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Genetic and Biochemical Studies on Protein Phosphorylation in the Circadian Clock of Drosophila Melanogaster

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GENETIC AND BIOCHEMICAL STUDIES ON PROTEIN PHOSPHORYLATION IN THE CIRCADIAN CLOCK OF *DROSOPHILA MELANOGASTER*

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Saul Kivimäe

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GENETIC AND BIOCHEMICAL STUDIES ON PROTEIN PHOSPHORYLATION IN THE CIRCADIAN CLOCK OF *DROSOPHILA MELANOGASTER*

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Circadian rhythms in physiology and behavior are observed in almost all phyla. Genetically encoded internal clocks generate such rhythms. Identification of gene products required for the generation and maintenance of endogenous circadian near 24-hr rhythms has led to a paradigm of multiple interlocked transcriptional/translational feedback loops as the basis for molecular circadian oscillators in all studied model systems. Protein phosphorylation plays an essential role, regulating the stability, activity and subcellular localization of proteins that constitute the biological clock.

This study investigates the role of the protein kinase Doubletime, a *Drosophila* ortholog of casein kinase 1ε, in the fruit fly circadian clock. For the first time enzymatically active Doubletime protein is produced and direct phosphorylation of clock protein Period is demonstrated. Phosphorylation sites are identified and their functional significance is tested in a cell culture system. An *in vivo* analysis of a Period mutant that eliminates one of the identified phosphorylation sites is also carried out. The analysis suggests that phosphorylation dependent regulation of Period protein stability, transcriptional repressor activity and possibly subcellular localization may all be regulated in an integrated fashion that involves two sequence motifs in the center of the Period protein with high affinity for phosphorylation by Doubletime.

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LIST OF ABBREVIATIONS

aa	amino acid
°C	degrees Celsius
lac Z	β-galactosidase
µl	10^{-6} liter
bp	base pair
CLD	cytoplasmic localization domain
CT	circadian time in hours after onset of subjective day
DD	constant darkness
ECL	enhanced chemiluminesence
EMS	ethyl methanesulfonate
FASPS	familial advanced sleep phase syndrome
GST	gluthathione-S-trasfrease
hr	hour
KDa	kilo Dalton
l	liter
LD	Light:Dark cycle, 12h:12h
LRE	light-response element
M	Molar, mole per liter
min	minute
ml	10^{-3} l
mM	10^{-3} Molar
ng	10^{-9}g
nt	nucleotide
ON	over night
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RNAi	ribonucleic acid interference
SCN	suprachiasmatic nucleus
SD	standard deviation
SFM	serum free media
UAS	upstream activating sequence (yeast transcription factor GAL4 binding sites)
UTR	untranslated region
UV	ultraviolet
wt	wildtype
ZT	zeitgeber time, time in hours in an entrainment cycle, ZT 0 is onset of light in an LD cycle
PAS domain	*P*er-*A*rnt- *S*ingle-minded domain (PER, ARNT and SIM are founder proteins for the PAS domain homology)

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CHAPTER 1: OVERVIEW AND BACKGROUND

1.1 Biological timing and its ecological advantage

Most organisms live in a continuously changing environment that varies in a rhythmic fashion related to the Earth's rotation around its axis. This planetary movement produces a daily solar cycle with a periodicity of approximately 24 hrs. Alternating change in exposure to sunlight generates dramatic and predictable changes in the environment. Organisms have acquired internal timekeeping mechanisms to actively follow daily changes in the environment leading to many physiological and behavioral responses to vary with a period that is close to 24 hrs or circadian.

Circadian rhythms are evident in a vast number of behaviors exhibited by organisms in all branches of the evolutionary tree from cyanobacteria to humans. Cyanobacteria segregate photosynthesis and nitrogen fixation temporally such that these processes occur 180 degrees out of phase from each other to avoid inhibition of nitrogen-fixing nitrogenase enzyme by molecular oxygen (Mitsui et al., 1986). Most animals have daily activity patterns. The majority of mammals are night-active or nocturnal (e.g. mice or bats) initiating their activity at twilight. Birds however are mostly day-active or diurnal. Many higher plants exhibit daily leaf movements. Many species of plants restrict their flowering time to a specific and relatively short time period during the day to maximize species-specific cross-pollination by insects. Honeybees in turn maximize their foraging efficiency by appearing at the food source at a time that coincides with the

flowering time. Such activity rhythms persist in constant conditions without timing cues indicating that an internal clock is responsible for initiating the pattern.

An internal pacemaker mechanism that couples the organism to external cycles provides several advantages over passive responses to changing environmental conditions. Inner time keeping allows anticipation of coming environmental changes and preparation in advance for predictably reoccurring events. Developmental events in many species are gated to a particular time of day maximizing the survival of offspring. For instance new fruit flies eclose from their pupal cases near dawn at the time of maximal humidity. Such timing is not dependent on the humidity in the environment as temporal control of eclosion is also evident in constant laboratory conditions suggesting again an internal clock for initiating the behavior at particular time of day.

Continuous monitoring of ongoing time in the daily cycle by an internal clock is also exploited to sequence an internal temporal program of physiological processes that peak at different times of day. Animals and plants that are kept in artificial light-dark cycles that substantially differ from 24 hrs show impaired performance (reviewed in (Pittendrigh, 1993).

The ubiquitous presence of circadian rhythms in life strongly suggests adaptive value. Few experimentally controlled examples are available that demonstrate direct requirement for clock function in survival of populations over several generations in a competitive environment. One of the best examples are co-culturing experiments of cyanobacterial strains that have equivalent growth rates but differ in their clock free-running rhythms due to mutations in clock components (Ouyang et al., 1998). A wildtype strain with 25-hr rhythm was used along with two strains that either had long rhythm (30-

hr) or short rhythm (23-hr) clock. Pairs of the three strains were initially mixed at equal concentrations and cultured at different light-dark (LD) cycles of either a 22-hr day of 11-hr light and 11-hr darkness or a 30-hr day of 15-hr light and 15-hr darkness. 27 days later the ratio of the strains was determined in the grown cultures. Invariably the strain that had its endogenous rhythm closer (in resonance) to the imposed light-dark cycles (30-hr strain in LD 15 hrs :15 hrs day cultures and 23-hr strain in LD 11 hrs :11 hrs day cultures) had taken over the culture. The competitive advantage is however only evident in a cycling environment. Co-cultures grown in a stable environmental condition of constant light showed no preferential selection of rhythmic strains over strains with a disrupted clock (Woelfle et al., 2004).

1.2 Anatomical basis and defining features of circadian rhythms

In several model organisms neuronal centers have been identified that are required for persistent physiological and behavioral rhythms. The anatomical basis of circadian pacemakers in marine mollusks *Aplysia* and *Bulla* has been extensively studied. In these organisms the eyes contain a circadian pacemaker that is capable of producing self-sustained circadian rhythms even when cultured in isolation from the rest of the organism (Block and McMahon, 1984). In insects surgical lesion studies and tissue transplantation experiments have found circadian pacemakers in either optic lobes of crickets and cockroaches or in cerebral lobes in flies and moths (Helfrich-Forster, 2004). In mammals the circadian pacemaker resides in the suprachiasmatic nucleus (SCN) of the

hypothalamus. Surgical removal of the SCN causes arrhythmia in behavior (Ralph et al., 1990). Transplantation studies proved that the SCN determines the period of the behavioral rhythms. Experiments with short period mutant hamsters with 20-hr behavioral rhythms showed that donor SCNs obtained from mutant animals could restore behavioral rhythms in wildtype SCN-lesioned hamsters and with the period of the donor (Ralph et al., 1990). In addition to neurons many other cell types have functional circadian oscillators. Monitoring of circadian gene activity in ex-vivo organ cultures shows the widespread presence of circadian clocks in animals (Plautz et al., 1997; Yamazaki et al., 2000; Yoo et al., 2004). In higher plants no central circadian pacemaker appears to be present despite quite complex tissue organization (Thain et al., 2000).

Several criteria have been postulated as formal properties of circadian oscillator clocks. Firstly, the observed output rhythms of an organism must be persistent in constant conditions that eliminate environmental time cues. Secondly, the period length of the oscillation needs to be stable over a wide range of temperatures to effectively measure time of day rather than temperature. Thirdly, the clock mechanism has to be entrainable by cycling environmental factors in order to synchronize to external rhythms. In addition to having an input from the environment, the clock also has to have means to communicate the time of day information in order to generate overt cycling behavior that is in the right phase with the environment.

Under constant conditions the periodicity of overt rhythms observed in many organisms slightly deviates from 24 hrs depending on the species and experimental conditions such as illumination or developmental stage. The period length of such free-running rhythms is remarkably precise from one day to the next deviating very little from

its mean period length. The basis for the persistence of the near 24-hr oscillations is poorly understood. In multicellular organisms intercellular interactions contribute to the stability of circadian clocks. Dissociated neurons from the mammalian circadian pacemaker SCN show a distribution of period lengths in their firing rhythms with a mean period length that correlates with overall output rhythms (Liu et al., 1997). Neurons in the intact SCN fire in synchrony with a near 24-hr rhythm that reflects the overt rhythm of the animal. Neuronal activity is required to establish synchrony between individual SCN neurons (Yamaguchi et al., 2003). In unicellular organisms such as cyanobacteria the intracellular clockwork produces stable 24-hr rhythmicity with no apparent interaction between neighboring individual cells (Mihalcescu et al., 2004).

The second essential feature of circadian rhythms is temperature compensation. The rates of most biochemical reactions double or triple with every 10°C increase in temperature while the overt free-running circadian period length of an organism is very similar at different ambient temperatures. The advantage of temperature compensation is apparent for organisms that do not control their body temperature but it also occurs in homoeothermic animals. Many warm-blooded animals hibernate or show daily bouts of torpor that can lead to dramatic changes in body temperature. Temperature compensation is critical to maintain a near 24-hr period length of their internal clock. A clock that would change its period length depending on the fluctuating ambient temperature of any particular day would lose its key function in maintaining the appropriate phase angle between the internal clock and the external time of day. The mechanism how circadian rhythms are maintained constant over a wide range of temperatures is unknown. A general idea was postulated in the early days of modern circadian research that suggests

temperature compensation to be a function of two counter imposed reactions. While the rate of both reactions increases with temperature the product of one reaction inhibits the second reaction that is primarily determining the period length of the clock. Thus a rise in temperature would increase the inhibitor levels that would keep the rate of the second reaction constant or temperature compensated (Sweeney and Hastings, 1960). Despite the apparent resistance of the period length of the clock to temperature changes, the phase of the oscillation is sensitive to temperature pulses and cycles.

The third property of circadian rhythms is their ability to be entrained by cycling environmental factors. Light and temperature transitions are the most common entrainment cues that reset the internal clock to synchronize it to external time. Light and temperature pulses can change the phase of the onset of a biological rhythm. The magnitude and direction of the shift is dependent on the circadian phase in which the stimulus is given. Phase response curves, plots of phase shifts in response to stimulus as a function of circadian time, describe such phase dependent responses. For example in *Drosophila melanogaster*, light pulses administered in the early subjective day have little or no effect on the phase of the rhythm. Light pulses produce delays in late evening and early night whereas phase advances are generated when stimulus is presented in late night (e.g. (Curtin et al., 1995; Martinek et al., 2001)). A true entrainment is displayed when the reset phase of the rhythm is maintained after removal of the entraining agent and return to constant conditions. In mammals light signaling occurs via the retinohypothalamic tract that connects the retina with the SCN. Outer-retinal cells with rod and cone photoreceptors as well as melanopsin-expressing retinal ganglion cells located in the inner layers of the retina are required for photic entrainment (Berson et al.,

2002; Hattar et al., 2002; Panda et al., 2003). Multiple photoreceptor systems are required for entrainment in other organisms as well. In *Drosophila* mutations that eliminate the compound eyes and extra-retinal structures affect photic entrainment synergistically. Flies with combined mutations that eliminate cryptochrome function in addition to all eye structures show an almost complete failure to entrain to light-dark cycles (Helfrich-Forster et al., 2001; Mealey-Ferrara et al., 2003).

1.3 Molecular basis of circadian clocks

The first clock mutants were isolated in *Drosophila melanogaster* in an EMS mutagenesis screen. Three mutations in one genetic locus termed *period* were identified that either lengthened, shortened or completely abolished eclosion rhythms (Konopka and Benzer, 1971). Locomotor activity rhythms were affected in a similar way suggesting that a common underlying regulatory mechanism was affected for both behaviors. At the same time mutations in another model system, *Neurospora crassa*, were generated also by chemical mutagenesis. Similarly to *Drosophila* mutants long and short rhythm alleles of the same genetic locus called *frequency* were recovered (Feldman and Hoyle, 1973). The first mammalian circadian clock mutant called *tau* was found in Syrian hamsters. This mutation had risen spontaneously and shortens wheel running activity rhythms by four hours (Ralph and Menaker, 1988). A chemical mutagenesis screen conducted in mice led to the discovery of the second mammalian clock mutation called *Clock* (Vitaterna et al., 1994). This mutation abolishes activity rhythms when homozygous and

lengthens the period in heterozygous animals. Other mammalian clock genes have subsequently been found by searching for homologs of clock genes discovered initially in *Drosophila*. Circadian clock components have also been isolated from cyanobacteria and *Arabidopsis*.

In each phylum clock genes are organized into transcriptional/translational feedback loops that oscillate between gene activation and repression with a near 24-hr periodicity. Interestingly, most of the proteins that make up the clocks are not conserved between the different model systems across phyla suggesting multiple origins of molecular clocks converging on similar general mechanisms to generate and maintain circadian gene activity. Some structural similarities in different molecular clockworks are however clearly identified. PAS domains are found in animal as well as in fungal clock proteins. This may however also reflect the versatility of the PAS domain as an environmental sensor present in a wide spectrum of organisms from bacteria to humans. Casein kinase 2 (CK 2) is also used widely; it regulates circadian rhythms in plants, animals as well as in fungi. In clock systems the transcriptional/translational feedback loops are extensively regulated by protein kinases. Most clock proteins are phosphorylated in a time of day dependent manner to regulate their stability, activity or subcellular localization. While in certain cases cycling clock gene transcription can be eliminated without losing behavioral output rhythms with normal periodicity, protein phosphorylation appears to be essential for a functioning clock. Protein phosphorylation is believed to be important in generating delays in different steps of the molecular cycles to maintain cycling of clock gene-directed transcription with an unusually long near 24-hr periodicity and prevent dampening of the feedback loops into steady state.

Drosophila and *Neurospora* clockworks have long been the leading model systems to learn about the molecular components and the underlying mechanisms of molecular clocks. More recently, the mammalian clock has greatly benefited from findings in flies due to structural homologies between many proteins in the two clockworks. Despite the structural homologies in fly and mammalian clocks the functions of similar proteins is not necessarily conserved. Below an overview is given of the three best-studied molecular clockworks of *Drosophila*, *Neurospora* and mammals.

1.3.1 *Drosophila* clockwork

The *Drosophila* clock gene *period* was the first gene involved in circadian timekeeping to be cloned (Bargiello et al., 1984; Reddy et al., 1984). It functions as a PAS domain-containing transcriptional repressor and forms dimers with the product of the second clock gene cloned in fruit flies, *timeless* (tim). *tim* was identified by interaction with PER as well as by positional cloning of a novel arrhythmic mutation (Gekakis et al., 1995; Myers et al., 1995). Both proteins localize to the nucleus each night where PER down regulates the mRNA production from both of their genes. The cycling nuclear localization of PER is blocked by the arrhythmic *timeless* null mutation (Vosshall et al., 1994). Thus, *timeless* function has been proposed as the regulator of PER subcellular localization.

The mRNA and protein levels of both *per* and *tim* oscillate with a circadian period. Peak levels of mRNA of both genes occur around CT14 (Hardin et al., 1990;

Sehgal et al., 1995). Protein levels of PER and TIM are at their highest in the middle of the night at CT18 with a 4-hr delay with respect to mRNA accumulation (Hunter-Ensor et al., 1996; Myers et al., 1996; Siwicki et al., 1988; Zeng et al., 1996; Zerr et al., 1990).

Transcriptional feedback loops of the *Drosophila* molecular clock are shown in figure 1. The cycling expression of *per* and *tim* is regulated by two PAS domain-containing helix-loop-helix type transcription factors *Cycle* (CYC) and *Clock* (CLK) identified in an EMS mutagenesis screen (Allada et al., 1998; Rutila et al., 1998). In *Clk* and *cyc* mutants the mRNA levels of *per* and *tim* are constitutively produced at low levels (Allada et al., 1998; Rutila et al., 1998). The mRNA and protein levels of CLK cycle with a circadian rhythm with the peak levels produced around dawn (Bae et al., 2000; Bae et al., 1998; Darlington et al., 1998). CYC mRNA and protein however are constitutively produced (Bae et al., 2000; Rutila et al., 1998). In cell culture experiments CLK and CYC are able to activate *per* and *tim* promoters given the presence of their binding sites (Darlington et al., 1998; Hogenesch et al., 1998). This activation of gene activity by CLK and CYC is suppressed by the activities of PER and TIM (Darlington et al., 1998). In the middle of the night PER-TIM dimers translocate to the nucleus. PER and TIM can bind to the CLK-CYC complex which inhibits their DNA binding activity without disrupting the dimer of CLK and CYC (Lee et al., 1999). The repressive activity is to be carried out primarily by PER monomers, as TIM appears to be dispensable for transcriptional inhibition *in vivo* (Rothenfluh et al., 2000). Thus a transcriptional feedback loop is formed that regulates cycling expression of PER and TIM as negative regulators of their own expression through inhibiting CLK and CYC activity.

A second transcriptional feedback loop regulates the oscillating transcription of the *Clk* gene that cycles in antiphase compared to *per* and *tim*. PER and TIM are required for high-level expression of *Clk* mRNA (Bae et al., 1998). This requirement is lost in *Clk* mutant flies. Such genetic interaction suggests the presence of a *Clk* repressor that itself is expressed under CLK control (Glossop et al., 1999). The PAR domain containing bzip protein Vrille (VRI) fulfills the requirements for the *Clk* repressor. Overexpression of VRI leads to long behavioral rhythms or arrhythmicity. Its mRNA is expressed under CLK control and oscillates in phase with *per* and *tim* (Blau and Young, 1999). In flies overexpressing *vri*, *Clk* mRNA levels are suppressed and *in vitro* VRI binds to sites in the *Clk* promoter (Cyran et al., 2003; Glossop et al., 2003). Another PAR domain containing bzip transcription factor Pdp1 (PAR domain protein 1) functions to activate *Clk* promoter. Pdp1 mRNA and protein are also expressed with a circadian rhythm under CLK-CYC control but with a later peak phase than *vri* around CT 18. Loss of Pdp1 stops the clock indicating its essential role in oscillator function (Cyran et al., 2003). Pdp1 can also activate *Clk* promotor in reporter assays.

Pdp1 and VRI compete for the same binding sites in the *Clk* promoter (Cyran et al., 2003). The changing balance between initial VRI-dependent suppression and subsequent Pdp1-dependent activation later in the cycle determines the cycling expression of *Clk*. The newly produced CLK at the end of the night and early morning is inhibited by the feedback of high levels of PER induced by the previous wave of CLK activity. The two feedback loops regulating *per* and *Clk* expression are linked and simultaneously restarted by CLK activity after PER is degraded during the day.

In addition to regulation at the transcriptional level many clock components in *Drosophila* are also regulated posttranscriptionally and posttranslationally. Constitutive transcription of *per* and *tim* genes still allows behavioral rhythms to be manifested (Frisch et al., 1994; Kaneko et al., 2000; Vosshall and Young, 1995; Yang and Sehgal, 2001). Comparison of *per* transcription and mRNA accumulation rates shows a delay specific to the transcript buildup phase in the morning suggesting regulated mRNA turnover (So and Rosbash, 1997; Stanewsky et al., 1997). *per* RNA splicing is also a regulated process. There is a temperature-sensitive spliced intron in the 3' UTR of *per* mRNA. In cold temperatures this intron is preferentially removed which correlates with earlier rise in *per* mRNA and protein levels (Majercak et al., 1999). Such temperature dependent regulation may regulate seasonal activity.

Most proteins involved in the transcriptional feedback loops are phosphorylated in a time of day dependent manner. Three kinases, CK 1ε/*dbt*, CK 2 and GSK-3/*shaggy*, have been identified as clock components in *Drosophila*.

Mutations in the gene *doubletime*, a *Drosophila* ortholog of CK 1ε, have been found to shorten or lengthen the period of the behavioral rhythms or abolish them altogether (Price et al., 1998; Rothenfluh et al., 2000; Suri et al., 2000). DBT is constitutively expressed at the mRNA and protein level and is an essential gene for fly development regulating cell survival and proliferation (Kloss et al., 1998; Kloss et al., 2001; Zilian et al., 1999). Loss of DBT activity is associated with high levels of hypophosphorylated PER indicating its role in determining the rate of PER degradation (Price et al., 1998). DBT has been shown to interact with PER *in vitro* and *in vivo* and create a stable complex with PER throughout the circadian cycle (Kloss et al., 1998;

Kloss et al., 2001). PER phosphorylated by DBT is recognized by the Slimb protein (Ko et al., 2002). Slimb is a component of the SCF ubiquitin ligase complex, which marks proteins for proteosomal degradation in a phosphorylation-dependent manner (Deshaies, 1999). Fly mutants that have reduced levels of Slimb accumulate hyperphosphorylated forms of TIM and PER proteins suggesting that the stability of both proteins is Slimb-dependent (Grima et al., 2002). Overexpression of Slimb leads mostly to loss of locomotor activity rhythms with a few animals displaying long period rhythmicity (Grima et al., 2002; Ko et al., 2002). Reduction of Slimb levels also lengthens behavioral rhythms (Grima et al., 2002). Long period rhythms in both overexpression and under-expression conditions have been explained by a hypothesized Slimb function both in cytoplasm and in the nucleus (Grima et al., 2002). Enhanced PER degradation in the cytoplasm would delay nuclear translocation of PER and thus delay the overall cycle. Reduction of Slimb function may delay the degradation of nuclear PER, which also would delay the start of the next cycle.

The short and long period length alleles of DBT enhanced or attenuate, respectively, PER degradation in the nucleus further demonstrating the importance of timely PER degradation as a critical determinant in establishing 24-hr rhythmicity. In addition to protein degradation, DBT appears to affect also the timing of nuclear translocation of PER. A short period mutant dbt^{S} shows delayed PER nuclear translocation that is apparently independent of PER protein stability (Bao et al., 2001). The mechanism by which DBT affects the nuclear entry of PER is currently unknown. In cultured cells reduction of DBT activity by RNAi has been demonstrated to reduce the transcriptional repressor activity of PER (Nawathean and Rosbash, 2004). DBT appears

to affect different regulatory aspects of PER function in multiple steps throughout the circadian cycle.

Another protein kinase required in the clock mechanism, CK 2, was identified by mutations in the catalytic subunit (*timekeeper*) and in the regulatory β-subunit (*andante*) (Akten et al., 2003; Lin et al., 2002). Both mutations of CK 2 lead to long locomotor activity rhythms. At the molecular level the protein levels of PER and TIM are elevated in the mutants. In cultured *Drosophila* S2 cells CK 2 has been shown to cooperate with DBT positively regulating the transcriptional repressor activity of PER (Nawathean and Rosbash, 2004). Reduction of CK 2 activity in the mutant animals may elevate PER and TIM levels due to inefficient repression of CLK dependent transcription. Additionally, the nuclear translocation time of PER and TIM is delayed in CK 2 mutants. The expression and activity levels of CK 2 itself are constant over the circadian cycle.

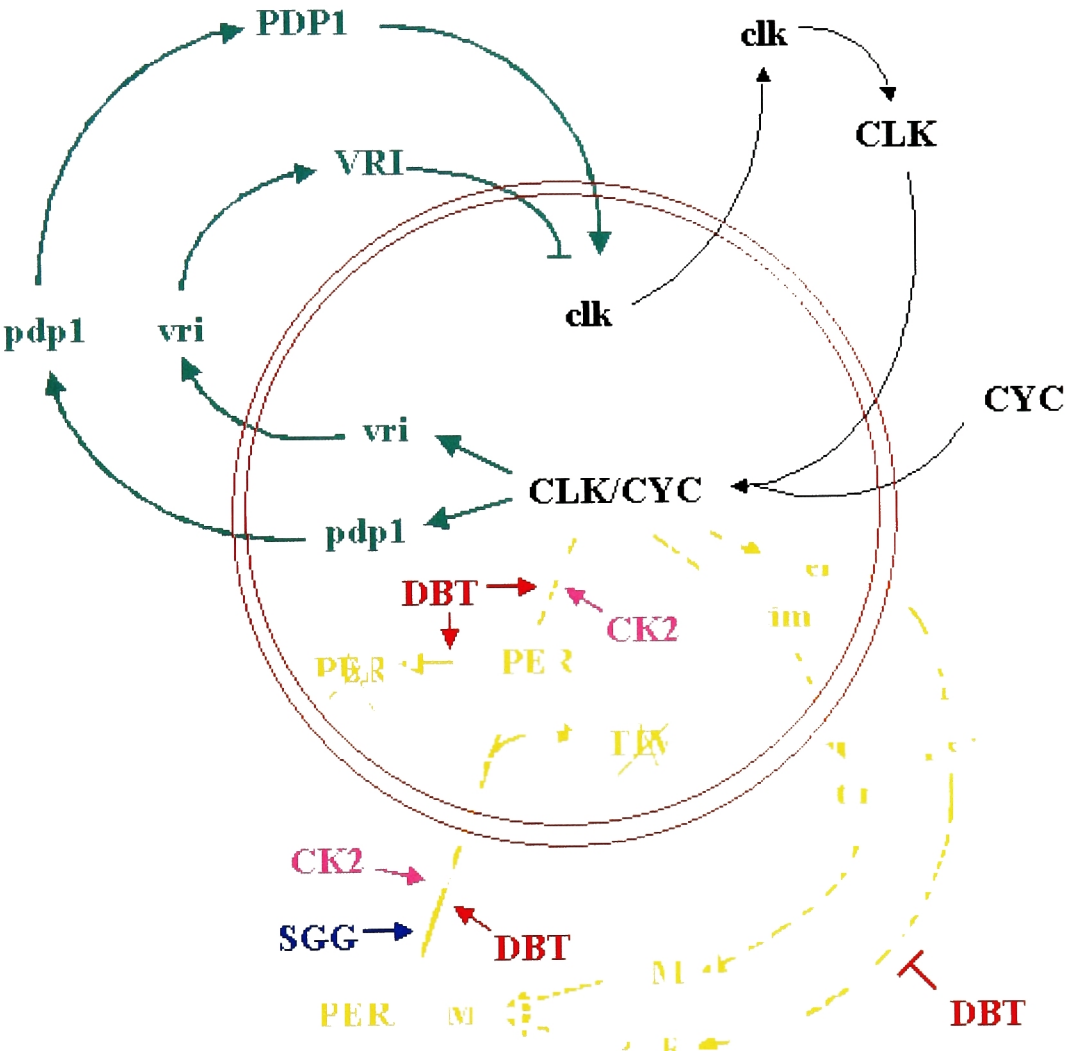
An overexpression screen of transposon-tagged *Drosophila* genes revealed a role for *shaggy* (*sgg*), a developmentally required protein kinase and a fly homolog of GSK-3, in the circadian clock. Like *dbt* and CK 2, the mRNA and protein levels of *sgg* do not cycle. Raising or lowering the levels of SGG leads to shortening or lengthening, respectively, of the period length of locomotor activity rhythms (Martinek et al., 2001). Overexpression of SGG is correlated with a faster rate of nuclear translocation of the PER-TIM complex and hyperphosphorylation of TIM *in vivo* (Martinek et al., 2001). Thus, at least one of SGG functions in the clock is to control the rate of PER nuclear entry. *In vitro* SGG phosphorylates TIM. Direct phosphorylation may serve as a signal for nuclear translocation. Additionally, hyperphosphorylated forms of TIM that are found in SGG overexpressing flies, are specifically light sensitive (Martinek et al., 2001)

Figure 1. Model of *Drosophila* clockwork.

Regulatory relationships between the genes and proteins in the negative and positive transcriptional feedback loops are shown. Positive feedback loop is color coded in green, negative feedback loop is in yellow. Lines ending with bars indicate negative regulation, arrows designate positive regulation. Protein names are in upper case, genes and mRNAs are written in lower case. Two red concentric circles represent the nuclear membrane. Briefly, CLK-CYC heterodimer activates *per* and *tim* genes as well as *vri* and *pdp1* genes during late day, early evening. With a 4-hr delay relative to their mRNAs, PER and TIM proteins are produced and after heterodimerization translocate to the nucleus in the middle of the night. In the nucleus PER represses CLK-CYC dependent gene activation when released from complex with TIM causing a decline in *per* and *tim* mRNA levels. This closes the negative feedback loop that controls transcription of CLK-CYC target genes. At the same time rhythmic *Clk* expression is regulated by a positive feedback loop that involves repression of *Clk* expression by VRI and subsequent activation by PDP1. Protein accumulation phases of VRI and PDP1 are temporally segregated. VRI accumulates in early evening whereas PDP1 accumulates in late evening. In the early morning highly accumulated CLK is initially inhibited from activating its target genes by PER monomers from the previous night. By midday PER monomers have been degraded and new cycle of transcription can start. CYC protein is constitutively expressed.

PER monomer stability is negatively regulated in the cytoplasm and nucleus by DBT. Association with TIM stabilizes PER. In addition, DBT and CK 2 regulate nuclear translocation of PER/TIM dimers and enhance PER transcriptional repressor activity in the nucleus. SGG positively regulates nuclear translocation of PER/TIM dimers.

Abbreviations: per- *period*, tim – *timeless*, clk – *Clock*, dbt – *doubletime*, sgg – *shaggy*, vri – *vrille*, pdp1 – PAR domain protein 1, cyc – *cycle*, ck2 – casein kinase 2.


PDP1
clk
CLK
VRI
clk
pdp1
vri
CYC
vri
CLK/CYC
pdp1
DBT
CK2
CK2
SGG
DBT
DBT

suggesting a possible role for SGG in light resetting as well.

Protein dephosphorylation also plays a critical role in *Drosophila* clock. *twins* (*tws*) and *widerborst* (*wdb*), two regulatory subunits of protein phosphatase 2A (PP2A), are transcriptionally under circadian clock control. Reduction of expression levels of either gene in cultured cells leads to destabilization of transfected PER (Sathyanarayanan et al., 2004). Overexpression of *wdb* lengthens the period of locomotor activity rhythms *of* mutant animals whereas overexpression of *tws* leads to shortened rhythms that degrade into arrhythmia. PP2A activity appears to be required also for appropriately timed nuclear translocation of PER. In a strong *tws* hypomorphic mutant, PER nuclear translocation is delayed compared to wildtype in addition to low protein levels. Altering expression levels of *mutagenic star* (*mts*), the catalytic subunit of PP2A, has effects similar to manipulations of *tws* levels. Overexpression of *mts* shortens while decrease of activity by overexpression of a dominant negative *mts* lengthens the period of behavioral rhythms. PER protein in *mts* overexpressors can be detected exclusively in the nucleus. Similar to *tws* hypomorphic allele, overexpression of dominant negative *mts* leads to very low levels of PER.

1.3.2 *Neurospora* clockwork

frequency gene was the first clock component isolated and cloned in *Neurospora* (Feldman and Hoyle, 1973; McClung et al., 1989). The *frq* mRNA and protein levels oscillate with a circadian rhythm and this cycling expression is necessary for clock

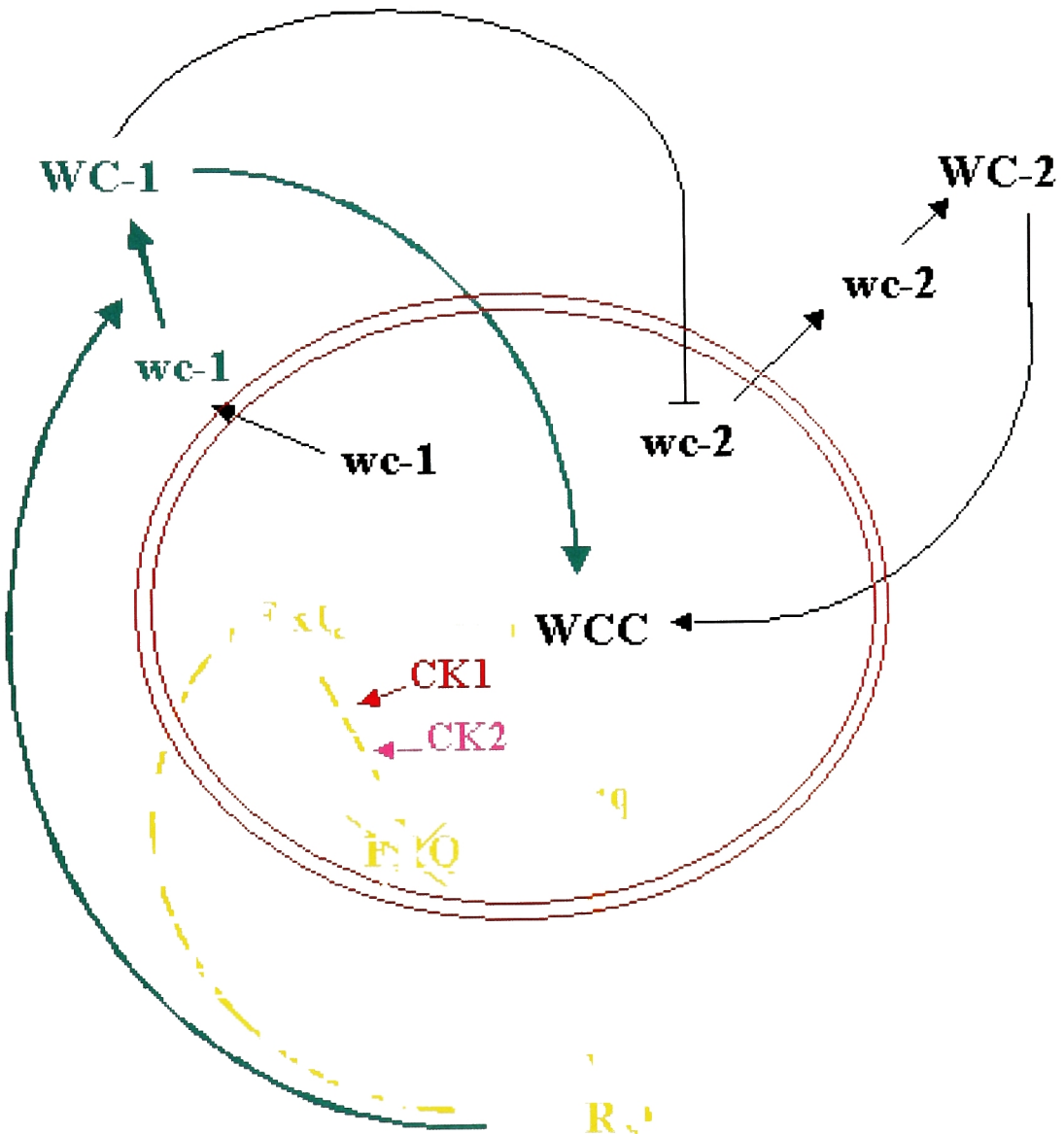
function. Transcriptional/translational feedback loops in *Neurospora* molecular clock are shown in figure 2. Two transcription factors *white collar-1* (*wc-1*) and *white collar-2* (*wc-2*) are essential for cycling *frq* expression (Crosthwaite et al., 1997). WC-1 and WC-2 heterodimerize via their PAS domains and form a white collar complex (WCC) that binds to two sites in the *frq* promoter (Ballario et al., 1998; Froehlich et al., 2002; Froehlich et al., 2003). WCC activates *frq* promoter during late night at a time when *frq* mRNA and protein are at low levels. As *frq* mRNA levels rise during the morning with peak levels around CT4, newly translated FRQ starts to appear and reaches maximum levels with a 4-hr delay compared to its mRNA around CT8 (Aronson et al., 1994; Garceau et al., 1997). The accumulation of FRQ correlates with declining activity of WCC. FRQ levels continue to rise due to continuing translation. In the nucleus FRQ binds to WCC, which correlates with the inhibition of *frq* gene transcription. Thus FRQ feeds back and negatively regulates its own gene forming a negative feedback loop reminiscent of PER-dependent feedback in the *Drosophila* clock.

The *wc-1* and *wc-2* mRNA and WC-2 protein are constitutively produced (Denault et al., 2001; Lee et al., 2000). The WC-1 protein content however is cycling in antiphase to FRQ (Lee et al., 2000). Production of WC-1 is positively regulated by FRQ generating a positive feedback loop, which ultimately contributes to reinitiation of FRQ synthesis in the next cycle. In addition to FRQ dependent positive posttranscriptional regulation, WC-1 is independently regulated by WC-2 via protein stabilization through interactions between their PAS domains (Cheng et al., 2002). Separate from the enhancement of WC-1 synthesis, FRQ also promotes *wc-2* mRNA expression. WC-2

Figure 2. Model of *Neurospora* clockwork

Positive and negative feedback loops are color coded like in Figure 1. Two concentric red circles represent nuclear membrane. Lines ending with bars indicate negative regulation, arrows designate positive regulation. Protein names are in upper case, genes and mRNAs are written in lower case. Briefly, WCC that contains WC-1 and WC-2 proteins activates *frq* gene transcription. During late day FRQ feeds back by binding to WCC and inhibits its activity, causing a decline in *frq* transcription. At the same time FRQ positively affects WC-1 translation from constitutively produced *wc-1* mRNA. Newly formed WCC is kept inactive most of the night by bound FRQ. By late night, progressive phosphorylation of FRQ by several kinases has led to its ubiquitination-dependent degradation. This in turn allows WCC to reactivate transcription. *wc-2* mRNA and protein are constitutively produced

Abbreviations: frq – *frequency*, wc-1 – *white collar 1*, wc-2 – *white collar 2*, CK 1 – casein kinase 1, CK 2 – casein kinase 2.


WC-1
WC-2
wc-2
wc-1
wc-1
wc-2
WCC
CK1
CK2

protein binds both WC-1 and FRQ allowing heteromeric complex formation (Denault et al., 2001). Homodimerization between FRQ proteins is required for its interaction with WCC (Cheng et al., 2001)

The rise of WC-1 levels coincides with high levels of FRQ that is progressively phosphorylated prior to degradation (Garceau et al., 1997; Liu et al., 2000). Newly formed WCC is kept inactive in complex with phosphorylated FRQ. Interaction between FRQ and WCC displaces the complex from its binding sites in *frq* promoter (Froehlich et al., 2003). Progressive phosphorylation of FRQ eventually leads to its degradation in the night allowing WCC to bind DNA again and restart the cycle at late night.

Protein phosphorylation has been shown to be important in determining the kinetics of the circadian cycle by regulating FRQ stability. General kinase inhibitor 6-dimethylaminopurine affects FRQ phosphorylation and increases its protein stability (Liu et al., 2000). Phosphorylation occurs at multiple sites leading to mobility shift in Western blot analysis of phosphorylated FRQ (Liu et al., 2000). A number of protein kinases have been implicated in regulating FRQ stability. Casein kinase 1ε homologs in Neurospora, CK-1a and CK-1b can phosphorylate FRQ *in vitro*. Furthermore, CK-1a is associated with hyperphosphorylated FRQ *in vivo* (Gorl et al., 2001). The kinases target PEST-like sequences in FRQ. Deletion of one of the motifs, PEST-1, leads to increased FRQ stability that still shows molecular rhythms in abundance although with a greatly lengthened period of 28 hrs (Gorl et al., 2001). Another protein kinase, calcium/calmodulin dependent protein kinase (CAMK-1) potently phosphorylates FRQ *in vitro*. However, removal of CAMK-1 *in vivo* has subtle effects of lengthened conidiation rhythm with a phase delay in initiation of conidiation in constant darkness (Yang et al.,

2001). Much stronger effects are obtained by inactivation of casein kinase 2, a third kinase identified biochemically to phosphorylate FRQ. Disruption of CK 2 catalytic subunit stabilizes FRQ and abolishes its cycling expression (Yang et al., 2002). In addition to protein stability CK 2 dependent phosphorylation appears to negatively affect FRQ interaction with WCC. In a CK 2 disrupted strain hypophosphorylated FRQ shows increased interaction with WCC (Yang et al., 2002). Phosphorylated FRQ is degraded through the ubiquitination-dependent pathway reminiscent of *Drosophila* and mammalian PER proteins. FWD-1, a Slimb homolog of F-box and WD40 repeat containing protein, is required for normal FRQ degradation. In a FWD-1 disruption strain hyperphosphorylated forms of FRQ accumulate indicating a failure in periodic turnover of FRQ protein (He et al., 2003). FWD-1 binds FRQ and is required for cycling FRQ degradation at the molecular level as well as for expression of conidiation rhythms (He et al., 2003).

Protein phosphatases in *Neurospora* are also implicated in clock control. Mutations in both PP1 and PP2A affect the function of FRQ apparently by distinct mechanisms (Yang et al., 2004). The *Neurospora* PP2A enzyme containing a mutant regulatory subunit RGB-1 causes low expression of *frq* mRNA and overall protein levels, while mutant PP1 seems to regulate FRQ protein stability by directly affecting the degradation.

1.3.3 Mammalian clockwork

At first glance the mammalian circadian oscillator seems very similar to the one in *Drosophila*. Orthologs of most fly clock proteins have been found to be part of the

mammalian pacemaker. Like in *Neurospora* and fly clocks interlocked positive and negative transcriptional feedback loops drive oscillatory transcription of key clock genes. The transcriptional feedback loops in the mammalian molecular clock are shown in figure 3. Two PAS domain containing basic helix-loop-helix DNA binding proteins Clock and BMAL1 function to rhythmically activate transcription (Bunger et al., 2000; Gekakis et al., 1998; King et al., 1997). The *Clock* gene was originally found in a forward genetic screen looking for mutations with altered wheel running activity rhythms in mice. The mutation causes long period rhythmicity in heterozygous animals and loss of rhythms in homozygotes (Vitaterna et al., 1994). The mutant *Clock* gene has a premature stop codon that eliminates the C-terminal transcriptional activation domain resulting in a dominant negative form of the protein (King et al., 1997; King et al., 1997). The *Clock* gene product dimerizes with BMAL1 and the heterodimers bind specifically to E-box sequences to activate gene expression (Gekakis et al., 1998; Hogenesch et al., 1998). Unlike its ortholog in flies, Clock protein in mammals is produced constitutively (Maywood et al., 2003). However, both BMAL1 mRNA and protein cycle in their abundance. In the SCN neurons the peak levels of *Bmal1* mRNA are expressed in the middle of the night (Maywood et al., 2003). The maximum in protein levels of BMAL1 is delayed to early morning hours in the pacemaker cells. In peripheral organs the mRNA and protein levels are delayed by approximately six hours relative to the SCN.

The Clock-BMAL1 heterodimers activate rhythmic transcription of three *Period* genes (*Per1*, *Per2* and *Per3*), homologs of insect *per*, and two *Cryptochrome* genes (*Cry1* and *Cry2*), members of the blue-light photoreceptor/photolyase family (Jin et al., 1999; Kume et al., 1999). Transcript levels of *Per* genes peak in mid to late circadian day,

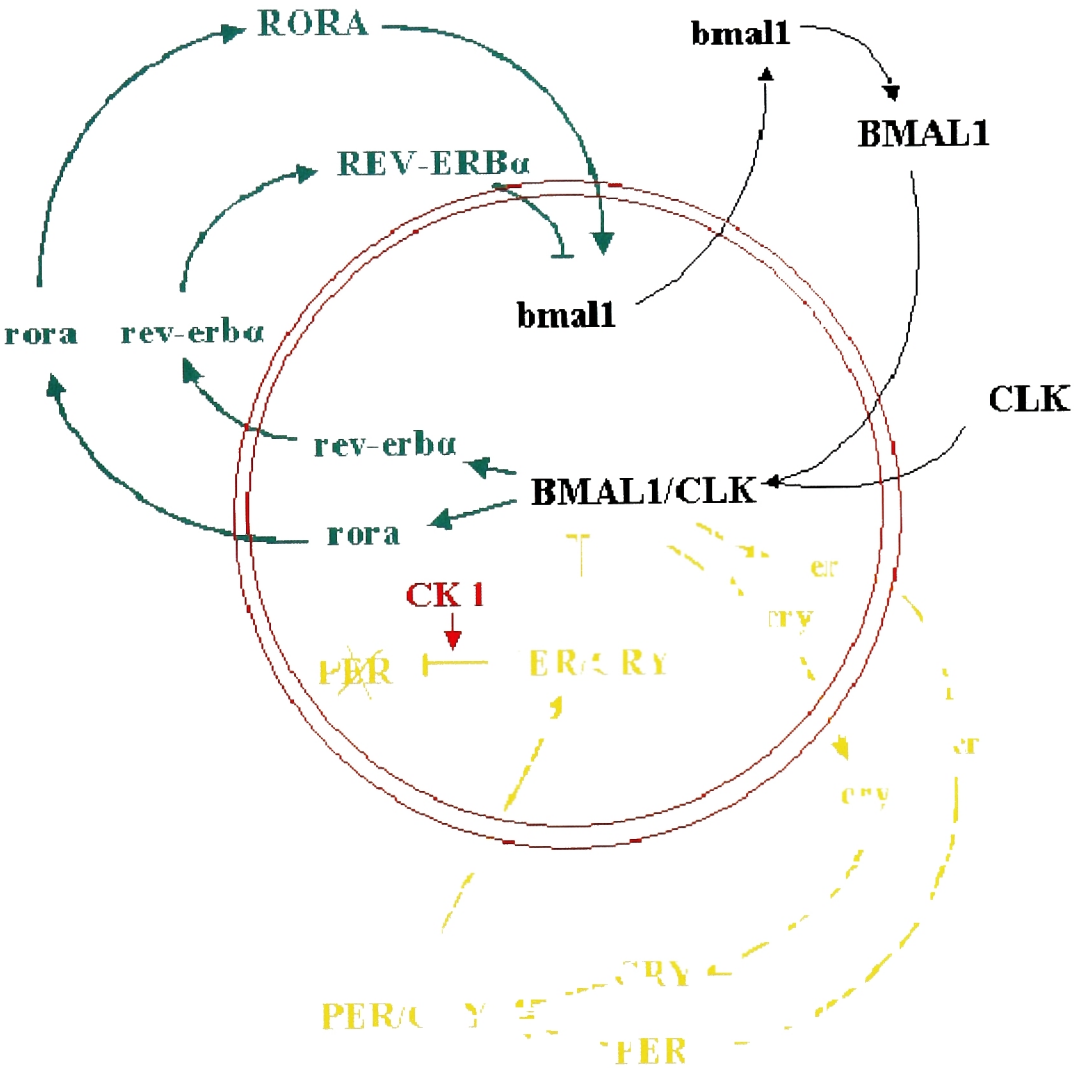
antiphase to the *Bmal1* mRNA while the *Cry* transcripts are delayed by about 4h. The PER1 and PER2 proteins form heteromultimeric complexes with CRY1 and CRY2 that when translocated to the nucleus negatively feed back to the Clock-BMAL1 complex (Kume et al., 1999). The inhibitory activity is carried out primarily by the CRY proteins while association between the PER and CRY proteins is required for their nuclear translocation (Griffin et al., 1999; Kume et al., 1999; Lee et al., 2001). Single knockouts of either *Cry1* or *Cry2* shorten or lengthen, respectively, activity rhythms while the combined loss leads to loss of both behavioral and molecular rhythms (van der Horst et al., 1999; Vitaterna et al., 1999). Loss of *Per1* function leads to subtle shortening of locomotor activity rhythms that can degrade into arrhythmicity over time in constant darkness (Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001). A more dramatic phenotype is apparent in *Per2* mutant mice. Animals lacking functional *Per2* have locomotor rhythms shortened by about 2 hrs that also tend to degrade to arrhythmicity in constant conditions (Bae et al., 2001; Zheng et al., 1999). *Per1* and *Per2* double mutant mice immediate lose locomotor activity rhythms in constant conditions that is accompanied by elimination of rhythmic clock directed gene expression (Zheng et al., 2001). *Per3* is not required for core clock function as its function can be eliminated in either *Per1* or *Per2* knockout background without noticeable additional effects, likely serving as an output component (Bae et al., 2001). Interestingly *Per1* and *Per2* have differential effects on the expression of clock output genes suggesting there may be diversity in the protein complexes that bind circadian promoters during the negative feedback in the nucleus (Zheng et al., 2001).

Figure 3. Model of mammalian clockwork

Positive and negative transcriptional feedback loops are color coded like in Figure 1. Two concentric red circles represent the nuclear membrane. Lines ending with bars indicate negative regulation, arrows designate positive regulation. Protein names are in upper case, genes and mRNAs are written in lower case. Briefly, in the negative feedback loop, CLK-BMAL1 heterodimers activate the transcription of *Cry* and *Per*. PER and CRY proteins are produced in the cytoplasm and after heteromultimerization translocate to the nucleus during the night. In the nucleus PER and CRY protein complexes interact with CLK-BMAL1 heterodimers on clock-controlled promoters and inhibit gene activation causing a decline in *Per* and *Cry* mRNA levels. At the same time cycling BMAL1 expression is regulated by a positive feedback loop that involves rhythmically expressed Rev-Erb/ROR family of transcription factors. REV-ERBα (likely to function redundantly with REV-ERBβ) promotes repression of *Bmal1* transcription while RORA activates (probably redundantly with other ROR family proteins) *Bmal1* transcription. By midday PER proteins have been targeted for degradation by phosphorylation-dependent ubiquitination. Repressive protein complexes at circadian promoters are disassembled and new cycle of transcription can start. Unlike in flies, Clk mRNA and protein in mammals are constitutively expressed.

Most clock proteins in mammals are hyperphosphorylated during the night in the nucleus. CK1 can phosphorylates PER and also CRY proteins when in multimeric complexes.

Abbreviations: per – *Period* (represents in this model both *per1* and *per2* genes), cry – *Cryptochrome* (represents here both *Cry1* and *Cry2* genes), clk – *Clock*, bmal1 – Brain and Muscle ARNT1 like, rora *retinoic acid-related orphan receptor a*, CK1 – casein kinase 1ε and δ isoforms.


RORA
bmal1
BMAL1
REV-ERBα
bmal1
rora
rev-erbα
CLK
rev-erbα
BMAL1/CLK
rora
CK 1

The positive action of Clock-BMAL1 and negative feedback from PER-CRY are connected by nuclear receptor family of proteins Rev-Erbα and Rora that are required for cycling *Bmal1* transcription (Preitner et al., 2002; Sato et al., 2004). *Rev-Erbα* gene is activated by Clock-Bmal1 heterodimers that activate also *Per* and *Cry* genes (Preitner et al., 2002). Rev-Erbα mRNA and protein are expressed in the same phase in the middle of the circadian day. Rise of Rev-Erbα causes *Bmal1* transcription to decrease to trough levels at the end of the day (Preitner et al., 2002). Rev-Erbα binds to retinoic acid-related orphan receptor response elements in the *Bmal1* promoter and inhibits transcription. In Rev-erbα deficient animals *Bmal1* mRNA cycling is dampened and not repressed during the day (Preitner et al., 2002). Rora, a transcriptional activator of the RevErb/Ror family participates in the activation of the *Bmal1* gene in the SCN (Sato et al., 2004). *In vitro* and in luciferase reporter assays RevErbα and Rora compete for the same binding sites in the *Bmal1* promoter (Sato et al., 2004). Rora peak levels in the SCN are delayed compared to RevErbα suggesting successive repression and activation by RevErbα and Rora, respectively (Sato et al., 2004; Ueda et al., 2002). Both the *RevErbα* and *Rora* deficient mice have a subtle 20-30 min period shortening in activity rhythms (Preitner et al., 2002; Sato et al., 2004). These surprisingly similar phenotypes have been speculated to be a result of reduced amplitude of Bmal1 rhythms (Sato et al., 2004). The weak behavioral phenotype suggests that other factors contribute to *Bmal1* expression. In fact other members of the RevErb/Ror family that all can bind to the same DNA binding sites are also under clock control (Preitner et al., 2002; Ueda et al., 2002). *Rorb* knockout mice display behavioral rhythms with a lengthened period (Andre et al., 1998). RevErbβ cycles in the same phase with RevErbα in the SCN and Rorb shows low amplitude rhythms in

the SCN with a delayed phase similar to Rora. The third activator in this protein family, *Rorc*, is absent in the SCN (Ueda et al., 2002). In the peripheral clock in the liver Rorc cycles while Rora is constitutively produced and Rorb is undetectable (Preitner et al., 2002; Ueda et al., 2002). Different RevErb/ROR family members may act redundantly which could explain the weak behavioral phenotypes of the single knockouts.

Both Clock-BMAL1 and RevErb/ROR family proteins together control a subclass of clock-controlled genes exemplified by *Cry1*. *Cry1* promoter contains both E-boxes and RevErb/ROR binding sites (Etchegaray et al., 2003). Maximal Clock-Bmal1 binding to E-boxes in the *Cry1* promoter occurs surprisingly in the middle of the day while peak transcription takes place in early night. Rhythmic RevErbα binding to *Cry1* promoter, which extends the activation of transcription, explains the apparent discrepancy in activator binding and actual peak activation of *Cry1* promoter (Etchegaray et al., 2003). In *RevErbα* knockout mice *Cry1* mRNA peaks predictably prematurely and the *Clock* mutant mice show reduced *Cry1* levels (Kume et al., 1999; Preitner et al., 2002). Expression of *Per* genes is unaltered in *RevErbα* deficient mice but also reduced in the *Clock* mutant (Jin et al., 1999; Preitner et al., 2002). Such combinatorial control produces the delayed phase of *Cry1* expression compared to the *Per* genes that are also activated by Clock-BMAL1 heterodimers.

Clock-dependent transcriptional activation is accompanied by chromatin modification in mammals. Histone H3 acetylation is cycling on Clock responsive promoters in synchrony with RNA polymerase activity on these promoters (Etchegaray et al., 2003). Clock protein associates specifically with a histone acetyltransferase p300 in a circadian fashion with peak levels of interaction in the middle of the day. Rhythmic

chromatin acetylation is accompanied also by cycling histone H3 phosphorylation in *Per1* and *Per2* promoters (Etchegaray et al., 2003).

Many of the transcription factors involved in circadian feedback loops are themselves phosphorylated with a circadian rhythm. Protein phosphorylation serves several functions including regulation of nuclear translocation, protein stability and activity. Two casein kinase 1 isoforms CK 1ε and CK 1δ have been placed in the clock mechanism by genetic and biochemical studies. A pointmutation in CK 1ε was identified in the Syrian hamster mutant *tau* (Lowrey et al., 2000). These animals have a fast running circadian clock with a 20-hr period of locomotor activity rhythms and also show defective responses to entraining light treatment (Ralph and Menaker, 1988). The mutated kinase in these animals has reduced activity *in vitro* but it remains unclear how this contributes mechanistically to the observed phenotype. The phosphorylation pattern of Per proteins and Clock is similar in the mutant and normal hamsters. In the mutant the molecular cycle is compressed to a 20-hr period with no 4-hr asymmetry in protein accumulation that could account for the period change (Lee et al., 2001). Another piece of genetic evidence for CK 1 as a clock protein comes from the identification of defective phosphorylation of human Per2 in familial advanced sleep phase syndrome (FASPS) patients (Toh et al., 2001). People affected by FASPS display advanced activity onset and offset (Jones et al., 1999). A point mutation in hPer2 associated with FASPS changes the first serine in five consecutively positioned serines that are aligned in a manner that is consistent with 5 tandemly positioned CK 1 phosphorylation sites. Normally phosphorylation of the first serine could prime CK 1 for the downstream serines for processive phosphorylation but when mutated could slow down the phosphorylation of

the 5 serines. Consistent with this hypothesis CK 1ε phosphorylates the mutated sequence with a slower rate *in vitro* (Toh et al., 2001). Again, like in the *tau* hamsters it is not clear how the mutation leads to advances in circadian behavior in the patients. The apparent similarities in the phenotypes of both the *tau* hamsters and FASPS patients may indicate a similar mechanistic basis of the faster clock in both conditions. CK 1ε and CK 1δ have also been shown to directly associate with clock protein complexes *in vitro* and *in vivo* via direct associations with Per proteins (Camacho et al., 2001; Keesler et al., 2000; Lee et al., 2001; Takano et al., 2004; Vielhaber et al., 2000).

It is unclear how the behavioral phenotypes described above are generated by defective clock protein phosphorylation. However, several regulatory processes have been identified, primarily using cultured cells that help to define the roles of phosphorylation in the circadian clock. Nuclear entry or exit of Per proteins, apparently depending on the cell line used, can be forced by overexpression of CK 1ε (Takano et al., 2004; Takano et al., 2000; Vielhaber et al., 2000). Mammalian clock proteins are phosphorylated predominantly during the night in the nucleus (Lee et al., 2001). Per proteins are highly phosphorylated at late night when bound to heteromultimeric complexes with Clock-BMAL1 dimers at circadian promoters and repressing transcription (Lee et al., 2001). Clock and BMAL1 are also phosphorylated primarily when in the nucleus (Lee et al., 2001). CK 1 dependent phosphorylation of Per proteins leads to destabilization and degradation (Akashi et al., 2002; Keesler et al., 2000). Phosphorylated Per proteins are ubiqutinated and targeted to the proteosome (Akashi et al., 2002; Yagita et al., 2002). Protein-protein interactions regulate the phosphorylation of clock proteins. For example CK 1ε phosphorylates Cry and Per proteins only once they

form heterotrimeric complexes (Eide et al., 2002). Thus one of the benefits of large clock protein complexes at night on circadian promoters may be formation of scaffolds that bring together substrates and kinases. Reporter assays suggest that BMAL1 dependent transcription may also be modulated by phosphorylation. In cultured cells repressing CK 1ε or CK 1δ activity reduces BMAL1 dependent transactivation of *Per1* promoter (Eide et al., 2002). BMAL1 can be phosphorylated *in vitro* by mitogen-activated kinase (MAPK) (Sanada et al., 2002). In cell culture MAPK activity moderately inhibits avian BMAL1 activity (Sanada et al., 2002).

1.4 Entraining input to circadian oscillators

In most organisms light is the strongest clock-resetting agent. The mechanism of light dependent phase resetting varies between different clock models. Three anatomical pathways have been postulated that mediate light-dependent resetting of the *Drosophila* clock. These include poorly understood retinal and extra-retinal pathways that mediate the phase resetting of *Drosophila* pacemaker by light. Best understood entrainment pathway is a cell autonomous cryptochrome (CRY) photopigment dependent degradation of TIM. Short light pulses in the early night delay the phase of behavioral rhythms whereas advances are produced during late night. These behavioral responses can be explained with the subcellular localization of TIM at the time of degradation. Considering TIM as a transporter of PER to the nucleus, degradation of TIM in the accumulation phase would delay the nuclear translocation of PER and thus the

transcriptional repression phase of the cycle. A transition from delays to advances occurs at the time of TIM and PER nuclear translocation in the middle of the night. This can be explained by the release of PER from the complex with TIM upon light exposure which has been demonstrated to enhance the repressor activity of PER and thus advance the end of the molecular cycle (Kloss et al., 2001; Myers et al., 1996; Rothenfluh et al., 2000).

CRY binds a pterin chromophore and a flavin molecule that is required for redox process dependent conformational change upon light exposure. A conformationally active form of CRY binds TIM and targets it for ubiquitination (Lin et al., 2001; Naidoo et al., 1999). An unknown protein kinase is also involved in light induced TIM degradation as it is phosphorylated at tyrosine residue(s) prior to degradation (Naidoo et al., 1999). Light activation causes CRY itself to be degraded as well (Lin et al., 2001). CRY mRNA and protein levels are under circadian control cycling in the phase of CLK with maximum levels just after dawn (Emery et al., 1998).

In the absence of functional CRY circadian rhythms can still be entrained with lower efficiency indicating alternative pathways for photoentrainmant. Extra-ocular photoreception from anatomically diverse sources appears to contribute to the photoentrainment of *Drosophila* clock. Genetically ablating the function of conventional photoreceptors with a phospholipase C mutant $norpA^{P41}$ causes diminished but persistent responsivness to the entraining light indicating extra-ocular photoreception. Flies with a combination of *cry* and *norpA* loss of function mutations retain reduced entrainability suggesting photopigments additional to CRY are involved in the entrainment of rhythms (Stanewsky et al., 1998). Phase shifting of circadian locomotor activity by light exposure is lost with elimination of both *cry* function and the genetic removal of external

photoreceptor cells and a structure known as the Hofbauer-Buchner eyelet located underneath the compound eye that makes neuronal contacts with the pacemaker cells in the brain (Helfrich-Forster et al., 2001).

In the *Neurospora* clock light exposure leads to WCC dependent activation of *frq* transcription (Crosthwaite et al., 1995). Transcriptional activation by WCC is mediated by two light-response elements (LRE) in the *frq* promoter (Froehlich et al., 2002; Froehlich et al., 2003). DNA bound WCC undergoes a multimerization step upon light exposure that promotes its activity. The FAD bound WC-1 protein in WCC functions as a blue light receptor (Froehlich et al., 2002; He et al., 2002).

In mammals the circadian pacemaker tissue in the SCN is not directly accessible to light. Its light responses are mediated by retinal ganglion cells expressing photoreceptor melanopsin as well as by conventional visual photoreceptor cells that innervate the SCN via the retinohypothalamic tract. Light pulses activate calcium dependent signaling cascades that ultimately lead to the phosphorylation of transcriptional activators like CREB, which activates genes with Ca/cAMP response elements. Among these genes are clock genes *Per1* and *Per2* that mediate phase resetting (Travnickova-Bendova et al., 2002).

1.5 Circadian clock directed gene expression

Genome-wide expression analysis described in more than dozen studies from *Arabidopsis*, *Drosophila*, *Neurospora* and mammals has shown that hundreds of genes

covering a wide variety of functions from basic metabolism to synaptogenesis are under the control of the circadian oscillators (reviewed in (Duffield, 2003). In mouse liver for example glycolysis, glyconeogenesis, fatty acid metabolism and cholesterol metabolism are all under circadian control (Panda et al., 2002). In many cases the mRNAs of rate limiting enzymes for these pathways are transcribed with a circadian rhythm. Furthermore, within circadianly controlled pathways genes are transcribed in similar phases allowing the components of the pathway to be expressed at the same time (Panda et al., 2002). Interestingly circadian control of gene expression is tissue specific rather than gene specific. In any tissue approximately 10% of the transcripts are under circadian control. Overlap between two sets of circadian mRNAs in two tissues is only about 10%. About half of the remaining transcripts are present in both tissues but they are rhythmically expressed only in one of the tissues (Storch et al., 2002) Given that the circadian control of gene activity is tissue specific and each tissue has a mostly separate set of genes under clock control then most of the genome may be under circadian regulation when the whole organism is considered.

Although transcriptional control appears to be the dominant mechanism for controlling clock output genes, alternative ways can be hypothesized. Despite constitutive expression, substantial amount of CK 1 and DBT cycles between cytoplasm and nucleus with a circadian period due to a stable interaction with the PER proteins (Kloss et al., 2001; Lee et al., 2001). Such rhythmic change in subcellular localization could provide means for circadian posttranslational control of protein activity or stability. An example of such control may be circadian binding and phosphorylation of $GABA_A$ receptor by CK 1ε and CK 1δ that leads to receptor inhibition in the SCN (Ning et al., 2004)

Aims of this thesis

It is becoming increasingly evident that protein phosphorylation plays an essential role in the functioning of circadian clocks in all model organisms. In each model system several protein kinases have been identified but the mechanisms of their action are poorly understood.

In this thesis genetic and biochemical approaches were taken to investigate how casein kinase 1 like protein kinase Doubletime affects the *Drosophila* circadian clock. Aims were set to identify its phosphorylation sites in the circadian clock protein Period and investigate the function the identified Doubletime target sites in a cultured cell model system and in living flies.

CHAPTER 2: MATERIALS AND METHODS

Maintenance of cultured cells

Sf9 cells (Invitrogen) were maintained at 28 ^{0}C in serum free media Sf-900 II SFM according to supplier's recommendation. A new batch of cells was used every 3-4 months. S2 cells were maintained at room temperature in Drosophila SFM media (Invitrogen) supplemented with 15% fetal bovine serum and 1.5g L-glutamine/500ml.

Cloning of plasmid constructs

Fragments of PER coding sequence were amplified by PCR and cloned between EcoRI and XhoI sites in pGEX6P-1 (Amersham) expression plasmid for bacterial expression. For GST-SGG and GST-CYC full-length open reading frames of *sgg* and *cyc* were amplified by PCR and cloned into pGEX-6P-1 between SmaI and NotI sites and EcoRI and XhoI sites, respectively. GST-TIM construct have been described before (Martinek et al., 2001).

For GST-DBT baculovirus construction, the *dbt* open reading frame was PCR amplified and cloned as an EcoRI-XhoI fragment into the pGEX-6P-1 vector. From the resulting contruct, the GST-DBT coding sequence was released as SspI fragment and ligated into the EcoRV site in the pFastBac-1 vector (Invitrogen). Correct orientation relative to the polyhedrin promoter was verified with diagnostic digests. The resulting pFastBac-1 vector encoding for GST-DBT was used to transform DH10Bac competent cells (Invitrogen) and recombinant baculovirus DNA was selected and purified following the Bac-to-Bac system (Invitrogen) instructions. Baculoviruses encoding for DBT

mutants were constructed analogously except point mutations changing the relevant amino acids were first introduced into *dbt* coding sequence.

For S2 cell expression, *per* cDNA was cloned into pAct plasmid (gift from Dr. S.Kidd) that contains the actin promoter region upstream of the insert cDNA and 3' regulatory sequences of the *Drosophila* actin gene. Point mutations into the *per* coding sequence were introduced by following the QuickChange II (Stratagene) mutagenesis protocol. Expression plasmids encoding for luciferase, *Clock* and *lacZ* under actin promoter control have been described in the literature (Rothenfluh et al., 2000). per-luc reporter construct contains luciferase coding sequence under *per* gene promoter cloned into pCasper-4 plasmid (Brandes et al., 1996).

Expression and purification of recombinant PER proteins from *E.coli*

Proteins were expressed in BL21pLysS cells (Novagen). Protein expression was induced with 1mM IPTG at cell density OD_{600}=0.6-0.8 for 3-4 hrs at 37°C. After induction cells were pelleted and resuspended in STE buffer (150 mM NaCl, 50mM Tris pH 8.0, 1mM EDTA) and sarcosyl in STE was added to a final concentration of 1%. Resuspended cells were frozen once. Thawed lysate was passed through 18-gauge syringe needle 5 times to fragment DNA. Shared lysate was centrifuged at 15000g for 30 min at 4°C. After centrifugation Triton X-100 in STE was added to the supernatant to 2% final concentration and incubated with glutatione sepharose for 30 minutes at 4°C. Bound protein was washed once with HND600 buffer (600mM KCl, 20mM Tris pH 7.5, 5mM EDTA, 10% glycerol, 0.5% Tween 20) and 4 times with HND100 (same as HND600

except with 100mM KCl). Bound proteins were stored at –20°C in 1:1 mix of 50% glycerol/HND600.

Expression and purification of recombinant DBT proteins

Sf9 cells were transfected with baculovirus DNA containing GST-DBT expression cassette using Lipofectamine (Invitrogen) according to manufacturer's recommendations. 72 hrs after transfection cell culture media was collected and transfected cells were lysed and tested for GST-DBT protein expression by Western blot analysis. Anti-GST antibody (Santa Cruz Biotech) was used to detect recombinant protein expression. Cell culture media from cells tested positive for recombinant protein expression was used to infect cells for 48 hrs to produce GST-DBT baculovirus stocks. Viral stocks were stored at 4°C. For protein expression 0.3-1 ml of viral stock was used to infect approximately $1.5x10^7$ adherent cells in a 150-cm^2 tissue culture flask in 25 ml of media. Cells were lysed 48 hrs after infection in HND100 buffer on ice for 10 minutes in the presence of protease inhibitors III (Calbiochem). Lysates were cleared by centrifugation at 15000g for 10 minutes. After centrifugation extracts were incubated shaking with glutathione sepharose at 4°C for 30 minutes. Resin with bound protein was washed 3 times with lysis buffer and once with PreScission protease cleavage buffer (150mM NaCl, 50mM Tris pH 7.5, 1mM EDTA). Recombinant DBT protein was cleaved off the glutathione sepharose in overnight incubation with PreScission protease (Amersham) in 0.5 ml of protease buffer following the conditions recommended by the manufacturer. Supernatants from protease cleavage were concentrated about 20 fold by ultrafiltration in Microcon centrifugal filters with molecular cutoff 30kD (Millipore). Resulting concentrates were used within hours

for kinase assays. The activity was diminished to almost undetectable levels in 24 hrs after purification.

In vitro protein phosphorylation

Glutathione sepharose beads bound by GST-PER fusion proteins were washed once with kinase buffer (50mM Tris pH 7.5, 10 mM $MgCl_2$, 5mM DTT) and resuspended in kinase buffer. DBT protein purified from Sf9 cells was added (1/10 total reaction volume) to resuspended beads after addition of 0.5µl radioactive ATP labeled with P-32 at γ-phosphate (3000Ci/mmol) and 100µM unlabeled ATP. Reactions were carried out in 30 µl and proceeded for 1 minute at room temperature. Reactions were stopped by adding equal volume of 2x SDS-PAGE loading buffer (125mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol (v/v). Phosphorylated proteins in SDS-PAGE loading buffer were boiled for 5 minutes and were resolved in 10% SDS-PAGE.

Reactions with casein were carried out in similar conditions, except DBT proteins were not cleaved off the glutathione sepharose beads. 100 ng of casein was used in each reaction.

Luciferase assay

Luciferase assays were carried out essentially as described (Rothenfluh et al., 2001). S2 cells were transfected in 24-well plates at 60-80% confluency using Effectene transfection reagent (Qiagen) following manufacturer's recommendations. Cells were transfected with 50 ng pAct-PER, encoding for either wildtype or mutant PER, 5 ng pAct-Clk, 10 ng per-luc, 20 ng pAct-lacZ and 115 ng of pAct plasmid without an insert.

Effectene transfection reagent (Qiagen) was used to deliver DNA into the cells following manufacturer's protocol. Cells were harvested 24-36 hrs after transfection and lysed in Cell Culture Lysis Reagent (Promega). Extracts were mixed with Luciferase Assay Reagent (Promega) in 1:5 ratio and luciferase activity was measured using liquid scintillation counter LS6000IC (Beckman) in single photon collection mode. Luciferase activity was normalized to cotransfected beta-galactosidase activity in each sample.

Protein extraction and Western analysis

Total protein from fly heads was extracted in HE buffer (20mM Tris pH 7.5, 100mM KCl, 10mM EDTA, 0.1% Triton, 1mM DTT) supplemented with protease and phosphatase inhibitors (Calbiochem). Protein concentration was measured with BCA protein measurement kit (Pierce) according to manufacturer's protocol.

For detection of PER and TIM proteins 20 μg of total protein was resolved in 6% SDS-PAGE. Following antibodies at indicated dilutions in 5% nonfat milk were used for protein detection: anti-TIM 1:2000 (Myers et al., 1996), anti-PER 1:20000 (gift from Dr. J. Hall) or anti-PER 1:600 (Chemicon). Appropriate species-specific horseradish peroxidase coupled secondary antibodies (Jackson Laboratories) were used at 1:10000 dilution. Antibody binding was detected by ECL Western Blotting Detection Reagent (Amersham). Primary antibody incubations were carried out overnight at 4°C. Secondary antibody incubations were carried out at room temperature for 1 hr. Blots were washed after antibody incubations in 1x TBST (50mM Tris pH 8.0, 150mM NaCl, 0.5% Tween 20) 3 times, each 10 minutes. Secondary antibody incubations were also carried out in 1x TBST.

RNA isolation and Northern analysis

RNA was isolated from fly heads by homogenization in RNA STAT-60 extraction reagent (TEL-TEST, Inc.) according to manufacturer's protocol or with RNeasy RNA isolation kit (Qiagen) according to animal cell RNA isolation protocol with additional centrifugation after initial homogenization of fly heads and prior to column binding. 2-20 μg of total RNA was resolved in 1% formaldehyde gel following RNeasy Northern gel protocol and transferred to NytranN membrane (Schleicher and Schuell) in 10x SSC. Membranes were UV crosslinked in Stratalinker (Stratagene). Pre-hybridization was carried out for 0.5-2 hrs and hybridization was performed overnight in 10 ml of Ultrahybe buffer (Ambion). Blots were washed 4 times in washing buffer (0.2x SSC, 1% SDS) at 65^0C. Signal was detected and quantified with Typhoon 9200 imager (Amersham).

Radioactive probes were generated using Decaprime II DNA labeling kit (Ambion) according to the manufacturer's protocol. Full cDNA sequences were used to make probes for detection of *per*, *tim*, *vri*, *clk*, *dbt*, *cyc* and *rp49* mRNAs. For *pdp1ε* mRNA detection sequence specific for the ε isoform was used as a template for probe synthesis.

RNase protection assay

RNase protection assays were carried out using RPAIII kit (Ambion) according to manufacturer's protocol. For hybridization, 2-5 μg of total RNA from fly heads was used. For probe generation, a fragment of *per* genomic sequence containing last 110 nucleotides (nt) from intron 4 and first 311 nt from exon 5 was amplified by PCR and

cloned into pBluescript plasmid between EcoRI and Xho sites in the direction which allowed antisense strand to be transcribed by T7 polymerase. This probe protects 311 nt of *per* mRNA that includes the per-short domain. In *perΔS* flies the protected fragment is 51 nt shorter. Probe synthesis was carried out using MaxiScript kit (Ambion) according to manufacturer's protocol.

Fly culture and locomotor behavior analysis

Drosophila melanogaster were reared on standard medium at room temperature (~25°C). Monitoring and analysis of locomotor activity of individual flies was performed in constant darkness at 25°C using the *Drosophila* Activity Monitoring System IV (Trikinetics).

CHAPTER 3: PERIOD PHOSPHORYLATION BY DOUBLETIME *IN VITRO*

3.1 Background

The *Drosophila* clock protein PERIOD (PER) is known to be progressively phosphorylated in the circadian cycle (Edery et al., 1994). The majority of the phosphorylation occurs during the lights-off phase of the cycle when PER is primarily located in the nuclei of clock-containing cells. Mutations in the *doubletime* gene alter the phosphorylation pattern of PER (Price et al., 1998; Suri et al., 2000). Several lines of genetic and biochemical evidence suggest that Doubletime protein may directly phosphorylate PER. The *doubletime* gene sequence shares a high degree of similarity with the kinase domain of the mammalian CK1 family of protein kinases (86% amino acid sequence identity in the predicted kinase domain) (Kloss et al., 1998). Also, the DBT protein interacts with the PER protein both *in vitro* and *in vivo.* The mammalian CK1ε readily phosphorylates Drosophila PER protein *in vitro*, however when expressed in the genetic background of *doubletime* loss-of-function, the mammalian enzyme fails to complement (Shimoda and Saez, unpublished results). The failure of genetic complementation by the orthologous mammalian enzyme raised the question whether DBT directly phosphorylates PER. DBT could affect the PER phosphorylation pattern indirectly.

This chapter describes experiments that demonstrate direct phosphorylation of PER by the DBT protein kinase. Three regions in the PER protein are phosphorylated by

DBT *in vitro*. Results are shown that map the phosphorylated regions in PER and identify individual residues that are phosphorylated by DBT.

3.2 Production of enzymatically active DBT

Initially efforts were made to synthesize enzymatically active DBT protein in bacterial cells. Several expression constructs were tried employing His-tag, GST-tag or an intein-tag in the N-terminus of the DBT coding sequence. Multiple protein induction conditions were used at different temperatures and IPTG concentrations. All these efforts failed to produce enzymatically active DBT protein. Interestingly, production of active mammalian CK1ε in parallel in the same conditions was successful suggesting a specific problem emanating from the DBT protein primary sequence. An effort was made to express DBT protein in insect cells after failure in the bacterial expression experiments. Baculoviral expression in Sf9 cells was chosen for DBT over-expression in an effort to recover kinase activity. Two baculoviruses were constructed that were capable of expressing either the wildtype or a mutant DBT protein with an ATP binding lysine 38 mutated to an alanine (K38A). Lysine 38 in the mammalian CK1 is involved in ATP binding by the kinase and mutations in this residue render the kinase inactive (Longenecker et al., 1996). Recombinant proteins were expressed as N-terminal GST-fusion proteins to facilitate their purification. GST-DBT proteins were purified 48 hrs after infection and tested for kinase activity with casein as an *in vitro* substrate. The fusion protein containing the wildtype DBT sequence produced strong phosphorylation of

casein (Fig.4, lane 1) while the DBT mutant deficient in ATP binding showed very weak activity (Fig. 4, lane 2). This result demonstrates that Sf9 cells produce enzymatically active DBT kinase.

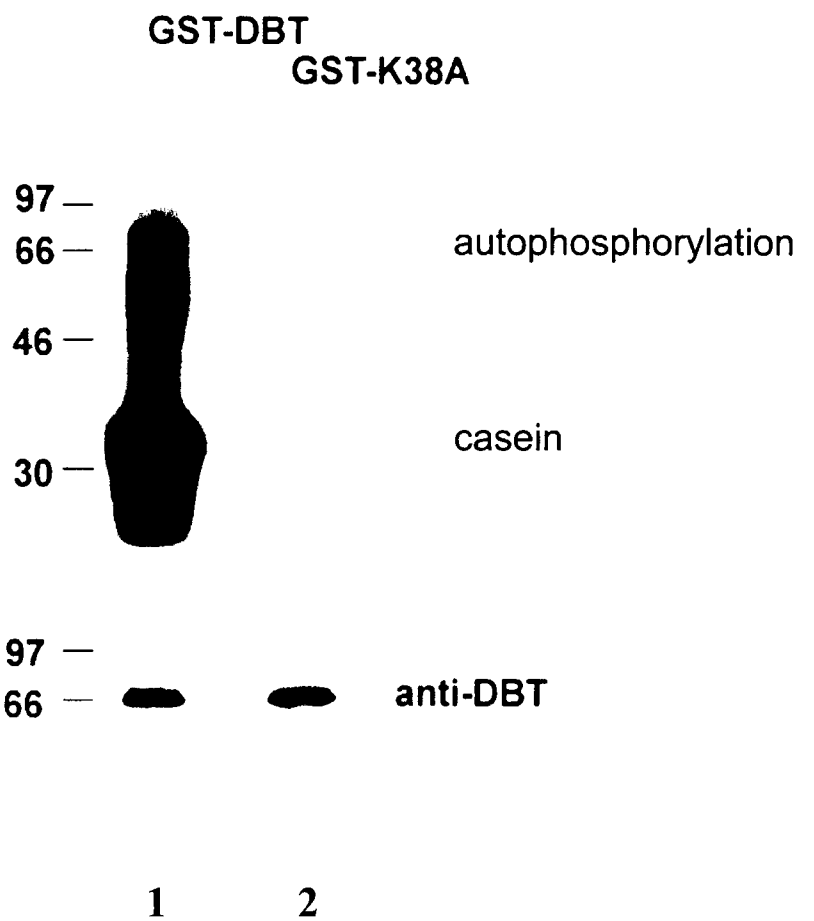


Figure 4. Recombinant DBT purified from Sf9 cells has protein kinase activity. GST-DBT (lane 1) and GST-DBT(K38A) (lane 2) were expressed and purified from Sf9 cells. Purified proteins were incubated with casein and radioactive P-32 labelled γ-ATP in kinase buffer. Top panel shows an autoradiograph of incorporated radioactive phosphoate. Casein is strongly phosphorylated by GST-DBT (lane 1, top panel). Casein phosphorylation is drastically reduced by a mutation of lysine 38 changed to alanine in GST-DBT (top panel, lane 2). Lower panel indicates equal amount of GST-DBT (lane 1) and GST-DBT(K38A) (lane 2) used in the kinase assay as detected by Western blot with anti-DBT antibody.

3.3 Comparison of enzymatic activities of DBT mutants affecting circadian clock function

Several *dbt* mutants have been recovered from *in vivo* mutagenesis screens that affect the periodicity of locomotor activity rhythms in *Drosophila* (Price et al., 1998; Suri et al., 2000). A mutant dbt^S with an amino acid substitution of proline to serine at residue 47 (P47S) in the kinase domain, speeds up the circadian clock by about 6 hours resulting in a 18-hr period locomotor behavior phenotype. Another mutant, dbt^L, with an amino acid substitution of methionine to isoleucine at residue 80 (M80I) leads to lengthening of locomotor activity behavior to a 29-hr period. Both of these mutations affect the accumulation pattern of PER in clock containing cells. dbt^S shows enhanced degradation of PER protein whereas the dbt^L shows extended stability of PER. A third mutant dbt^{AR} with a substitution of histidine 126 to tyrosine (H126Y) leads to arrhythmia. PER protein is overaccumulated in this mutant and displays an intermediate phosphorylation pattern. All these mutations map to the kinase domain of DBT. Since DBT functions as a protein kinase, it is important to determine if the period changing mutations affect its biochemical activity.

This section describes experiments that compare wildtype and mutant DBT proteins in their ability to phopshorylate casein. Mutations that affect clock function in flies were introduced into the baculoviral construct described above for wildtype DBT expression. Mutant and wildtype kinases were purified side-by-side and short time point kinase assays were performed using approximately equal amount of protein for each reaction and casein as a substrate. All the mutations decreased the kinase activity of DBT

but to various extents (Fig. 5). The most dramatic reduction was observed in the presence of the dbt^{AR} mutation (Fig. 5, lane 1). When normalized to the kinase protein levels, the dbt^{AR} showed 10% of activity when compared to the wildtype. The dbt^{L} mutation reduced the activity down to 30% of wildtype. Interestingly the dbt^{S} mutation that speeds up the clock *in vivo*, also reduced DBT activity. The reduction of kinase activity by dbt^{S} was much more moderate in contrast to the other mutations leading to a loss of activity by 15% when compared to the wildtype kinase. Similar results have been recently obtained with a Xenopus casein kinase 1ε with mutations that mimic these *dbt* mutations (Preuss et al., 2004).

The reduction of kinase activity by both the period shortening and lengthening mutations of *dbt* suggests a more complex regulation of the clockwork by DBT. It is possible that multiple substrates exist in the clock for DBT that may be phosphorylated at different times of day and defects in phosphorylation can lead to opposing behavioral phenotypes. PER itself is regulated by phosphorylation at multiple steps of the circadian cycle. Defective phosphorylation may have opposing phenotypic effects depending which aspect of PER regulation is affected.

3.4 DBT selectively phosphorylates PER

Availability of enzymatically active DBT allowed to test for direct PER phosphorylation by DBT . A selection of full-length or partial sequences of clock proteins were fused to GST and fusion proteins were produced and purified from *E.coli*.

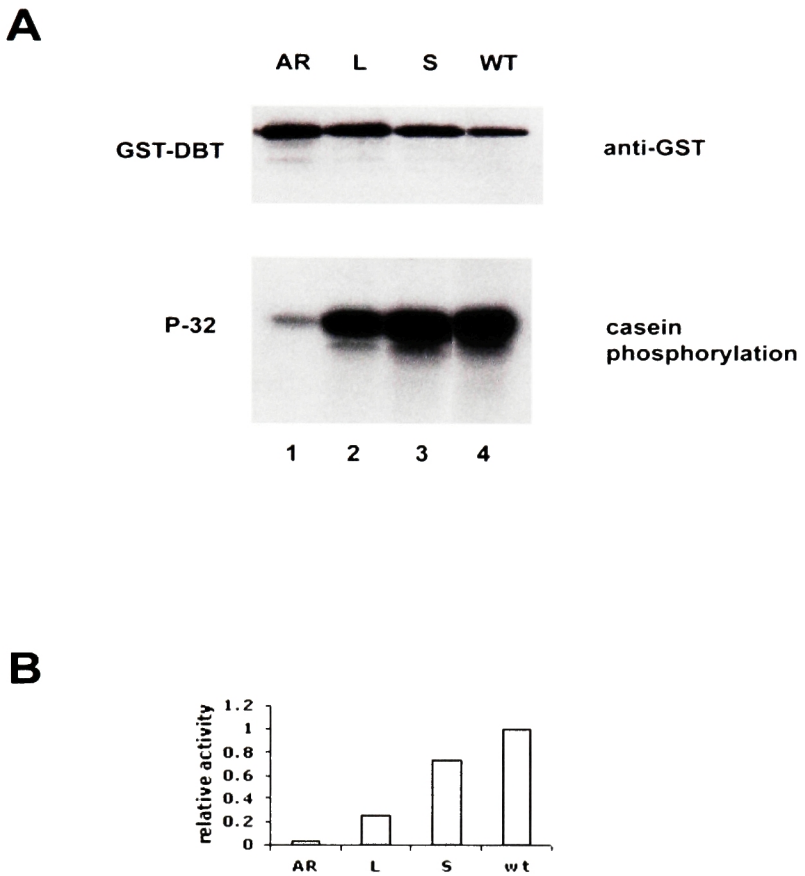


Figure 5. DBT mutations affecting behavioral rhythms also affect its kinase activity. Either wildtype DBT (WT) or DBT mutants found in flies with behaviorally arrhythmic (AR), long- (L) or short- (S) period rhythms were expressed and purified as GST fusion proteins from Sf9 cells and used to phosphorylate casein. (A, lower panel) Equal amount of casein was phosphorylated with wildtype GST-DBT (lane 4) or with mutant GST-DBT proteins: GST-DBT^{AR} (lane 1), GST-DBT^{L} (lane 2), GST-DBT^{S} (lane 3). (B) Relative kinase activity of GST-DBT fusion proteins was measured as a ratio of casein phosphorylation signal to total GST-DBT levels measured by anti-GST Western blot (A, top panel). Relative kinase activity of wildtype GST-DBT was set as 1.

Figure 6 shows that DBT specifically phosphorylated GST-PER fusion protein encoding for the first 640 amino acids of *Drosophila* PER (Fig 6, lane 11). No phosphorylation of two overlapping fragments of TIM covering amino acids 222-1150 or N-terminal half of CLK or full-length CYC and SGG were observed (Fig 6, lanes 1,3,5,7 and 9, respectively). This experiment established for the first time that PER could be directly phosphorylated by DBT. Next, a more comprehensive mapping of the phosphorylation of PER by DBT was undertaken.

3.5 Identification of DBT phosphorylation regions in PER.

A panel of overlapping GST fusion proteins were generated that covered the entire 1224 amino acids of *Drosophila* PER. The fusion proteins were phosphorylated with recombinant DBT. Phosphorylation reactions were carried out for 1 minute at room temperature with sub- to low-nanogram amounts of kinase in an effort to capture phosphorylated regions with highest affinity for DBT. Figure 7 shows phosphorylation of 17 PER fragments that define 3 regions in PER as *in vitro* targets for DBT.

The phosphorylation of PER 1-640 initially observed can be broken down into two regions. A fusion protein containing the N-terminal 365 amino acids of PER was phosphorylated by DBT (Fig 7, lane 7). A sub-fragment of this region containing amino acids 170-365 was not phosphorylated (Fig 7, lane 9). Thus the C-terminal boundary of this phosphorylation region is defined by amino acid 170. An N-terminally truncated protein that deletes the first 148 amino acids and ends with amino acid 365 (GST-149-

365) (Fig 7, lane 8) retained a comparatively similar amount of phosphorylation as the original fusion protein containing amino acids 1-365 (Fig. 7, lane 7). This suggests that the N-terminal phosphorylation region is likely to be located between residues 149 and 170. An effort was also made to generate fusion proteins with a fixed N-terminal endpoint in the beginning of PER and a set of deletions progressing N-terminally from residue 365 but all these fusion proteins were unstable in *E.coli*, which precluded complementary mapping of the N-terminal boundary of this phosphorylation region.

Sequences between amino acids 170 and 580 were not significantly phosphorylated by DBT (Fig 7, lanes 4, 6, 9). Also PER 650-700 was not phosphorylated by DBT (Fig 7, lane 2). Strong phosphorylation of PER 570-645 was however observed. Thus, a second phosphorylation region in the first 640 residues of PER resides between amino acids 580-645.

DBT also phosphorylated a fusion protein encoding residues 650-1224, which approximately corresponds to the C-terminal half of PER (Fig.7, lane 17). Despite the presence of a multitude of C-terminal degradation products of this protein, only the highest mobility forms were significantly phosphorylated. This suggested that the phosphorylation occurs close to the very C-terminus of PER. Shorter sub-fragments collectively encoding for residues 947-1136 were not phosphorylated by DBT (Fig 7, lanes 12, 13, 14). The effective phosphorylation of GST-PER 1101-1224 defined the last ~ 100 residues as the C-terminal phosphorylation region for DBT. In conclusion, DBT phosphorylates three sub-regions of PER: 149-170 aa in the N-terminus, 580-645 aa in the center of the protein and 1136-1224 aa in the C-terminus. All of these regions have

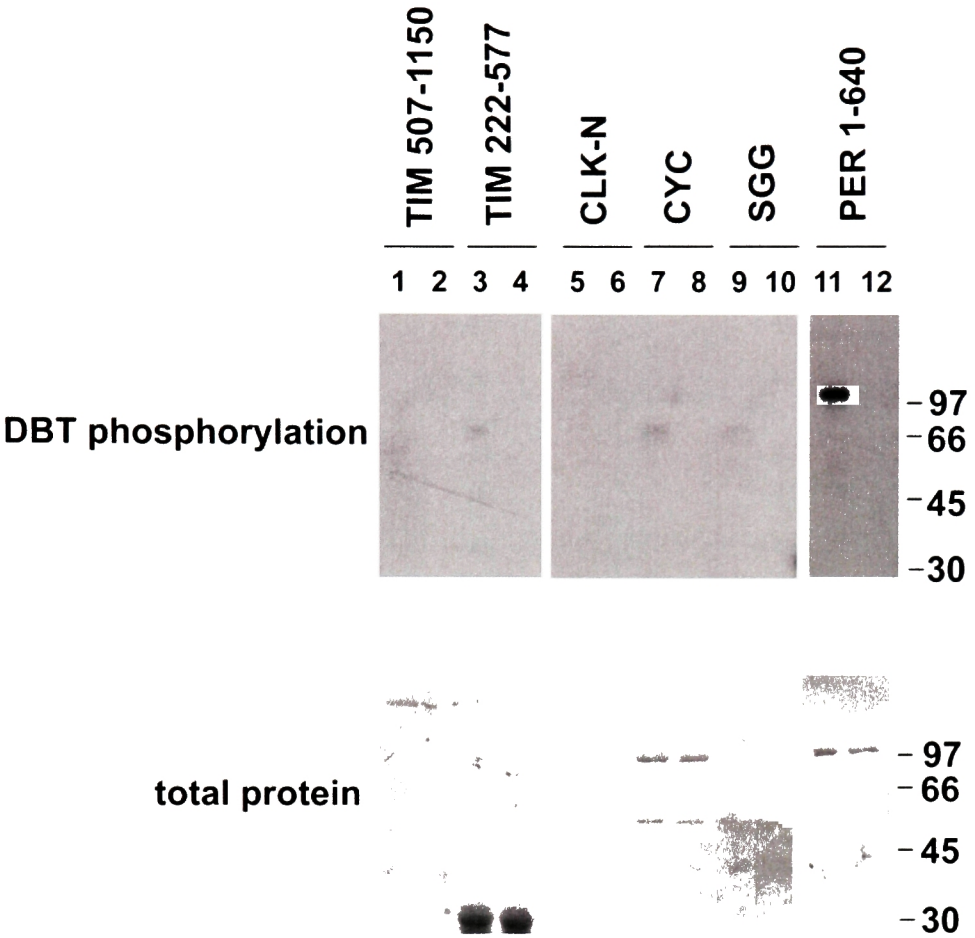


Figure 6. DBT preferentially phopshorylates PER.

Fragments or full-length sequences of proteins required for circadian clock function in *Drosophila* were fused to GST and expressed and purified from *E.coli*. Purified fusion proteins were phosphorylated with either wildtype DBT (odd-numbered lanes) or inactive DBT(K38A) mutant as a negative control (even-numbered lanes). Top panel shows autoradiograph of incorporated phosphate, lower panel shows total protein stained with amido black. Molecular weight sizes are indicated at the right of each panel. Sequences fused to GST as substrates for phosphorylation: lanes 1-2, TIM amino acids 507-1150; lanes 3-4 TIM amino acids 222-577; lanes 5-6 CLK amino acids 1-600; lanes 7-8 full-length CYC; lanes 9-10 full-length SGG; lanes 11-12 PER amino acids 1-640.

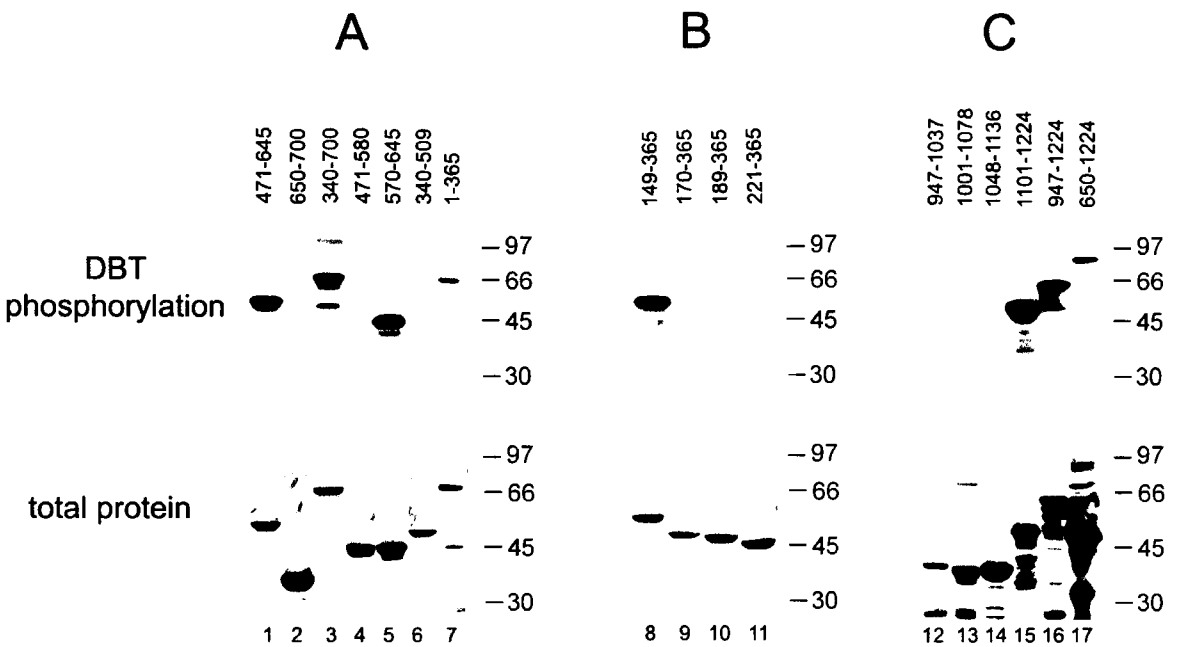


Figure 7. DBT phosphorylates three regions in PER.

Different fragments of PER were fused to GST and expressed and purified from *E.coli.* Each fusion protein was phosphorylated with recombinant DBT and resolved in 10% SDS-PAGE and transferred to nitrocellulose. Top panel in (A),(B) and (C) shows signal from incorporated radioactive phosphate. Lower panel in (A),(B) and (C) show total protein stained with Ponceau S. Numbers on top of each lane indicate amino acids in PER included in each fusion protein. (A) Shows mapping of phosphorylation in the middle of PER with minimal phosphorylated region between residues 570-645. (B) Shows mapping of phosphorylation in N-terminal fragments of PER with minimal region between residues 149-170. (C) Shows mapping of phosphorylation to C-terminal end of PER with minimal phosphorylated region between residues 1101-1224. Molecular weight markers are shown at the right of each panel.

relatively high levels of serines and threonines (17-26%), many of them arranged in SXXS sequences (two serines separated by two random residues) where the second serine is considered a preferred site of phosphorylation by CK 1. An effort was made to computationally predict potential phosphorylation sites in the identified target regions and mutagenesis was performed to mutate combinations of serines and threonines in predicted CK 1 phosphorylation motifs, but phosphorylation was never completely eliminated. An alternative approach of positively identifying phosphorylated residues by mass-spectrometry was therefore carried out.

3.6 Identification of individual DBT phosphorylation sites in PER

Protein phosphorylation experiments with overlapping PER fusion proteins described in the previous section identified relatively short regions in PER that were phosphorylated by the DBT kinase. Three fusion proteins were chosen for subsequent phosphopeptide analysis experiments in order to define individual phosphorylation sites in each individual phosphorylation region. Fusion protein GST-PER149-365 was chosen for identification of N-terminal phosphorylation sites. To identify the phosphorylation sites in the middle of the protein, the GST-PER580-645 fusion protein was used. For C-terminal sites the GST-PER1101-1224 fusion protein was used. All three proteins were phosphorylated by recombinant DBT as in the initial phosphorylation region mapping experiments. The phosphorylated proteins were resolved in acrylamide gels and protein bands were stained and excised for subsequent analysis at the Rockefeller University

Protein Research Facility according to the facility procedures. Phosphorylated proteins were digested with trypsin and the resulting peptides were resolved into approximately 100 fractions for each protein. Fractions that contained the majority of the radioactivity were treated individually with alkaline phosphatase and propanethiol. Treated fractions were analyzed by mass-spectrometry and peptide masses of treated and non-treated samples were compared. Alkaline phosphatase treatment hydrolyzes the phosphate off the phosphorylated residue and thus leads to reduction of the peptide mass by 80 Da per phosphorylated residue. As an independent test phosphopeptides were reacted with propanethiol. This would result in a substitution reaction at the phosphorylation site and reduce the peptide mass by 21 Da per phosphorylated residue. Peptide fractions that responded to both treatments were subjected to Edman sequencing to identify the position of individual phopshorylated amino acids in the peptides.

The bulk of the radioactivity in the N-terminal phosphorylation region was contained in one long peptide shown in Table 1, that contained the last 16 amino acids of GST sequence that were followed by the first 27 amino acids of the PER sequence in the fusion protein GST-PER143-365. The long stretch of amino acids derived from the GST portion of the fusion protein in front of PER sequence in the peptide precluded the analysis by Edman sequencing. The PER sequence in this peptide corresponds exactly to the phosphorylation region mapped by *in vitro* phosphorylation experiments using overlapping GST-PER fusion proteins. The primary sequence between residues 149 and 170 in PER contains five serine residues at positions 149, 151, 153, 164 and 169. These residues are the predicted substrates for DBT in the N-terminus of PER.

Most of the phosphorylation in the fusion protein covering the middle portion of PER, GST-PER580-645 was distributed over four tryptic peptide fractions. Three different peptides shown in Table 1, responded to alkaline phosphatase and propanethiol treatment in these fractions. Amino terminal sequencing of these peptides showed that the modified residues correspond to serines 589, 607, 629 and threonine 610 in the full-length PER protein sequence. None of the peptides were phosphorylated at multiple sites according to mass-spectometry data, however this doesn't indicate the absence of such peptides. Serine 604 was not amenable for sequencing analysis due to its position at the end of a longer peptide. This residue is part of a CK 1 consensus sequence that also includes serine 607, which was phosphorylated. In conclusion, for further analysis the residues 589, 604, 607, 610, 613 and 629 would be considered as phosphorylation sites for DBT in PER. In further experiments the amino acid interval between residues 604 and 629 is called perSD (*per*-Short Downstream) domain to distinguish it from the previously defined upstream per-short domain.

Phosphorylation in the C-terminal fusion protein GST-PER1101-1224 was mostly consolidated into two fractions that each contained one phosphopeptide shown in Table 1. Amino terminal sequencing of these peptides revealed one phosphorylated residue in each peptide. In the full-length PER protein the identified phosphorylated residues correspond to serine 1134 and threonine 1219.

Figure 8 shows schematic representation of the identified DBT phosphorylation sites in PER relative to the previously recognized functionally important domains. With the exception of serine 589 none of the phosphorylation sites map to these domains.

Table 1. Identified phosphopeptides from GST-PER fusion proteins phosphorylated by DBT.

DBT substrate	Identified phosphopeptides
GST-PER143-365	*SDLEVLFQGPLGSPEF-* -(149)- **S**G**S**E**S**EADRVEGVAK**S**EAAQ**S**-169
GST-PER571-645	588-D**S**VMLGEISPHHDYYDS-604 606-**S**S**T**E**T**PP**S**YNQLNYNENLLR-625 626-FFN**S**KPVTAPAELDPPKTEPP-647
GST-PER1101-1224	1128-SSPIP**S**VMGDYNSDPPCS-1146 1210-IMEHPEEDQ**T**QHGDG-1224

First column indicates GST-PER proteins phosphorylated by DBT, numbers show the amino acids sequence in PER fused to GST. The second column indicates phosphopeptides recovered from each phosphorylated GST-PER fusion protein in respective rows (numbers indicate the position of each peptide relative to full-length PER protein sequence). Identified phosphorylation sites are underlined, except for the peptide from GST-PER143-365 where predicted sites are underlined. GST derived sequence in the N-terminus of this peptide is shown in italics. Each peptide was phosphorylated only once according to mass-spectometry. The sequencing results shown here likely represent phosphorylation in a heterogeneous pool of peptides.

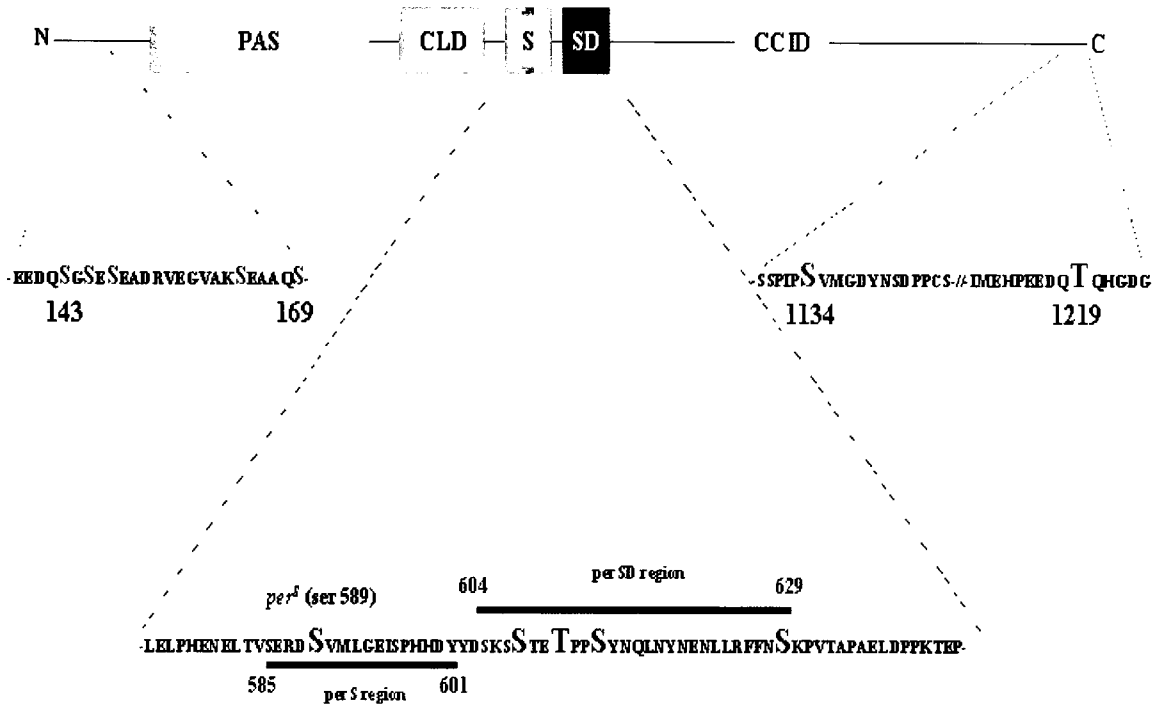


Figure 8. Distribution of phosphorylated regions in PER relative to previously known functional domains or motifs.

Colored blocks show functional domains found in PER. Three regions phosphorylated by DBT are shown as segments of PER protein sequence with phosphorylated residues indicated in red (serines between amino acids 143-169 and serine 604 are predicted but not shown to be phosphorylated). PAS indicates position of PAS domain (contains aa ~220-450) (Crews et al., 1988); CLD, cytoplasmic localization domain that promotes PER cytoplasmic localization in the absence of TIM (aa 452-512)(Saez and Young, 1996); S, per-short domain, causes short period behavioral rhythms when mutated (indicated as a green bar below the protein sequence, 585 and 601 indicate the first and last amino acid of the motif) (Baylies et al., 1992); SD, per-short downstream domain, contains many DBT *in vitro* phosphorylation sites (indicated by the purple bar above the protein sequence, 604 and 629 indicate the first and last amino acids of the motif)(this study); CCID, CLK-CYC inhibitory domain, represses CLK dependent transactivation when overexpressed in cultured cells (Chang and Reppert, 2003). (N and C indicate the amino- and carboxy-terminal ends of the schematic PER).

CHAPTER 4: ANALYSIS OF PERIOD PHOSPHORYLATION SITE MUTANTS IN CULTURED S2 CELLS

4.1 Background

The *in vitro* phosphorylation experiments described above revealed a number of DBT phosphorylation sites in PER. These phosphorylation sites were organized into three regions: up to 5 sites between residues 149-169 in the N-terminus, two sites in the C-terminus and 5 sites in the center of the protein within and next to the per-short domain. Most of the residues are localized to areas that have not been implicated before in the regulation of PER with the exception of serine 589 that shortens behavioral rhythms when mutated (Baylies et al., 1992; Konopka and Benzer, 1971; Rutila et al., 1992). To examine the biological relevance of the DBT target sites in PER, alanine mutagenesis of these residues in full-length PER was carried out and the mutant proteins were functionally tested for transcriptional repressor activity in S2 cells.

Schneider 2 (S2) cultured *Drosophila* cells offer an expression system that has been used successfully to study many features of the molecular clock. S2 cells do not express endogenously PER, TIM or CLK protein and thus the assays that measure the properties of these proteins can be set up without the concern of endogenous background activity. The CYC protein and the protein kinases implicated in clock control are expressed in these cells. Thus far the full circadian gene expression program has not been initiated in these cells but certain elements of the molecular clock cycle can still be analyzed. Previous studies have developed assays that measure PER and TIM

interdependent nuclear translocation as well as PER dependent repression of CLK transactivation of *per* gene promoter in S2 cells (Darlington et al., 1998; Saez and Young, 1996). Reduction of DBT kinase levels has also been reported to increase PER protein levels and reduce its transcriptional repressor activity in S2 cells (Nawathean and Rosbash, 2004). Thus S2 cells represent a suitable system to investigate the functional relevance of the *in vitro* DBT phosphorylation sites in PER.

4.2 Transcriptional repressor activity of PER phosphorylation site mutants in cultured S2 cells

Point mutations were generated in PER that mutated to alanine the serine and threonine residues that were found to be phosphorylated by DBT *in vitro*. Selected sites were mutated individually and a number of sites were mutated combinatorially to investigate possible interactions between different phosphorylation sites. The mutations were generated in the full-length *per* cDNA and cloned into an expression vector downstream of the actin gene promoter. This allows constitutive expression of PER. The mutant PER proteins were tested in a transcriptional repressor assay (Darlington et al., 1998). This assay measures CLK dependent activation of *per* gene promoter comparatively in the absence and presence of PER. A standard luciferase assay is employed to measure the effect of PER mutants on the CLK activity so that relative luciferase levels that are expressed under the control of *per* promoter report the CLK dependent activation of transcription.

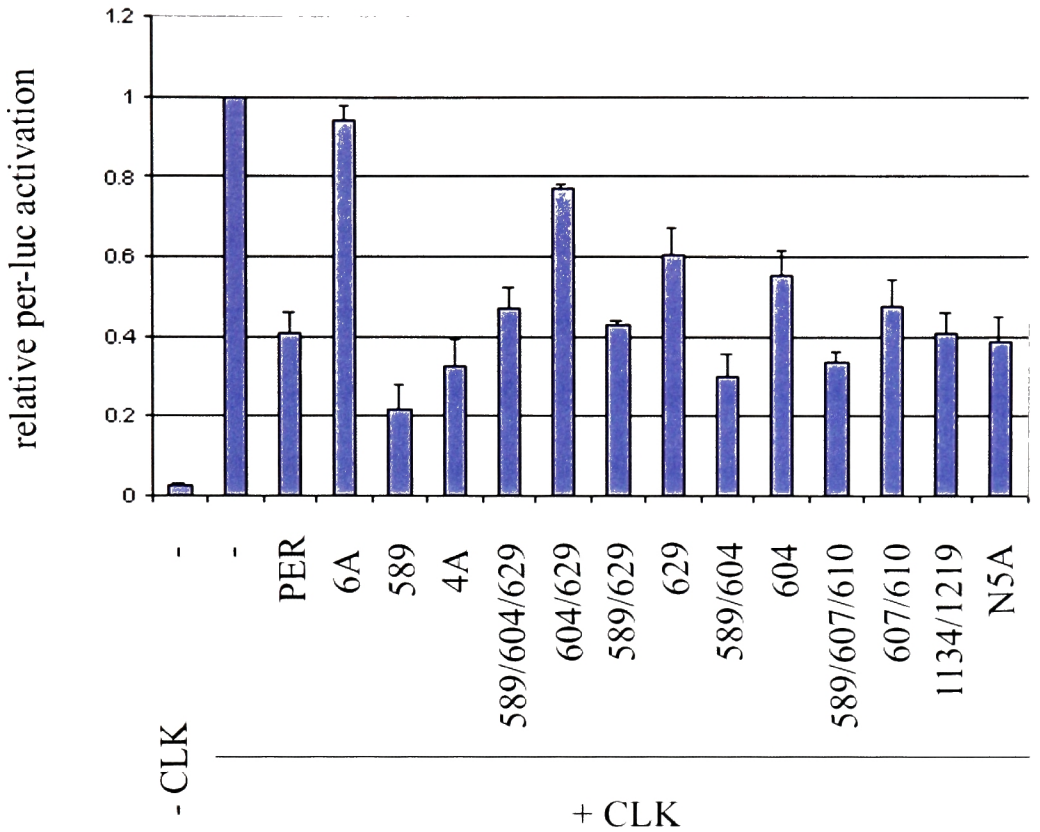


Figure 9. Repression of CLK dependent activation of per-luciferase reporter by PER phosphorylation site mutants.

Bar graphs indicate relative luciferase activity levels from S2 cells cotransfected with *Clk* and either wildtype or mutant *per* expression plasmids along with the per-luc reporter. Normalized luciferase activities were plotted as relative activation compared to no *per* expression (second bar). Values are averaged over three experiments; error bars indicate standard error of the mean. Numbers below the graphs indicate positions of serine or threonine residues in PER that were mutated to alanine (PER mutant 6A has amino acids 589, 604, 607, 610, 613, 629 mutated to alanine; PER mutant 4A has amino acids 589, 607, 610, 613 mutated to alanine; PER mutant N5A has amino acids 149, 151, 153, 164, 169 mutated to alanine). A significant difference in repression ($P<0.05$, t-test) was observed between wt PER and all the mutants except 589/629, 607/610, 589/607/610, N5A and 1134/1219 which showed no significant difference.

13 PER mutants were tested in the transcriptional repressor assay for which results are shown in Figure 9. C-terminal sites (Ser 1134 and Thr 1219) were mutated together in combination to alanine residues. Also the N-terminal candidate sites (Ser 149/151/153/164/169) were all mutated to alanine in one combinatorial point mutant. 11 mutations were generated that mutated residues in the perSD domain either alone or in combinations as shown in Figure 9. Serine 589 in the per-short domain was also mutated either alone or in combination with the residues in the perSD domain. All mutants were placed under the control of actin promoter and coexpressed with a CLK expressing plasmid along with the luciferase reporter. Strong activation of the luciferase reporter was observed by coexpressing CLK. This activation was repressed by 60% when wildtype PER was also coexpressed. The mutations in the N-terminal and C-terminal phosphorylation region had no significant effect on the ability of PER to repress CLK dependent transactivation (compare mutants N5A and 1134/1219 to wildtype PER in Figure 9). The serine 589, when mutated to alanine, enhanced the repressor activity of PER. In contrast all the mutations in the perSD domain reduced the repressor activity of PER. The inhibitory effect varied depending on the residues that were mutated. Strongest effects were seen in combinatorial mutations in the perSD domain, for example when residues 604 and 629 were mutated together in one construct (mutant 604/629 in Figure 9), stronger reduction in PER dependent repression was observed when compared to mutants that had both residues mutated individually (compare mutant 604/629 with 604 and 629 in Figure 9). Almost no repressor activity was exhibited by a combinatorial PER mutant with all the phosphorylation sites in perSD domain along with serines 589 and 604 mutated to alanine (mutant 6A in Figure 9). An antagonistic relationship between the

per-short domain serine 589 mutation to alanine and the perSD domain mutations was observed in combinatorial mutants. When the repressor activity of the mutants was measured that had the perSD residues mutated in combination with the serine 589 then the combinatorial mutants always showed intermediate activity when compared to serine 589 mutated alone or perSD domain residues mutated separately from serine 589 (compare mutant 589/604/629 to 604/629 and 589; 589/629 to 629 and 589; 589/604 to 604 and 589; 589/607/610 to 607/610 and 589 in Figure 9).

The functional analysis of mutated DBT *in vitro* phosphorylation sites presented here suggests that the residues in the per-short domain and perSD domain are relevant for PER dependent down regulation of CLK activity. The amino acids phosphorylated by DBT *in vitro* in the N- and C-terminus did not have an effect in this assay when mutated but they may be relevant for some other aspect of PER regulation that is not revealed in the S2 tissue culture cell model.

As most of the DBT *in vitro* phosphorylation sites are located in previously uncharacterized regions of PER no animal models are currently available for further study. An exception is the per-short domain for which *in vivo* mutants are available. The next set of experiments was carried out to investigate the role of the per-short domain into which one of the *in vitro* DBT phosphorylation sites, serine 589, maps.

CHAPTER 5: ANALYSIS OF *PERΔS* MUTANT *IN VIVO*

5.1 Background

Biochemical analysis of PER phosphorylation by DBT *in vitro* indicated that three regions in PER are preferentially targeted by the kinase. Two regions fall into the N-terminus between residues 149-164 and at the very C-terminus, marked by residues 1134 and 1219. Functionally these regions have not been examined in earlier studies of PER function. A third cluster of phosphorylation sites is located in the center of the protein including residues 589-629. Functional significance of residues 607, 610, 613 and 629 that were identified as phospho-acceptor sites for DBT *in vitro* had not been examined before. The experiments described above show that alanine mutagenesis of these residues decreased the transcriptional repressor function of PER whereas an opposite effect of enhanced repressor activity was observed with serine 589 mutated to alanine.

Serine 589 is mutated in a short 19-hr period mutant per^S indicating its functional significance in the clock mechanism (Baylies et al., 1987; Yu et al., 1987). The per-short domain is a 17 aa motif between residues 585-601 in PER that when mutated produces short period behavioral phenotypes (Baylies et al., 1992). Previous studies have examined the biochemical basis of the short period mutations by measuring circadian PER protein accumulation in two mutants of the per-short domain, per^S and per^T Western analysis suggests that these mutations affect the protein stability of PER in the later part of the circadian cycle when PER is primarily a nuclear protein (Fig. 10).

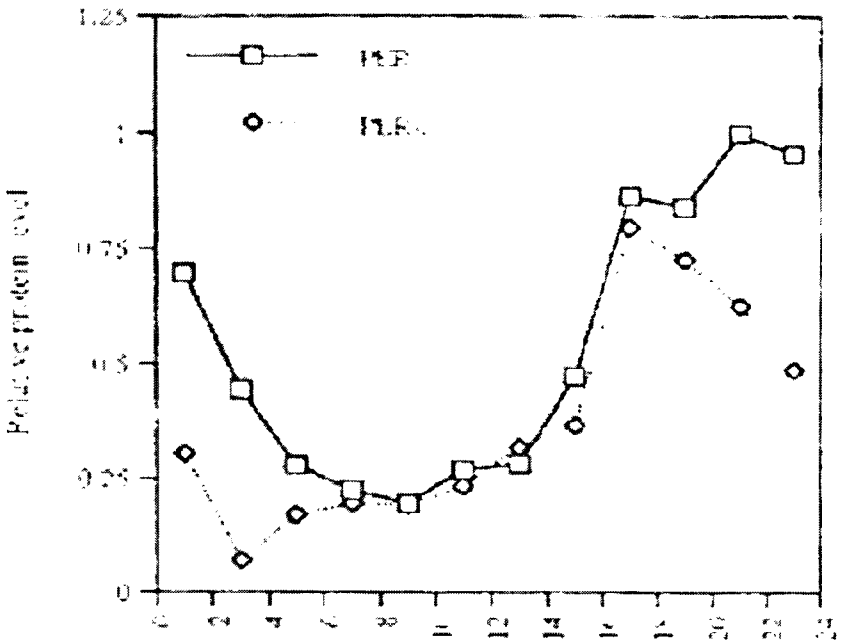


Figure 10. Early decrease of PER protein levels in the *per*^{S} mutant (from Marrus et al., 1996).

PER protein levels were measured through 24-hr cycle in LD. Solid line curve (PER) indicates PER levels in wildtype flies. Dashed curve (PER-S) indicates PER levels in the *per*^{S} mutant. Vertical axis shows relative PER levels measured by Western blot; peak levels in wildtype are set to 1. Horizontal axis shows hours in the 24-hr cycle when protein levels were measured (0-12 hrs lights on, 12-24 hrs lights off).

Note the premature decrease of PER protein abundance in the *per*^{S} mutant at the end of the 24-hr cycle when PER is primarily in the nucleus (Curtin et al., 1995).

In both mutants PER protein levels decline prematurely during the night, more prominently in the phenotypically "shorter" per^T mutant (Hamblen et al., 1998; Marrus et al., 1996). The premature decline in PER levels is thought to relieve its repressive activity on CLK dependent transcription and thus allow earlier start of the next cycle leading to a clock with a faster period length.

Previous studies have linked the per-short domain with DBT dependent regulation of PER. per^S and per^T mutations specifically rescue rhythms in a hypomorphic dbt^{AR} mutant background that cannot sustain functional clock on its own (Rothenfluh et al., 2000). The genetic interaction of per^S and per^T with dbt^{AR} suggests that the per-short domain inhibits DBT dependent phosphorylation of PER.

Functionally DBT has been shown to affect all aspects of PER regulation. Loss of DBT activity leads to an overaccumulation of PER indicating a role in protein stability regulation. DBT also regulates the transcriptional repressor activity of PER. Reduction of DBT levels by RNAi in S2 cells reduces PER dependent repression of CLK-CYC activity while leading to increased levels of PER protein (Nawathean and Rosbash, 2004). In addition to protein stability and transcriptional repressor activity DBT regulates the nuclear entry timing of PER. A short period allele dbt^S delays nuclear translocation of PER by an unknown mechanism (Bao et al., 2001). It is possible that these different aspects of PER regulation are each modulated by DBT-dependent phosphorylation independently by distinct phosphorylated regions. Alternatively, different aspects of PER regulation may all be affected by one set of phosphorylation sites which regulates PER repressor activity, protein stability and nuclear translocation in a more integrated fashion.

In this chapter molecular and genetic analysis of flies was carried out that express a mutant PER protein with the 17 amino acids long per-short domain deleted and thus also lacking serine 589, one of the identified *in vitro* DBT target sites. Since the per-short region has been suggested to regulate PER protein stability, it was hypothesized that elevated expression of PER lacking the per-short domain should suppress the short period behavioral phenotype observed in per^S and per^T. A transgenic line was identified that carried two transgenes expressing a PER mutant with the entire per-short domain deleted, termed perΔS. This *per* mutant was favored over the individual short period point mutants in per-short domain since transgenes expressing PER with the deletion display stronger locomotor activity period shortening phenotypes than transgenes carrying the per^S point mutation.

5.2 *perΔS* transgenes dominantly dosage dependently shorten the period of locomotor rhythms

Transgenic fly lines were created by genetic recombination that carried one or two *perΔS* transgenes or were heterozygous for one transgene. The *perΔS* transgenes contained the entire genomic locus of *per* with a 51 nt in-frame deletion removing the per-short domain coding sequence of amino acids 585-601 (Vosshall and Young, unpublished). The transgenes were located on chromosome 3 and were placed into wildtype or *per* null background. Six lines were tested in locomotor activity assay in constant darkness to determine the period length of their locomotor activity rhythms.

Table 2 shows the results from the locomotor activity assays. A progressively shortening period length reaching arrhythmia was observed as the number of *perΔS* transgenes was increased. Animals with two *perΔS* transgenes in *per* null background (Table 1, A) showed no appreciable rhythms in their locomotor activity whereas in wildtype background (Table 1, D) 30% of the flies with two *perΔS* transgenes had retained rhythmicity with a widely variable short period of 18-20 hrs, with a mean period of 19.2 hrs. Flies either homozygous (Table 1, B) or heterozygous (Table 1, C) for one *perΔS* transgene showed respectively 17.9-hr or 20-hr activity rhythms in *per* null background. These phenotypes were suppressed in wildtype background to 21.4-hr and 22.7-hr period lengths, respectively (Table 1, E and F). The results obtained in this analysis were contrary to the hypothesized suppression of short period phenotypes by overexpression of PER without the per-short region. If the per-short domain solely affected protein stability, progressively longer periods would have been expected, as more *perΔS* expression would have compensated for the instability of the mutant PER. A simple notion of premature PER degradation in the nucleus being responsible for short rhythms appears to be an insufficient explanation to describe the effects of the short period PER mutants. A possible explanation is that the per-short domain also regulates other aspects of PER regulation in addition to protein stability.

Table 2. *perΔS* transgenes dominantly dosage dependently shorten the period length of locomotor activity rhythms.

Genotype		tau ± S.D.	n	#AR (%)
A, *per*^{0}; P[*perΔS*]x2	(2 transgenes)		32	32 (100)
B, *per*^{0}; P[*perΔS*]	(1 transgene)	17.9 ± 0.3	24	1 (4)
C, *per*^{0}; P[*perΔS*]/+	(1 transgene heteroz.)	20.0 ± 0.2	25	
D, *per*$^{+}$; P[*perΔS*]x2	(2 transgenes)	19.2 ± 1.4	20	14 (70)
E, *per*$^{+}$; P[*perΔS*]	(1 transgene)	21.4 ± 0.2	26	
F, *per*$^{+}$; P[*perΔS*]/+	(1 transgene heteroz.)	22.7 ± 0.2	20	

Transgenic flies homozygous for one or two *perΔS* transgenes or heterozygous for one *perΔS* transgene were tested in locomotor activity assays in DD. The average period of locomotor activity rhythms (tau) of each genotype is indicated (S.D., standard deviation; n, total number of individuals tested; #AR(%), number of arrhythmic animals (percentage of total flies tested), P[*perΔS*]x2 indicates the presence of two *perΔS* transgenes). The effect of the transgenics was tested in *per* deficient (*per*^{0}) (A-C) and in wild type background (D-F). Note for comparison, wildtype flies exhibit locomotor activity rhythms period lengths of 23.5 ± 0.1 hrs.

5.3. Expression of clock gene mRNAs in arrhythmic and short period *perΔS* transgenics

To understand the basis of the behavioral phenotypes of the *perΔS*-expressing flies a molecular analysis of clock gene mRNA expression was carried out. Transgenic lines homozygous for one or two *perΔS* transgenes in a *per* null background were selected for analysis of mRNA expression of rhythmically expressed clock genes.

Animals were entrained in 24 hr light-dark (LD) cycles (12 hrs light – 12 hrs darkness) for 5 days and then transferred to constant darkness (DD). Total RNA from adult fly heads was isolated every 4 hours over the last entrainment day and the first day in constant darkness. Expression of *Clock*, *timeless*, *vrille* and *pdp1*ε mRNAs was measured by Northern blot. *per* expression was monitored by RNase protection with a probe spanning the per-short domain allowing independent measurement of mRNA expression from the endogenous *per* gene and the *perΔS* transgenes.

Quantitation of two independent time course experiments is shown in Figure 12. Representative mRNA expression profiles from one of the time courses from wildtype and transgenic animals are shown in Figure 11. The peak levels of expression of all the circadian genes monitored, were down regulated in the transgenic animals. The extent of the down regulation and cycling of mRNAs varied depending on the number of *perΔS* transgenes present and was also influenced by light. Stronger effects were observed in animals with two transgenes consistent with loss of behavioral rhythms in these animals.

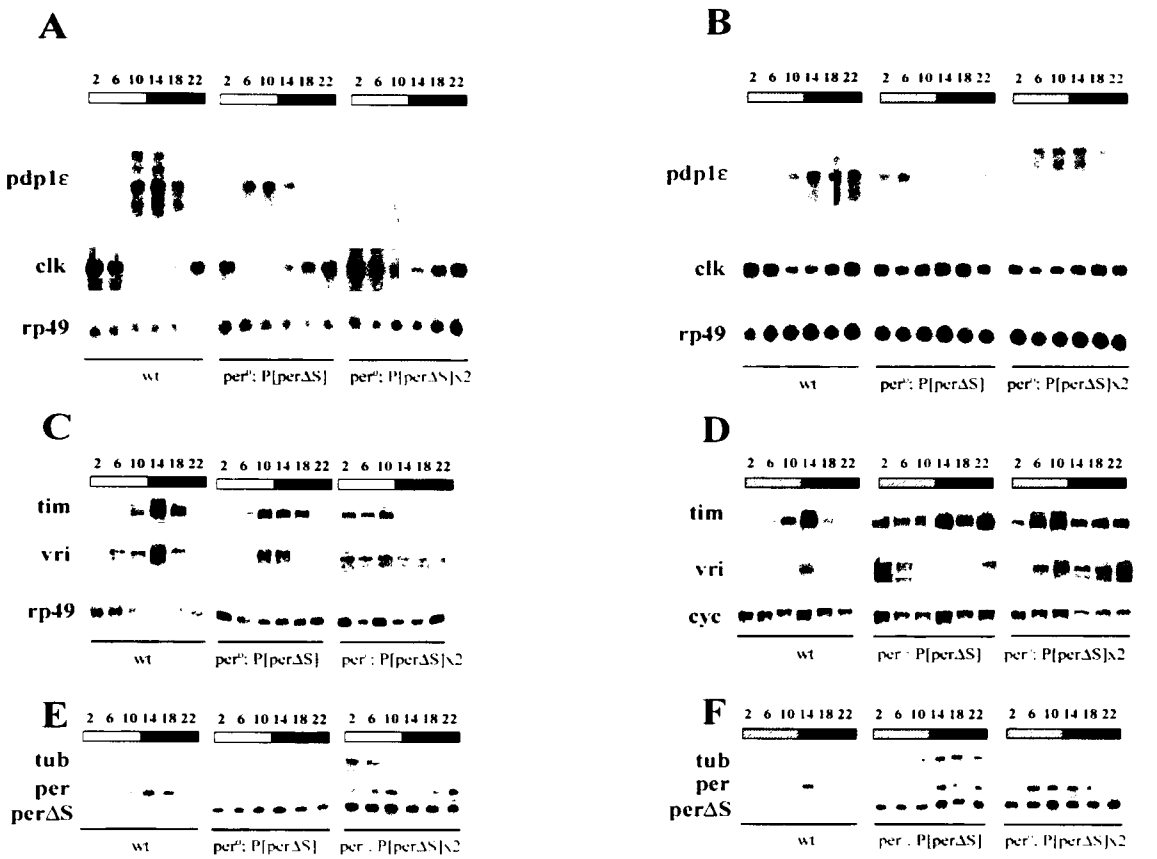


Figure 11. mRNA expression of cycling clock genes in flies homozygous for one or two *perΔS* transgenes.

One day in LD conditions (indicated by altering open and closed horizontal bars in panels A, C and E) and first day in DD (subjective day indicated by hatched bars, in panels B, D and F) were assayed. Genes indicated by their abbreviated names left of their respective Northern blots were assayed from three genotypes shown at the bottom of each panel (wildtype, left column; one *perΔS* transgene, middle column; two *perΔS* transgenes, right column in each panel). Numbers indicate hours in a 24-hr cycle when RNA was collected. In E and F RNase protection assay results are shown for *per* and *perΔS*. Top row of bands in E and F represents protected fragment from tubulin (tub) mRNA, below the top row of bands represents protected fragment from endogenous *per* gene mRNA, bottom row of bands in *perΔS* containing genotypes corresponds to a protected fragment from transgenic *perΔS*. Constitutive tubulin, rp49 and *cyc* mRNAs were measured for normalization.

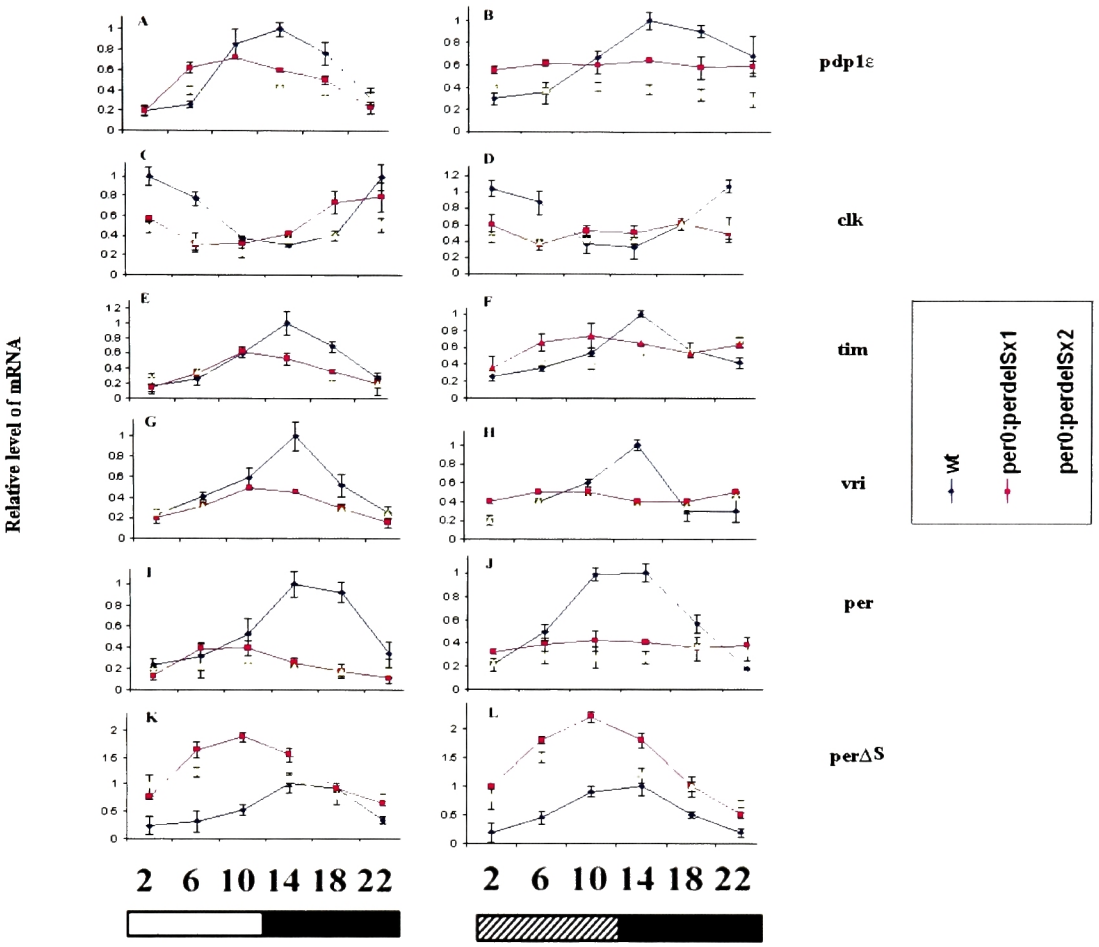


Figure 12. Quantitation of clock gene mRNA expression from two independent time course experiments.

mRNA expression of cycling genes making up the clock mechanism in flies was measured by Northern blot or RNase protection from two time course experiments (one of which is shown on Fig 10). An average of the normalized values was obtained and blotted. Genes assayed are indicated at the right to their respective graphs. Graphs in the left column are from time courses in LD (indicated by altering open and closed horizontal bars), right side graphs are from DD time courses (subjective day indicated by a hatched bar). Numbers on the vertical axis indicate relative expression (maximal expression in wildtype is 1). Numbers below the columns indicate hours in a 24-hr cycle when RNA was collected. Blue curves indicate wildtype time courses; pink curves indicate time courses from flies homozygous for one *perΔS;* yellow curves indicate time courses from flies homozygous for two *perΔS* transgenes. Error bars show standard error of the mean.

First, mRNA expression levels were measured in light-dark cycles. In wildtype flies the expression of *per*, *tim*, *vri* and *pdp1* genes peaks during early night at ZT 14 (see wildtype in Fig. 12, I, E, G, A, respectively). In contrast, expression of these genes in flies with one *perΔS* transgene showed an advanced phase with peak expression time in late day at ZT 10 (Fig. 12, panels I, E, G, A). The peak expression levels were lower leading to weaker amplitude oscillations in mRNA abundance. Corresponding alterations were observed in the *Clk* mRNA accumulation profile. In wildtype flies *Clk* gene expression oscillates in antiphase to *per*, *tim*, *vri* and *pdp1ε*. The phase relationship is maintained in one *perΔS*-transgene flies with peak *Clk* expression shifted to ZT22 from its normal early morning maximum (compare wildtype vs one-transgene flies in Fig. 12, C). Further reduction of expression levels was observed in animals containing two *perΔS* transgenes. Overall mRNA levels of *per*, *tim*, *vri* and *pdp1ε* were similar to trough levels observed at dawn and early morning in wildtype flies when maximal repression of these genes is detected (two-transgene curve in Fig. 12 I, E, G, A, respectively). Consistent with the lack of behavioral rhythms no cycling was observed in the expression of *per* and *pdp1ε* mRNAs. A very low amplitude oscillation was detected in *tim* and *vri* mRNA expression with a peak at ZT 6 which is even further advanced when compared to ZT 10 peak in animals with one *perΔS* transgene (Fig. 12 E, G). *Clk* transcript in flies with two transgenes was cycling in LD at low levels with a shallow broad peak from mid to late night (Fig. 12 C). In constant darkness cycling expression of endogenous clock gene transcripts had much lower amplitude in flies with one *perΔS* transgene.

An unexpected qualitative change of *pdp1ε* mRNA pattern was observed in two *perΔS*-transgene animals suggesting change in splicing pattern of the at least five transcripts that were detected (compare right and left panels in Fig. 11 A and B).

Expression levels of the mutant *perΔS* transcript were also measured. *perΔS* mRNA showed oscillatory expression in both animals with one and two *perΔS* transgenes with overall high level of expression (Fig. 12 K, L). The observed increase in mRNA expression from the transgenic *perΔS* genes on the chromosome 3 is probably caused by the lack of dosage compensation that regulates the endogenous *per* gene on chromosome X. Trough levels of *perΔS* were comparable to peak expression of endogenous *per* mRNA in wildtype flies (Fig. 12 K, L).

5.4 PERΔS and TIM protein expression in arrhythmic and short period *perΔS* transgenics

PER protein functions in the *Drosophila* clock as a transcriptional repressor down regulating the expression of genes activated by CLK-CYC heterodimers. The expression analysis of *per*, *tim*, *vri* and *pdp1* genes that are normally activated by CLK-CYC heterodimers, suggested that PERΔS strongly repressed the expression of these genes. *perΔS* transcripts were shown to be elevated both in one-transgene and two-transgene line. To test whether elevated PERΔS protein expression is associated with the enhanced repression of CLK-CYC target genes, anti-PERΔS Western blots were performed from flies with

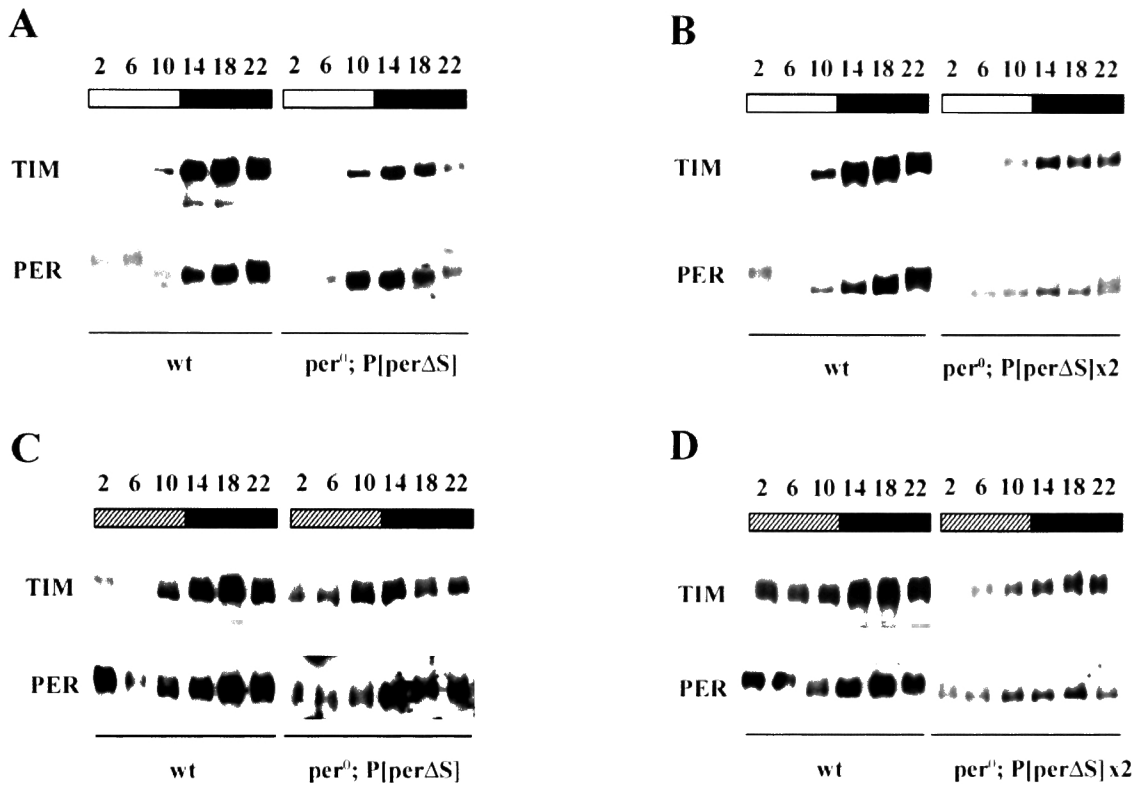


Figure 13. TIM and PERΔS protein expression in flies with one or two *perΔS* transgenes.

Last day in light-dark entrainment (indicated by alternating open and closed horizontal bars in panels A and B) and first day in constant darkness (panels C and D, subjective day indicated by hatched bars) were assayed. Numbers indicate hrs in a 24-hr cycle. Genotypes assayed are indicated at the bottom of each panel (wt, wildtype; P[perΔS]x2 indicates presence of two *perΔS* transgenes, P[perΔS] indicates the presence of one *perΔS* transgene). TIM protein signal is shown as indicated (top of each panel). PER protein was detected in wildtype extracts and only PERΔS was detected in transgenics due to *per* null background (bottom part in each panel).

one or two *perΔS* transgenes in *per* null background. TIM protein levels were also measured from these flies.

In flies with one *perΔS* transgene both TIM and PERΔS proteins were expressed rhythmically in LD cycles and also in DD. TIM levels were reduced compared to wildtype flies both in LD cycles and DD (Fig. 13 A, C). This was expected as *tim* mRNA expression was repressed in these flies. The protein accumulation profile of TIM was altered when compared to wildtype animals. The protein peak for TIM was advanced by 4 hours from ZT/CT 18 to ZT/CT 14 (Fig. 13 A, C).

PERΔS peak levels were likewise advanced by 4 hours compared to PER in wildtype flies (Fig. 13 A, C). The total PERΔS levels were not significantly increased however when compared to PER levels in wildtype flies. This was unexpected since a high level of *perΔS* mRNA expression was found in these flies. The lack of protein overexpression in the presence of elevated mRNA levels suggests lower stability for the PERΔS protein.

In the two *perΔS*-transgene lines TIM protein levels were further reduced compared to the one-transgene flies both in LD cycles and in DD (Fig. 13 B, D). The protein accumulation is still rhythmic which is unexpected given the behavioral arrhythmicity displayed by these animals in locomotor activity assays. Remarkably, the protein mobility reflecting the phosphorylation state was dramatically altered compared to wildtype and the one *perΔS*-transgene flies (Fig. 13 B, D). In wildtype flies, in addition to rhythmic changes in abundance, both PER and TIM proteins also show progressively slower protein mobility (e.g. Fig. 13 B in wt), which is contributed to protein phosphorylation (Edery et al., 1994; Zeng et al., 1996). Both proteins are

hypophosphorylated at daytime but get progressively phosphorylated when translocated to the nucleus in the night. The phosphorylation is reflected in progressively slower mobility in protein gels. In flies with two *perΔS* transgenes TIM was found to be constitutively migrating in the low mobility state suggesting constitutive hyperphosphorylation (Fig. 13 B, D). Lack of hypophosphorylated high mobility TIM species is observed in both light-dark conditions and in constant darkness. This observation would support a hypothesis that constitutive repression of clock gene mRNA expression might be caused by constitutive nuclear translocation of PERΔS in flies with two *perΔS* transgenes since TIM phosphorylation is associated with nuclear localization of the TIM-PER heterodimer.

Equally striking effects are observed for PERΔS protein profile in the arrhythmic two *perΔS* transgene flies. PERΔS protein in these flies is also cycling in abundance but the overall protein levels are dramatically reduced both in LD cycles and in DD (Fig. 13 B, D). Almost no hyperphosphorylated forms of PERΔS can be detected while mRNA analysis showed elevated levels of expression. This suggests that the PERΔS protein may be very rapidly phosphorylated and turned over.

5.5 Suppression of arrhythmic behavioral phenotype associated with *perΔS* overexpression by reduction of DBT activity or overexpression of SGG kinase inhibitor Dishevelled.

Several kinases have been identified that when mutated can affect the circadian phosphorylation profiles of PER and TIM. DBT and CK 2 can phosphorylate PER while TIM is the proposed target for Shaggy (SGG) (Lin et al., 2002; Martinek et al., 2001). To test whether PER and TIM hyperphosphorylation is relevant for the arrhythmia observed in flies homozygous for two *perΔS* transgenes the activities of their respective kinases DBT and SGG were separately genetically down regulated in the arrhythmic line with two *perΔS* -transgenes.

In order to reduce DBT activity, the wildtype *dbt* allele was replaced with the strongly hypomorphic dbt^{AR} allele by genetic recombination. The DBT^{AR} protein has reduced kinase activity *in vitro* (Figure 5, lane 1) and *in vivo* causes over accumulation of PER similarly to *dbt* loss-of-function (Rothenfluh et al., 2000). To reduce SGG activity, Dishevelled (DSH) protein was overexpressed in clock containing cells using the UAS-GAL4 two-component expression system. In Wnt/wg signaling pathway SGG phosphorylates β-catenin in a multiprotein "degradation complex" to mark it for proteosomal degradation (Moon et al., 2004). Upon wingless signaling, DSH is recruited to this protein complex resulting in the inhibition of SGG dependent phosphorylation of β-catenin (Wharton, 2003). Overexpression of DSH lengthens locomotor activity period rhythms in flies (Lino Saez, unpublished data), similarly to reduced SGG levels (Martinek et al., 2001). This however is specific to overexpression conditions. *dsh* loss-

of-function appears to have no effect on molecular rhythms of clock proteins suggesting that *dsh* is not part of the circadian clock (Lino Saez, unpublished data). Overexpression of *dsh* is used here as a tool to manipulate SGG activity.

Locomotor activity measured from flies containing the recombinant chromosomes that have been manipulated to reduce either DBT or SGG activity in the background of two *perΔS* transgene is shown in Table 3. As stated earlier flies that are homozygous for two *perΔS* transgenes are arrhythmic in their daily activity (Table 3, A). However when the weak kinase activity allele of dbt^{AR} is introduced into this genetic background over 80% of the animals regained rhythms in their locomotor activity indicating DBT dependent phosphorylation contributed to the PERΔS dependent loss of behavioral rhythms (Table 3, B). The rescued rhythms in the reduced DBT activity background had short period length around 18 hrs. Period lengths of individual flies varied from 16.5-18.8 hrs as indicated by the relatively large standard deviation (Table 3, B). Overexpression of *dsh* also rescued locomotor activity rhythms in otherwise arrhythmic background of two *perΔS* transgenes. The rescue was somewhat less efficient than with dbt^{AR}, leading to the average rhythms of 16 hrs in 80% of *dsh* overexpressors. Individual rhythms varied between 15.5-17.5 hrs. These data suggest that SGG activity may also contribute to the arrhythmia seen in the homozygous background of two *perΔS* transgenes. It is plausible that SGG activity may contribute to the constitutive phosphorylation of TIM observed in arrhythmic two-transgene flies.

Table 3. *dbt*^{AR} or *dishevelled* overexpression rescues behavioral rhythms in the homozygous two-*perΔS* transgenics.

Genotype	tau ± S.D.	n	#AR (%)
A, *per0*; P[*perΔS*]x2		15	15 (100)
B, *per0*; P[*perΔS*]x2 *dbt*^{AR}	18.1 ± 1.1	31	5 (18)
C, *per0*; *tim*(UAS)*Gal4*: UAS*dsh* P[*perΔS*]x2	16.0 ± 0.6	15	3 (20)

The hypomorphic *dbt*^{AR} allele was placed into the homozygous background of two *perΔS* transgenes by genetic recombination. DSH protein was overexpressed in clock containing cells using *dsh* transgene downstream yeast transcription factor Gal4 binding sites (UAS*dsh*) (Penton et al., 2002), GAL4 was expressed under the control of *tim* promotor (Blau and Young, 1999). The average period of locomotor activity rhythms (tau) of each genotype is indicated (S.D., standard deviation; n, total number of individuals tested; #AR (%), number of arrhythmic animals, percentage of total; P[*perΔS*]x2 indicates the presence of two *perΔS* transgenes).

CHAPTER 6: DISCUSSION

Initial studies of *dbt* mutations suggested that PER is the primary target of DBT in the molecular clock. Loss of *dbt* expression led to hypophosphorylation and over accumulation of PER *in vivo* (Price et al., 1998). Mutations of *dbt* that shorten or lengthen behavioral rhythms led to correspondingly earlier or later degradation of PER in fly heads (Price et al., 1998). The primary sequence of *dbt* suggested that DBT is a molecule similar to the casein kinase 1 family of protein kinases in mammals (Kloss et al., 1998). Until shown here, direct evidence for DBT kinase activity and PER phosphorylation was not available due to the absence of specific enzymatic activity in bacterially produced recombinant DBT preparations. Intriguingly, the highly homologous mammalian casein kinase 1ε (86% primary sequence identity in the kinase domain) is active in bacterial expression systems. It is possible that the DBT protein folding is more sensitive to its environment compared to the mammalian enzyme. Alternatively DBT may require a posttranslational modification for active conformation that is absent in bacterial cells. As shown in Chapter 3 of this thesis, recombinant DBT expressed in insect cells is enzymatically active when purified as a GST-fusion protein and when released from the GST protein into solution. This activity is short-lived however which may reflect the sensitive nature of its protein folding. Experiments described in the first part of Chapter 3 show DBT activity against casein and the proposed physiological substrate PER. Both proteins are successfully phosphorylated by DBT. Mutations of *dbt* that are known to affect the behavioral rhythm also have an effect on the basic kinase activity of DBT. The periods of locomotor activity and eclosion rhythms are shortened or

lengthened by dbt^S or dbt^L mutations, respectively (Price et al., 1998). Biochemically both mutations reduce the kinase activity of DBT or Xenopus CK 1ε when casein is used as a substrate (this study, (Preuss et al., 2004) The lack of correlation between behavioral phenotypes and the biochemical properties of the DBT/CK 1ε mutants is currently unexplained. It is possible that a number of DBT targets exist either in PER, as suggested by *in vitro* phosphorylation experiments described here, or in other proteins important for clock function. They may have different regulatory functions and could be phosphorylated at different times of day leading to opposing effects in the progression of the molecular cycle. It is conceivable that phosphorylation of different DBT sites are differentially affected by the dbt^S and dbt^L mutations.

Three distinct regions in PER are targeted by DBT *in vitro*. In the N-terminus of PER an interval of 20 amino acids between residues 149-169 contains 5 serines that are candidate phosphorylation sites for DBT. This region is located N-terminal to the PAS domain in PER. There are no mutations in *per* that affect this region and no function has been attributed to this sequence. DBT binding *in vitro* has been localized to the first 300 amino acids of PER which would include the phosphorylated region (Kloss et al., 1998; Preuss et al., 2004). The relationship, if any, between DBT binding to the N-terminus of PER and the identified phosphorylation region is not known.

In the C-terminal phosphorylation region the two identified DBT phosphorylation sites, serine 1134 and threonine 1219, localize also to a region that has not been characterized functionally before.

The phosphorylation sites identified in the third phosphorylation region in the middle of PER between residues 580-645 however localize to a domain that has been

previously shown to affect behavioral rhythms. The first circadian clock mutant per^S was identified as a point mutation at serine 589, shown here to be an *in vitro* substrate for DBT (Baylies et al., 1987). The original mutation changes serine 589 to a glutamine residue. This mutation leads to period shortening to 19 hrs in locomotor activity assays (Konopka and Benzer, 1971). Biochemically this mutation leads to premature degradation of PER protein in the nucleus similarly to the dbt^S mutation, which has a comparable 18-hr behavioral phenotype. The similarity of per^S and dbt^S associated phenotypes makes it tempting to consider a similar mechanism for the basis of the short period phenotype produced by these mutations. The per^S mutation eliminates a candidate phosphorylation site in PER that could be a signal that prevents PER degradation and perhaps the dbt^S mutation selectively reduces the kinase activity towards serine 589. Considering the degradation pattern of PER throughout the circadian cycle a testable prediction would be that serine 589 is phosphorylated in the accumulation phase of the PER protein in the early night. This hypothesis could be tested once phosphorylation state-specific antibodies have been developed for this residue. A targeted random mutagenesis of the sequence surrounding the per^S site revealed a 17 amino acid region between residues 585-601, called per-short domain, that when mutated at almost any position yields short period behavioral phenotypes (Baylies et al., 1992). The broad distribution of short period phenotypes in this region suggests a structural role in determining the pace of the molecular clock. Localization of a DBT target site into this region suggests that the function of this motif may be regulated in a phosphorylation dependent manner.

The distributed nature of short period mutants in this region that involve also residues which cannot be targets for protein kinases raises an alternative possibility of PER regulation by phosphorylation in this motif. The phosphorylation of serine 589 could instead of stabilizing PER induce instability. It is conceivable that the per-short domain represses PER destabilization by phosphorylation and the point mutations in this region that shorten behavior rhythms mimic the effect of phosphorylation at serine 589. According to this hypothesis the phosphorylation would have a structural role changing the conformation of the region that makes PER susceptible for further phosphorylation leading to subsequent degradation or altering its properties as a transcriptional repressor. The timing of phosphorylation at serine 589 in this case would be different from the "stabilization" model. The phosphorylation of PER at serine 589 should correlate with hyperphopshorylated PER in the late night and early morning hours when overall PER levels start to decline.

The remaining phosphorylation sites identified in this region map right downstream of the per-short domain, hereby called perSD (*perS* *D*ownstream) domain. The function of these residues has not been studied before. The arrangement of 4 of the downstream sites (serines 604, 607, 610, 613) resembles the CK 1 phosphorylation site motif in human PER2 protein that has been implicated in the familial advanced sleep phase syndrome (FASPS) when mutated. In human PER2 protein there are 5 serines spaced in a CK 1 consensus sequence like arrangement where every third residue is a serine. These 5 serines are thought to be progressively phosphorylated by CK 1ε starting with the first one that initiates phosphorylation of the next downstream serine until all 5 serines are phosphorylated. In FASPS patients the first, priming serine is mutated to

glycine (Toh et al., 2001). This mutation is considered to inhibit the phosphorylation of the remaining serines and thus lead to altered hPER2 levels or activity. A similar mode of phosphorylation could be envisioned also for *Drosophila* PER. The mass-spectrometry data however supports distributive phosphorylation rather than progressive, since multiply phosphorylated peptides within the perSD domain were not observed and single site phosphorylation was observed in all three of the positions that were examined (Table 1). However, the mass-spectometry data does not indicate the absence of multiply phosphorylated peptides, as recovery of phosphopeptides in the mass-spectometer is poorly predictable. In conclusion, the *in vitro* phosphorylation experiments showed direct phosphorylation of PER by DBT and the localization of phosphorylation sites suggested possible phosphorylation dependent regulation of a centrally positioned regulatory motif previously implicated in the regulation behavioral rhythms.

Functionally PER is a transcriptional repressor that inhibits CLK-CYC dependent gene activation (Darlington et al., 1998). As mentioned above, with the exception of serine 589 that is mutated in the short period mutant per^{S}, the DBT phosphorylation sites identified *in vitro*, do not map into any previously characterized region in PER. To assess the functional significance of the phosphorylation sites, the identified phospho-acceptor amino acids were mutated to alanines in the full-length PER protein and tested in a previously established cell culture based PER transcriptional repressor activity assay (Darlington et al., 1998). Mutagenesis of phosphorylation sites in the N- and C-terminal phosphorylation regions did not have any significant effect of PER transcriptional repressor activity (Fig. 9). However when mutated in the per-short domain and in perSD domain, the repressor activity of PER was affected. A point mutation that changed the

serine 589 to an alanine enhanced PER repressor activity. In contrast, all mutations in the perSD domain sites reduced PER ability to repress CLK dependent transactivation. The effect of the perSD domain mutations varied in strength depending on the position mutated. A stronger reduction of PER activity was observed when multiple mutations were combined in the perSD domain. Combinatorial point mutants with serine 589 mutated along with one or more residues in the perSD domain showed intermediate phenotype when compared to mutants that had either only serine 589 or only perSD domain sites mutated. The transcriptional repressor assay results suggest functionally opposing roles for the per-short and perSD domains. Protein-protein interactions may direct which sequence motif determines the behavior of PER protein at particular time of day. For example serine 589 phosphorylation may correlate with TIM bound stable PER associated with low transcriptional repression. An alternative and perhaps a more interesting model could be postulated that proposes cooperation between the two sequence motifs (Fig. 14). As discussed above, the per-short domain may act as a structural motif that inhibits phosphorylation of the perSD domain sites by DBT. When the per-short domain is phosphorylated, its protective function is lost and downstream sites in the perSD domain would become phosphorylated. This in turn would enhance the transcriptional repressor activity of PER. In this model again short period mutations in the per-short region would functionally mimic its phosphorylated state.

In previous studies per-short domain mutations have not been examined in association with transcriptional repressor activity. Cell culture experiments shown here suggested that the per-short region mutations may also affect the transcriptional repressor activity in addition to earlier reported protein stability regulation (Hamblen et al., 1998;

Marrus et al., 1996). To test if the short period *per* mutants specifically affect PER protein stability or also its repressor activity, overexpression of PER with defective per-short domain was carried out *in vivo*. It was hypothesized that overexpression of destabilized per-short domain defective PER should rescue the short period length behavioral phenotypes in flies as higher expression level would compensate for decreased protein stability. Transgenic fly strains were analyzed behaviorally that expressed a mutant PER with the per-short domain deleted (PERΔS) either from one or two transgenes (Table 2). The rhythms were progressively shortened as the *perΔS* dose was increased. In the most extreme case, flies homozygous for two *perΔS* transgenes in *per* null background had become arrhythmic. The strong dosage dependent shortening has not been observed with multiple copies of wildtype *per* transgenes or duplications of *per* locus. Earlier studies have found that the addition of extra copies of wildtype *per* genes has a limited effect. One additional copy of wildtype *per* shortens locomotor activity rhythms by an hour, a second copy shortens the rhythm by an additional 30 minutes but period shortening beyond the total 1,5 hrs is not observed when three or more per genes are present (Ashmore et al., 2003; Smith and Konopka, 1982)

Molecularly at the mRNA expression level, increasing dose of PERΔS expression led to progressively lower level expression of cycling clock genes (Fig. 12) that was accompanied by dampening of transcript cycling and advancement of peak phases of expression. The mRNA expression data suggests that PERΔS represses clock gene expression more efficiently than wt PER. Animals with one *perΔS* transgene showed no recognizable cycling in clock gene expression in DD. In LD conditions however, these animals retained cycling clock gene expression suggesting that light could counteract the

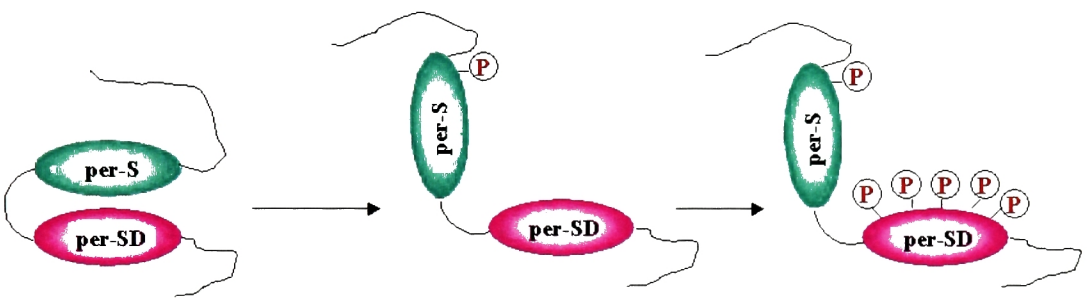
Figure 14. Model of the putative function of *in vitro* identified DBT phosphorylation sites in the per-short and perSD domains in *Drosophila* PER.

A schematic of Drosophila PER protein is shown with the per-short (per-S) and per-short downstream (perSD) domains highlighted. Phosphates incorporated by protein phoisphorylation are indicated with circled Ps.

Based on the observations of enhanced transcriptional repression of clock genes in PERΔS expressing flies (Fig. 12) and reduced transcriptional repression by DBT phosphorylation site mutants in the perSD domain in cultured cells (Fig. 9) it is proposed that PER phosphorylation takes place in two regulatory steps leading to unstable hyperphosphorylated PER that carries out the transcriptional repressor function. In this model per-short and perSD domains have opposite regulatory functions regarding PER activity as a transcriptional repressor and its protein stability. In the unphosphorylated state the per-short domain inhibits phosphorylation of the perSD domain, which results in stable PER protein with low transcriptional repressor activity. In the intermediate phosphorylation state serine 589 in the per-short domain is phosphorylated and this leads to change in conformation or protein-protein interaction of the per-short domain. The phosphorylation of per-short domain allows subsequent multisite phosphorylation in the perSD domain that is required for PER transcriptional repressor activity and also results in rapid degradation of PER.

INACTIVE REPRESSOR

ACTIVE REPRESSOR



HYPOPHOSPHORYLATED PER

- low transcriptional repressor activity
- stable protein

HYPERPHOSPHORYLATED PER

- high transcriptional repressor activity
- unstable protein

repressive activity of *perΔS*. In LD, the investigated mRNAs showed dampened amplitude cycling with a 4 hrs advanced phase of peak accumulation. This is similar to previously observed light effects of per^S mRNA profile (Marrus et al., 1996). Behaviorally per^S mutant exhibits short 19-hr period activity rhythms that are somewhat longer than flies with one *perΔS* transgene (17.9-hr period, Table 2, B). Peak levels of per^S mRNA in constant darkness are advanced by 4-6 hrs compared to wildtype (Marrus et al., 1996). In LD conditions the advanced peak is extended to overlap with the wildtype accumulation peak. This is contributed to light dependent delay in the declining phase of per^S mRNA (delay in repression) (Marrus et al., 1996). In animals with two *perΔS* transgenes cycling expression of clock gene mRNAs was not generally apparent in either LD or DD conditions. Very low amplitude oscillations with a peak at ZT 6 in flies with two *perΔS* transgenes could still be detected for *tim* and *vri* mRNAs. This could be related to their ability for high amplitude cycling. In normal flies *tim* and *vri* cycle with one of the highest amplitudes among clock controlled genes (Claridge-Chang et al., 2001). The repressive effects of *perΔS* expression are less severe for *Clk* gene expression. This could be because PER does not directly regulate *Clk* promoter. The observed dampening of *Clk* mRNA oscillations is expected given the lower levels of expression of its regulators *vri* and *pdp1ε*. The expression of *perΔS* mRNA from the transgenes was cycling with an advanced phase accumulation profile. *perΔS* mRNA abundance was higher than normally the peak *per* mRNA levels in wildtype flies. *perΔS* expression was higher in animals with one transgene than with two. This is likely due to higher levels of repressor PERΔS feeding back to the promoters of the transgenes. The expression of *perΔS* mRNAs is oscillating in both one-transgene and two-transgene flies in LD and

DD. This is unlike the endogenous clock genes for which no cycling or very low amplitude cycling is observed in two-transgene flies. The important difference between *perΔS* and endogenous clock gene mRNAs is their relative expression level. Higher expression levels may facilitate higher amplitude cycling of *perΔS* mRNA (Fig. 12).

A more prominent transcriptional repression in flies with two *perΔS* transgenes was observed as would be expected if higher levels of PERΔS protein would be present in these animals. However when PERΔS protein levels were measured by Western blot from flies with one and two *perΔS* transgenes, the overall PERΔS levels were not elevated in one-transgene flies (Fig. 12, panels A, C). Furthermore, in flies homozygous for two *perΔS* transgenes that are behaviorally arrhythmic, the PERΔS protein levels were dramatically reduced (Fig. 13, panels B, D). PER degradation is known to require protein phosphorylation by DBT (Price et al., 1998). This result combined with high *perΔS* mRNA levels (Fig. 12) suggests rapid phosphorylation and degradation of PERΔS protein. Genetically this could be tested in future experiments by crossing the two *perΔS* transgenes into a temperature sensitive proteosome subunit mutant background (Belote and Fortier, 2002). At restrictive temperature hyperphosphorylated forms of PERΔS protein should accumulate that could be readily detected by Western blot.

The arrhythmic behavior of the flies with two *perΔS* transgenes can be rescued back into short period rhythms when reducing DBT activity by introducing a hypomorphic dbt^{AR} allele into the arrhythmic background (Table 3). This behavioral rescue suggests that the low level of PERΔS observed in the animals with two *perΔS* transgenes is due to its rapid DBT dependent phosphorylation and degradation. It also suggests that enhanced transcriptional repression in the arrhythmic two *perΔS* containing

animals could be due to enhanced PERΔS phosphorylation by DBT. An autoregulatory feedback may be operating where PERΔS through its transcriptional repressor activity feeds indirectly back to its own post-translational regulation. Such feedback loop could limit the time in the circadian cycle when predominantly phosphorylated forms of PER are in the nucleus facilitating the restart of the next transcriptional cycle in the clock. It is currently unknown if DBT activity is constitutive or controlled by the circadian clock. However, cycling phosphorylation in the presence of constitutive kinases can be achieved by rhythmic dephosphorylation. Protein phosphatase 2A (PP2A) has been shown to affect the stability and subcellular localization of PER (Sathyanarayanan et al., 2004). Furthermore, its regulatory subunits *tws* and *wdb* are transcribed under clock control. Strongly hypomorphic allele tws^{60} leads to very low levels of PER (Sathyanarayanan et al., 2004). It is possible that abnormally potent repression by PERΔS downregulates the expression of PP2A subunits in the two-transgene flies to the level that abolishes cycling in DBT dependent phosphorylation leading to the rapid constitutive phosphorylation and degradation. According to this model loss of per-short region would trigger premature PER phosphorylation that leads to premature repressive activity and low steady state protein levels.

In wildtype flies the state of TIM phosphorylation is indicative of the subcellular localization of the PER-TIM complex. Hyperphosphorylated TIM is associated with nuclear localization of the TIM-PER complex. Interestingly, TIM in the flies with two *perΔS* transgenes was also constitutively hyperphosphorylated (Fig. 13). TIM hyperphosphorylation in the arrhythmic flies with two *perΔS* transgenes could be dependent on SGG activity as overexpression of DSH protein, known to inhibit SGG

activity in Wnt signaling, resulted in rescue of behavioral rhythms (Tabel 3). As the subcellular localization of PERΔS and TIM in these flies is currently unknown, two possibilities could be considered to explain the abnormal TIM phosphorylation state. PERΔS may repress one or more genes that encode proteins, which inhibit SGG activity or TIM phosphorylation leading to constitutive TIM phosphorylation and therefore constitutive nuclear translocation of PERΔS-TIM complexes. Interestingly, in the per^S mutant the early decline of mutant PER levels is suppressed by light (Marrus et al., 1996). It is possible that light counteracts premature nuclear translocation of TIM-PER^S complexes. Thus far, the timing of nuclear translocation of PER^S *in vivo* has only been measured in light-dark conditions (Curtin et al., 1995). Alternatively the per-short domain deletion may affect the interaction between the two proteins. One of the two regions in PER that mediates its interaction with TIM maps just 75 amino acids upstream of the per-short domain. In this scenario TIM and PERΔS interaction would be very short lived after nuclear entry and dissociation would lead to hyperphosphorylation of both proteins. In wildtype flies PER-TIM complex dissociation is delayed after nuclear entry in the middle of the night (Zeng et al., 1996). This delay contributes to the delayed onset of PER dependent repression of transcription. In PERΔS expressing flies the peak levels of clock gene mRNA expression were advanced and overall transcript levels were lower. This could indicate premature onset of PERΔS dependent repressive activity, which would prevent mRNA accumulation to high levels and simultaneously would bring the peak forward. In flies with two *perΔS* transgenes the better repression could produce effectively more phospho-PERΔS leading to enhancement of the phenotypes observed in flies with one *perΔS* transgene. It would be important to measure directly the nuclear

translocation timing of PERΔS in the two-transgene animals to test the possibility of constitutive nuclear localization.

In conclusion, *in vitro* phosphorylation site mapping shows that DBT phosphorylates PER at multiple sites. When mutated to alanines and tested in a functional assay, the *in vitro* phosphorylation sites in the central part of PER influence PER transcriptional repressor activity. The *in vitro* phosphorylation sites map into or right downstream of a previously recognized per-short domain that when mutated causes behavioral rhythms to shorten. To test the proposed function of the perSD domain in the context of a fully functional clock an *in vivo* analysis of *per* transgenics with mutated candidate phosphorylation sites is required The per-short domain had previously been shown to function genetically as an inhibitor of DBT dependent phosphorylation of PER. Removal of this domain leads to an apparently hyperactive PER molecule that may be phosphorylated more effectively *in vivo* and displays an enhanced transcriptional repressor activity. The residues mapped as *in vitro* phosphorylation sites downstream of the per-short domain are required for transcriptional repressor activity of PER. The per-short domain may function as a structural inhibitor that could normally delay the phosphorylation of the downstream sites and thus delay the onset of transcriptional repression by PER. A following model is proposed for the aberrant function of PERΔS. It is proposed that in the per-short domain deletion mutant *perΔS* a time delay is eliminated between nuclear accumulation of hypo- and hyperphosphorylated states of PER. The elimination of per-short domain leads to an immediate PER hyperphosphorylation and full transcriptional repressor activity when located to the nucleus. The short rhythms observed in flies with one PERΔS transgene are a result of premature onset of negative

feedback on PER regulated promoters. The arrhythmia in flies with two PERΔS transgenes is proposed to be caused by constitutive negative feedback by PERΔS. A possible constitutive nuclear translocation is proposed that is caused by an indirect effect of enhanced PERΔS repressor activity on TIM phosphorylation.

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