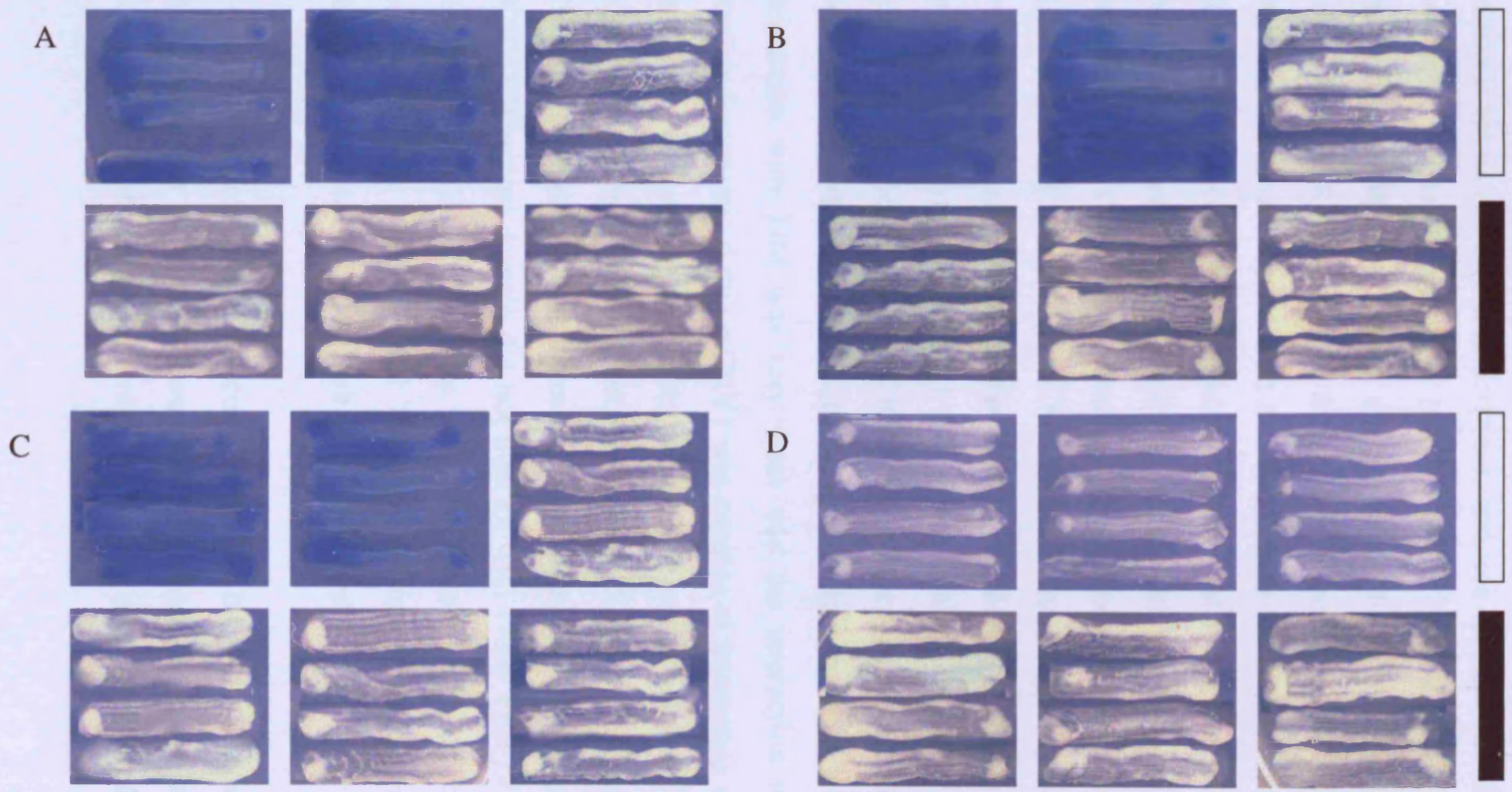


Fig 5.3 Yeast plate assay results for point mutations of dCRY tested against PER(233-685) and TIM: A=dCRY, B=dCRYS526D, C= dCRYV531K and D=dCRYE530P were tested as bait against PER233-685 and TIM, using empty pJG4-5 as a control, in both light and dark conditions. Protein interaction is indicated by a blue colour. The L and D at the far left of each row signify the light conditions the assays were grown in: L=light, D=dark. All yeast assays were repeated using independently transformed yeast strains.

Chapter 6: Final Conclusions

I have attempted to further elucidate the role of dCRY in the central circadian clock of *Drosophila*, specifically focusing on the mechanism by with dCRY is light regulated in its interaction with *Drosophila* PERIOD and TIMELESS. The previous evidence that my work was based on, demonstrated that the C-terminal domain of dCRY was essential for its repression in darkness, and that on removal of the last 22 amino acid residues a constitutively active form of dCRY (dCRYΔ) was produced. The notion that a discrete light-regulatory domain might lie in the C-terminal portion that was absent from dCRYΔ warranted further investigation, and it was decided to replace the dCRY C-terminal domain with a non-homologous domain from a protein similar to dCRY, but which was not light regulated. mCRY1 was selected as the reciprocal protein in a C-terminal domain swap that should, if a the light-regulation domain was intrinsic to the C-terminal domain, add a light-regulation domain to mCRY1 and a light-independent domain to dCRY. In the simplest theory would be that the light-regulation domain would confer light repression onto the mCRY1 protein which, while dCRY with the mCRY1 C-terminal domain would simply be constitutively active. Using yeast assays mCRY1 was challenged with both *Drosophila* PER and TIM, and found to interact with both in a light independent manner, though the interaction with TIM was very weak and the interaction with PER very strong. Having demonstrated that mCRY1 was capable of interacting with the components of the *Drosophila* clock, the chimerics were constructed with exchanged C-terminal domains between dCRY and mCRY1 and challenged in yeast assays with PER and TIM in light and dark conditions. The initial results were surprising: mCRY1 with a dCRY C-terminal domain did not interact with either PER or TIM in either light or darkness whereas dCRY with a mCRY1 C-terminal domain became light regulated, interacting with both PER and TIM, but not in darkness as mCRY1 does. In that sense this was the opposite of what was expected.

To identify which parts of the constructs were interacting with PER and TIM several more constructs were made consisting of truncated forms of dCRY and mCRY1. Using this methodology it was found that the C-terminal domain of mCRY1

interacted with PER and TIM whereas the photolyase domain of dCRY did not. The simplest explanation is that the interaction domain is within the mCRY1 region of the dCRY-mCT chimera, and that light regulation is conferred by the dCRY photolyase domain. That a light signal should be conferred by the photolyase domain is not in itself surprising, but the dCRYΔ data had strongly indicated that the C-terminal domain of dCRY was required for repression in darkness to occur. In the absence of this region it is hard to understand how the interaction domain of mCRY1 is repressed in darkness and released in light, when mCRY1 itself is not light regulated (in the central clock). The answer could lie in the fact that in mammalian peripheral clocks mCRY1 may have a photoreceptor function, but this is merely unsupported speculation. That the dCRY photolyase domain conveys a light signal had previously been found in experiments with transgenic flies that expressed dCRYΔ in an essentially dCRY null background. These flies would show a lengthening of period and eventual arrhythmia under constant light when over-expressing dCRYΔ, but remain rhythmic if dCRYΔ is not expressed. Therefore, dCRYΔ, in the absence of the C-terminal domain, is still conveying a light signal.

The creation of transgenic flies expressing mCRY1 and dCRY-mCT was the next step from the yeast assays, and provided more robust data. It was established that mCRY1 could not serve as a photoreceptor in *Drosophila*, and the over-expression of mCRY1 only increased the period in darkness by a statistically significant, but still subtle, increase of about 26 minutes. The exact cause of this is not known, and would most likely require detailed ICC work to fully identify, but based upon the interaction between mCRY1 and PER it had been hypothesised prior to the fly studies that a lengthening of period might be seen, as PER was sequestered by its interactions with mCRY1 thus delaying the clock cycle. The fly studies with lines over-expressing dCRY-mCT proved far more interesting, as these lines produced an almost identical behavioural phenotype to dCRYΔ fly lines: a lengthening of period in darkness and retaining rhythmicity in constant light for several days. In fact, these characteristics of dCRYΔ flies were even more distinct in the dCRY-mCT flies. Once again, this was a surprise result. The yeast data had suggested a light regulated protein. Analysis of these flies at the molecular level by Western blot analysis, using among the others the anti-dCRY antibody generated as part of this work, and detailed in Chapter 3, revealed some very interesting features. Western blots on samples of flies

taken over the circadian period showed that TIM cycling was reduced and that hyperphosphorylated TIM was absent, which is the same TIM profile as seen in dCRYΔ flies. However, the significant difference between dCRYΔ and dCRY-mCT flies was that whereas dCRYΔ is constantly degraded, even in darkness, dCRY-mCT is stable throughout the day, and shows no evidence of degradation even in light. It would appear that the presence of the mCRY1 C-terminal domain protects the protein from the light induced degradation that is a principal feature of dCRY. dCRY-mCT is therefore at many times higher levels in the pacemaker cells than dCRYΔ, and remains without being degraded even in light. The exact mechanism by which dCRY-mCT is then constantly active is a subject for further investigation, but certainly activation by light is detached from degradation, hence it is possible activated dCRY-mCT remains and accumulates over time. Sufficient levels of activated dCRY-mCT present in the night may provide long enough 'light signal' to generate the lengthening of period seen in these flies. The very high levels of dCRY-mCT may also mean that any trans-acting factor necessary for repression of dCRY activity is overwhelmed, hence the repression mechanism fails to regulate dCRY-mCT. These are two possible mechanisms by which the effects seen may be explained. Only further investigation will confirm the nature of the effect.

Immediate further work would be to do a PER profile on dCRY-mCT fly lines using Western blot analysis, which was not done for this investigation due to time constraints. Pulse experiments should also be conducted for dCRY-mCT fly lines to assess sensitivity to light. For mCRY1 expressing flies, the profile of mCRY1 over the circadian period should be determined by Western blot analysis, and similarly for PER and TIM in these flies. ICC work could be used to distinguish any difference in location for mCRY1 and dCRY-mCT and the effects on PER and TIM location and distribution also. For these studies it might be possible to use the dCRY antibody that was generated as part of this work. It has been suggested in Chapter 4 that the likely interaction domain of mCRY1 with PER and TIM is in the homologous domain that was swapped to prevent disruption of the protein's secondary structure. If this is true then the dCRY interaction domain may well be the corresponding region, which lies just N-terminal to the C-terminal domain. The use of site directed mutagenesis and more chimerics could be used to map this domain precisely.

Finally, based upon some previous data that suggested that the region of dCRY from residue 519-530 was essential for PER and TIM interaction and light regulation, the idea of post translational modification of the C-terminal of dCRY as a system of regulation was explored through site directed mutagenesis of site that were putative targets of common protein factors found in both *Drosophila* and yeast. That dCRY is light regulated in yeast is an important consideration, as it is only explainable if whatever mechanism that serves to regulate dCRY in *Drosophila* also exists in yeast. Should this mechanism involve a trans-acting partner to modify dCRY, this must be common to both *Drosophila* and yeast. Three such potential motifs were found in the C-terminal domain of dCRY, and mutations were introduced to modify them. The mutation that disrupted the secondary structure of this region gave a dCRY version that did not interact with either PER or TIM in yeast assay. The version of dCRY where serine was replaced with aspartic acid at position 526, to place a negative charge at a putative phosphorylation site, gave a form that was light regulated, but which had a greater affinity for both PER and TIM, but especially TIM. The replacing of V with K at position 531 had the opposite effect, and reduced dCRY interaction with both PER and TIM, but especially with TIM. Whether these changes in PER/TIM affinity are due to some modification of the mechanism by which dCRY activity is repressed is not something that can be elucidated from this data. However, that changes in affinity have been produced is certain, and the significance of this region of dCRY is further supported. This work was only the beginning, and there is a great deal more required to uncover the process of dCRY regulation.

To follow on from these studies, transgenic fly lines expressing dCRYS526D and dCRYV531K should be created, and the flies assessed at both the behavioural and molecular levels, as yeast assays provide a robust indicator but only as an initial assessment and to support subsequent fly data. That this region of dCRY warrants further investigation is clear, particularly as to whether a trans-acting factor does bind to an of these motifs, and if so how this fits in with the circadian cycle, and how the modification effects dCRY.

This work has served to provide further understanding of the regulation and interactions of the unique photoreceptor of the *Drosophila* circadian clock, and has gone some way to highlighting that past models of dCRY regulation may have been

too simplified. It is clear that dCRY regulation is complex, and that this study has not elucidated them. However, it has gone some way to provide understanding from which future studies can be based. This work has begun a line of inquiry, rather than taking an existing one further, and has taken a novel approach to dissecting the role of dCRY.

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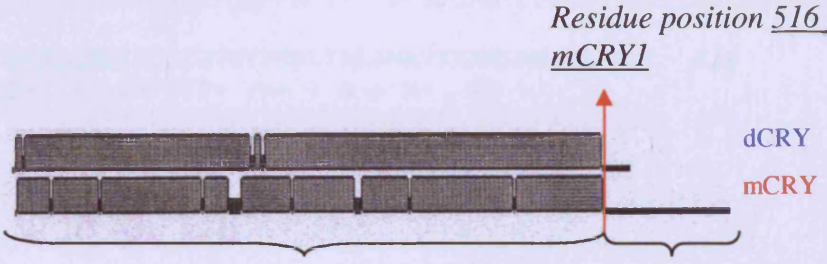
Appendix 1

A BLAST alignment (Tatiana and Madden 1999) between *Drosophila* CRYPTOCHROME (dCRY1) and mouse CRYPTOCHROME (mCRY1) reveals similarity principally in the region between residues 1-516 of dCRY with the region 1-491 of mCRY1.

A: a visual representation of the comparison between dCRY1 and mCRY1 reveals a level similarity with unique C-terminal domains.



Similarity indicated graphically through a solid bar format.

Dissimilarity indicated graphically through a thin line.

B: The alignment of the amino acid sequences of dCRY1 (shown in blue) and mCRY1 (shown in red). The numbers at the start and end of each sequence signifies the position number of the first and last residues respectively relative to the first residue of the proteins (position 1). The line between the two sequences indicates the similarities between the sequences. If the query and the subject have the same amino acid at a given location, the residue itself is shown. Conservative substitutions, as judged by the BLAST algorithm, are indicated with +.

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5 GANVI-WFRHGLRLHDNPALLAALADKDQGIALIPVFIFDGESAGTKNVGYNRMRFLLDS
  G N + WFR GLRLHDNPAL   +   D    +  V+I D   AG+ NVG NR RFLL
2 GVNAVHWFRKGLRLHDNPALKECIQGAD---TIRCVYILDPWFAGSSNVGINRWRFLLQC

LQDIDDQLQAATDGRGRLLVFEGEPAYIFRRLHEQVRLHRICIEQDCEPIWNERDESIRS
L+D+D  L+        RL V  G+PA +F RL ++   + ++ IE D EP    ERD +I+
LEDLDANLRKLNS---RLFVIRGQPADVFPRLFKEWNITKLSIEYDSEPFGKERDAAIKK

LCRELNIDFVEKVSHTLWDPQLVIETNGGIPPLTYQMFLHTVQIIGLPPRPTADARLEDA
L  E  ++ + ++SHTL+D   +IE NGG PPLTY+ F   V  +  P    AD    D
LATEAGVEVIVRISHTLYDLDKIIELNGGQPPLTYKRFQTLVSKME-PLEMPADTITSDV

TFVELDPEFCRSLKLFEQLPTPEHFNVYG----DNMGF----LAKINWRGGETQALLLLD
+ P                + +H   YG    + +GF    L+   W GGET+AL  L+
```

```
IGKCMTPL-----------SDDHDEKYGVPSLEELGFDTDGLSSAVWPGGETEALTRLE

ERLKVEQHAFERGFYLPNQALPNIHDSPKSMSAHLRFGCLSVRRFYWSVHDLFKNVQLRA
L  E+ A+   F    P     ++  SP  +S +LRFGCLS R FY+ + DL+K V+  +
RHL--ERKAWVANFERPRMNANSLLASPTGLSPYLRFGCLSCRLFYFKLTDLYKKVKKNS

CVRGVQMTGGAHITGQLIWREYFYTMSVNNPNYDRMEGNDICLSIPWAKPNENLLQSWRL
       +    + GQL+WRE+FYT + NNP +D+MEGN IC+ IPW K N   L  W
-------SPPLSLYGQLLWREFFYTAATNNPRFDKMEGNPICVQIPWDK-NPEALAKWAE


GQTGFPLIDGAMRQLLAEGWLHHTLRNTVATFLTRGGLWQSWEHGLQHFLKYLLDADWSV
G+TGFP ID  M QL  EGW+HH  R+ VA FLTRG LW SWE G++ F + LLDADWS+
GRTGFPWIDAIMTQLRQEGWIHHLARHAVACFLTRGDLWISWEEGMKVFEELLLDADWSI

CAGNWMWVSSSAFERLLDSSLVTCPVALAKRLDPDGTYIKQYVPELMNVPKEFVHEPWRM
AG+WMW+S S+F +        CPV   +R DP+G YI++Y+P L   P +++++PW
NAGSWMWLSCSSFFQQFFHCY--CPVGFGRRTDPNGDYIRRYLPVLRGFPAKYIYDPWNA

SAEQQEQYECLIGVHYPERIIDLSMAVKRNMLAMKSLRNSL  516
Q+  +CLIGV+YP+ +++ + A + N+  MK +   L
PEGIQKVAKCLIGVNYPKPMVNHAEASRLNIERMKQIYQQL  491
```

Appendix 2

Spectral data for transgenic flies expressing mCRY1 in a 12:12 light:dark regimen giving the circadian period in hours.

The control lines:

	w^{1118};+/UAS-HAmcry1.1	*w^{1118};+/UAS-HAmcry1.2*	*w^{1118};+/UAS-HAmcry1.3*	*w^{1118};;UAS-HAmcry1.4/+*	*w^{1118};+/UAS-HAmcry1.5*	*yw;tim-GAL4/+*
	24.22	23.9	24	24.16	24.04	24.1
	23.86	24.12	23.88	24.16	23.88	24.16
	23.94	23.9	24.04	23.94	23.94	24.04
	24.04	23.86	23.84	24	24.16	24.16
	24.04	23.94	23.88	23.9	24.16	23.88
	24	24.04	24	23.94	24	24.16
	23.94	23.9	24	24.08	23.84	24.16
	23.94	24.08	23.88	23.9	23.88	24.2
	24	23.9	24.04	24	24.1	24.1
	24	23.9	23.94	24.34		23.94
	24.4	23.68	23.94	24		24.16
	24.04	24.12		24		
	24.12	23.9		23.9		
	23.82	24.12		24.08		
	23.94	24.08		23.94		
	24	24		24.12		
	24.04	23.86		24.04		
	23.82	24		23.9		
	24	23.86		23.9		
	24	24.22		24.16		
	23.9	24				
	24	24.16				
	24					
	23.86					
	24.04					
	24.1					
	24.04					
	23.78					
	23.94					
	23.88					
	23.94					
	23.88					
	24					
	23.94					
	24					
	23.84					
	23.94					
	23.94					
	23.84					
Average	**23.97**	**23.98**	**23.95**	**24.02**	**24**	**24.1**
StDEV	**0.11**	**0.13**	**0.07**	**0.12**	**0.12**	**0.1**

The over-expressing lines:

	yw;tim-GAL4/UAS-HAmcry1.1	*yw;tim-GAL4/UAS-HAmcry1.2*	*yw;tim-GAL4/UAS-HAmcry1.3*	*yw;tim-GAL4/+;UAS-HAmcry1.4/+*	*yw;tim-GAL4/UAS-HAmcry1.5*
	23.94	24.04	24	23.94	24.1
	24.04	24.08	24.1	23.94	24.2
	23.94	24.08	24	23.9	24
	24.16	24	24.04	24	24
	23.9	24.04	23.88	23.94	24.32
	24	23.94	24	24	24.1
	24	23.78	24.1	24.04	24.16
	24.04	24.04	24.1	23.86	24
	24	23.94	24.04	23.9	24.16
	23.86	24.04	23.78	23.9	23.94
	23.9	24	24	23.94	24.04
	24.1	24.04	24.04	24	24.2
	24.04	24	24.26	24.04	23.94
	24.26	23.86	24.2	23.86	24
	23.88	23.94		23.9	23.94
	24.16	23.86		23.86	24.1
	23.94	23.9		23.86	23.94
	24.2	23.9		23.9	24.2
	24.1	24.08		23.78	
	23.88			24	
	24.16			24.08	
	24.04			23.94	
	24.04			24	
	24.16			23.94	
	24.04			23.94	
	24.16			24.12	
	24.1			24.04	
	23.84			24	
	24.32			24	
	24.04			24.04	
	23.88			24	
	24.04				
	24				
Average	**24.04**	**23.98**	**24.04**	**23.96**	**24.07**
StDEV	**0.12**	**0.09**	**0.12**	**0.08**	**0.11**

Appendix 3

Spectral data for transgenic flies expressing mCRY1 in a constant darkness regimen giving the circadian period in hours.

	w^{1118};+/UAS-HAmcry1.1	*w^{1118};+/UAS-HAmcry1.2*	*w^{1118};+/UAS-HAmcry1.3*	*w^{1118};;UAS-HAmcry1.4/+*	*w^{1118};+/UAS-HAmcry1.5*	*yw;tim-GAL4/+;*
	23.67	24.1	24.35	23.99	24.62	24.88
	24.32	23.57	23.73	24.21	24.53	24.65
	25.36	24.1	24.44	24.32	24.26	24.32
	24.43	24.32	24.35	24.43	23.82	24.1
	24.32	24.88	24.17	24.1	24.26	24.32
	24.77	24.54	24.08	24.1	24.53	24.87
	24.32	24.77	24.08	24.43	24.26	24.26
	25.72	24.1	23.99	23.36	24.35	24.62
	24.77	24.65	23.82	24.65	23.31	24.17
	24.21	24.54	23.39	24.65	24.08	24.08
	24.54	24.21	23.14	23.26	24.08	24.9
	23.99	24.77	23.65	24.69	24.59	24.35
	24.32	24.09	24.08	23.52	23.24	24.54
	23.91	25	23.9	25	24.49	25.11
	23.91	24.19	23.71	24.49	24.09	23.42
	23.56	24.09	24.59	24.29	23.9	23.74
	23.99	23.61	23.99	24.09	24.59	24.34
	23.91	23.99	24.29	24.59	23.8	23.8
	24.17	24.29	24.59	24.59	23.43	23.52
	24.17	23.43	23.9	24.79	24.29	23.66
	23.23	23.8	23.9	23.24	23.99	25
	24.35	24.69	24.19	23.8	23.99	24.59
	23.91	24.49	24.29	24.69	24.59	24.69
	24.17	23.7	24.59	23.61	23.8	24.54
	23.06	23.9	24.19	25	23.61	24.04
	24.19	23.43	23.61	23.8	24.79	24.79
	23.9	23.06	24.59	23.71	23.9	23.33
	23.9		23.9	24.19		24.69
	23.52					24.69
	24.09					
	23.99					
	24.09					
	24.69					
	23.99					
	23.71					
	23.8					
	24.09					
	23.8					
	23.61					
	24.29					
	23.8					
	24.19					
Average	**24.11**	**24.16**	**24.05**	**24.2**	**24.12**	**24.35**
StDEV	**0.49**	**0.49**	**0.37**	**0.51**	**0.42**	**0.49**

yw;tim-GAL4/UAS-HAmcry1.1	*yw;tim-GAL4/UAS-HAmcry1.2*	*yw;tim-GAL4/UAS-HAmcry1.3*	*yw;tim-GAL4/+;UAS-HAmcry1.4/+*	*yw;tim-GAL4/UAS-HAmcry1.5*
25.85	23.67	24.62	24.1	24.17
26.1	23.67	24.72	24.21	23.39
26.49	24.77	24.53	24.54	24.35
25.6	23.99	25	23.99	23.99
25.72	24.77	24.26	23.88	24.62
25.12	23.78	23.99	25	24.44
24.32	23.67	24.53	24.77	24.72
24.1	23.78	23.73	24.77	24.53
23.57	24.29	24.53	24.21	24.62
24.65	24.79	24.62	24.77	24.35
24.32	23.52	24.44	25.12	24.26
24.72	24.59	24.35	23.99	23.73
24.35	24.59	24.35	23.99	24.62
24.9	25.54	24.59	23.57	24.44
24.44	24.69	25.11	23.67	23.73
24.08	23.8	25.11	24.79	24.53
24.44	24.19	25.43	25.32	23.91
24.9	24.28	24.9	25.54	23.99
23.31	23.99	24.19	24.49	25.21
24.81	24.79	24.44	24.69	24.49
23.82		25.11	25.21	24.59
23.73		25.43	25.11	23.9
24.62		24.59	24.39	24.9
24.44		25.11	25.32	24.29
24.72		24.49	24.09	24.79
25.1		24.79	24.49	24.59
24.53		24.64	24.68	25.21
24.35		24.19	25	25.11
24.72				25.43
24.44				25.2
23.99				24.09
25.1				25.21
24.62				24.69
24.9				25.43
23.9				24.49
24.09				
24.39				
24.39				
24.9				
24.9				
25.43				
24.69				
24.49				
24.59				
24.9				
24.49				
24.39				
25.11				
24.19				
24.49				

	25.11				
Average	**24.65**	**24.26**	**24.64**	**24.56**	**24.51**
StDEV	**0.61**	**0.54**	**0.41**	**0.53**	**0.5**

Appendix 4

Spectral data for transgenic flies expressing mCRY1 in a *cry*^{b} background in a constant dark regimen giving the circadian period in hours.

w;UAS-HAmcry1.1/+;cry^{b}/cry^{b}	*yw;tim-GAL4/UAS-HAmcry1.1;cry^{b}/cry^{b}*	*w;UAS-HAmcry1.3+;cry^{b}/cry^{b}*	*yw;tim-GAL4/UAS-HAmcry1.3;cry^{b}/cry^{b}*	*w;UAS-HAmcry1.4/+;cry^{b}/cry^{b}*	*yw;tim-GAL4/UAS-HAmcry1.4;cry^{b}/cry^{b}*	*yw;tim-GAL4/UAS-dcry;cry^{b}/cry^{b}*	*yw;tim-GAL4/+;cry^{b}/cry^{b}*
24.39	24.39	23.9	24.9	23.64	24.39	24.39	23.64
24.9	24.39	23.43	24.39	24.38	24.39	24.38	24.18
24.39	24.39	23.9	24.39	24	24.9	24.14	24.18
24.39	24.39	24.39	24.39	24.76	24.14	24.14	24.38
24.9	24.39	24.39	24.39	24.38	24.39	24.9	24.18
24.9	24.39	23.9	24.9	24.38	24.38	24.14	24.38
23.43	24.14	23.9	24.38	24	24.39	24.9	24.56
23.9	24.39	24.9	24.9	24	24.39	24.9	24.18
24.9	24.39	24.39	24.39	24.76	24.39	24.39	24.38
24.39	24.39	24.14	24.38	24.38	24.39	24	24.38
24.9	24.9	24.39	24.39	24.38	24.39	24.38	24.76
23.9	24.39	24.39	24.14	24	24.39	24.76	24.76
23.9	24.9	24.39	24.9		24.9	24	24.38
24.9	24.9		24		24.39	24	24.38
24.39	24.39		24.38		24.9	23.82	
24.39	24.9		24.38		24	24.56	
24.39	24		24.38		24		
24.39	24.38		24.38		24.38		
24.38	24.38		24		24.38		
24.38	24		24.38		24.38		
24.38	24		24.38		24		
23.64	24.18		24.76		24.38		
23.64	24		24.38				
23.64			24.38				

	24.38							
	23.64							
	24							
	24.38							
	24.38							
	24.38							
	24.38							
Average	**24.30**	**24.39**	**24.19**	**24.44**	**24.26**	**24.39**	**24.36**	**24.34**
StDEV	**0.42**	**0.28**	**0.37**	**0.25**	**0.33**	**0.25**	**0.36**	**0.28**

Appendix 5

Spectral data for transgenic flies expressing mCRY1 in a *cry*^{b} background in constant light regimen giving the circadian period in hours.

w;UAS-HAmcry1.1/+;cry^{b}/cry^{b}	*yw;tim-GAL4/UAS-HAmcry1.1;cry^{b} /cry^{b}*	*w;UAS-HAmcry1.3+;cry^{b}/cry^{b}*	*yw;tim-GAL4/UAS-HAmcry1.3;cry^{b}/cry^{b}*	*w;UAS-HAmcry1.4/+;cry^{b}/cry^{b}*	*yw;tim-GAL4/UAS-HAmcry1.4;cry^{b}/cry^{b}*	*yw;tim-GAL4/+;cry^{b} /cry^{b}*	*yw;tim-GAL4/UAS-dcry;cry^{b}/cry^{b}*	*Cry^{b}xTG4*
24	24.55	24	24.55	24.26	24	24.94	Arrhythmic	24
24.26	24	23.74	24	24	24.55	25.26		24.54
24	23.48	24	23.74	24.26	24	25.6		24
23.74	24	24	24.26	24.26	24	25.95		24.26
23.74	24.54	24.26	23.48	24.26	24	25.6		24.54
24.26	24	24	24.26	24.26	23.48	25.6		24.26
24	24	24.26	23.74	24	24	25.6		24
24	24	24.55	23.74	24.62	24.26	25.6		24
24.26	25.6	24.55	25.26	24.94	24	24.94		24
24.94	25.6	25.6	25.6	25.6	24.76	25.26		24.38
25.6	24.3	25.6	24.62	24.62	24	25.6		24
25.6	25.6	26.3	24.94	25.26	24	25.26		23.64
24.94	25.26	25.95	25.95	23.7	24.38	26.3		24
24.94	25.26	24.94	24.94	24.3	24.38	24.94		
25.6	25.6	24.94	23.7	23.13	24.76	24.62		
25.95	25.26	25.95	24.3	24.94	24	23.64		
25.26	26.3	25.6	25.95	24.62	24	25.16		
25.26	24	25.6	25.26	23.7	24	24.38		
25.26	23.64	26.3	24.94	23.41	24	23.64		
25.26	25.16	26.3	25.6	24	23.64	24.38		
25.6	25.16	25.6	25.26	24.38	24.38	25.57		
25.26	24	25.6	24	24.38	24.38			
25.95	24.38	24.38	24.3	24.76				
24.94	24	24.38	24.3	24.76				
25.6	24.38	24.84	25.6	24				
24.94	24.38	24.38	25.95	26				

	25.6	24.76	24	23.7				
	25.6	24.76	24	25.26				
	25.26			23.13				
	24			24.94				
	24			25.26				
	24.94			25.6				
	24			24				
	25.26			24.3				
	24.62			24.94				
	25.6							
	24.3							
	24							
	25.26							
	24.3							
	25.26							
	24.94							
	24.38							
	24.38							
Average	**24.84**	**24.64**	**24.92**	**24.67**	**24.40**	**24.14**	**25.14**	24.12
StDEV	**0.66**	**0.72**	**0.84**	**0.79**	**0.63**	**0.32**	**0.69**	0.26

Appendix 6

Spectral data for transgenic flies expressing dCRY-mCT in a 12:12 light:dark regimen giving the circadian period in hours.

yw;tim-GAL4/UAS-HAdcry-mct2	*w^{1118};UAS-HAdcry-mct2/+*	*yw;tim-GAL4/UAS-HAdcry-mct6*	*w^{1118};UAS-HAdcry-mct6/+*	*yw;tim-GAL4/+;UAS-HAdcry-mct7/+*	*w^{1118};;UAS-HAdcry-mct7/+*	*yw;tim-GAL4/+*
23.9	23.9	24.12	23.9	23.91	23.7	23.9
24.12	24.12	24.12	23.9	23.9	23.9	23.9
24.12	24.12	24.36	24.12	23.9	23.91	23.9
24.12	23.91	24.12	23.9	24.12	23.7	24.12
23.91	24.12	24.12	23.9	24.12	23.9	24.12
23.9	24.12	24.36	23.9	23.9	23.9	24.36
23.9	24.12	24.12	23.9	23.9	24.36	24.12
24.12	24.12	24.12	23.9	24.12	23.9	24.12
24.12	23.9	24.12	23.9	24.36	23.9	24.12
23.9	23.9	24.12	23.9	23.9	24.36	23.9
23.9	24.12	24.36	24.12	24.36	24.12	24.12
23.9	23.9	24.36	23.9	24.12	24.12	23.9
24.12	24.12	23.9	24.12	23.9	23.9	24.12
23.9	23.9	24.12	23.9	23.9	24.36	24.12
24.12	24.12	23.7	23.9	23.9	24.36	24.12
		24.36	23.9	24.12	24.12	23.9
		24.12	24.12	23.9	24.12	23.9
		24.12	23.9			24.12
		24.12	23.9			24.12
		24.12	23.9			24.12
						23.7
						24.12
						24.12
Average	**24.00**	**24.03**	**24.15**	**23.94**	**24.02**	**24.04**	**24.05**
StDEV	**0.11**	**0.11**	**0.16**	**0.09**	**0.16**	**0.22**	**0.14**

Appendix 7

Spectral data for transgenic flies expressing dCRY-mCT in a constant dark regimen giving the circadian period in hours.

yw;tim-GAL4/UAS-HAdcry-mct2	*w^{1118};UAS-HAdcry-mct2/+*	*yw;tim-GAL4/UAS-HAdcry-mct6*	*w^{1118};UAS-HAdcry-mct6/+*	*yw;tim-GAL4/+;UAS-HAdcry-mct7/+*	*w^{1118};;UAS-HAdcry-mct7/+*	*yw;tim-GAL4/+*
27.69	24	26.34	25.12	26	24	24.38
25.71	23.48	26.34	24.55	27.86	23.64	24
26.34	25.12	27	24	25.57	24	24
27	24	25.12	24	25.57	24.38	25.12
25.71	23.48	26.34	24	26.9	24	25.71
26.34	24.55	27	24.55	25.16	24	24.55
25.71	24	26.34	24	25.57	23.64	24
26.34	23.64	25.71	24.38	25.16	23.64	26.34
26.44	24	27.69	24.38	25.57	24.38	24.76
28.36	24	25.71	23.64	26	24	24.38
26.9	24.38	26.18	24	25.57	24	24.38
30	23.64	25.71	24	25.12	24.38	24.76
27.37	23.64	26.18	24	25.57	24	24.38
28.36	24.38	26.18	23.64	26.44	24	23.64
28.89	24	26.18	23.64	25.57	24	24.76
26.9	24.38		24	25.57	24	24
26	23.61		23.28	25.57	24.83	24.38
28.36	23.61		23.64	26	23.61	24.38
27.86	24		24	25.57	23.61	24.38
27.86	23.61		23.28	26.18	23.23	24.76
27.37	24		23.64	25.71	23.61	24.76
26.9	24		23.64	24.83	23.61	24.76
26	23.61		24.76	25.26	23.23	24.38
26.44	24		23.64	26.18	23.23	
26.18	24		24	26.18	23.61	
25.71	24		24.41	25.71	23.61	
27.17	24		23.61	25.71		

	25.71	24		23.61	25.71		
	26.67	24		23.61	26.67		
	27.17	24		23.61	25.71		
	26.67	24		24	26.18		
	26.67	23.61		24	26.18		
	26.18	24		24	26.18		
	25.71	23.61		23.61	25.71		
	25.71			24	25.71		
	26.18			24	26.18		
	27.17			24			
	25.26			24			
	26.67			23.61			
	26.67			24			
	27.69			24			
	26.67			23.61			
				24			
				24			
				23.61			
Average	**26.83**	**23.95**	**26.27**	**23.93**	**25.84**	**23.86**	**24.56**
StDEV	**1.00**	**0.34**	**0.62**	**0.37**	**0.56**	**0.38**	**0.58**