Circadian Expression and Promoter Analysis of the Zebrafish *Period-4* gene

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Abstract

In most organisms, light plays a key role in the synchronization of the circadian timing system with the environmental day–night cycle. Light pulses that phase-shift the circadian clock also induce the expression of *period* genes in vertebrates. Here, we report the cloning of a zebrafish *period* gene, *zfperiod4*, which is repressed by light. High amplitude rhythms of *zfperiod4* expression are detected under light dark (LD) cycles in zebrafish larvae as well as in the zebrafish cell line PAC-2 that contain a directly light-entrainable clock. The expression of *zfperiod4* is detected during the first day of development and we show that the presence of a LD cycle is essential to subsequently establish a robust circadian rhythm in gene expression. We have developed a transient and stable transfection protocol for PAC-2 cells. In this way we have established an *in vivo* luciferase reporter assay for *zfperiod4* expression in this cell line. High-definition bioluminescence traces have enabled us to accurately measure phase-shifting of the clock by light. We have also exploited this model to study how four E-box elements in the *zfperiod4* promoter regulate expression. Mutagenesis reveals that the integrity of these four E-boxes is crucial for maintaining low basal expression together with robust rhythmicity and repression by light. In the context of a minimal heterologous promoter, the E-box elements also direct a robust circadian rhythm of expression that is significantly phase-advanced compared with the original *zfperiod4* promoter and lacks the light-repression property. These results reveal flexibility in the phase and light responsiveness of E-box-directed rhythmic expression, depending on the promoter context. Finally, a preliminary pharmacological analysis implicates the involvement of the MAP Kinase, cAMP, and PKC signaling pathways in the maintainance of the amplitude as well as entrainment of circadian clock rhythms.

Key words: Circadian, Clock, Zebrafish, Period gene, Luciferase, PAC-2, Cell lines

1. Introduction

The temporal organization of metabolism, physiology and behavior in most plants and animals is dominated by rhythms. Ultradian rhythms have a cycle length shorter than 24 hours and include eye blinks (24 eye blinks per minute in humans), respiration movements, heartbeat and sleep patterns. In contrast, many rhythms have cycle lengths significantly longer than 24 hours, ranging from days, months to even years and these are termed infradian. Infradian rhythms include circalunal, circatidal and circannual rhythms that correspond to the waxing and waning of the moon, the ebb and flood of the tides and seasonal changes respectively. However, the most extensively studied of all rhythms are those with a period length enduring approximately one day, the so-called circadian rhythms (circa-diem; around one day). It is now experimentally well established that an endogenous, self-sustaining, circadian oscillator or clock, controls these rhythms (Pittendrigh, 1993). Since under constant conditions this generates a rhythm with a period length of approximately 24 hours, this clock needs to be constantly reset (entrained) by environmental signals such as light, nutrient availability and temperature to ensure that it remains synchronized with the natural 24-hour cycle. These synchronizing signals are termed "zeitgebers" meaning time-givers (Pittendrigh, 1993). The circadian timing system can be considered to consist of three parts. 1. A circadian oscillator that autonomously generates circadian rhythms, 2. an input pathway that relays environmental signals to the pacemaker for entrainment and 3. an output pathway whereby the pacemaker directs circadian rhythms in physiology and activity (Figure.1). During the last 20 years significant progress has been made in understanding the cellular and molecular basis of this system using a variety of model systems (Harmer et al., 2001). Most significant progress has been made by studies in *Drosophila*, *Neurospora* and *Cyanobacteria*, primarily through their advantages as genetic models, well suited to large-scale mutant screening (Wager-Smith and Kay, 2000). More recently, progress has been made in understanding the molecular basis of the vertebrate circadian timing system helped in part by the discovery of many

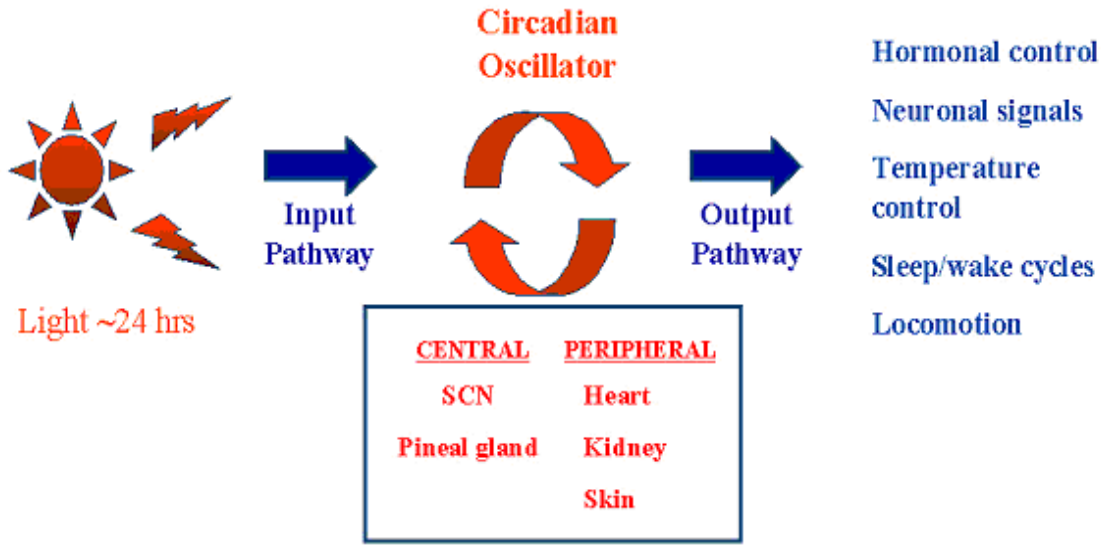


Figure.1

Schematic representation of the vertebrate circadian timing system. Light information, which is a major zeitgeber, is conveyed via the input pathway to the circadian pacemaker. This is composed of both central and multiple peripheral oscillators. Via, the output pathway these pacemakers drive oscillations in various aspects of physiology.

basic similarities between the *Drosophila* and vertebrate circadian clocks (Wager-Smith and Kay, 2000). Since the *Drosophila* circadian timing system is the best understood and more relevant to the study of vertebrate circadian clocks, in the following sections I will focus on summarising our current understanding of the *Drosophila* and vertebrate systems. I will first review our knowledge of the input pathway, circadian oscillator and output pathway. Then I will explain the current models for the molecular basis of the clock mechanism before discussing peripheral clocks and the zebrafish circadian timing system in more detail.

1.1 Light input pathways

A fundamental property of the circadian oscillator is its ability of its phase to be reset by light in order to remain synchronized with daily changes in the photoperiod of the environment (Roenneberg et al., 2003). Light is frequently considered as the most important zeitgeber although temperature also plays a significant role in many systems, such as *Neurospora* (Liu et al., 1998). Experimentally, the phase shifting effects of light are most often measured by maintaining animals for several days in constant darkness before delivering a short light pulse and then subsequently determining the new phase of the rhythm (Johnson, 1999). The mechanism and the factors through which light entrains the clock have been extensively studied.

1.1.1 Multiple light input pathways to the *Drosophila* clock

In *Drosophila*, genetic analyses have shown the existence of several light input mechanisms (Foster and Helfrich-Forster, 2001, see figure.2). Evidence for the involvement of ocular photoreception came from the characterization of the *norpA* mutation (no receptor potential A, (Pearn et al., 1996)). This mutation affects Phosphoinositide-specific phospholipase C and thereby disrupts inositol phosphate signaling involved in the phototransduction cascade (Pearn et al., 1996). Mutant flies impaired in compound eye and ocelli function fail to entrain to light dark (LD) cycles with very low light intensities and also take a

longer time to re-entrain their activity rhythm to a phase change in the LD cycle (Helfrich-Forster et al., 2001). A second system involves cryptochrome (CRY), which is a protein related to the blue light photoreceptor molecules in higher plants as well as the 6-4 photolyase DNA repair enzyme family (Cashmore et al., 1999; Devlin and Kay, 1999) (see also section 1.4.1). Characterization of the fly mutant, cry^{baby}, revealed that they also take a longer time to re-entrain their rhythm to a change in the phase of the LD cycle which suggested a role for this protein in the light input pathway to the clock (Emery et al., 1998; Stanewsky et al., 1998). In *Drosophila*, the interaction of CRY with the central clock feedback loop mechanism, specifically with the timeless (TIM) protein, is thought to underlie its function (Ceriani et al., 1999)(See also section 1.4.1). This light dependent interaction represents the initial step in circadian phototransduction and renders the period-timeless (PER-TIM) complex inactive and unable to participate in negative feedback. Subsequently the TIM protein is degraded (Ceriani et al., 1999). Cryptochrome expression in the Lateral Neurons (LNs) is thought to underlie its function as a circadian photoreceptor (Emery et al., 2000). The third photic input pathway was implicated by the observation that *norpA* / cry^{baby} double mutants still showed some entrainment to LD cycles. The location but not the identity of the photoreceptor that constitute the third system has been analysed by characterizing glass mutant flies ($glass^{60j}$). These mutant flies lack the ocelli, the Hofbauer-Buchner (H-B) eyelets and all retinal photoreceptor cells (Helfrich-Forster et al., 2001). In $Cry^{baby}/glass^{60j}$ double mutants all three indicated light input pathways are inactivated and consequently these mutants are blind in the circadian sense (Helfrich-Forster et al., 2001). This points to the Hofbauer-Buchner eyelets been the site of a third circadian photoreceptor.

1.1.2 Ocular and extraocular light input pathways in vertebrates

In mammals, perception of light relies upon the retina. A wide range of experimental evidence points to the integrity of the retina as being crucial for the ability of the mammalian clock to perceive and be entrained by LD cycles

(Foster, 1998). A report in humans that light shined behind the knee may be able to entrain circadian rhythms has not been substantiated (Barinaga, 2002; Campbell and Murphy, 1998). Transgenic studies in mouse have demonstrated that the ablation of the main classes of visual photoreceptor cells in the retina: the rods and cones does not eliminate entrainment of the circadian clock by light (Freedman et al., 1999; Lucas et al., 1999). More recent studies have implicated a subset of photoreceptive retinal ganglion cells (RGCs) that directly project to the SCN, as the principal circadian photoreceptors (Berson et al., 2002; Hattar et al., 2002). These cells express the photoreceptor protein melanopsin. Knockout studies have confirmed the importance of this protein in circadian photoreception (Hattar et al., 2003; Panda et al., 2003). However, the rod and cone photoreceptors also appear to play some role in circadian photoreception depending upon the light intensity (Hattar et al., 2003). Both glutaminergic as well as pituitary adenylate cyclase-activating peptide (PACAP) signaling have been implicated in conveying the signal from the retinal photoreceptor to the clock via the retino hypothalamic tract (RHT) (Hannibal, 2002; Hattar et al., 2003). These signals result in induction of immediate early genes such as *c-fos* and c-*jun*, as well as the clock genes *mPer1* and *mPer2* within the SCN (Kornhauser et al., 1996; Zylka et al., 1998). This induction appears to be mediated by the MAPK signalling pathway via CREB-mediated transcription (Obrietan et al., 1998). Other candidate photoreceptor molecules include the cryptochromes. In mammals, mCRYs are expressed in both the outer and inner nuclear layer (ONL and INL) as well as the ganglion cell layer (GCL) of the retina (Sancar, 2000). Characterization of the biochemical functions of mCRYs has shown that they can act as potent repressors of the core oscillator components, Clock (CLK) and MOP3/BMAL (Griffin et al., 1999). However, knockout studies in mouse reveal that they seem to function as components of the core clock mechanism (van der Horst et al., 1999) (See section 1.4.2). Thus, unlike the situation in *Drosophila*, they do not appear to function as a component of the light input pathway.

Non-mammalian vertebrates possess a wider array of photoreceptive tissues, with several different types of extra-retinal photoreceptive organs developing from the embryonic forebrain (Menaker et al., 1997). For example 1) all non-mammalian vertebrates possess an intra cranial pineal organ or pineal body (*epiphysis cerebri*) which contains photoreceptors. 2) many bony fish and lampreys have an intracranial parapineal organ closely linked with the pineal. 3) amphibians and reptiles possess an extracranial 'thirdeye' termed the frontal organ in frogs and 4) all non-mammalian vertebrates also possess deep brain photoreceptors, located in the 3rd ventricle of the diencephelon. Of these, the deep brain and pineal photoreceptors have been directly demonstrated to play a role in the regulation of circadian clocks (Foster and Soni, 1998). The deep brain photoreceptors were first linked to circadian entrainment as a result of studies on house sparrows by Menaker and colleagues in the 1960s and early 1970s. Removal of both the eyes and pineal did not block photoentrainment (Menaker and Underwood, 1976). Subsequent studies showed that the brain photoreceptors contribute to photoentrainment in many species of birds, fish, reptiles and amphibians (Menaker and Underwood, 1976). The pineal photoreception is demonstrated in all non-mammalian species studied so far and illumination of non-mammalian pineals in *in vitro* studies has shown that the pineal photoreceptors regulate melatonin synthesis in a circadian manner (See review, (Menaker and Underwood, 1976). Such extra retinal photoreceptors appear to be absent in mammals.

Finally, in zebrafish widespread direct photosensitivity of peripheral tissues has been demonstrated by the observation that clock gene rhythms of expression in cultured cell and tissues can be directly entrained by exposure to LD cycles in a similar manner to that documented in *Drosophila* (Whitmore et al., 2000). This suggests the presence of a widely expressed photoreceptor molecule in fish. Candidates for this photoreceptor include cryptochromes (Cermakian et al., 2002) as well as novel opsins (Moutsaki et al., 2003).

1.2 Pacemakers

1.2.1 Pacemaker structures in *Drosophila*

In *Drosophila*, lateral neurons (LNs) represent the site of central pacemaker function (see Figure.2). These neurons are located bilaterally between the optic lobes and the central brain (Helfrich-Forster, 1998; Kaneko, 1998). In the adult they consists of two groups of more ventrally located cells: five small LNvs (s-LNv) and four large LNvs (l-LNv) projecting to the dorsal protocerebrum and to the second optic lobe neurophil (medulla) respectively (Kaneko 2000; Stanewsky et al., 1997). Both of these LNv groups characteristically express the *pigment dispersing factor (pdf*), a neuropeptide gene which is expressed with a circadian rhythm (Renn et al., 1999). The dorsal brain region receiving input from the LNs is also connected to another group of clock-gene-expressing neurons, the "dorsal neurons". Several lines of evidence suggest that the brain is the site of pacemaker for adult locomotor activity rhythms. Transplantation of the fast-clock *per* mutant (per^{s}) brain into a aperiodic *per* mutant (per^{01}) abdomen restored rhythmicity, with a short period (19h) that was characteristic of the donor's behavioral rhythm (Handler and Konopka, 1979). More recent molecular studies have suggested the presence of pacemakers in most peripheral tissues. Surprisingly, expression of the *period* gene and other clock genes is not restricted to the lateral neurons or other brain regions implicated in pacemaker function but has been identified in most tissues (Hall, 1995). Transgenic flies expressing a construct containing the *period* gene promoter driving expression of the luciferase reporter gene (*per*-luc) have demonstrated circadian rhythms of *period* expression in cultured *Drosophila* dissociated body segments (head, thorax, or abdomen) (Plautz et al., 1997). *Per*-driven bioluminescence rhythms were also shown to be present in the proboscis, antennae, anterior wing margins and legs (Plautz et al., 1997). Subsequently, antennal neurons have been shown to be both necessary and sufficient for circadian rhythms in olfactory responses, which demonstrates that a peripheral tissue can function as an autonomous pacemaker in *Drosophila* (Krishnan et al., 1999; Tanoue et al., 2004).

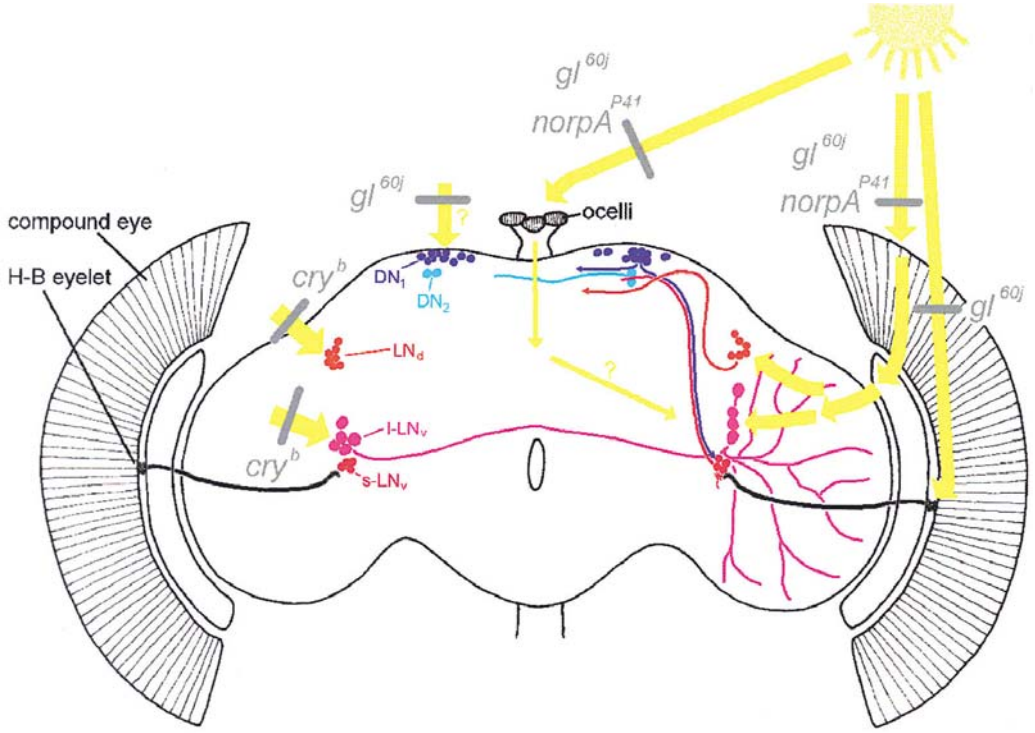


Figure.2

Cellular targets of the multiple light-input pathways utilized by *Drosophila*'s circadian system. A frontal view of the fly's adult brain is shown with the *per*/*tim*-expressing dorsal neurons (DN1 and DN2) in blue and the lateral neurons (LN_d, I-LN_v, and s-LN_v) in red. The projections of these neurons are indicated in the right hemisphere, whereby the arrows represent neurites whose exact terminal endings are not yet identified (Kaneko and Hall, 2000). The right hemisphere shows the light-input pathways through retinal and extraretinal eye structures; the ocelli, the compound eyes, and the Hofbauer-Buchner eyelet (H-B eyelet). In the left brain hemisphere, the light inputs to the DN1s and LNs via cellular photopigments are depicted. Question marks indicate places where either the function of cells as photoreceptors is not clear or the pathways toward the *per*/*tim*-expressing cells are not yet identified. The grey bars represent interruptions of pathways by the individual mutations specified. In $norpA^{P41}$; cry^{b} all known photoreceptive structures are impaired, except the H-B eyelet. In the gl^{60j} cry^{b} mutant, all indicated light-input pathways are disrupted and consequently these mutants are blind in the circadian sense.
Figure adapted from (Helfrich-Forster et al., 2000).

1.2.2 Central and peripheral pacemakers in vertebrates

In mammals, the suprachiasmatic nucleus (SCN) located in the anterior hypothalamus has been shown to be the site of the central circadian pacemaker responsible for the generation and regulation of rhythms in behaviour, hormonal secretion and various physiological functions (Klein, 1991). This small, paired nucleus consisting of only several thousand cells lies dorsal to the optic chiasma and lateral to the third ventricle. Each nucleus contains two sub-divisions: a) a core receiving direct and indirect visual input from neurons in the RHT, a dense projection from the midbrain raphe, and input from the hypothalamus and thalamus and b) a shell receiving input from the basal forebrain, thalamus and the brainstem (Moore and Silver, 1998). All SCN neurons appear to function as circadian oscillators (Liu et al., 1997) and each nucleus is capable of independently generating circadian rhythms. Analysing clock gene expression in SCN of behaviorally "split" hamsters under constant light revealed antiphase oscillation in the left and right nuclei (de la Iglesia et al., 2000). The split condition arises after prolonged constant light treatment and represents a separation of a normal circadian activity rhythm into two separate component rhythms (de la Iglesia et al., 2000). Various SCN neuronal cell types have been identified by their differential expression of various neural peptides (e.g. Argenine Vasopressin (AVP), Vasoactive Intestinal Polypeptide (VIP)) (Klein, 1991). Recent studies documenting clock gene expression suggest that the nucleus can also be subdivided into different regions in terms of light responsive gene expression and various clock functions (de la Iglesia et al., 2004). A pacemaker has also been identified in the retina that controls the local synthesis of the hormone melatonin (Tosini and Menaker, 1996). In addition, the pineal gland in lower vertebrates such as birds and reptiles is the site of an independent circadian clock, that drives rhythms of melatonin release and directly responds to entraining light signals in culture (Menaker et al., 1997; Menaker and Wisner, 1983; Takahashi et al., 1980). However, the pineals of certain fish, lizards and birds do not oscillate in culture (Takahashi et

al., 1989; Underwood, 1990). One of the most extensively studied pineal systems is of the chicken where light exposure *in vitro* can modulate N-acetyltransferase activity and melatonin production (Deguchi, 1979; Hamm et al., 1983; Wainwright and Wainwright, 1980). It has also been reported that dissociated chick pineal cell cultures can express a circadian oscillation of melatonin release (Robertson and Takahashi, 1988).

1.3 Output pathways

1.3.1 Clock outputs in *Drosophila*

In *Drosophila,* circadian gene expression in the LNvs has been extensively studied in an attempt to understand how the clock mechanism regulates the locomotor activity of flies. In these neuronal cells, *Drosophila* Clock (dCLK) and Cycle (dCYC) proteins directly regulate transcription of the bZIP transcription factor gene *Vrille* (*vri*), which forms part of the central oscillator (Blau and Young, 1999; Cyran et al., 2003) (see section 1.4.1). One transcriptional target of VRI is the neuropeptide PDF, which appears to play a role in regulating activity rhythms in flies (Blau and Young, 1999). Genetic analysis has been used to identify other genes involved in clock outputs. For example the *Lark* gene has been implicated in regulating the circadian rhythm of eclosion. The LARK RNA binding protein has been postulated to mediate post transcriptional regulation of genes encoding other downstream elements of this clock output pathway (Newby and Jackson, 1996). High-density oligonucleotide-based arrays have been used to carry out a global examination of clock-controlled genes. DNA microarray analysis now makes it possible to simultaneously monitor changes in expression of thousands of genes (Lockhart and Winzeler, 2000). Mc Donald and Rosbash described 134 genes that oscillate under DD conditions and display altered levels in the Clk^{jrk} mutant background (McDonald and Rosbash, 2001). Claridge-Chang and colleagues on the other hand analysed cycling profiles under LD and DD conditions in different genetic backgrounds (Claridge-Chang et al., 2001). They identified

158 cycling genes, some of which are involved in learning and memory, vision, olfaction, locomotion, detoxification and metabolism. To gain deeper insight into clock-controlled processes in the whole fly, Ceriani and colleagues probed high-density arrays with RNA isolated from both head and body fractions (Ceriani et al., 2002). They found that although the number of cycling genes is similar between the two samples, only a small proportion of genes cycle in both structures. This indicates that the clock controls different aspects of physiology throughout the fly.

1.3.2 Multiple clock outputs in the vertebrate system

The SCN is connected to many regions of the brain by direct and indirect neuronal projections. These neuronal pathways have been implicated as important clock output pathways (Klein, 1991). For example, adrenergic projections via the supracervical ganglion (SCG) project to the pineal gland and direct melatonin synthesis. This hormone that is synthesised by a multistep pathway from tryptophan via serotonin, is produced exclusively at night under the control of noradrenaline release and constitutes the major endocrine output of the clock (Korf et al., 1998). High affinity receptors for melatonin are expressed in various structures within the brain including the SCN where they are predicted to mediate feedback by melatonin on the clock (von Gall et al., 2002b). A high density of receptors is also found in the pars tuberalis where they are involved in the regulation of pituitary function in response to seasonal variation in day length (von Gall et al., 2002a). Silver and coworkers have shown that a diffusible substance from transplanted fetal SCN tissue can restore weak circadian rhythmicity in locomotor activity in SCN-lesioned hamsters (Silver et al., 1996). Thus, diverse hormonal and neuronal signals appear to represent the principal output pathways conveying circadian timing information to the animal. However, the precise nature of these signals remains unclear.

The existence of clocks in most peripheral tissues, as in *Drosophila*, implicates the direct regulation of gene expression by circadian clock components as an important clock output pathway. Panda and colleagues have used high-density

oligonucleotide arrays to examine the circadian expression of more that 7000 known genes and 3000 expressed sequence tags (ESTs) in the SCN and liver (Panda et al., 2002). This analysis revealed nearly 650 circadian regulated transcripts, most of which cycle either in the SCN or liver but not in both. This analysis also revealed cycling of a number of genes that are involved in pathways already experimentally linked to circadian clock outputs. It appears that the CLK/BMAL1/E-box system mediates cycling transcription of many of these clock output genes (Panda et al., 2002). Circadian clock regulated gene expression has also been examined by expression profiling of serum shock synchronized fibroblasts (Grundschober et al., 2001). This work revealed that approximately 2% of all expressed genes followed a robust circadian rhythm of gene expression. These genes included cell cycle regulatory factors. An alternative transcriptional output from the clock involves DBP, a PAR domain transcription factor (Wuarin and Schibler, 1990). The transcription of DBP (albumin-D box binding protein) is activated by CLK/BMAL1 through E-boxes and inhibited by the mPER and mCRY proteins (Ripperger et al., 2000; Yamaguchi et al., 2000) (see also section 1.4.2). The expression of the *dbp* gene oscillates with a high amplitude in liver, kidney, pancreas, heart, muscle and lung (Fonjallaz et al., 1996; Lavery and Schibler, 1993; Wuarin et al., 1992). Many transcriptional targets of this gene for example the cholestrol-7-alpha hydroxylase gene therefore are also expressed with a circadian rhythm (Lavery and Schibler, 1993).

1.4 Molecular organization of the Clock

1.4.1 *Drosophila*

The principle way that molecular components of the clock have been identified is by forward genetic screening in a range of model systems, most notably *Drosophila*. Specifically, natural or induced mutants have been characterized which show altered circadian rhythmicity and subsequently genetic analysis

and cloning of the mutated loci, has resulted in the description of "clock genes".

The first clock mutant "*Period*" was identified in *Drosophila* based on its perturbation of fly circadian locomotor and eclosion rhythms (Konopka and Benzer, 1971). The *period* mutation was also found to affect ultradian rhythms such as the frequency of the male courtship song (Kyriacou and Hall, 1980). Three types of *period* mutations were originally described based upon the properties of circadian rhythms under free running conditions in constant darkness. The "short" period allele (per^s) shows a significant reduction in *period* length, the "long" period allele (per^l) shows a longer period length and the *period* null allele (per^0) are arrhythmic (Konopka and Benzer, 1971). Subsequent analyses lead to the cloning of the first clock gene in *Drosophila* the *Period* (*per*) gene. The dPER protein shares a domain with a family of proteins including ARNT (Aryl hydrocarbon receptor nuclear translocator) and SIM (Single Minded Protein). The so-called PAS domain is thought to be involved in protein-protein interactions. The next fly clock gene that was cloned was *timeless* (*tim*) identified by its ability to bind to dPER and by positional cloning of a mutant gene that was also responsible for altered activity rhythms (Gekakis et al., 1995; Myers et al., 1995). However, the roles of dPER and dTIM remained poorly understood until two other *Drosophila* clock components were discovered. The *Clock* (*dClk*) and *cycle* (*dCyc*) genes were identified through a genetic screen for mutations that affect circadian rest: activity rhythms in flies (Allada et al., 1998; Rutila et al., 1998). *dClk* and *dCyc* encode transcription factors that directly activate *dper* and *dtim* expression by binding to E-box elements within their promoters (Darlington et al., 1998; Lee et al., 1999a). Both dCLK and dCYC contain a PAS domain as well as a basic helix-loop-helix domain (bHLH) that is thought to mediate sequence specific DNA binding. The *Vrille (vri)* transcription factor that is important for embryonic development has also been implicated as a central clock component. This PAR domain factor (Proline and Acidic Rich) was first identified in a screen for clock controlled genes and its rhythmic expression was shown to be

regulated by dCLK and dCYC. Subsequently a second PAR domain factor was identified by its homology with VRI, the PAR Domain Protein 1 (pdp1) and together with VRI was implicated in the regulation of cyclic *dClk* mRNA expression (Cyran et al., 2003). The *cry* gene was identified initially by the characterization of the cry^{baby} mutation, which eliminated *period* controlled luciferase cycling in whole adult flies. In peripheral tissues of this mutant no rhythmic clock gene expression was detected, however in the lateral neurons robust PER-TIM cycling continued. Consistently, a circadian rhythm in locomotor activity was still present in this mutant although they exhibited poor synchronization with LD cycles. The role of *cry* in the *Drosophila* circadian machinery is still not fully understood. Biochemically, dCRY is a flavin/pterin containing protein related to the 6-4 photolyases and plant blue light photoreceptors (Emery et al., 1998; Emery et al., 2000; Stanewsky et al., 1998). There is direct evidence that while CRY functions as the circadian photoreceptor in the LNs, it is also a component of the clock mechanism, at least in the peripheral tissues (Stanewsky et al., 1998). One clock component not implicated as a transcriptional regulator was *Doubeltime* (*dbt*). It was identified by genetic screening for abnormal locomotor activity rhythms (Kloss et al., 1998; Price et al., 1998). $Doubletime^{short}$ or dbt^{S} mutants exhibited free running periods of 18h, whereas dbt^{L} showed a lengthened period of up to 27h. Cloning and characterization of the locus affected by these mutations revealed that DBT encodes a protein kinase resembling mammalian *Casein kinase 1ε*.

The various clock components described above appear to be organised into a transcription translation negative feedback loop where the protein products of one or more clock genes indirectly regulate transcription of their own genes (see Figure.3). The first loop of this model starts with activation of *dper* and *dtim* expression by dCLK/dCYC at about noon. dPER/dTIM protein then feeds back to inhibit dCLK/dCYC activity during the second half of the night (Allada et al., 2001). In the second loop, dCLK/dCYC also activates *vri* and *Pdp1* transcription at about noon. *vri* and *Pdp1* RNA and protein accumulate with different kinetics such that VRI protein accumulates first and represses *dClk*

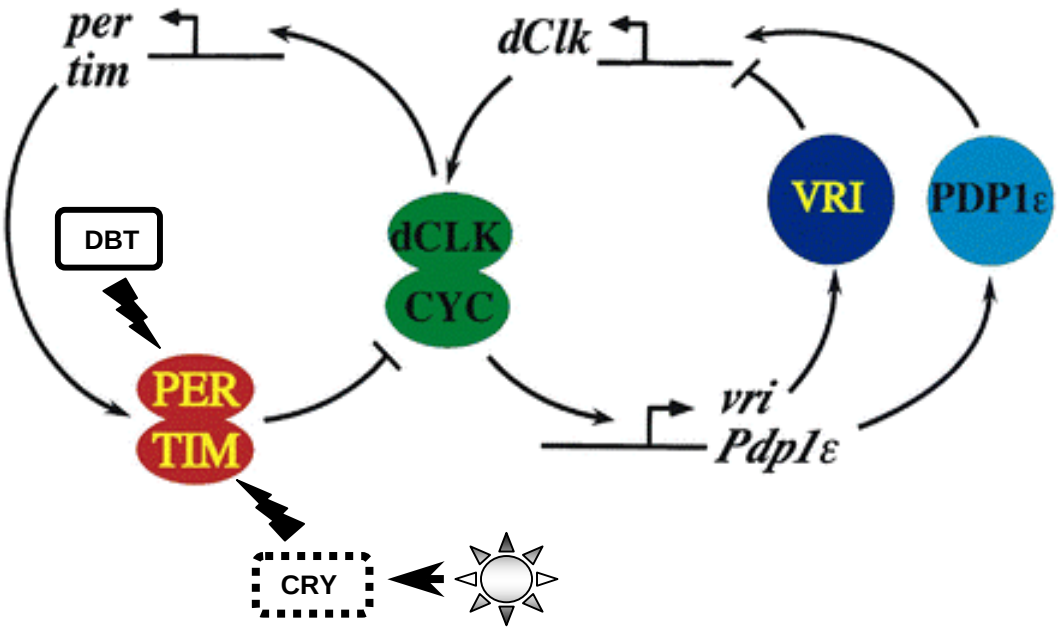


Figure.3

Model of the circadian oscillators in *Drosophila.* The cycle begins with the activation of transcription of the *per* and *tim* genes by the dCLK-CYC hetero dimer binding to E-box motifs. Upon reaching a certain threshold concentration the dPER/dTIM heterodimer enters the nucleus and shuts off its own synthesis. At the same time it can activate transcription of the *dClk* gene. Light-dependent degradation of dTIM is mediated by CRY, and the stability of dPER seems to be affected by the DBT kinase. The second loop involves the activation of *vri* and *Pdp1* transcription by dCLK/CYC. The phase dependent regulation of PDP1 and VRI protein activates and represses *dClk* gene. Newly produced dCLK protein is inactive due to the presence of dPER repressor. Once dPER is degraded, dCLK/CYC reactivates *dper/dtim* and *vri/pdp1* transcription to start a new cycle.

expression. Then PDP1 protein accumulates and activates *dClk* transcription after VRI-mediated repression ends in the middle of the night. However, newly produced dCLK protein is inactive due to the presence of the dPER repressor. Once dPER is degraded, dCLK/dCYC reactivates *dper*/*dtim* and *vri*/*Pdp1* transcription to start a new cycle. The two loops are linked together by dCLK/dCYC and restart simultaneously (Cyran et al., 2003). *dCry* appears to play a role as circadian photoreceptor. In the LNs dCRY binds to the dTIM protein in a light dependent interaction and then subsequently results in the down regulation of dPER-dTIM. In peripheral clocks *dCry* appears to play a more direct role as a core central component as well as a light receptor although its precise function here remains unclear (Hardin et al., 2003). The DBT protein functions by directly phosphorylating monomeric dPER protein and thereby reducing its stability. In this way there is a delay in the increase in dPER/dTIM complex repression function that introduces an important delay step in the transcriptional translational feed back loop (Price et al., 1998).

1.4.2 Vertebrates

The mammalian circadian clock shares similar components and a similar basic organization with the fly clock. Many mammalian homologues of *Drosophila* clock genes have now been described and reverse genetic analysis tends to suggest they function in a similar fashion. Only a limited number of vertebrate clock genes have been identified using forward genetic analysis in the mouse and hamster, *Clock* and *Casein Kinase1ε* respectively (King et al., 1997; Lowrey et al., 2000).

The positive elements of the mouse clock, as in the fly, are the bHLH/PAS transcription factors mCLK and BMAL1 (Figure.4). *mClock* was originally identified in a genetic screen for mice with aberrant locomotor activity rhythms (King et al., 1997; Vitaterna et al., 1994). BMAL1 was identified by virtue of its interaction with mCLK (Gekakis et al., 1998; Hogenesch et al., 1998) and subsequent experiments have shown that disruption of *bmal1* by homologous recombination causes immediate loss of locomotor rhythms in mice held in

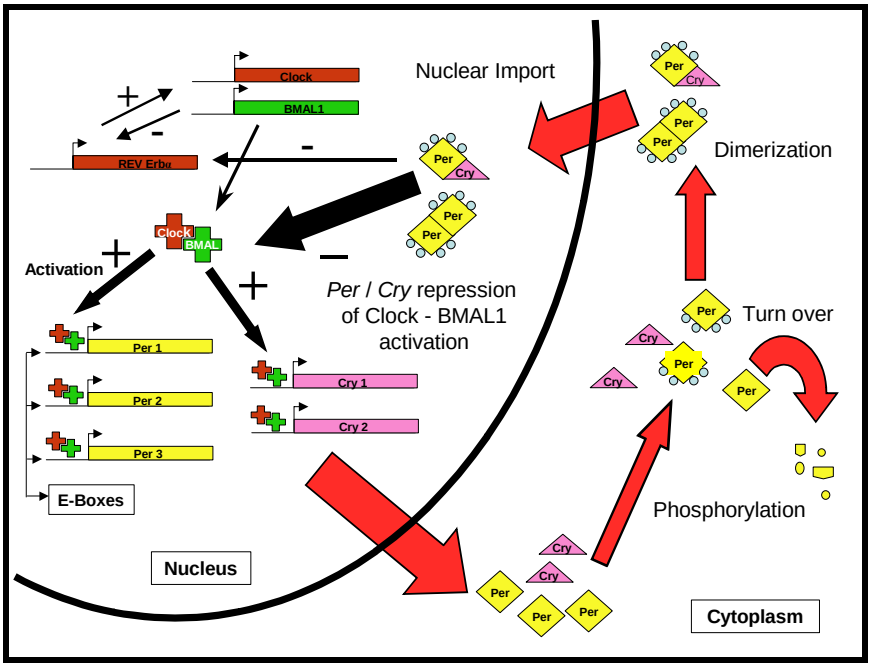


Figure.4

Molecular organization of the cell autonomous circadian clock mechanism. The CLK and BMAL bHLH PAS domain proteins activate the expression of the *Per* and *Cry* genes by forming a heterodimeric complex and binding to the circadian E box enhancer sequence. The CRY & PER proteins are translated in the cyctoplasm and then form a complex that is translocated into the nucleus and disrupts directly the transactivation mediated by the CLK-BMAL heterodimer. In this way PER &CRY act to reduce their own transcription. The lowering of the PER/CRY proteins reactivates this positive component of the clock and so starts the circadian cycle again. The degradation of the PER protein is thought to involve phosphorylation by Casein Kinase 1ε. *REV-Erbα* is an orphan nuclear receptor that belongs to the core clock mechanism. It activates *Clock* and *Bmal1* and is repressed by these proteins. It is also repressed by the negative factors. It forms a secondary loop and adds on precision in timing to the central clock mechanism.

constant conditions (Bunger et al., 2000). Three mammalian *period* gene homologs have been identified based upon amino acid similarity with the dPER protein (Zylka et al., 1998). This suggests either specialization or redundancy of function of the three family members. All three mPER proteins function as negative regulators of mCLK and BMAL1, however this action is not as strong as the inhibition seen in *Drosophila* (Kume et al., 1999). Systematic knockout studies have revealed that the three *per* genes play distinct roles in the circadian clock mechanism (Bae et al., 2001; Zylka et al., 1998). Whereas *mper1* and *mper2* seem to be essential, *mper3* is dispensable for circadian rhythmicity (Shearman et al., 2000). Both *mper1* and *mper2* are expressed with a circadian rhythm and are rapidly induced in the suprachiasmatic nucleus by light pulses delivered during the subjective night but not during the subjective day (Shearman et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998). Also, repression of *mper1* expression in the suprachiasmatic nucleus has been observed during phase-shifting of the clock by forced changes in running wheel activity (Maywood et al., 1999). The precise contribution of these genes to clock entrainment by light remains unclear (Albrecht et al., 2001; Bae and Weaver, 2003; Cermakian et al., 2001). A homologue of the *Drosophila timeless* gene was identified also by sequence homology (Sangoram et al., 1998). However, attempts to address the role of *mtim* in the circadian clock failed because its inactivation by homologous recombination lead to an embryonic lethal phenotype (Gotter et al., 2000). Recent functional evidence does point to the involvement of *mtim* in generating circadian rhythmicity in the SCN and places *mtim* on the negative arm of the molecular feedback loop (Barnes et al., 2003). Photoreceptor function has not yet been shown for the two mouse *cryptochrome* gene homologues: mCRY1 and mCRY2 (Harmer et al., 2001). mCRY1 and mCRY2 were originally identified by sequence homology with cryptochromes from other species (Hsu et al., 1996). They are rhythmically expressed and form complexes with the mPER proteins which are thereby translocated into the nucleus (Kume et al., 1999). Knockout studies

clearly support a role for the mammalian cryptochromes as central clock components (van der Horst et al., 1999).

As in *Drosophila*, phosphorylation of the mPER proteins seems to be important in regulating their rate of turnover and thereby ensuring a delay in their accumulation and in their negative action within the feedback loop (Lee et al., 2001). Thus, Casein Kinase 1ε which is associated with, phosphorylates and thereby regulates the stability of the mPER proteins is an important clock component (Lee et al., 2004). Indeed, mutation affecting the phospho acceptor site for *casein Kinase 1ε* in the hPER2 protein is responsible for the familial advanced sleep syndrome (Toh et al., 2001). Furthermore, a mutation in the *casein Kinase 1ε* gene in the hamster has been shown to be responsible for the *tau* mutant phenotype where there is a characteristic reduction in circadian clock period length in constant conditions (Lowrey et al., 2000). REV-ERBα represents an additional clock component (Preitner et al., 2002). This belongs to a large family of transcription factors called "orphan nuclear receptors", nuclear-receptors for which a ligand has yet to be identified (Mangelsdorf et al., 1995). The expression of REV-ERBα is activated by CLK and BMAL and repressed by factors from the negative limb. REV-ERBα in turn represses expression of CLK and BMAL, thereby regulating the activity of the positive limb of the feed back loop.

1.5 Peripheral clocks in vertebrates

With the cloning of the first clock genes in vertebrates and *Drosophila,* our understanding of the molecular basis of circadian rhythmicity improved dramatically. One of the initial surprising observations was that clock gene expression was detected in most cell types and was not restricted to specialized central pacemaker tissues (King et al., 1997; Zylka et al., 1998). *Clock* gene mRNAs accumulated in a circadian manner not only in the SCN but also in peripheral tissues such as the liver, muscle, kidney and lungs. Subsequently, the view that the circadian clock is the function of a small number of specialized

tissues has changed significantly with the discovery of independent functional circadian clocks in a wide range of cell types and organs in mice, rats and zebrafish (Plautz et al., 1997; Whitmore et al., 2000; Whitmore et al., 1998; Yamazaki et al., 2000). These clocks have now been termed "peripheral" clocks.

Yamazaki and colleagues documented circadian rhythms in various cultured mammalian tissues (Yamazaki et al., 2000). Using a transgenic rat expressing the firefly luciferase gene under the control of the mouse *per1* promoter (*per-luc*), they demonstrated that the lungs, liver, kidneys and skeletal muscle all showed circadian rhythms in reporter gene activity once dissected and placed in culture (Yamazaki et al., 2000). Importantly, rhythmicity in these tissues dampens after only a few cycles, while the SCN continues to oscillate for at least 32 days in culture. Furthermore, ablation of the SCN has been shown to abolish or desynchronize rhythmic clock gene expression in peripheral tissues (Sakamoto et al., 1998). This suggests that the presence of the SCN pacemaker is essential for the maintenance of peripheral clock function. More recent work has addressed this issue in mouse, studying the expression of the endogenous *Per2* locus by integrating a luciferase reporter in a knock-in experiment. In this study a more robust oscillation in luciferase expression was detected in various cultured organs (Yoo et al., 2004). The pathways and the mechanisms whereby the SCN communicates with the peripheral clocks is still unclear. Importantly, glucocorticoids phase-shift circadian rhythms of gene expression in mouse peripheral tissues such as liver, kidney and heart, without affecting the clock in SCN neurons (Balsalobre et al., 2000a). It has also been shown that circadian temperature differences comparable to day-night temperature changes in the environment can phase shift peripheral clocks in mice without affecting the light-controlled central clock in the SCN (Brown et al., 2002). In addition temporal feeding restriction under light-dark or constant dark conditions can change the phase of circadian gene expression in peripheral cell types by up to 12 hours while leaving the phase of cyclic gene expression in the SCN unaffected (Damiola et al., 2000). Food-induced phase resetting proceeds faster

in liver than in kidney, heart, or pancreas, but after 1 week of daytime feeding, the phase of the circadian gene expression is similar in all peripheral tissues examined (Damiola et al., 2000). In the case of the peripheral clocks of zebrafish, direct entrainment by light/dark cycles has been demonstrated in *in vitro* organ and cell cultures as previously reported in *Drosophila* (Plautz et al., 1997; Whitmore et al., 2000). This result predicts the existence of photopigments and clock input pathways in many extra-retinal cell types and suggests a less centralized organization of the circadian timing system in zebrafish.

1.6 Clocks in mammalian cell cultures

An important step in understanding the regulation of peripheral clocks was the discovery that serum shock treatments can induce circadian rhythms of gene expression in cultured rat-1 fibroblasts and other mammalian cell lines (Balsalobre et al., 1998). Subsequently, treatment with activators of various signalling pathways was found to mimic the effects of serum. Thus, for example, glucocorticoids, forskolin (cAMP activator), growth factors, calcymycin (calcium ionophore) and phorbol esters were all found to induce circadian gene expression in rat-1 fibroblasts (Akashi and Nishida, 2000; Balsalobre et al., 2000b; Nonaka et al., 2001; Yagita and Okamura, 2000). Recently, it has become possible to monitor circadian clock gene expression rhythms in the rat-1 fibroblast cell lines in real time. Rat1 cells have been stably transfected with a construct containing the rat *period1* promoter linked to the luciferase reporter gene (Izumo et al., 2003). Their analysis has confirmed that the circadian clocks induced in these cells are temperature- compensated. The establishment of an immortalised cell line derived from fetal rat SCN was reported in 1999 (Allen et al., 2001; Earnest et al., 1999). These SCN 2.2 cell lines show a robust circadian rhythm in clock gene expression and uptake of 2-deoxyglucose (Earnest et al., 1999). Transplantation of the SCN 2.2 cells, restored the circadian activity rhythm in arrhythmic, SCN-lesioned rats (Izumo et al., 2003). Furthermore, co-culturing of the SCN 2.2 cells with the

mammalian NIH3T3 fibroblast cell line established a robust circadian rhythm of clock gene expression in the 3T3 cells but with a 4-12 hours delay in the phase relative to the SCN 2.2 cells (Allen et al., 2001). These results demonstrate that the cell culture system can be a useful tool to dissect the regulatory pathways linking the SCN and peripheral pacemakers.

1.7 Zebrafish as a model system to study the circadian clock

Zebrafish offer several advantages as a model system to study the circadian clock. The ease of access to early stages of embryonic development makes this an attractive vertebrate to study the origin and function of the clock during early development. Furthermore, it's proven utility in large scale genetic analysis potentially makes it a powerful system in which to identify genes encoding circadian clock components or factors that function within the clock input and output pathways in vertebrates (Cahill, 2002). To date, clock genes have been isolated from zebrafish either by sequence homology with their mammalian counterparts (e.g. *Clock* 1, 2, 3 (Hirayama et al., 2003; Whitmore et al., 1998), *period3* (Delaunay et al., 2000), *Period2* (Hirayama et al., 2003), *Period4* (Vallone et al., 2004) and the *cryptochromes 1A, 1B, 2A, 2B, 3&4*, (Kobayashi et al., 2000) or by two hybrid screens for interacting partners of the CLOCK (CLK) protein (e.g. BMAL-1, 2 and 3 (Cermakian et al., 2000; Hirayama et al., 2003)).

Among the outputs of the clock in zebrafish, circadian rhythms of locomotor activity, visual system function and pineal melatonin synthesis have been described. The locomotor activity of larval zebrafish (5-18 days old) has been measured by a computerised video image analysis system. In wild type strains of fish, monitoring this as a clock output was found to be robust and reliable (Cahill et al., 1998). Larval zebrafish like the adults, are most active during the subjective day and the average free running period of the activity rhythm under constant infrared light was found to be 25.5h (Cahill et al., 1998; Hurd et al., 1998). Such behavioral rhythms have been used as an assay in a screen for

chemically-induced semidominant mutations that alter the period length of the rhythm in free running conditions.

Behavioral, physiological and molecular studies have also revealed that the circadian clock in zebrafish regulates several functional aspects of the visual systems. Using a behavioral assay, Li and Dowling (1998) have shown that the visual system is more sensitive during the day than during the night. It has been shown that the dopaminergic interplexiform cells and the olfactoretinal efferents that innervate these cells are necessary for the expression of several aspects of visual system rhythmicity (Li and Dowling, 2000a; Li and Dowling, 2000b). Other retinal rhythms that have been studied in zebrafish include the synthesis of the hormone melatonin (Cahill, 1996) and the expression of mRNA for inter-photoreceptor retinoid binding protein (IRBP; (Rajendran et al., 1996)).

The zebrafish pineal gland also contains a light-sensitive circadian oscillator that drives robust rhythms of melatonin synthesis and release *in vitro* (Cahill, 1996). The circadian rhythm in melatonin synthesis results, at least in part, from clock-control of the mRNA expression for arylalkylamine-N-acetyltransferase (AANAT), the penultimate enzyme in melatonin synthesis (Gothilf et al., 1999). Light has two distinct effects on zebrafish pineal melatonin rhythms, it resets the clock in a phase-dependent manner, and it acutely suppresses melatonin synthesis regardless of phase (Cahill, 1996).

Studies on the expression of rhythmically expressed clock genes in various adult zebrafish tissues have demonstrated the existence of multiple, independent peripheral clocks, which are directly light entrainable (Whitmore et al., 2000; Whitmore et al., 1998). Originally documented using adult heart and kidney cultures, light entrainable clocks have also been described in zebrafish embryonic primary cell lines (eg. PAC-2 and Z3 (Pando et al., 2001; Whitmore et al., 2000)).

Cell-autonomous light and circadian clock regulation of the vertebrate cell cycle has been shown for the first time and using zebrafish larvae and these zebrafish cell lines (Dekens et al., 2003b). Exposure of the larvae and cell lines

to a LD regime causes cells to enter S phase of the cell cycle at the end of the light phase. Such rhythms also persist for several days under free running conditions (DD) (Dekens et al., 2003b).

The existence of zebrafish cell lines that contain directly light-entrainable circadian clocks provides an attractive complimentary cell culture model for studies at the animal level. The cells also have culture properties that make them well suited for long term analysis for circadian clock. Firstly, cells in confluent cultures remain viable for up to 2 months without sub-culturing. In addition, the cells grow optimally at room temperature and do not require a CO_2 gassed humidified environment due to the buffering properties of their preferred medium: Leibovitz-15 (L-15). Recent studies have predicted blue light absorbing properties for the photopigment expressed in Z3 cells and have implicated the MAPK pathway in conveying the light signal to changes in clock gene expression (Cermakian et al., 2002).

1.8 Experimental Aims

The goal of this project was to investigate the mechanisms whereby light is able to directly regulate clock function in peripheral tissues and cells of the zebrafish. We planned to use the PAC-2 cells as an *in vitro* model system to study the regulation of clock gene expression directly in response to light. We aimed to establish an *in vivo* assay for clock gene expression, based on the reporter gene, luciferase. This type of assay has already been used extensively in *Drosophila*, *cyanobacteria* and *Arabidopsis* as well as other clock model systems and represents a powerful non-invasive assay of clock function which lends itself to high throughput analysis and is an ideal assay for genetic screening for clock mutants (Brandes et al., 1996; Millar et al., 1995; Millar et al., 1992).

Our first step would be to identify *zebrafish* clock genes that are acutely light regulated. We then planned to isolate the promoter regions of such genes and clone them upstream of the luciferase reporter gene. We would then stably

transfect these constructs into the PAC-2 cells and monitor bioluminescence *in vivo* using a scintillation counter based assay. This approach would provide the basis for a systematic promoter analysis to identify enhancer promoter elements mediating the direct transcriptional effects of light. The luciferase reporter cells should also enable a detailed characterization of the circadian clock and its regulation by light in the PAC-2 cells. As a complementary approach, we aimed to use the same luciferase-reporter construct to generate stable transgenic lines of zebrafish in order to provide *in vivo* validation of our cell culture results. We then planned to use a pharmacological analysis to study the signal transduction mechanism regulating clock functions.

2. Materials and Methods:

2.1 PCR and oligonucleotide primers

RT-PCR and long-distance PCR reactions were performed using the Perkin Elmer GeneAmp RNA PCR kit and XL PCR kits respectively according to the manufacturer's instructions. In the case of RT-PCR, typically 1 μg of total RNA was used as template for cDNA synthesis primed by random hexanucleotide primers. Long distance PCR was used to amplify targets from 0.4 μg of high molecular weight PAC-2 genomic DNA. In all cases, primers were designed as 27-mers (MWG Biotech, Ebersberg, Germany) with a base composition as close to 50% AT & 50% GC as possible. Genome Walking and RACE PCR were performed using the Genome Walker and Marathon cDNA amplification kits (Clontech) respectively, again according to the manufacturer's instructions. PCR reactions were temperature cycled using a Perkin Elmer 9700 thermal cycler.

The following pairs of oligonucleotide primers were designed using the sequence of *Xenopus period1* cDNA clones (Genbank Accession number: AF250547) (Zhuang et al., 2000) and an IMAGE *Xenopus* cDNA (BE679697) both aligned with the human *per1* cDNA sequence to identify highly conserved nucleotide sequences. These were used for the first long distance PCR with PAC-2 cell genomic DNA: sense 5′AGT GGC TGC AGC AGT GAA CAG TCT GCC 3′ and antisense 5′CCA AAG TAT TTG CTG GTG TTG CTG CTC 3′. The following temperature cycle parameters were used: Initial denaturation: 94°C/1 min.; then, 16 cycles 94°C/15 sec., 63°C/8 min; then 12 cycles 94°C/15 sec., 63°C / 8 min with the 63°C step increasing by 10 seconds with each cycle; and finally, 72°C/10 min. The products were analysed by Southern blotting using an *mper1* PAS domain probe (Tei et al., 1997). For *period2* a zebrafish EST cDNA with high homology with the chicken *period2* cDNA (AW280601.1) was amplified using the following primer sequences: Sense: 5'-GGA ACT TTC AGG AGA TCT GCA AAG GCG-3' and antisense:

5’-GTC TCC CAC GTG TAC AAT GGT GCT GC-3’ using RT-PCR cycling parameters recommended by the kit manufacturer.

2.2 Cloning and sequencing PCR-generated fragments

In the case of PCRs that yielded single products, reactions were purified directly on Quiaquick purification columns (Quiagen). In the case of multiple products, individual bands were purified by agarose gel electrophoresis followed by Quiaquick column purification from the agarose slices (gel extraction kit, Quiagen). Following purification, the DNA was ligated to the pGEM-Teasy vector (Promega) and then transformed into competent E.coli bacteria (TOP10F’, InVitrogen) using the manufacturer’s reagents and instructions.

Taq sequencing was performed using the central sequencing service of the Max-Planck Institute. The following temperature cycling parameters were used: 96°C/20 sec., 50°C/10 sec. and 60°C/4min. for 30 cycles

2.3 Plasmid DNA Extraction

Liquid cultures of ampicillin-resistant bacteria were prepared by inoculating 100ml aliquots of Luria Bertani (LB) medium using either single bacterial colonies or frozen bacterial glycerol stocks (Sambrook et al., 1989). Plasmid DNA was extracted from saturated overnight cultures using a column-based extraction system according to the manufacturer’s instructions (Midi prep, Quiagen) and the final DNA pellets were dissolved in 100–200µl of Millipore filtered, glass distilled water. Yields of DNA were calculated based on optical density measurements of dilutions made from the stocks. The optical density was measured at both 260nm and 280nm and so used to assess DNA purity as well as concentration (Sambrook et al., 1989). Glycerol stocks were prepared for each plasmid produced according to standard methods (Sambrook et al., 1989). Minipreps of plasmid DNA from 1.5ml cultureswere prepared using a standard “boiling method” described elsewhere (Sambrook et al., 1989).

2.4 Cell culture/Transfection

A subline derived from the zebrafish embryonic cell line PAC-2 (Lin et al., 1994) was propagated at 25°C in L-15 (Leibovitz) medium (Gibco BRL) supplemented with 15% Fetal Calf Serum (Biochrom KG), 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin (Gibco BRL) in an atmospheric CO_2, non-humidified cell culture incubator. These fibroblast-like cells grow optimally as an adherent monolayer culture on normal tissue culture-treated plastic substrates (Greiner). Cells were typically passaged once every two weeks, by first trypsinizing, to induce detachment from the substrate followed by dilution in culture medium at a ratio of 1:6 and then seeding in fresh culture flasks. Typically, cells proliferated and returned to confluence within one week of passaging and confluent cultures could be maintained for up to 1 month without significant loss of viability.

For transfection, cell cultures maintained in 185cm^2 flasks at 75% confluency were trypsinized by first washing with 1xPBS (without Calcium and Magnesium), then treating with diluted 1x trypsin for 5-10mins at room temperature and finally diluting the detached cells minimum 10 times in L-15 culture medium and transferring to a 14ml falcon tube. Cells collected in this way from one 185cm^2 flask were used for 2 transfections. After centrifugation (1500 rpm for 5 mins) at 4°C, cells were finally resuspended in 1ml L-15 medium without serum and 0.5 ml of cells were transferred into a 0.4cm electroporation cuvette (Peqlab) for each transfection. To this cuvette was then added a mixture of linearized plasmid DNA (25µg), with or without 2-3µg of a linearised neomycin resistance encoding plasmid (pcDNA3.1NEO) and, 6.5µg of non-linearised supercoiled pGEM-Teasy DNA as carrier DNA. As a control for neomycin resistance, cells were electroporated with the pGEM-Teasy carrier DNA alone. The mixture of cells and DNA were then subjected to electroporation at 0.29 KV, 960µF using a GenePulser II apparatus (BioRad). The electroporated mixture was then transferred into 10cm tissue culture dishes containing 10ml of L-15 medium and incubated overnight at 25°C to allow attachment of viable cells. The next day the medium was removed, attached

cells were rinsed with 5-10ml 1xPBS and then 10ml of fresh medium was added and the cells returned to the incubator for 3 days at 25^{o}C.

On the fourth day after transfection, the culture medium was supplemented with 800µg/ml neomycin, and then subsequently this medium was changed each 5-7 days. After cells from the control transfection (lacking neomycin resistance plasmid) had died completely, the neomycin concentration was reduced to 400µg/ml and subsequently the medium replaced each 5 days. After two more weeks of neomycin selection, typically several hundred clones of cells were visible on each plate. Individual colonies were picked from the plate by trypsinization in 10-20 µl trypsin solution and then transferred into a 96 well plate. Subsequently the clones were progressively expanded into 48 well, 6 well plates and finally into 25cm^2 flasks. Alternatively, all clones were trypsinized and pooled together for subsequent analysis. Individual clones or pools of clones were then maintained in culture medium supplemented with 250µg/ml neomycin.

The pcDNA3.1NEO neomycin resistance-conferring plasmid was generated in our lab and based on the commercial expression vector pcDNA 3.1/myc-HisA (InVitrogen). A BamHI-BglII fragment was deleted so removing the CMV promoter from the construct, and leaving the neomycin resistance gene under the transcriptional control of an SV40 early promoter and upstream of SV40 termination and polyadenylation signals. All disposable culture plastic ware was obtained from Greiner or Falcon (Becton Dickinson).

During the treatment with various activators and inhibitors of signaling pathways, cell viability was tested by trypan blue exclusion using a standard protocol (Sambrook et al., 1989).

2.5 *In Vivo* luciferase assay and data analysis

Using a confluent 25cm^2 flask, cells were washed twice in 1x PBS, trypsinized and then diluted in L-15 culture medium. In total, 3x10^4 (~100µl of a 1:80 dilution) cells per well were seeded into a 96-well fluoro-assay plate (Nunc).

The plate was then incubated overnight at 25^{o}C to ensure proper attachment of the cells to the surface of the well. The medium was then replaced with 250µl fresh culture medium containing 0.5mM beetle luciferin, potassium salt solution (Promega) and the plate sealed using an adhesive "Top Seal" sealing sheet (Packard). Plates were then transferred into a Packard Top-count NXT scintillation counter (2-detector model, Packard) and bioluminescence was measured and expressed as the frequency of photon emission (cps) counted during 5-8 sec/well at intervals of 30-40mins. Three independent pools, each plated in a minimum of eight duplicate wells represented each transfected plasmid. All assays were performed at least three times. In most experiments the plate was illuminated during the intervals between counting. To ensure uniform illumination of wells across the plate, each of the sample plate was positioned below a transparent, empty 96 well plate when inserted into the counter's plate-stacking unit. The counter was located in a thermostatically controlled dark room and illuminated using a tungsten light source (20 µW/cm^2) that was connected to a programmable timer. Luciferase assay data was analysed by first storing the data as ASCII data files using the Packard "Hologram" software system and then importing these files directly into Microsoft Excel using the Import and Analysis macro (I&A, Plautz and Kay, Scripps) Subsequently data was plotted graphically using the I&A software. Period length estimates were made following importing data directly into the CHRONO software (Till Roenneberg, University of Munich). Period estimates were made by linear regression following "peakfinder" analysis.

2.6 β-Galactosidase assay

Extracts were prepared from electroporated PAC-2 cells and assayed for β-galactosidase activity using a standard protocol (Sambrook et al., 1989). Protein concentrations in the extracts were determined using a standard Bradford protein assay protocol and then used to adjust the β-galactosidase

measurements to compensate for any differences in protein concentration between the samples (Sambrook et al., 1989).

2.7 RNA extractions, RNAse Protection assays (RPA) and probes.

RNA was extracted from confluent PAC-2 cell monolayers by lysing the cells in Trizol Reagent (Gibco, BRL). Addition of chloroform and subsequent centrifugation lead to phase separation under conditions where RNA remained water-soluble and proteins or DNA were partitioned in the lower, organic phase or at its interphase. Total RNA was subsequently isolated from the aqueous phase by isopropanol precipitation followed by centrifugation and then rinsing the pellet using 75% ethanol.

RNAse protection assays (RPA) were performed using a previously described protocol with slight modifications (Ausubel, 2002). Riboprobes were prepared by incorporating ^{32}PUTP (Amersham) in *in vitro* transcription reactions with a linearized plasmid DNA template using T7 or T3 RNA polymerase (Promega). Template DNA was then eliminated by digestion with RQ1 DNAse (Promega). Following a single phenol:chloroform extraction, the labeled riboprobe was then purified by passage through NucAway spin columns (Ambion). Reaction volumes for subsequent hybridizations and digestions were scaled down to allow each hybridization to be performed in a final volume of 10µl with 1µg total RNA. Aliquots of RNA were speedvac dried prior to hybridization with the riboprobes. The final RNAse-digested and purified RNA samples were analyzed by electrophoresis on 6% denaturing polyacrylamide gels.

For RPA analysis of *zfperiod4*, a 401nt riboprobe probe was prepared using a BamHI – BglII fragment from the 5' end of the coding region of the cDNA cloned in the vector pBluescript SK-, while for *zfperiod2* a 549nt probe was prepared directly from the RT-PCR generated cDNA fragment of *zfperiod2*, subcloned into pGEMTeasy (section 2.1).

2.8 Site-directed mutagenesis.

For introducing specific, single base substitutions into the *zfperiod4* promoter, we used a Quickchange site-directed mutagenesis kit (Stratagene) according to the manufacturers instructions. The plasmids pGL3basic 3.1Kb, pGL3basic 1.7Kb and pGL3basic 0.4Kb were used as templates for mutagenesis in combination with mutagenic primers (MWG biotech).

2.9 Raising zebrafish larvae

The zebrafish Tübingen strain was maintained and crossed according to standard methods (Westerfield, 2000). Fertilized eggs were collected within 2 hr of laying and rinsed well, and aliquots of 35 were transferred into 20 ml of E3 buffer (Dekens et al., 2003a) in 25cm^2 tissue culture flasks. Flasks were sealed and then submerged horizontally in large-volume, thermostatically controlled water baths to maintain a constant temperature. Larvae were illuminated with a tungsten light source (11μW/cm^2) connected to a programmable timer.

2.10 Western blot analysis

For western blot analysis, whole cell protein extracts were prepared from confluent 25cm^2 flasks of PAC-2 cells. The medium was aspirated, the cell monolayer was rinsed twice with 1xPBS and then extracts prepared by direct addition of 700μl of laemmli solution (2x Stock solution, (Sambrook et al., 1989)). Repeated pipetting of the cell lysate against the culture surface using a 1ml automatic pipette was performed to ensure complete lysis. The samples were heated for 5min at 95^{o}C to denature the proteins before storage at -20^{o}C. The quality and the quantity of the protein samples was checked by electrophoresis of a10μl aliquot on a10% SDS-polyacrylamide gel (including a 5% stacking gel) followed by staining with Coomasie blue using a standard protocol (Sambrook et al., 1989). A Miniprotean III gel system (BioRad) was

used for this and all subsequent electrophoresis and transfer steps according to the manufacturers instructions. Having adjusted the volumes of the extracts to ensure equal loading, samples were then reloaded on 8% or 10% polyacrylamide-SDS gel for electrophoresis and subsequently transfered to a nitrocellulose membrane (PROTRAN BA 83, Schleicher & Schuell) at 100V for 1 hr using the miniprotean III apparatus according to the manufacturer's recommendations. Transferred membranes were then subsequently blocked for two hours at RT in 1x Tris Buffer saline supplemented with 0.1% Tween 20 and 5% BSA. Primary antibodies (Phospho p44/42, Phospho PKC, New England BioLabs) were used at a concentration of 1:1000. Immunoreactive bands were visualized by using anti-rabbit IgG secondary antibody (1:5000). Signals were then detected using 1ml of ECL plus western blotting detection reagents (Amersham Biosciences) for each membrane and exposed on a scientific imaging film (Kodak Biomax) for between 5sec and 10min depending on the intensity of the signal.

2.11 Whole mount *In situ* hybridization

Freshly fertilised eggs (~35-40) were collected, cleaned and transferred into 20ml of E3 buffer with 0.2mM PTU (1-phenyl-2-thiourea) in 25cm^2 tissue culture flasks (Dahm and Nüsslein-Volhard, 2002). These flasks were then sealed and incubated in large volume thermostat-controlled water baths and illuminated with a tungsten light source (11 μW/cm^2) to ensure a constant incubation temperature of 25^{o}C. The developing embryos were then exposed to various lighting regimes before collection and fixation in 4% Para formaldehyde (PFA) O/N at 4^{o}C. The samples were then processed according to an established protocol (Oxtoby and Jowett, 1993). Hybridization was performed using dioxigenin (DIG)-labelled RNA sense and antisense probes (1:450). The *Per4* probe was synthesized by first cloning a 1.7kb Sph-I-Spe-I fragment of the *Per4* cDNA (nucleotide position 3501-1793) into the pGEM T-easy vector. T7 or Sp6 RNA polymerase was then used to synthesize the

antisense or sense probes respectively, after linearizing the plasmid at appropriate polylinker restriction sites. After incubation of the hybridized embryos with anti-dioxygenin-AP Fab fragment (at a 1:4000 dilution, Roche) the embryos were stained with a mixture of Nitroblue tetrazolium chloride/5-bromo-4chloro-3-indolyl phosphate (NBT/BCIP, Roche). 2-3 hrs of staining was sufficient to detect the alkaline phosphatase signal in embryos hybridized with the antisense probe. Samples were then washed several times in PBTX (1xPBS+0.3% TritonX-100) and fixed in 4% PFA for 1 hr. After gradual hydration, samples were incubated in 100% methanol overnight at 4^{o}C. They were then sequentially processed by first transferring into 25%, 50% and 75% glycerol in PBTX and finally stored at –20^{o}C.

2.12 Transient transfection

pBluescript II SK *Per4* I Sce-I plasmid was generated by cloning the 3.1kb *Period4* promoter sequence and luciferase reporter gene into the BamHI site of the pBluescript II SK (-) vector (Thermes et al., 2002). This vector was modified to carry two I-*SceI* meganuclease recognition sites flanking the multiple cloning site. To prepare the PAC-2 cells for transient transfection, 5 X 10^5 cells were plated into each 25cm^2 flask and incubated at 25^{0}C for 4 days (one 25cm^2 flask was used per transfection). The cell monolayer was washed twice with 1xPBS and then processed as described previously (see section 2.4). 10µg of the super coiled plasmid DNA was co-transfected with 10µg of pCMV I-SceI expression vector (Rouet et al., 1994) and 5µg of the carrier DNA. The electroporated cell aliquots (~500µl) were diluted with 1ml of L-15 medium and 250µl was plated into each well of a 96 multiwell plate and counted on the Packard scintillation counter.

2.13 Preparing transient transgenic embryos

To generate transient transgenic zebrafish larvae, 10ng of plasmid DNA were mixed with 5 Units of the *I-SceI* meganuclease enzyme + 1.5µl of the buffer

(1x final concentration) (BioLabs) in a final volume of 30µl. The samples were then microinjected using an established protocol (Dahm and Nüsslein-Volhard, 2002) into one cell stage of the embryos. They were incubated at 28^0C for 3 days and then the larvae were aliquoted into 96 well plates and sealed with "Topseal" a plastic. The bioluminescence was then counted on the Top-count NXT scintillation counter for 2 days on a LD cycle.

3 Results

3.1 Light-regulated clock genes

In mammalian systems, phase shifting signals such as light pulses characteristically result in rapid and transient induction of expression of the "*period*" clock genes in the SCN (Zylka et al., 1998). Three *period* gene homologs have now been described in zebrafish which show a circadian rhythm of expression and are acutely induced by light pulses (Pando et al., 2001). In the Zebrafish, many genes are encountered with more copies than their mammalian counterparts. This is thought to be the consequence at least in part, of a genome duplication that occurred during early teleost evolution (Postlethwait et al., 1998). It is therefore possible that additional *period* gene family members may exist in zebrafish as in the case of the cryptochrome genes where 6 have been described in zebrafish compared with two in the mouse (Kobayashi et al., 2000). We therefore chose to search for additional *period* gene homologs in zebrafish that might be regulated directly in response to light.

Our initial strategy was to clone *period1* and *period2* homologs from zebrafish since at that stage, only the sequence of *zfperiod3* had been published (Delaunay et al., 2000). We compared *Xenopus period1* cDNA database entries (Zhuang et al., 2000) with human *period1* (Tei et al., 1997) in order to identify the most conserved nucleotide sequences that could be PCR targets for cloning the corresponding zebrafish homolog. We designed 27mer primers based on these conserved regions (see section 2.1). For the *period2* homolog, we designed primers to amplify a zebrafish EST sequence (AW280601.1) that showed strong homology with *period2* cDNA sequences from chicken (Okano et al., 2001). We then used these oligonucleotides to prime long distance PCR on high molecular weight zebrafish genomic DNA (*period1* primers) or to amplify cDNA fragments by RT-PCR from PAC-2 cell total RNA (*period2* primers). In the case of the *period2* primers, we amplified a 549 bp cDNA fragment by RT PCR and for *period1*, a 13kb genomic fragment was successfully amplified, although attempts at RTPCR with the same primers

failed. Sequencing of the PCR fragments confirmed that the amplified *period2* coding sequence was identical to the zebrafish EST database entry, while the *period1* related genomic sequence was most similar to other *period1* vertebrate database entries (see Figure.5).

During this project, results were published by Pando and colleagues, documenting the expression patterns of *zfperiod1* and *2* in the zebrafish cell line, Z3, although no sequence data was presented (Pando et al., 2001). Both genes were reported to show high amplitude day-night rhythms of expression and an acute induction of mRNA expression in response to light pulses given after a prolonged exposure to DD conditions. Subsequently, the full length cDNA sequence and expression pattern of the zebrafish *period 2* homolog was reported (Delaunay et al., 2003). We therefore decided to compare the expression of our *period 1* gene homolog with that of the partially documented *zfperiod1* gene (Pando et al., 2001). We subcloned a cDNA fragment from our *period1* homolog, by RT PCR with primers based on the genomic DNA sequence. Then we used this to generate a specific ^{32}P-labelled antisense riboprobe for a RNAse protection assay (RPA) analysis. We initially analysed the expression profile of our *period1* gene homolog in PAC-2 cells and zebrafish larvae maintained under light-dark (LD, 12 hours light, 12 hours dark) cycles, constant darkness (DD) and during light exposure following an extended period in DD conditions. Our analysis revealed a high amplitude, 24-hour rhythm of expression under LD conditions with a peak at ZT3 in both the larvae and the cell line (3 hours after lights on) (Figure.6-A, B). On the 4th day in DD conditions which followed 3 days in a LD cycle, rhythmic expression persisted but with a dampened profile, expression tending towards levels intermediate between the peaks and troughs observed under LD cycles (Figure.6-C). This result is consistent with circadian clock regulation of this gene. Following 5 days adaptation to DD the cells were then re-exposed to light and after 3-5 hours, expression was strongly repressed until the end of the time course (Figure.6-D). Similar results were obtained with the larval RNA analysis except that a transient increase in expression occurred between 3 and 5

A)

```
ZFish: 21   KGVHM--QKNQELQSKKSPTKFVQKSPVVRPKDSAYPVNWRESQEEHRAAVQEELAFKDQ 194
            KG H+  +   +L+ K+ P+   Q  P  + KD         +  ++ A   EELA+K+Q
Chick: 577  KGQHIFTENKGKLEYKREPSAEKQNGPGGQVKDVIGKDTTATAAPKNVAT--EELAWKEQ 634

Zfish: 195  TVYSYQQISCLDSVIRYLESCNVPITVKRKCQXXXXXXXXXXDEDKQRNADSSMQVSEEP 374
             VYSYQQISCLDSVIRYLESCNVP T KRKC+          ++    NA
Chick: 635  PVYSYQQISCLDSVIRYLESCNVPGTAKRKCEPSSSVNSSVHEQKASVNA---------- 684

Zfish: 375  AHLKEQSGLSTLEVSKKPPGSGVVSPSLTPLALPSKPESVVSITSQCSYSSTIVHVGD 548
              ++     + L+ S K  G  VV   LT LALP KPESVVS+TSQCSYSSTIVHVGD
Chick: 685  --IQPLGDSTVLKSSGKSSGPPVVGAHLTSLALPGKPESVVSLTSQCSYSSTIVHVGD 740
```

B)

```
Xenopus: 1   agtggctgcagcagtgaacagtctgccagggagaaaaaccagaaagagctgatgaaggcc 60
             |||||||||||||||||||||||| ||| |||  |  |  ||||| || || ||||  ||
Human:   551 agtggctgcagcagtgaacagtcagcccgggcaaggactcagaaggaactcatgacagca 610
period 1 122  S  G  C  S  S  E  Q  S  A  R  A  R  T  Q  K  E  L  M  T  A

Xenopus: 61  cttaaagagctaaagattcggctcccatcagagaagaggctaaaaggcaagtctggcaca 120
             |||  ||||||| ||| |||| || ||  |||||    ||   || |||   ||||| ||
Human:   611 cttcgagagctcaagcttcgactgccgccagagcgccggggcaagggccgctctgggacc 670
period 1 142  L  R  E  L  K  L  R  L  P  P  E  R  R  G  K  G  R  S  G  T

Xenopus: 121 ctggcaagtctacagtatgccctgtcctgcgtcaaacaagtacgagcaaaccaagagtac 180
             ||||| |  || ||||| || ||| |||| ||||| || || |  || ||||| || |||
Human:   671 ctggccacgctgcagtacgcactggcctgtgtcaagcaggtgcaggccaaccaggaatac 730
period 1 162  L  A  T  L  Q  Y  A  L  A  C  V  K  Q  V  Q  A  N  Q  E  Y

Xenopus: 181 tcccag 186
             | ||||
Human:   731 taccag 736

period 1 182  Y  Q
```

C)

```
Xenopus: 251  agcagagagagcagcaacaccagcaaatactttgggagcatcgactcctccgag 304
              |||||  |||||||| |||| |||||||||||||| |||||||||||| ||||||
Human:   3449 agcagccagagcagccacacaagcaaatactttggcagcatcgactcttccgag 3502
period 1 1088  S  S  Q  S  S  H  T  S  K  Y  F  G  S  I  D  S  S  E
```

Figure.5

A) BLAST alignment of the amino acid sequence encoded by the zebrafish EST clone: AW280601.1, and the sequence for chicken *period2*. This represents the highest scoring alignment in a BLAST search of GenBank using the EST sequence. This strongly suggests that the zebrafish EST represents the *zfperiod2* gene.
B) Design of the 5' primer for PCR of the *zfperiod1* homolog. Alignment of the *Xenopus* cDNA fragment (AF250547) from the *period1* gene with the human *period1* full length cDNA (NM_002616) to identify a region of high nucleotide homology. The arrow indicates the position of the 5' primer based on *Xenopus* sequence.
C) Design of the 3' primer for PCR of the *zfperiod1* homolog. Alignment of the *Xenopus* IMAGE cDNA clone, BE679697, with the human full length *period1* cDNA sequence to identify regions of high nucleotide homology. The arrowhead indicates the position of the primer designed with the sequence complementary to the *Xenopus* sequence.

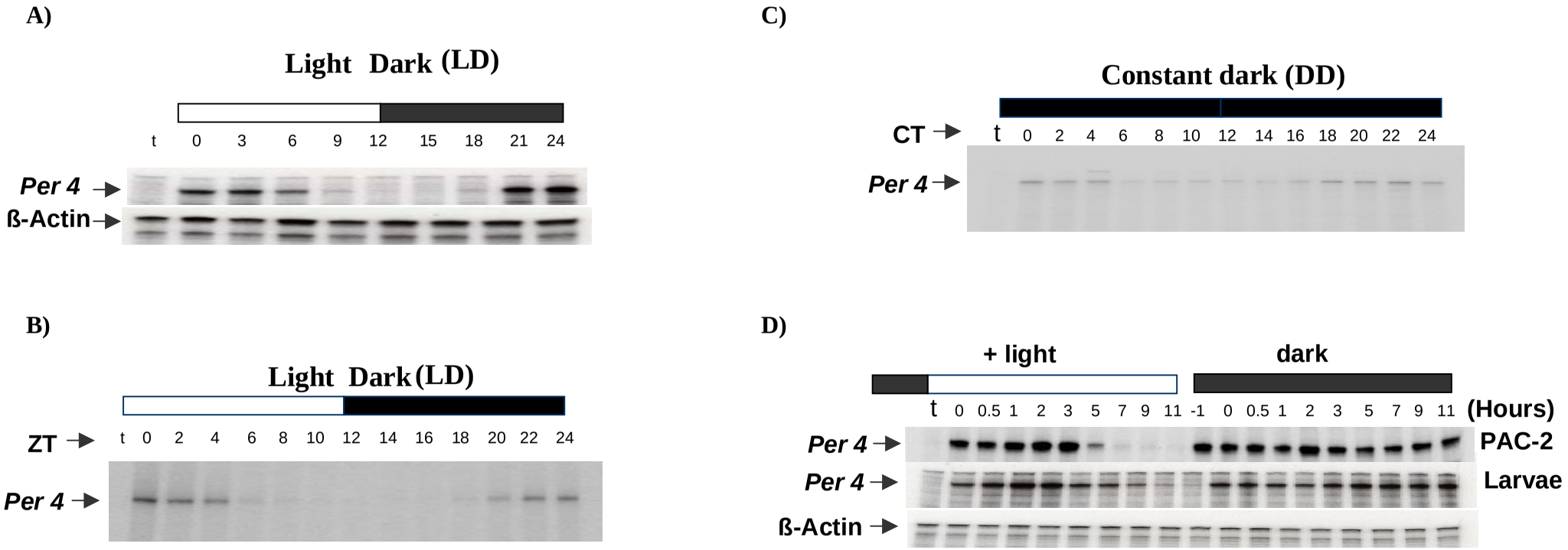


Figure.6

High resolution RNAse protection assay (RPA) of expression of the *zfperiod1* homolog transcript under LD conditions. A) Zebrafish were raised until the 6 days old larval stage under LD conditions and then harvested each 3 hours during 24 hours for RNA extraction. RPA analysis was then performed using the *zfperiod1* gene homolog-specific riboprobe. White and black bars above each panel represent the periods of light and dark conditions (12 hours of light, 12 hours of dark). The time for each point is labeled as zeitgeber time (ZT) – where ZT0 is defined as "lights on" and each zeitgeber unit is equivalent to 1 hour of clock time. B) Equivalent analysis of PAC-2 cells. Cells were incubated for 5 days prior to the 24 hour harvesting period. C) High resolution RPA analysis of PAC-2 cells maintained in DD conditions. Cells were maintained initially for 3 days in LD conditions and then for 4 days in DD. During the fifth day in DD, cells were harvested each 2 hours for 24 hours. D) Acute regulation of *Period* gene expression by light in zebrafish PAC-2 cells and larvae: RPA analysis of the expression of the *zfperiod1* gene homolog in PAC-2 cells and larvae exposed to DD for 5 days, then at time 0, illuminated for 30mins, 1, 2, 4, 6, 8 or 11 hours (+light). Controls remained in DD (dark) and were harvested at the same time points as the light-treated cells and larvae. A strong repression of expression occurs after 3 – 5 hours of illumination with different kinetics in the PAC-2 cells and larvae. Expression of ß-actin was assayed as a loading control in both larval and cell extracts.

hours after illumination prior to the strong repression that continued for the remainder of the time course (Figure.6-D). We also assayed the expression of our *period2* cDNA in the same RNA extracts and obtained results consistent with the published reports. Specifically, our data pointed to *zfperiod2* being a gene that is expressed only upon exposure to light and therefore little or no expression is detected under constant darkness conditions (data not shown). However, the expression properties that we observed for our *period1* homolog were significantly different from the published *zfperiod1* expression data (Pando et al., 2001). Specifically no repression of expression by light was documented for *zfperiod1*, only a transient induction upon light exposure that followed a period of adaptation to DD conditions was evident. In the absence of published sequence data, we were unable to compare directly the expression of our clone with that of the published *zfperiod1* gene. However, given the striking repression by light we chose to consider our clone as a novel *period* gene and so we have termed it *period4*.

Given the combination of light and clock regulation of *zfperiod4* expression we chose to characterize this gene in greater detail. As a first step, we used RACE-PCR to extend our initial cDNAs in order to obtain the full-length coding sequence (Figure.7-A). By comparison of this cDNA with the genomic sequence, we also determined the gene's exon-intron organization (Figure.7-B). Our sequence analysis confirms the high degree of similarity with *period1* homologs from other organisms (Figure.7-C). Also the position of introns within the *zfperiod4* gene matches well the location of introns in the mouse *period1* gene (Hida et al., 2000).

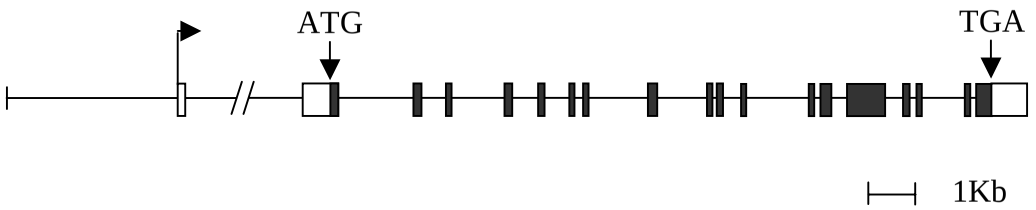
3.2 Expression of the zebrafish *Period4* gene during early embryonic development.

A characteristic property of the circadian system of the adult zebrafish is the presence of independent, light entrainable oscillators in most peripheral tissues (Whitmore et al., 2000; Whitmore et al., 1998). We wished to study the spatial pattern of *period4* expression in 5 days old larvae that were raised under an LD

A)

```
zfPer4: KDSAMGMETTEXXXXXXXXXXXXXXXXXXXXXXXXXEQDPPSTSGCSSDQSARVQTQKE 167
mPer1:  KDSAL-LETTESSKSTNSQSPSPPSSSIAYSLLSASSEQDNPSTSGCSSEQSARARTQKE 137

zfPer4: LMRALNELKIRLPPERKMKGRSSTLNALKYALSCVRQVRANREYYHQWNVEECHGCSLDL 227
mPer1:  LMTALRELKLRLPPERRGKGRSGTLATLQYALACVKQVQANQEYYQQWSLEEGEPCAMDM 197

zfPer4: STFTVEELDNITSEYTLKNTDTFTMAVSFLSGKVVYISPQGSSLLRSKPERLHGVLFSEL 287  PAS A
mPer1:  STYTLEELEHITSEYTLRNQDTFSVAVSFLTGRIVYISEQAGVLLRCKRDVFRGARFSEL 257

zfPer4: LAPQDVSTFYSNTAPCKLPAWASCIGSVSPPMECTQEKSMSCRISGDVSSSSDVRYYPFR 347
mPer1:  LAPQDVGVFYGSTTPSRLPTWGTGTSAGSGLKDFTQEKSVFCRIRGGPDRDPGPRYQPFR 317

zfPer4: LTPYLLTLRDSDMAFPQPCCLLIAERVHSGYEAPRIPLDKRIFTTSHTPSCVFQEVDERA 407  PAS B
mPer1:  LTPYVTKIRVSDGAPAQPCCLLIAERIHSGYEAPRIPPDKRIFTTRHTPSCLFQDVDERA 377

zfPer4: VPLLGYLPQDLVGTPVLLCIHPDDRHIMVAIHKKILQFAGQPFEHSPLRMCARNGEYMTI 467  PAC
mPer1:  APLLGYLPQDLLGAPVLLFLHPEDRPLMLAIHKKILQLAGQPFDHSPIRFCARNGEYVTM 437

zfPer4: DTSWSSFINPWSRKVAFIVGRHKVRTSPLNEDVFTPPRGLEERALTPDIVQLSEQIHRLL 527
mPer1:  DTSWAGFVHPWSRKVAFVLGRHKVRTAPLNEDVFTPPAPSPAPSLDSDIQELSEQIHRLL 497

zfPer4: VQPVHCGSSQ---GYGSLPSNGSHEHQPXXXXXXXXXXXXXXXXQLHKPMTFQQICKDV 584
mPer1:  LQPVHSSSPTGLCGVGPLMSPGP-LHSPGSSSDSNGGDAEGPGP---PAPVTFQQICKDV 553

zfPer4: HMVKTNGQQVFIDSRNRPPPKKHSTA-GALKAGQSAEVCRSLVXXXXXXXXXXXXXLIVQ 643
mPer1:  HLVKHQGQQLFIESRAKPPPRPRLLATGTFKA--KVLPCQSPNPELEVAPVPDQASLALA 611

zfPer4: KEPP-----TTFSYQQINCLDSIIRYLESCNVPNTVKRKCGXXXXXXXXXXXXKQQEA- 697
mPer1:  PEEPERKETSGCSYQQINCLDSILRYLESCNIPSTTKRKCASSSSYTASSASDDDKQRAG 671

zfPer4: ---PGNAKGPSVSLVDDSALLP----------PLALHNKAESVASVTSQCSFSSTIVHV 743
mPer1:  PVPVGAKKDPSSAMLSGEGATPRKEPVVGGTLSPLALANKAESVVSVTSQCSFSSTIVHV 731

zfPer4: GDKKPPESDIVMEEAPP--TPNTA-LPVTQPQFPPMATPSLPLSXXXXXXXXXXXXXXXX 800
mPer1:  GDKKPPESDIIMMEDLPGLAPGPAPSPAPSPTVAPDPTPD------------------- 771

zfPer4: XXXERLGLTKEVLSAHTQQEEQNFMCRFGDLSKLRVFDPTSAVRRRPNAPLSRGVRCSRD 860
mPer1:  -AYRPVGLTKAVLSLHTQKEEQAFLNRFRDLGRLGLDTSSV------APSAPGCHHGPI 824

zfPer4: YPAAXXXXXXXXXXXXXLKHQESSEQTGS-CSPAGPIRGLLPGVPALGRPSNPSIPMXXX 919
mPer1:  PPGRRHHCRSKAKRSRHHHHQTPRPETPCYVSHPSPVPSSGPWPPP---PATTPFP---- 877

zfPer4: XXXXXXXXXXXXXXVPNVQXXXXXXXXXXXXXXISHPVSDPSMQPGLRFPLQNSQXXXXX 979
mPer1:  ----------------------AMVQPYPLPVFSPRGGPQPLPPAPTSVSPATFPSPL 913

zfPer4: XXXXXXXXXXNYMFPQPSVGMAQPFYSPNSAFPFAAANMGSPAPCQIQTPIQRAHSRSST 1039
mPer1:  VTPMVALVLPNYLFPTPPSYPYGVSQAPVEGPPTPASH--SPSPSLPPPPL-------SP 964

zfPer4: PHSYSQRENGAEREGAESPLFQSRCSSP--LNLLQLEESPSNRFEVASGQQTTSPMVXXX 1097
mPer1:  PHR------------PDSPLFNSRCSSPLQLNLLQLEESPRTEGGAAAGGPGSS------ 1006

zfPer4: XXXXXXXXXXXXXXXVDSKTNENGETNESNQDAMXXXXXXXXXXXXXXXXXXXXXXXXX 1157
mPer1:  -----AGPLPPSEETAEPEARLVEVTESSNQDAL-----SGSSDLLELLLQEDSRSGTGS 1056

zfPer4: XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXKYFGSVDSS--ENSHSRKQTAE 1215
mPer1:  AASGSLGSGLGSGSGSGSHEGGSTSASITRSSQSSHTSKYFGSIDSSEAEAGAARARTEP 1116

zfPer4: GDGEAQLIKCVLQDPIWLLMANTDEKTMMTYQLPIRDRDSVLKEDRAALRAMQEHQPRFT 1275
mPer1:  GD---QVIKCVLQDPIWLLMANADQRVMMTYQVPSRDAASVLKQDRERLRAMQKQQPRFS 1173

zfPer4: EEQKSELSQVHPWIRTGRLPRAINISACAGCRSPPSVP 1313
mPer1:  EDQRRELGAVHSWVRKGQLPRALDVMACVDCGSSVQDP 1211
```

B)



C)

	Zf *Per 4* I	Zf *Per 4* P
mper 1	43%	52%
hPer 1	41%	50%
mPer 2	38%	50%
hPer 2	38%	50%
zfPer 2	38%	48%
zfPer 3	35%	46%
mPer 3	31%	44%

Figure.7

A) Sequence alignment of the *zfperiod4* and *mPer1* amino acid sequences using BLAST 2 sequence alignment software. Boxed amino acids are the two PAS domains A & B and the PAC domain, lying C-terminal to the PAS B domain. The '+' symbol represents alignment of non-identical amino acids with similar chemical properties. B) Genomic structure of the *zfperiod4* gene, shown by alignment of the cDNA and genomic PCR sequences. Black boxes represent the translated sequence of exons and open boxes, untranslated exon sequences. Lines linking the exons are introns, with the first, 12.5Kb intron represented by a broken line. An arrowhead indicates the start site of transcription. A scale bar beneath the scheme represents 1Kb. C) The percentage identity (I) and similarity (P) between the mouse, human and zebrafish sequences are shown in the left panel.

cycle using *in situ* hybridization. These larvae were treated with 1-Phenyl-2-thiourea (PTU) to avoid pigmentation of the skin and so facilitate the examination of internal staining (Dahm and Nüsslein-Volhard, 2002). Pools of larvae were harvested and fixed at the time points ZT3 and ZT12 representing the peak and trough points of the expression rhythm defined by our previous analysis (Figure.6-A). Both sense as well as antisense riboprobes were prepared from the *zfperiod4* cDNA in order to control for non-specific binding of the RNA probe. Using the specific antisense probe, we found that *zfperiod4* expression is predominantly localized in the brain region at ZT3 (Figure.8-A, Left panel) while no expression was detected at ZT12 (Figure.8-A, right panel), consistent with the results of our previous high resolution RPA analysis (Figure.6-A). At the peak point, ZT3, no expression was detected in the tail region of the larvae. This result pointed to the possibility that the clocks in peripheral tissues outside of the brain might not have matured by this stage of development or that the *in situ* hybridization assay was not sensitive enough to detect lower levels of expression in the tail region. So, to resolve this issue we used the more sensitive and quantitative RPA analysis to assay *period4* mRNA levels in RNA extracts prepared separately from the larval head and tail regions (Figure.8-B, C). Embryos were raised from fertilization at 25°C under a LD cycle and then harvested between day 4 and day 7 at stages corresponding to the early larval development. On each day, pools of ~35 larvae were harvested at ZT3 and ZT12, bisected using a scalpel and then RNA extracts prepared separately from the two body sections. RPA analysis revealed rhythmic *zfperiod4* expression in both the head and the tail region with lower levels and a lower amplitude evident in the tail region (Figure.8-B). No oscillation was observed with the ß-actin gene, which served as a loading control for this experiment (Figure.8-B). These results indicate that the failure of *in situ* hybridization to detect *zfperiod4* expression in the tail region of the larvae results from the sensitivity of our assay being insufficient to detect the lower expression in these sites. However, our results do imply the presence of

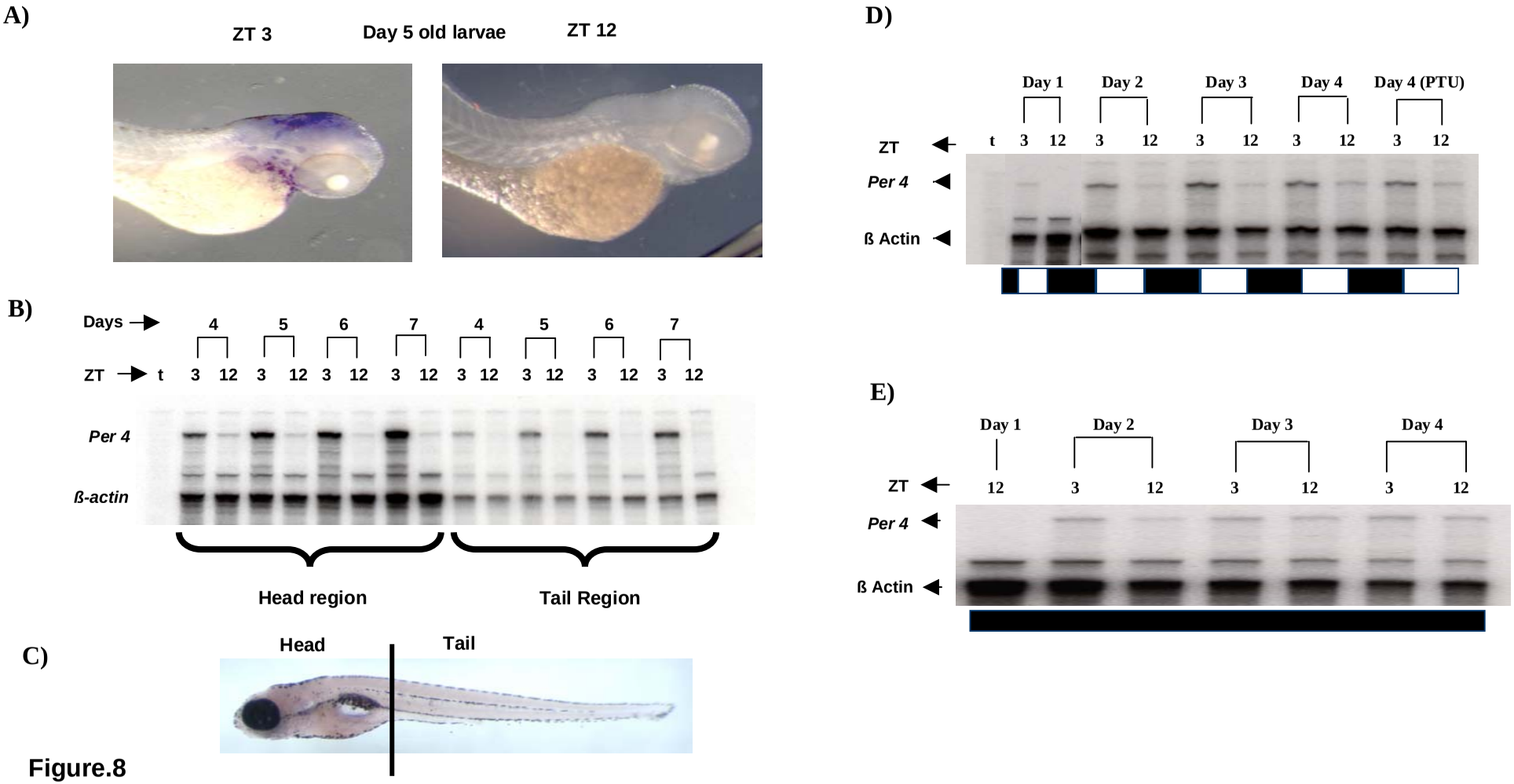


Figure.8

Developmental and spatial expression pattern of the *zfperiod4* gene in larvae. A) *In situ* hybridisation of 5-day old larvae probed using a *zfPeriod4* gene specific antisense riboprobe. Specific staining with alkaline phosphatase is found only in the brain region from larvae collected at ZT3 while no expression is detected at ZT12. B) RPA analysis of *zfperiod4* and *zfß-actin* expression in RNA extracts prepared from the head or tail region of developing larvae from day 4 to day 7 under a LD cycle at the ZT3 and ZT12 time points each day. Rhythmic *zfperiod4* expression was observed in both the head and the tail region, with significantly lower levels in the tail. C) Diagramatic representation of zebrafish larvae indicating our definition of the head and the tail regions D) *zfperiod4* gene expression during embryonic and early larval development under LD cycles. Pools of embryos or larvae (~35) were collected from Day 1 to Day 4, each day at ZT3 and ZT12 (times identified as representing the peak and trough points of *period4* rhythmic expression in 6 days old larvae (see Figure 6-A)) and RPA analysis was then performed for *zfperiod4* and *zfß-actin* expression. From day 2, a rhythmic pattern of *zfperiod4* expression was observed. Constant expression of the ß-actin gene confirmed equal loading E) Equivalent analysis of embryos raised from the late blastula stage in DD conditions. In this case, a shallow rhythm of expression was visible on day 2 however, on days 3 and 4 constant expression was observed.

significant tissue-to-tissue variability in the expression levels of this gene in the larva, with highest levels present in brain structures.

We next performed a systematic study of the expression of the *Period4* gene during early zebrafish development from just after fertilization until early larval development. To perform an accurate quantitative analysis we chose to assay the levels of the mRNA in whole embryo RNA preparations using RPA analysis. Embryos were raised in LD or DD conditions at 25°C and total RNA extracts were prepared from pools of ~35 embryos from day 1 to day 4 at ZT3 and ZT12 each day. ZT0 corresponds to "lights on" and on day 1 represents the time that the eggs were laid and fertilized (Figure.8-D). In embryos raised under LD conditions, a peak trough difference in expression was already visible from the first day, which then progressively increased in magnitude until the end of the time course (Figure.8-D). Under DD conditions, the same shallow ZT3 – ZT12 difference was visible on day 1 (Figure.8-E). Subsequently, an even shallower difference was seen on day 2 and then no significant oscillation was detected on the last two days (Figure.8-E). The RPA analysis using the β-actin probe confirmed equal loadings of RNA in the assay. This result implies that the presence of a LD cycle is essential for the maturation of high amplitude rhythms of clock gene expression during early zebrafish development but not for establishing the basal expression of clock genes.

3.3 Cloning of the *zfPeriod4* promoter

In order to explore the regulation of *zfperiod4* in more detail we chose to isolate the promoter and use this as the basis for a luciferase reporter gene construct. In the context of stably transfected PAC 2 cells, we planned to explore the regulation of this promoter by the endogenous, light entrainable clock. We initially identified the start site of transcription by performing 5' RACE PCR. We cloned and sequenced all the amplified products and then compared the sequence of the longest clones with the genomic sequence. This

analysis revealed the presence of a 5' untranslated region of 609 bp. We then designed nested, 27 mer PCR primers to perform Long Distance PCR on PAC-2 cell genomic DNA using a "Genome Walker" kit. After a series of three genome walker PCR reactions, a total of 3.1kb of DNA, 5' of the *zfperiod4* start of transcription was cloned. This entire 3.1kb region was then PCR-amplified using *period4*-specific, 27mer primers at the 5' and 3' extremities. This fragment was cloned first into pGEM Teasy for sequence analysis (see Figure.9) and then recloned into the Xho I-Hind III polylinker sites of the pGL3 basic expression vector (Appendix-1). This vector provides a polylinker for cloning promoter regions upstream of the firefly luciferase cDNA sequence.

3.4 Transfection of plasmid DNA into PAC 2 cells and selection

There had been no previous reports of the transfection of the PAC-2 cell line. We chose initially to test an electroporation-based protocol. We optimized transfection conditions by electroporating aliquots of PAC-2 cells with a commercial expression vector plasmid encoding β-galactosidase: pcDNA 3.1/myc-His lacZ (Invitrogen). A β-galactosidase assay performed with crude extracts from the transfected cells was then used to determine relative transfection efficiencies. 20 μg or 25 μg of the supercoiled DNA from pcDNA 3.1myc-His lacZ was transfected together with different amounts of carrier DNA (pGEM Teasy) at different voltages with a capacitance setting of 960 μF in 0.4mm cuvettes. Following transfection, cells were plated in 10cm tissue culture dishes and the following day, dead, detached cells were removed by washing with 1X PBS and then fresh medium was added to the cells. After 48 hours the cells were harvested, protein extracts prepared and then assayed for β-galactosidase activity. Results from a Bradford protein quantification assay were used to adjust the β-Galactosidase activity measurements to represent 20μg total protein extract per sample (Table.1). From these results we selected the following optimal conditions for all subsequent electroporations: 25 μg of linearized plasmid DNA, 10μg pGEM Teasy carrier DNA diluted in a final

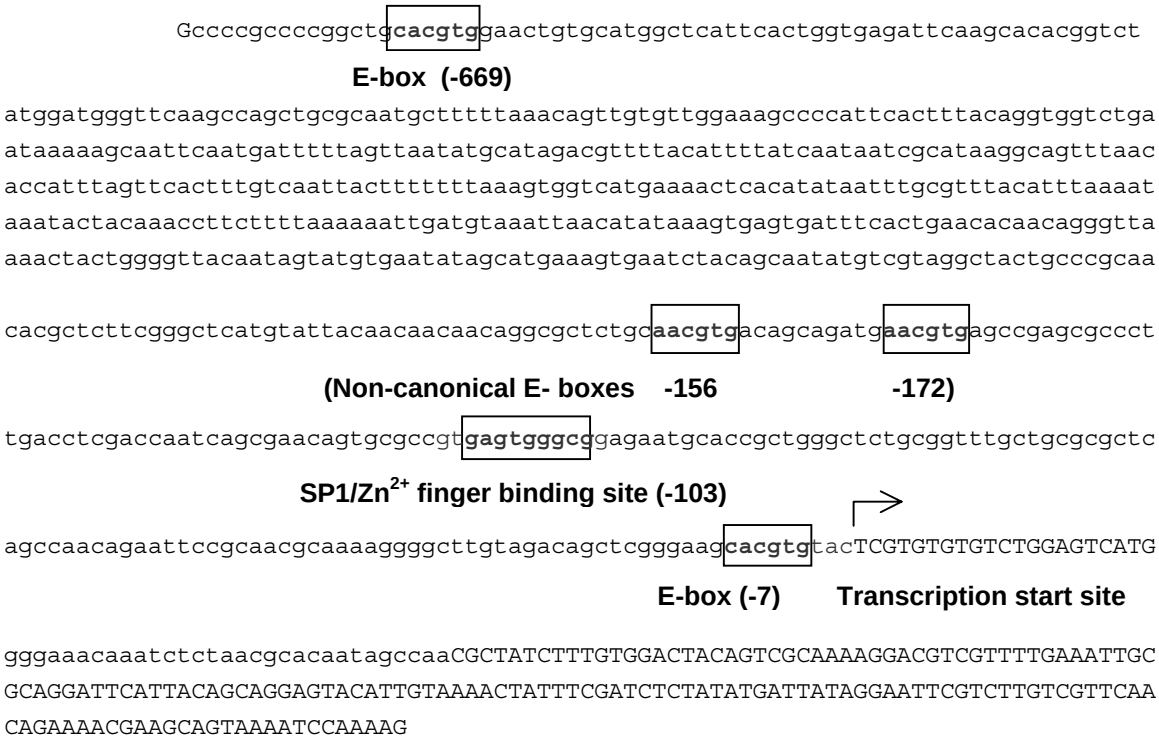


Figure.9

895bp genomic sequence flanking the *zfperiod4* transcription start site. Uppercase bases represent transcribed sequences, while lower case bases represent the upstream, non transcribed genomic region. An arrowhead indicates the start of transcription as defined by the 5' end of 5' RACE PCR clone sequences. The two consensus E-box elements, SP-1/Zn^{2+} finger binding site and, the two non-consensus E-box elements are highlighted in bold and by boxes

volume of 50µl with water, mixed with 0.5ml cell suspension, and then electroporated at 0.29KV, at 960 µF in 0.4mm cuvettes.

Our initial attempts to perform a transient transfection assay using the *zfperiod4* luciferase reporter construct resulted in failure to detect significant levels of bioluminescence in our *in vivo* luciferase assay. For this reason we chose to perform a stable transfection assay. Using the same electroporation conditions, we cotransfected the *zfperiod4* luciferase reporter plasmid together with a neomycin resistance-encoding plasmid pcDNA3.1NEO and subsequently selected for neomycin-resistant colonies. After one month of selection, approximately 200-300 resistant colonies, each consisting of several hundred cells, were visible on each plate. Control cells transfected with carrier DNA alone were all dead after 10 days from the start of selection.

Table.1

β-galactosidase Plasmid (0.8-1µg/µl stock)	**Carrier (pGEM Teasy DNA) (added as 1µg/µl stock and H_2O added to a final volume of 25µl)**	**Voltage (KV)**	**OD measured at 340nm. Values normalized for 20 µg protein**
20µg	10µg	0.23	0.035
20µg	10µg	0.24	0.029
20µg	10µg	0.25	0.1102
20µg	10µg	0.26	0.1396
20µg	10µg	0.27	0.098
20µg	10µg	0.28	0.3211
20µg	10µg	0.29	0.4555
20µg	10µg	0.30	0.4385
25µg	10µg	0.27	0.285
25µg	20µg	0.27	0.1944
25µg	25µg	0.27	0.216

Prior to picking the neomycin-resistant colonies, we confirmed luciferase expression, by adding 0.5 mM luciferin to the culture medium and then exposing the plates directly to X-ray film for 24 hrs in a dark room. The developed film revealed positive spots over each colony confirming the presence of bioluminescence originating from luciferase expression (Figure.10). Differences in intensity of the spots reflect differences in colony size but also possibly reflect differences in the number of plasmid copies or sites of plasmid integration between clones. For this reason, for the purpose of comparing the stable expression of different promoter constructs, we chose to pool together all the neomycin-selected colonies by trypsinization and subsequently analyzed the clones as a pool. Given that each pool consists of at least 200-300 colonies, this procedure should average out possible integration-specific effects. However, in addition we also picked individual, isolated clones from the plates, which represented a range of signal intensities. Several individual clones were then assayed for bioluminescence expression.

3.5 *In vivo* assay for *period4* expression

Cultures were assayed for luciferase activity *in-vivo* by first seeding them at confluence, in a 96 well plate. Then the medium was supplemented with 0.5mM luciferin and bioluminescence monitored using a Packard Topcount scintillation counter. This sensitive, 96 well plate scintillation counter has been widely used in other circadian clock studies e.g. with transgenic luciferase reporter lines of *Drosophila*, in order to monitor the low levels of bioluminescence typically obtained from such an *in vivo* luciferase assay (Brandes et al., 1996). It has the advantage that up to twenty 96 well plates can be counted in one experiment. A robotic plate handling system enables the counter to systematically monitor each well in each of the 96 well plates in a cycle, which can be repeated indefinitely. Data is sorted by the use of a bar code system that enables the counter to identify each plate being counted. This counter therefore, allows large numbers of samples to be counted automatically

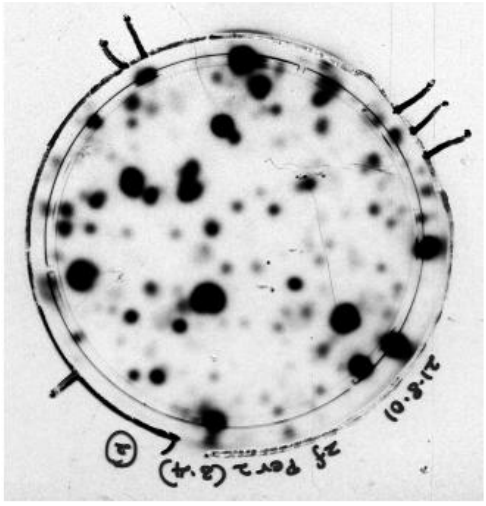


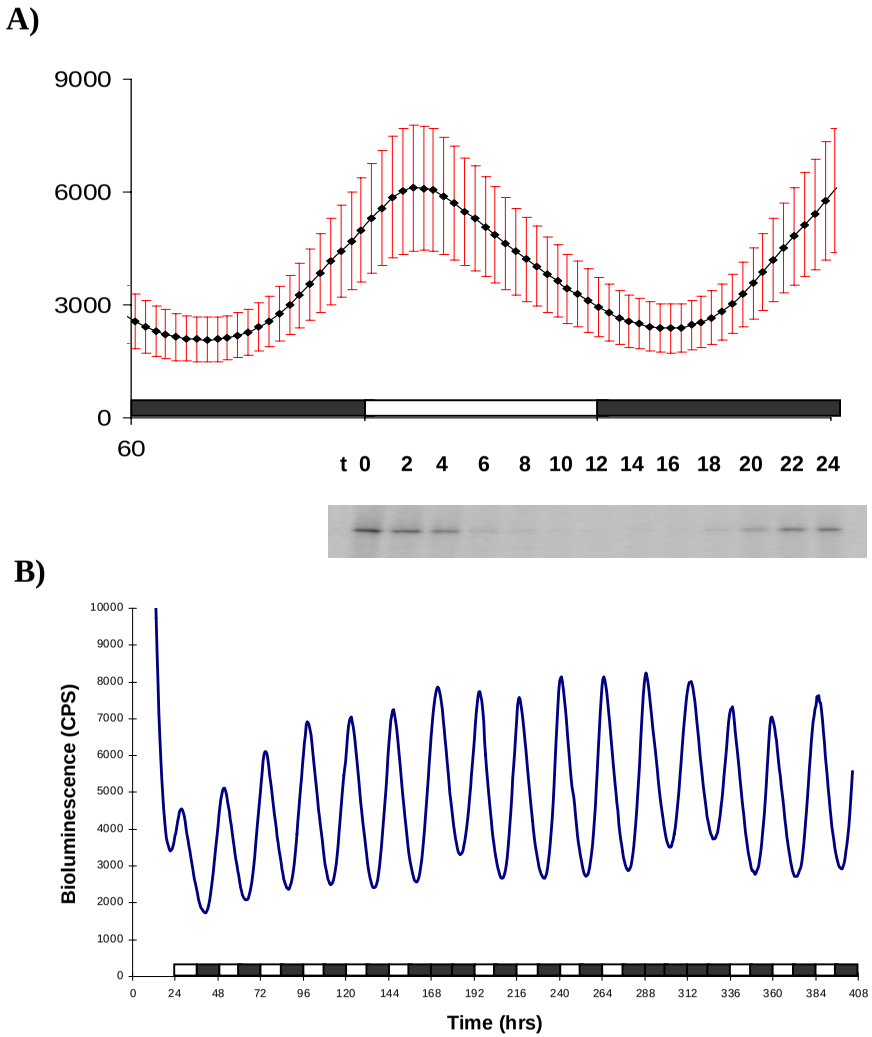
Figure.10

Visualizing luciferase positive, neomycin resistant colonies: The culture medium of a petri dish containing hundreds of neomycin-resistant, stably transfected PAC-2 cell clones was supplemented with 0.5 mM luciferin and then exposed on top of an x-ray film overnight. After developing the film, dark spots are visible which represent individual luciferase - positive colonies, due to light emitted from the luciferase-catalysed oxidation of luciferin. Differences in intensity may be due to variation in the number of copies of the gene inserted into the genome as well as the size of the colonies. The markings on the film and the plate are for alignment of the plate with respect to the film.

over an extended time period and therefore is ideal for a circadian clock assay. While plates are outside of the counting chamber they are held in "stacker" columns and so can be exposed to a LD cycle. Initially we maintained cells in an LD cycle and observed a robust daily rhythm of luciferase activity, which matched well our previous high resolution RPA data for expression of the endogenous *zfperiod4* gene (compare Figure.6-B and 11-A). A peak of expression was observed 3 hours after lights-on and a trough, 12 hours later at the beginning of the dark phase. This robust rhythm of expression continued for the duration of an extended experiment where cells were maintained for 16 days in LD cycle conditions (Figure.11-B). During two short periods of DD, the rhythms persisted but with a tendency to dampen slightly, consistent with a circadian clock regulation of zf*period4* expression. Return to an LD cycle however, within one or two days, restored the original rhythm amplitude. The observation of robust cycling for this extended time course also confirms the viability of the cell cultures during long periods at confluence. The initial "spike" of luciferase activity is an artefact, which follows the first addition of luciferin to the luciferase expressing cells. These artificially elevated levels are reduced progressively during the first 24 hours of the assay.

We next explored how changing the lighting regime affected expression of the reporter gene. Cells were exposed to an LD cycle for 3 days, then DD for 6 days, returned to an LD cycle for 3 days, then constant light (LL) for 3 days and finally returned to LD for 3 days (Figure.11-C). This analysis revealed the following properties.

1. Rhythmic expression established under an LD cycle persists under constant darkness, with a dampening profile tending towards a value in the midline of the LD cycle rhythm, a result consistent with our initial RPA analysis. We determined that the free running period length (τ) was 25.19 +/- 0.21 hours using CHRONO circadian rhythm analysis software (Vallone et al., 2004).



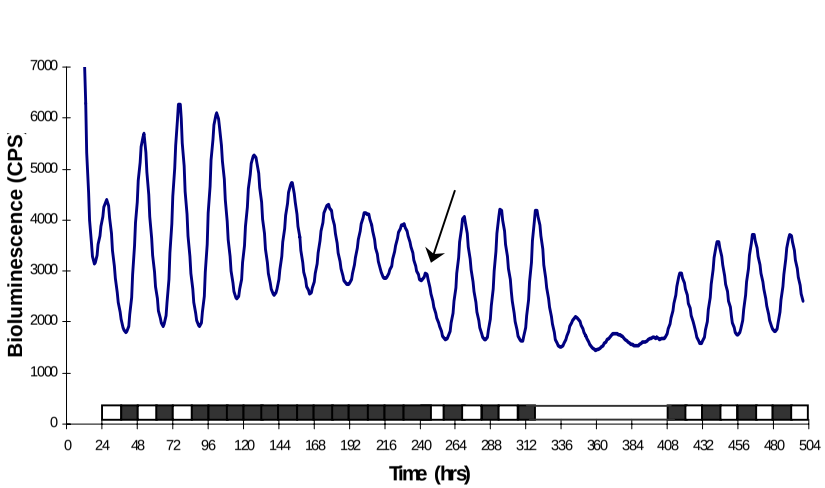


Figure.11

A) Alignment of the bioluminescence profile of the *zfperiod4* promoter-luciferase stable cell lines with the high resolution RPA analysis of endogenous *zfperiod4* expression in PAC-2 cells. This reveals a good correlation between the luciferase reporter activity and levels of the endogenous transcript. Changes in luciferase expression are delayed only by approximately 1 - 2 hours compared with the transcript levels. For the bioluminescence data, the scale on the 'y' axis represents bioluminescence measurements in counts per second (cps) plotted against time (ZT) on the 'x' axis. The white and black bar represents the duration of the light (12 hours) and dark (12 hours) periods. For each point, error bars represent the standard deviation. White and black bars show the light and dark periods. B) Rhythmic *zfperiod4* promoter luciferase reporter expression in cells maintained under LD conditions for 16 days.

The very high levels of bioluminescence recorded during the first 12 hours of the experiment represent a transient artifact observed immediately following the first addition of luciferin to the culture medium of luciferase-positive cells. During two short periods in constant darkness, the rhythms tend to dampen. C) Effect of different lighting regimes on the expression of the *zfperiod4*-luciferase reporter gene. Cells were maintained for 3 days under an L (12 hours); D (12 hours) cycle, then 6 days in DD, returned to LD for 3 days then 3 days in constant light (LL) conditions and finally 4 days in LD cycles. An arrowhead indicates the onset of the repression that occurs upon exposure to light following the period in DD conditions. In DD the dampening is relatively slow and tends towards the median of the peak and trough expression values observed in LD conditions, while in LL the dampening is more rapid and tends towards the trough values of expression under LD cycles.

2. After the first four hours following transfer from DD to the LD cycle luciferase expression is strongly repressed for the remainder of the 12 hour light period, consistent with RPA analysis of endogenous gene expression. Subsequently, during the dark phase expression increases once more and a 24 hour rhythm of expression is re-established. The normal phase relationship between this rhythm and the LD cycle is immediately established suggesting very strong phase shifting of the clock by light. Interestingly, while the trough values of the new rhythm are comparable with those under the original LD cycle, the peak values are lower and take several cycles to be restored. Thus there appear to be long term effects on the clock following exposure to DD conditions.

3. Exposure to LL leads to a strong dampening of the reporter gene rhythm, with expression tending towards the trough values observed under LD conditions, consistent with the notion that light exposure serves to repress *zfperiod4* expression.

Parallel analysis of independent clones revealed changes in rhythmic expression identical to those described above but with significant variation in the basal expression levels (data not shown). Given this variability we subsequently chose to pool neomycin-resistant colonies obtained from each transfection of all additional promoter constructs. The observed consistency of luciferase expression between independently-generated pools of cells for a given construct strongly suggests that this approach successfully "averages out" variability due to position and copy number integration effects (see Figure.11-A).

One potential complication in the interpretation of data from these luciferase-clock reporter cell lines is the possible direct entrainment of the cellular clock by photons emitted from within the same cell, by the luciferase catalysed oxidation of luciferin. The sub-cellular distances separating clock photoreceptors and luciferase-luciferin reaction complexes would be predicted to make this even more likely. We decided to test whether this was the case, by RPA analysis of endogenous *zfperiod2* gene expression in the cell lines. Given

the strong induction of *zfperiod2* in response to light pulses, we reasoned it would be a good indicator of "auto-entrainment" by light. We cultured two independent cell lines in a LD cycle for 3 days and then switched the cells into DD conditions for 3 days. Duplicate sets of cultures were maintained under identical conditions except that one set had luciferin included in the culture medium while the other set did not. On the fourth day we harvested aliquots of cells during a 24 hours time course and then assayed *zfperiod2* expression by RPA. We compared the results from cells including (luc+) and excluding (luc-) luciferin and did not encounter significant expression in either set of cell cultures (Figure.12). Thus we can infer that the luciferase-mediated reaction does not seem to influence the expression of *zfperiod2* in the cells in DD, at least within the limits of sensitivity of this assay. In addition, we compared the RPA analysis of the endogenous *zfperiod4* expression profile in DD with the bioluminescence profiles observed for the luciferase reporter construct in DD conditions. If bioluminescence was entraining the cellular clocks then one might anticipate that the rate of dampening of the luciferase reporter rhythm might be slower than that of the endogenous gene. Dampening of the endogenous *zfperiod4* rhythm in DD is similar to that of the bioluminescence rhythm. From these results, we conclude that the bioluminescence from the luciferase assay is insufficient either in intensity or wavelength, to directly influence the endogenous clock.

3.6 Phase response curve analysis

The rapid re-entrainment of the luciferase reporter rhythm following transfer of cells from constant darkness to a LD cycle hints at a rapid phase resetting effect of light on the PAC-2 cell clock. In order to confirm and quantify this effect we decided to perform a systematic analysis of the phase shifting effect of light using the luciferase-reporter cells. Depending upon which phase of the circadian cycle a light pulse is experienced, then the rhythm is either phase advanced or phase delayed. A systematic quantitative analysis of how light

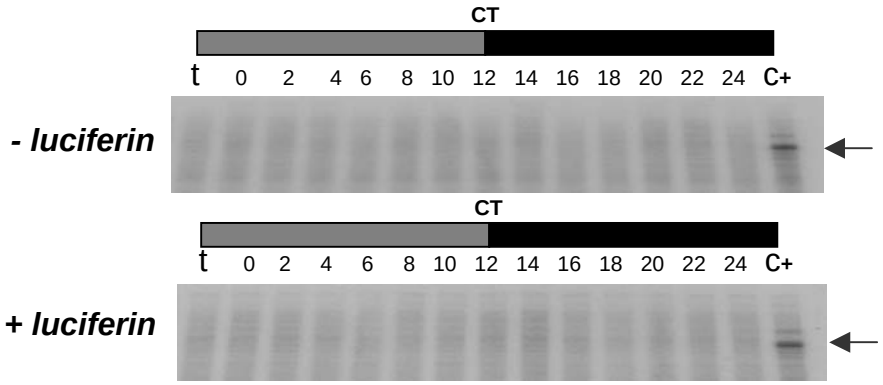


Figure.12

Comparison of the expression of the endogenous *zfperiod2* gene in luciferase reporter cell lines, with or without bioluminescence. Two separate pools of *zfperiod4*-luciferase cells were grown in constant temperature conditions on LD cycles for three days and then transferred to DD conditions for four days. On the fifth day in DD, cells were harvested at two-hour intervals for 24 hours to prepare RNA extracts for RPA analysis using a *zfperiod2* specific riboprobe. One pool of cells included culture medium supplemented with 0.5mM luciferin (+luciferin), while the other included normal culture medium (-luciferin). Time points are labeled as circadian time (CT) where time "0" represents 9:00am, the onset of the light period on the original entraining LD cycles. The dark and shaded bar shown above the samples indicates approximate subjective day (grey) and subjective night (black) for the cells, based on the timing of the original LD cycle. 't' represents a tRNA negative control reaction while 'c+' is a sample of RNA from wild type PAC-2 cells maintained on an LD cycle and harvested at ZT3 – the peak time of *zfperiod2* expression. The presence or absence of luciferin does not influence the very low basal expression of *zfperiod2* observed during the time course of the experiment. An arrowhead indicates the *zfperiod2* specific protected fragment

pulses phase shift a circadian clock can be performed to generate a so-called Phase response curve (PRC) (see Figure.13). Phase response curves have been calculated for many different plants, animals and unicellular organisms (Johnson, 1999) and they represent a valuable resource with which to predict various aspects of clock behavior including how rapidly a given clock will entrain to changes in the phase of the LD cycle. Experimentally, the conventional way to measure a PRC is to first adapt the animal to constant darkness over a period of several days and then, non-invasively, measure the phase of the free-running circadian clock rhythm. Subsequently it is exposed to a light pulse and after 48 hours, the new phase of the rhythm is compared with the original phase in order to measure the phase advance or delay that has occurred. The delay between giving the light pulse and measuring the new phase is important since there are frequently transient effects on the clock rhythm that may ultimately not reflect the stable re-entrainment of the pacemaker (Pittendrigh, 1993). The magnitude of the phase advance or delay is then plotted against the subjective time that the light pulse was delivered to obtain the phase-response curve. To date, such a PRC analysis has not been performed on a cell culture clock.

Given the high definition of the PAC-2 *zfperiod4* luciferase bioluminescence traces, it was relatively easy to very accurately measure the phase of the clock rhythms with a large number of samples. The luciferase reporter cells were plated in 10 plates in medium supplemented with luciferin. All plates were exposed for 3 days to an LD cycle and on the fourth day, were individually sealed in lightproof boxes. After 3 complete days in DD, individual plates were light pulsed for 1 or 4 hours, at 3 or 4 hour intervals respectively using a tungsten light source (20 $\mu W/cm^2$). One control plate remained in DD. Following the final light pulse, all plates were counted for 3 days in DD. Stable phase shifts for each light-treated plate relative to the DD control on the third day were then calculated. The time of onset of each light pulse was expressed in circadian time (CT) where CT0 is defined as the beginning of the subjective day and CT12, the beginning of the subjective night (Figure.13). 24 CT hours

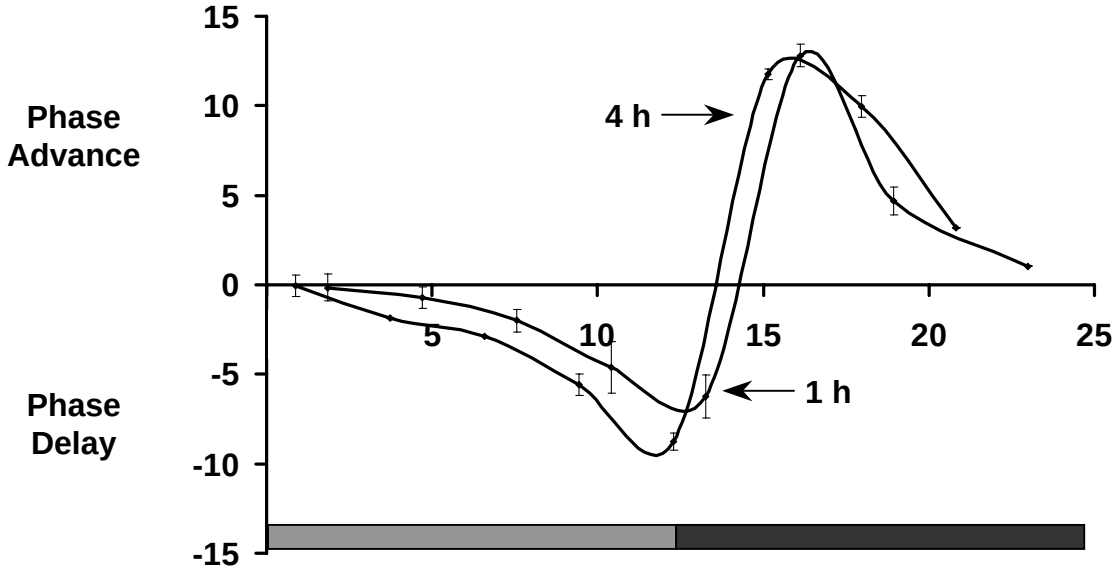


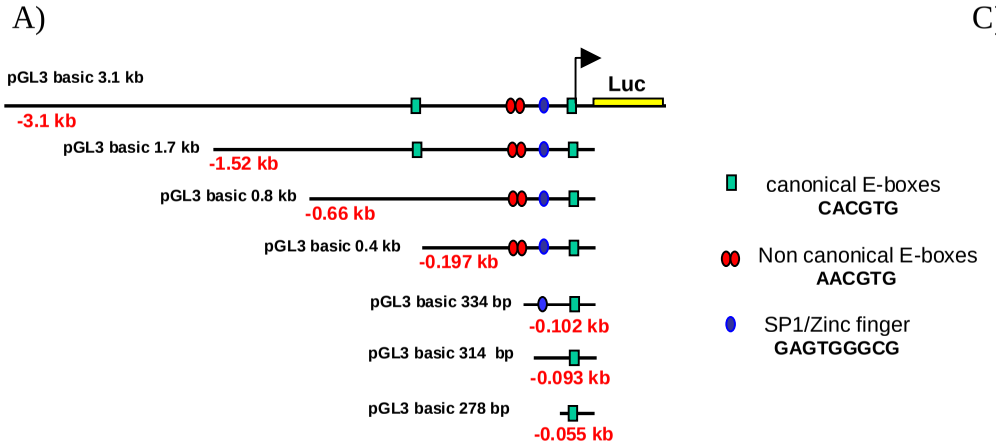
Figure.13

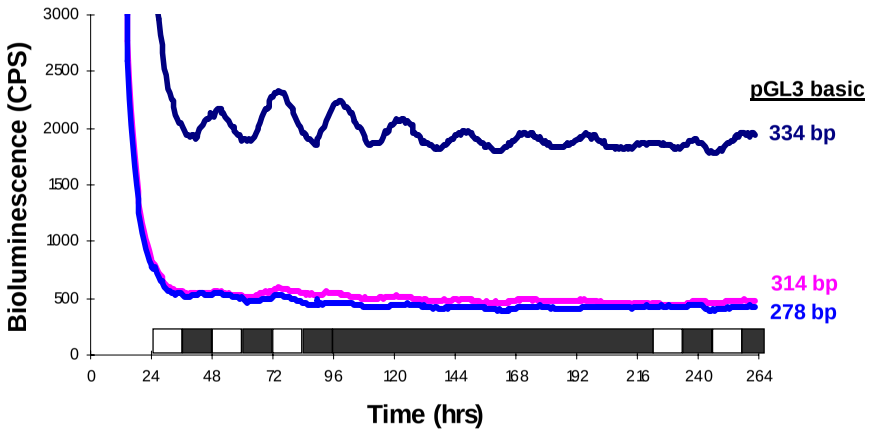
Phase-response curve analysis of PAC-2 cells for 1 or 4 hr light pulses (arrowheads). On the ‘x’ axis, represents the time of onset of each light pulse. On the ‘y’ axis positive values represent phase advance and the negative value phase delays. Calculations were based on data obtained from 16 independent culture wells for each of four independent experiments. Mean phase shifts are plotted together with error bars indicating standard deviation.

is the duration of one free running period. In terms of the *zfperiod4* luciferase rhythm, CT0 is the time point 3.2 "real" hours before each peak. Phase shifts were expressed as circadian hours, by multiplying "actual" hour times by 24/τ.
The phase shifting properties of 1 and 4 hour light pulses were comparable (Figure.13), with very large phase shifts induced by light pulses during the early subjective night (Johnson, 1999). These properties are consistent with our results showing that the clock entrains rapidly to large phase shifts in the entraining LD cycle.

3.7 Promoter analysis

We next wished to determine which regions of this 3.1kb promoter fragment were responsible for conferring rhythmic expression in LD and DD conditions, as well as the repression in response to light. We first determined the complete sequence of the 3.1kb fragment and compared this with the Genomatix mathinspector transcription factor binding site database. This analysis revealed the presence of two canonical E-box elements (CACGTG) and two non-canonical E-boxes (AACGTG) (see Figure.9). One of the canonical E-boxes was positioned just upstream from the start site of transcription (position –7), while the other was located further upstream (position –669). The important role played by the E-Box element in transcriptional regulation within the circadian clock mechanism suggested that these elements might direct regulation of this promoter by core clock components. Interestingly however, neither of the canonical sites corresponds to the optimal binding site for CLK-BMAL heterodimers defined in previous studies (Hogenesch et al., 1998).
We next prepared a series of promoter deletions to systematically truncate the 3.1 kb *zfPeriod4* promoter as shown in Figure14-A. Truncations were performed using PCR with a nested series of 5'primers all containing an engineered Xho-I site and a common 3' primer -located downstream of the transcription start site incorporating an engineered HindIII site. Promoter fragments were directly amplified from PAC 2 cell-derived genomic DNA. The fragments obtained were cloned into the XhoI-Hind III sites of the pGL3basic





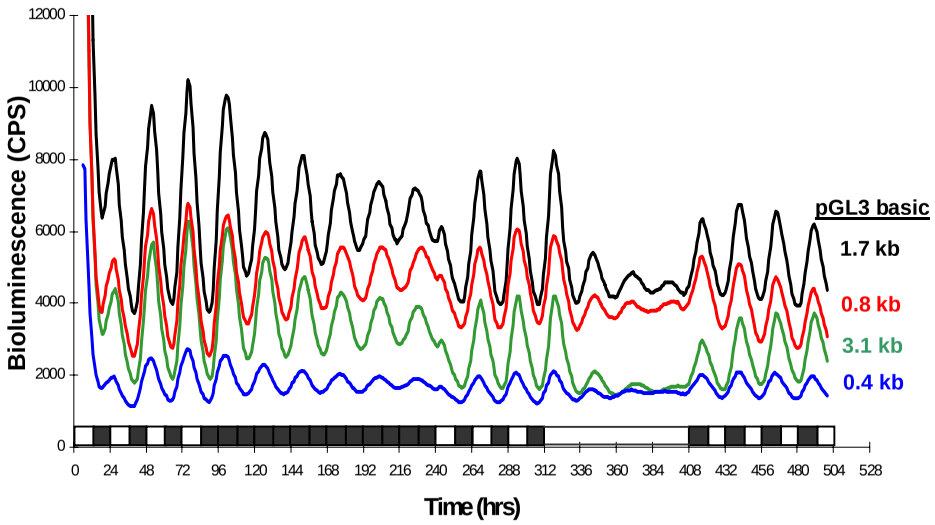


Figure.14

Zfperiod4 promoter analysis. A) Schematic representation of deletions prepared from the *zfperiod4* promoter. E-box enhancer elements identified by sequence analysis are represented by symbols. Green rectangles, Canonical E boxes; Red ellipses, non-canonical E boxes and blue oval, a SP1/Zinc finger binding site. The arrowhead represents the transcription start site. "Luc" is the luciferase gene coupled to the 3'end of the promoter within the plasmid pGL3basic. The value shown below each construct (-1.52 kb, 0.66 Kb etc) is the distance between the 5' end of the fragment and the transcription start site. B) Bioluminescence profile of the pGL3basic 3,1Kb, 1.7kb, 0.8Kb and 0.4Kb constructs stably transfected into PAC-2 cells, with pools of independent clones analysed using the same lighting conditions as described in figure 11-C. C) Analysis of the pGL3basic 334bp, 314bp, and 278bp constructs except that these transfected pools of cells were maintained for 3 days in LD, 5 days in DD and then returned for 2 days to an LD cycle.

vector and cotransfected into PAC-2 cells with the neomycin resistance conferring plasmid: pcDNA3,1 NEO. Analyzing the luciferase expression in pools of transfected cells under different lighting conditions using various constructs revealed that a minimum of 197bp (pGL3basic 0.4kb) upstream of the transcription start site was sufficient to maintain all the promoter properties although with a significantly reduced amplitude compared with the pGL3 basic 3.1Kb control (Figure.14-B). Interestingly, the magnitude of the repression by light following 6 days in DD conditions seems to be directly proportional to the amplitude of the rhythmic expression observed in LD conditions. The 197bp "minimal promoter" fragment includes the consensus E-box enhancer elements identified by sequence analysis excluding only the upstream canonical E-box (-669). Further truncations severely attenuated the expression rhythms with the 55bp promoter fragment construct (pGL3 basic 278bp) essentially arrhythmic with very low basal expression levels (Figure.14-C). This indicates the presence of key regulatory elements between 55bp and 197bp upstream from the start site of transcription. In order to better define the location of these regulatory elements, a finer set of nested deletions was generated and plasmids carrying inserts with 102bp and 93bp of sequence upstream from the start of transcription (pGL3 basic 334bp and pGL3 basic 314bp respectively) were analyzed as before. With 102bp of upstream sequence, expression was comparable to that of the construct carrying 197bp, although with a lower amplitude. However, the 93bp insert reporter construct showed a significant reduction in the basal level of expression that was essentially arrhythmic, similar to that of the 55bp fragment construct (Figure.14-C). Thus, a 9bp region was revealed by the fine deletion analysis to contain promoter elements necessary for maintaining basal expression levels. Within this region lies a CG rich sequence similar to the consensus binding site for zinc finger transcription factors including Sp1 (see figure.9). Such elements are frequently encountered in constitutively expressed gene promoters (such as housekeeping genes) and appear to be important for regulating the basal levels of promoter activity (Cook et al., 1999).

In order to directly test the function of the E-box enhancer elements within the context of the 3.1Kb promoter, we next prepared a series of promoter constructs carrying point mutations in these elements in various combinations and assayed them, as before, in the context of stable PAC-2 cell lines. (Figure.15-A). Interestingly, single or double mutations affecting the canonical E-Boxes, do not abolish rhythmic expression of the *zfperiod4* promoter reporter construct in LD or DD conditions or the acute repression by light, however there is a significant increase in the basal level of expression (Figure.15-B). Mutation of all the canonical and non-canonical E-boxes further increases the basal expression and reduces significantly the amplitude of the expression rhythms and the magnitude of the repression by light (data not shown). Mutating all three E-boxes in the context of the pGL3 basic 0.4Kb construct completely abolishes rhythmic expression as well as the repression by light and significantly elevates the basal expression levels (Figure.15-C). These results strongly implicate the E-box elements in conferring a low basal level of expression in the promoter and maintaining rhythmic expression under LD and DD conditions.

3.8 Factors affecting E-box dependent regulation.

Our deletion analysis and mutagenesis experiment revealed that a 0.4kb promoter fragment was sufficient to direct all the rhythmic expression properties of the endogenous *zfperiod4* gene. Furthermore, E-boxes within this region play a key role in mediating the regulation by the clock and light as well as determining the basal levels of expression. In order to explore whether other promoter elements might influence the E-box regulatory function, we cloned these E-boxes along with their flanking sequence into a heterologous promoter pLUC MCS (Figure.16-A, see appendix-2). This vector has a synthetic TATA box sequence positioned immediately downstream of a multiple cloning site. Enhancer elements cloned into this vector regulate transcription that is initiated 20 bp downstream from the TATA element. The heterologous promoter drives

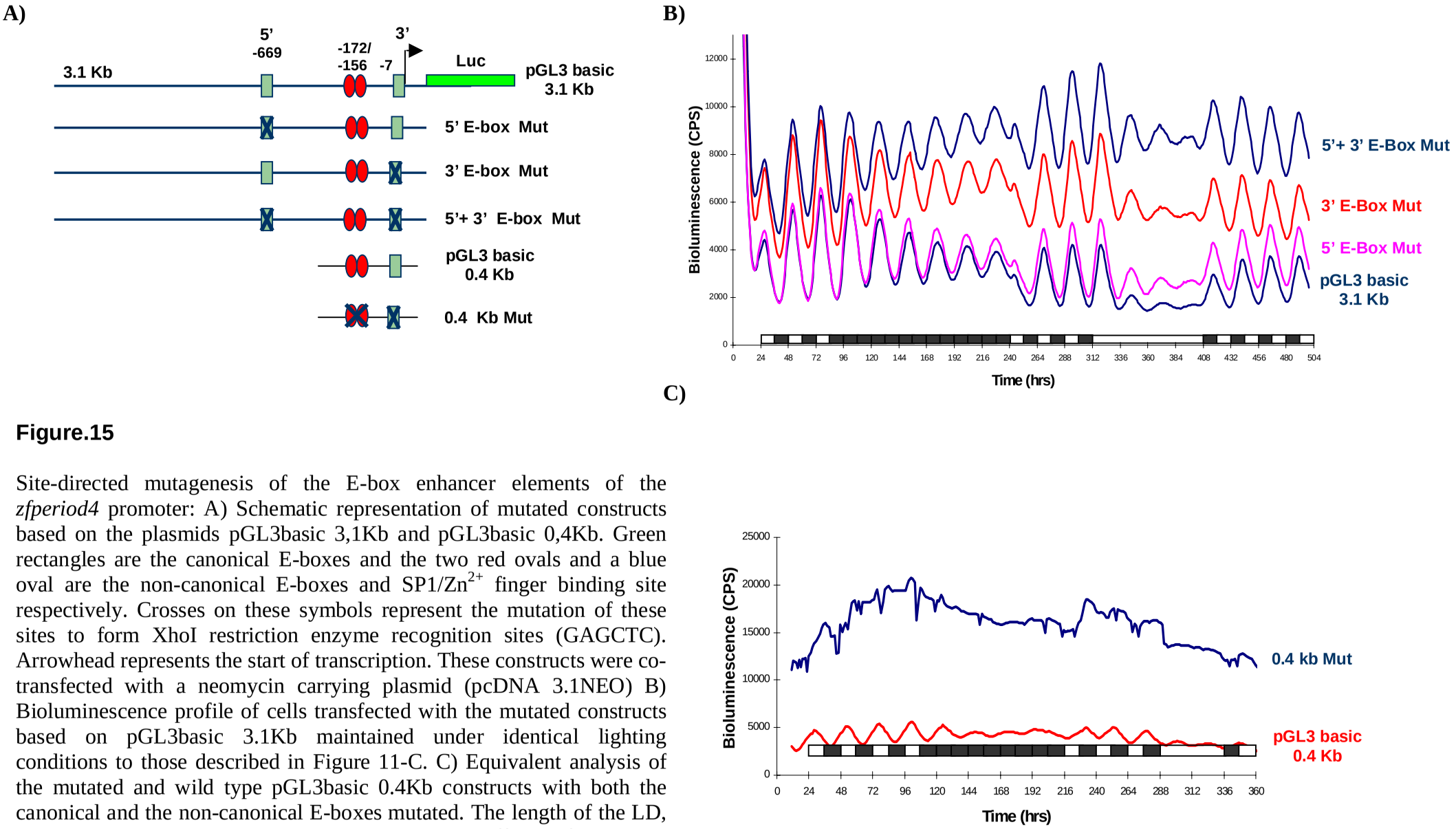


Figure.15

Site-directed mutagenesis of the E-box enhancer elements of the *zfperiod4* promoter: A) Schematic representation of mutated constructs based on the plasmids pGL3basic 3,1Kb and pGL3basic 0,4Kb. Green rectangles are the canonical E-boxes and the two red ovals and a blue oval are the non-canonical E-boxes and SP1/Zn^{2+} finger binding site respectively. Crosses on these symbols represent the mutation of these sites to form XhoI restriction enzyme recognition sites (GAGCTC). Arrowhead represents the start of transcription. These constructs were co-transfected with a neomycin carrying plasmid (pcDNA 3.1NEO) B) Bioluminescence profile of cells transfected with the mutated constructs based on pGL3basic 3.1Kb maintained under identical lighting conditions to those described in Figure 11-C. C) Equivalent analysis of the mutated and wild type pGL3basic 0.4Kb constructs with both the canonical and the non-canonical E-boxes mutated. The length of the LD, DD and LL periods in this case were slightly different from those described in Figure 11-C.

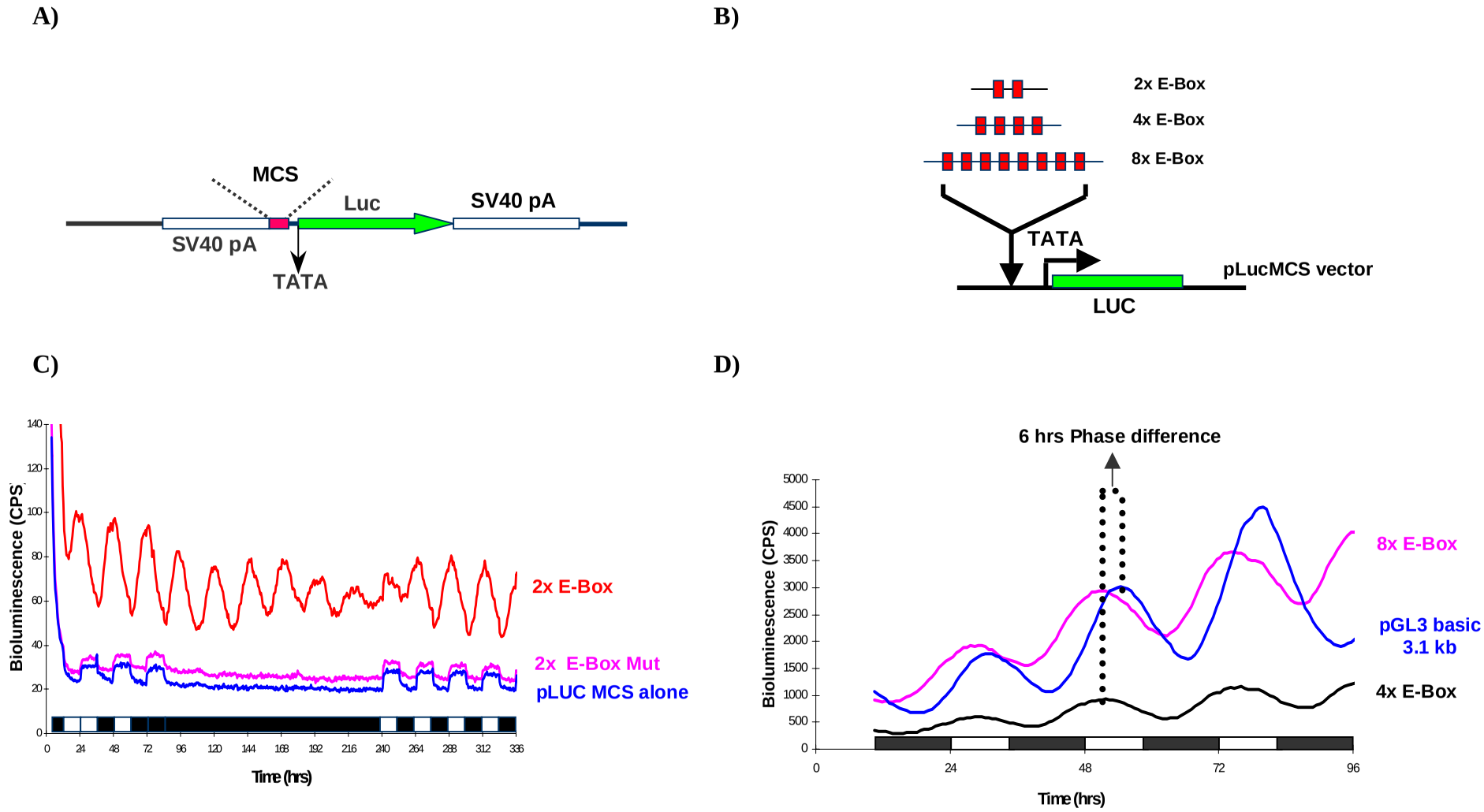


Figure 16

Influence of copy number and spacing of E-boxes upon clock and light regulated expression. A) Schematic of pLucMCS minimal promoter vector. The minimal TATA box promoter is located upstream of a luciferase (Luc) gene and downstream of a multiple cloning site (MCS), SV40pA provides the SV40 polyadenylation signal. SV40 polyA signal just upstream of the MCS protect the luciferase cassette from being incorporated into transcripts initiated upstream in the vector backbone sequences. B) Schematic of the different E-box constructs in the pLucMCS vector. C) Bioluminescence trace of pLucMCS heterologous promoter construct containing 2 multimerized canonical E-boxes from the *zfperiod4* promoter construct (position –7) along with an equivalent construct containing mutated E-boxes and the empty pLucMCS vector alone. White and black bars indicate the lighting conditions. D) Comparison of the bioluminescence traces of constructs containing 8 or 4 multimerized canonical E-boxes and the 3.1kb wild type *zfperiod4* promoter (see Fig.14-A). The dotted lines indicate the phase difference between the z*fperiod4* and heterologous promoter rhythms.

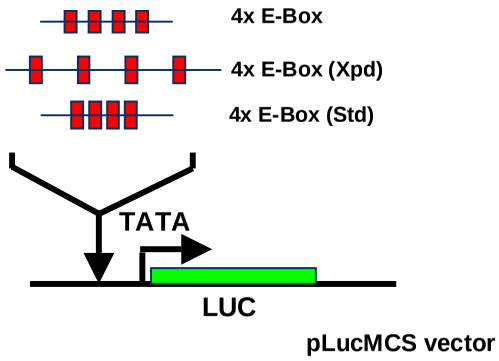
expression of a luciferase reporter gene. It is well documented that cloning multiple, multimerized copies of enhancer elements into heterologous promoters such as pLucMCS results in cooperative binding of regulatory transcription factors and therefore increases the magnitude of the transcriptional response. Given that the number and spacing of elements influences the level of cooperation we cloned different numbers of E-boxes with different spacing into the construct and examined the patterns of expression.

First we cloned two copies of the (-7) canonical E-box sequence into pLucMCS (2x E-box) (Figure.16-B). We also prepared a control construct where the two E-box sequences were eliminated by mutation to an XhoI recognition site (2x E-box-mut). These two contructs were stably transfected into PAC-2 cells together with the empty pLucMCS vector and then the luciferase expression profile was examined. The pLucMCS and 2x E-box-mut transfected cells produced bioluminescence similar to the background from an empty well (Figure.16-C). Abrupt, small step increases and decreases in bioluminescence that occur when the lights are switched on and off, result from autofluorescence from the plastic of the plate. However the expression of 2x E-box was significantly higher than the background and a low amplitude rhythm of expression was observed under LD and DD conditions (Figure.16-C). The ability of this heterologous promoter to direct a circadian rhythm of expression is consistent with the role of the E-box predicted by the previous promoter analysis. Interestingly, the phase of this rhythm was advanced relative to the rhythm of the pGL3 basic3.1Kb control construct. Increasing the number of (-7) canonical E-boxes in the promoter to four, (4x E-box) lead to a significant increase in the basal level of expression and a more robust rhythm of expression in LD and DD (Figure.16-D). Interestingly, a phase advance of about 6 hours was evident compared with the pGL3 basic3.1Kb control rhythm (Figure.16-D, pGL3 basic 3.1kb and 4x E-box traces). Finally, we cloned eight (-7) canonical E-boxes (8x E-box) into pLuc MCS and compared its expression with that of 4x E-box and the pGL3 basic3.1Kb control. We found a further

increase in the basal level of expression and rhythm amplitude but no change in the 6 hours phase difference relative to the pGL3 basic3.1Kb rhythm (Figure.16-D). This result indicates that the number of E-box copies included in the heterologous promoter influences the rhythm amplitude and overall expression levels. However, a characteristic phase advance of the E-box reporter construct rhythms relative to the *zfperiod4* promoter is unaffected by increasing the number of copies of the E-box sequence.

We next explored the influence of the spacing between the E-box enhancers upon the expression pattern of the heterologous promoter. In the previous E-box constructs, elements were separated by 10bp. We next prepared a similar construct werethe spacing of four E-box elements were either increased to 20bp (4x E-box (Xpd)) or decreased to 6bp (4x E-box (Std)). Following stable transfection, we compared the luciferase expression of pGL3 basic3.1Kb and the 4x E-box construct (Figure.17-A). We entrained the cells for three days in LD cycles, then exposed them for two days to DD conditions, returned them to an LD cycle for three days, transferred them to LL conditions for two days and finally returned them to LD for one day. All E-box constructs showed a similar pattern of rhythmic expression, however increasing and decreasing the spacing between the E-boxes significantly reduced the amplitude of the rhythm with the 4x E-box (Std) (6bp spacing) construct showing the lowest rhythm amplitude (Figure.17-B). Importantly, alterations in the spacing of the E-boxes does not affect the 6 hr phase delay of the expression rhythm compared with the pGL3 basic3.1Kb construct (Figure.17-C). A difference in the rhythmic expression profile between the heterologous E-box and *zfperiod4* promoter constructs was observed under constant light conditions. Under LL where the *zfperiod4* promoter was strongly repressed, the 4x E-box heterologous promoter construct expression was strongly dampened but to a high basal level corresponding to the peak values of the rhythm observed during the previous LD cycles (Figure.17-B and C). Consistently, following two days adaptation to DD conditions, exposure to light does not lead to a strong repression of expression. Levels decrease transiently and then subsequently increase during the

A)



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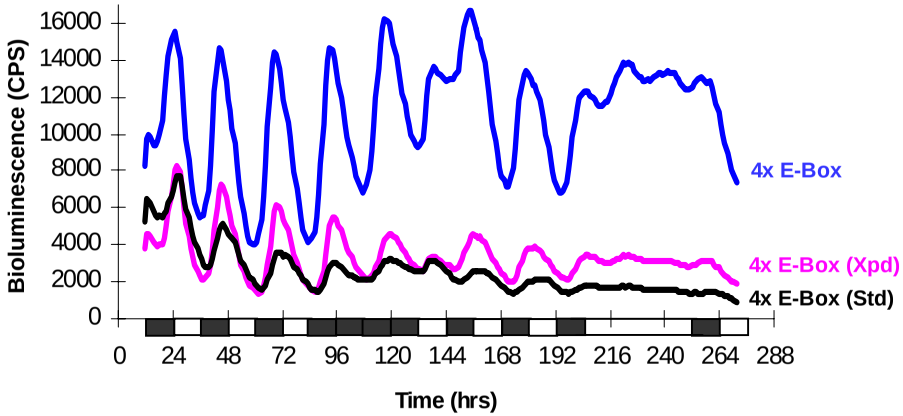

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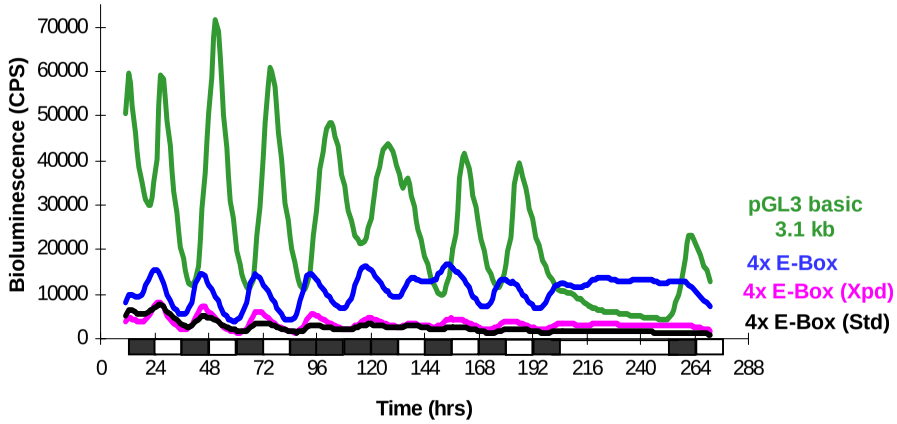


Figure.17

A) Schematic of the different E-box constructs in the pLucMCS vector. B) Bioluminescence trace comparison between the pGL3 basic 3.1Kb construct (see Figure.14-A), and pLucMCS promoter constructs containing 4 canonical E-boxes with a 10bp, 20bp (Xpd) or 6bp (Std) spacing between each E-box (Green, blue, pink and black traces respectively). C) The data shown in panel B plotted on a smaller scale, without the pGL3 basic 3.1 kb promoter construct.

remainder of the 12-hour light period (Figure.17-C). These results show that the promoter environment of the E-boxes within the *zfperiod4* gene significantly influences the phase of the circadian rhythm of expression which they direct as well as its response to light.

3.9 Establishing transgenic lines expressing *Period4-luciferase* in zebrafish larvae.

In order to study the expression of the *zfperiod4* luciferase construct *in vivo*, we attempted to establish stable transgenic lines of zebrafish. We therefore linearized the pGL3basic3.1Kb plasmid and micro-injected 1-cell stage embryos with the restriction enzyme digest at a final plasmid DNA concentration of 10ng/µl mixed with phenol red dye. Surviving eggs were bleached after 24 hours and then dechorionated by pronase treatment. Embryos were then raised for 5 days at 27°C under a LD cycle. We initially assayed for transient transgenesis by aliquoting individual embryos into a 96 well plate in E3 buffer supplemented with 0.5mM luciferin. The embryos were then assayed for bioluminescence during 24 hours using the Packard Topcount scintillation counter. In each experiment, 200 – 300 injected larvae were assayed but none were found to be bioluminescent. Injections were repeated 5 times but still no evidence was found for transient expression of the construct. In order to test for the possibility that stable integration might enable expression of the construct, we recovered the larvae from the 96 well plate assay and raised them to adulthood. These fish were then crossed in an attempt to identify transgenic founders with germ line transmission of the construct. From 200 adults tested, none were found to be positive.

We next decided to test an alternative approach to establishing a stable transgenic line. In a recent report it was documented using medaka that the I-Sce I *Saccharomyces cerevisiae* intron encoded homing endonuclease can be used to mediate high efficient stable and transient transgenisis in fish (Thermes et al., 2002). Specifically a reporter construct is engineered to include the 18bp recognition sites for I-Sce I and is co-injected with the I-Sce I enzyme.

Cleavage of the injected DNA by I-Sce I prevents the formation of concatemers and is thought to increase the efficiency of DNA integration into the genome. The enzyme is unlikely to cut zebrafish genomic DNA since the 18 bp recognition site is expected to be found only once in $7x10^{10}$ bp of random sequence DNA. As a result of the specific plasmid cleavage *in vivo*, transient expression of the construct is more efficiently established and a larger proportion of cells in the developing embryo contain the plasmid thereby also increasing the probability of germ line integration. The same system has also been reported to improve the efficiency of transient expression following transfection of cell cultures. However, in this case a plasmid encoding the I-SceI meganuclease (see figure.18-A) is cotransfected with the reporter vector. We therefore initially tested whether by using the I-SceI system, we could improve the efficiency of transient transfection of the PAC-2 cells.

3.9.1 Co-expression of the *I-SceI* meganuclease leads to efficient transient expression of *zfperiod4*-luciferase in PAC-2 cells.

The *zfperiod4* promoter linked to the luciferase reporter gene was cloned into the modified vector: pBluescript II SK(-) I-SceI, where two I-SceI recognition sites have been introduced, immediately flanking the polylinker sequence of pBluescript (Figure.18-B). The *Per4 I-SceI* supercoiled plasmid DNA was then cotransfected by electroporation into PAC-2 cells with the expression vector encoding a modified version of *I-SceI* meganuclease that includes a nuclear localization sequence (NLS) and the influenza hemagglutinin epitope tag (HA) (Rouet et al., 1994). One day after transfection, the medium was supplemented with luciferin (0.5mM) and the cells were then immediately assayed for bioluminescence. In contrast to our previous negative results from attempts to transiently express the pGL3 basic3.1Kb plasmid, we encountered a robust oscillation of expression for 3 days under LD cycles which then continued for 2 days under DD conditions (Figure.18-C). Subsequent return of the cells to a LD cycle resulted in repression of *Per4* I-Sce I expression beginning four hours after lights on and then a re-established high amplitude rhythm of expression

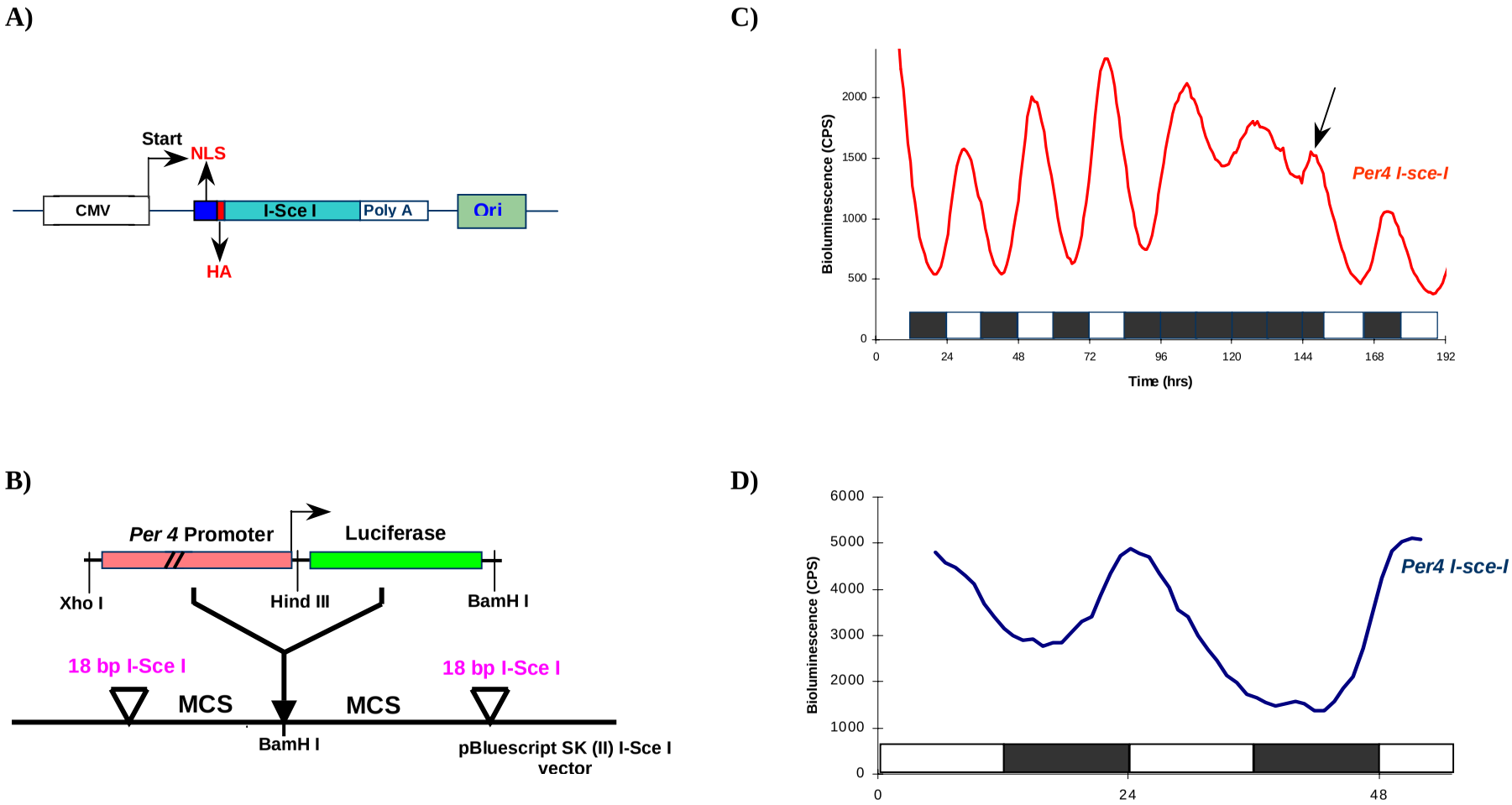


Figure.18

Transient transfection and transgenics. A) pCMV-I-Sce I expression vector encoding a modified I-Sce I protein of 276 amino acids. Expression is directed by a cytomegalovirus promoter/enhancer cassette (CMV) and a eukaryotic origin of replication is provided by SV40 sequences (Ori). Within the I-Sce I open reading frame, HA represents the influenza hemagglutinin epitope tag; NLS, the SV40 nuclear localization signal and Poly A, a human growth hormone polyadenylation site (Rouet et al., 1994). B) pBluescript II SK(-) I-Sce I cloning vector that carries two 18bp I-Sce I meganuclease recognition sites flanking the multiple cloning site (MCS). An arrow indicates the cloning of the *zfperiod4* promoter and luciferase gene from the 3.1kb, *zfperiod4* plasmid into the BamHI site of this vector that is flanked by the two I-Sce I meganuclease recognition sites. This generated a new plasmid *Per4* I-Sce I C) Transient expression of *Per4* I-Sce I in the PAC-2 cells. A representative bioluminescence trace of PAC-2 cells transiently transfected with the luciferase reporter construct. Black and white bars indicate the lighting conditions. The arrow mark represents the time when the reporter expression starts to be downregulated by light was turned on after a period in DD conditions. D) Transient expression in transgenic larvae. Bioluminescence trace of an individual 5 days old larva previously microinjected at the one cell stage with the *Per4* I-Sce I reporter construct. This was raised for 5 days at 25°C under a LD cycle, then luciferin was added to the E3 buffer and it was transferred to a 96 well plate. Bioluminescence was then monitored for two days also under LD conditions at 25°C.

for two LD cycles (Figure.18-C). The pattern of rhythmic luciferase expression in these transiently transfected cultures was similar to that of PAC-2 cells stably transfected with the pGL3basic*3.1kb zfperiod4* luciferase plasmid. Interestingly however, the cell rhythms dampened more rapidly in DD conditions. Transient expression persisted for a remarkably long time. In mammalian cells, maximum transient expression is observed between 48 and 72 hours following transfection and then expression declines rapidly (Sambrook et al., 1989). The success of this transient transfection assay lead us to test transient expression of the same construct in zebrafish embryos.

3.9.2 Transient transgenesis in zebrafish larvae

We co-injected the pBluescript *Per4-I-SceI* plasmid DNA with the I-SceI enzyme into one-cell stage embryos. After 5 days under a LD cycle the surviving larvae were aliquoted into a 96 well plate, luciferin was added to the E3 buffer and then they assayed for bioluminescence during two days under continuing LD cycle conditions. 40% of injected larvae showed a robust 24 hours rhythm of bioluminescence that matched the timing of the mRNA expression rhythm of the endogenous *zfperiod4* gene (Figure.18-D). Many embryos (50%) initially showed extremely high levels of bioluminescence that then rapidly declined during the course of the assay to ultimately establish a very low, non-oscillating expression profile. In only 10% of the injected embryos did we fail to detect any bioluminescence expression. As a result of the dramatic improvement in transient expression of the *zfperiod4* reporter construct, we recovered 200 – 300 luciferase positive larvae and are now raising them to adulthood. We will then screen them for germ line transmission and stable expression of the construct. Given the improvement in transient transgenesis, we are optimistic that stable transgenic lines will be obtained by this approach.

3.10 Pharmacological analysis

The daily entrainment of the circadian clock by zeitgebers is essential to ensure its synchronization with the 24 hour day night cycle of the environment. The levels and activity of clock components are all factors that contribute to the entrainment and maintenance of the clock mechanism. Thus, signal transduction pathways that modify clock component function are emerging as essential regulators of the circadian clock (Harmer et al., 2001). Identifying the key regulatory pathways is an important step in ultimately understanding how zeitgebers are perceived and entrain the clock.

The ability to study circadian gene expression in mammalian cell cultures has helped contribute significantly to our understanding of the role of specific signalling pathways in the vertebrate clock. The luciferase reporter PAC-2 cell lines represent an attractive cell model system to study how signal transduction pathways regulate the clock. The clock can be monitored at the level of gene expression in real time over long periods. Furthermore, the *in vivo* luciferase assay in PAC-2 cells lends itself particularly well to large scale, high throughput analysis. We chose to treat our luciferase reporter clones with activators and inhibitors of the MAPK, PKC and cAMP signaling pathways (see appendix-3) and subsequently examine the effects on rhythmic reporter gene expression under different lighting regimes. We thereby tested how each pathway contributed to the maintenance of rhythmic gene expression and its entrainment by light-dark cycles.

3.10.1 Testing for toxicity

Before testing the effect of the compounds on the luciferase reporter gene expression in the PAC-2 cells, the toxicity of these compounds was first tested. Cell clones were plated at confluence in 96 well transparent tissue culture plates and the compounds were added at various concentrations to the cell culture medium. After incubation for 3-4 days we first examined the cultures for any significant increase in cell detachment that normally occurs following

cell death. We also checked carefully for changes in cell morphology that could indicate a cytotoxic effect. In the event of abberant morphology such as a highly refractile cell border, that often precedes death and detatchment (our own observations), we stained the culture monolayers with trypan blue to check for exlusion of the dye from the cells – an indicator viability. Only the drugs that resulted in no signs of toxicity were examined further. In this way, we excluded use of the calcium ionophore, calcymycin and the PKA inhibitor, H-89 which at all concentrations tested, resulted in significant cell death.

3.10.2 Experimental design

We tested the effects of the activators and inhibitors on two different PAC-2 cell clones: DAP-49, a clonal cell line that carries the stably transfected full length *Period4* promoter-luciferase reporter plasmid (pGL3 basic 3.1kb) and 4x E-Box (-7), stably transfected with the heterologous promoter - luciferase reporter construct that contains 4 multimerized E-box elements. In this way, we compared the activity of a complex clock and light-regulated promoter with that of the minimal E-box enhancer reporter construct. These clones were tested under two different lighting regimes in the presence or absence of the various compounds. In regime 1, 12 hours after addition of luciferin (0.5mM) to the culture medium, cells were exposed to an LD cycle for one day and then on the second day at ZT11 the medium was supplemented with the compounds. At the end of the third day, the LD cycle was reversed following exposure to a 24 hours light period and then cultures were exposed for three more days to the reversed LD cycle. This regime tests for the ability of the gene expression rhythm to entrain to major changes in the lighting cycle. In untreated DAP-49 controls, under regime 1, the bioluminescence shows an initial robust oscillation which after reversing the LD cycle requires 48 hours to reentrain. The first peak following reversal of the LD cycle is broader, however amplitude and does not match the phase to the new LD cycle. A similar profile was evident for the 4x E-box clone, but with a shallower peak following the LD cycle phase reversal and a phase delay of about 6 hours with respect to DAP-49

rhythm. In regime 2, following addition of luciferin to the culture medium, clones were first exposed for 3 days to a LD cycle, and at the end of the third light period, at ZT11 the culture medium was supplemented with the compounds. The subsequent dark period marked the beginning of a 72-hour period of constant darkness. Thereafter, the cultures were exposed to a 12-hour light period and then returned for either 2 days to a LD cycle or 1 day in LD and 2 days in DD. These lighting conditions test for the maintenance of clock regulated rhythmic gene expression under free running, DD conditions. Under these conditions, with the control non-treated cells a robust rhythm in expression continued, with the same 6hr phase difference between the DAP-49 and 4x E-box clones.

3.10.3 The cAMP pathway influences the amplitude but not the entrainment of *period4* promoter and E-box-directed rhythmic expression

First we tested the effect of forskolin (5, 10, 15μM) on expression of the luciferase reporter constructs. This cell-permeable compound serves as a reversible activator of adenylate cyclase, the enzyme catalysing the conversion of ATP to cAMP. Thereby forskolin specifically induces the cAMP signalling pathway. When exposed to forskolin under lighting regime 1 (Figure.19-A), the DAP-49 clone showed an identical rhythmic expression profile compared to the untreated control cells at all three concentrations. Thus forskolin treatment did not influence the phase reversal, however, we found a 1.25-1.8 fold decrease in the peak expression between the treated and untreated cells (Figure.19A). Also under regime 2, the pattern of rhythmic expression for the forskolin treated cells was comparable with the untreated controls but with a consistent reduction in rhythm amplitude. This effect was even more pronounced in the case of the 4x E-Box clones, where a 2.5-4 fold reduction in the amplitude of rhythmic expression was observed under both the lighting regimes although the phase of the rhythms in forskolin treated cells continued to match that of the untreated 4x E-box controls (Figure.19-C, D).

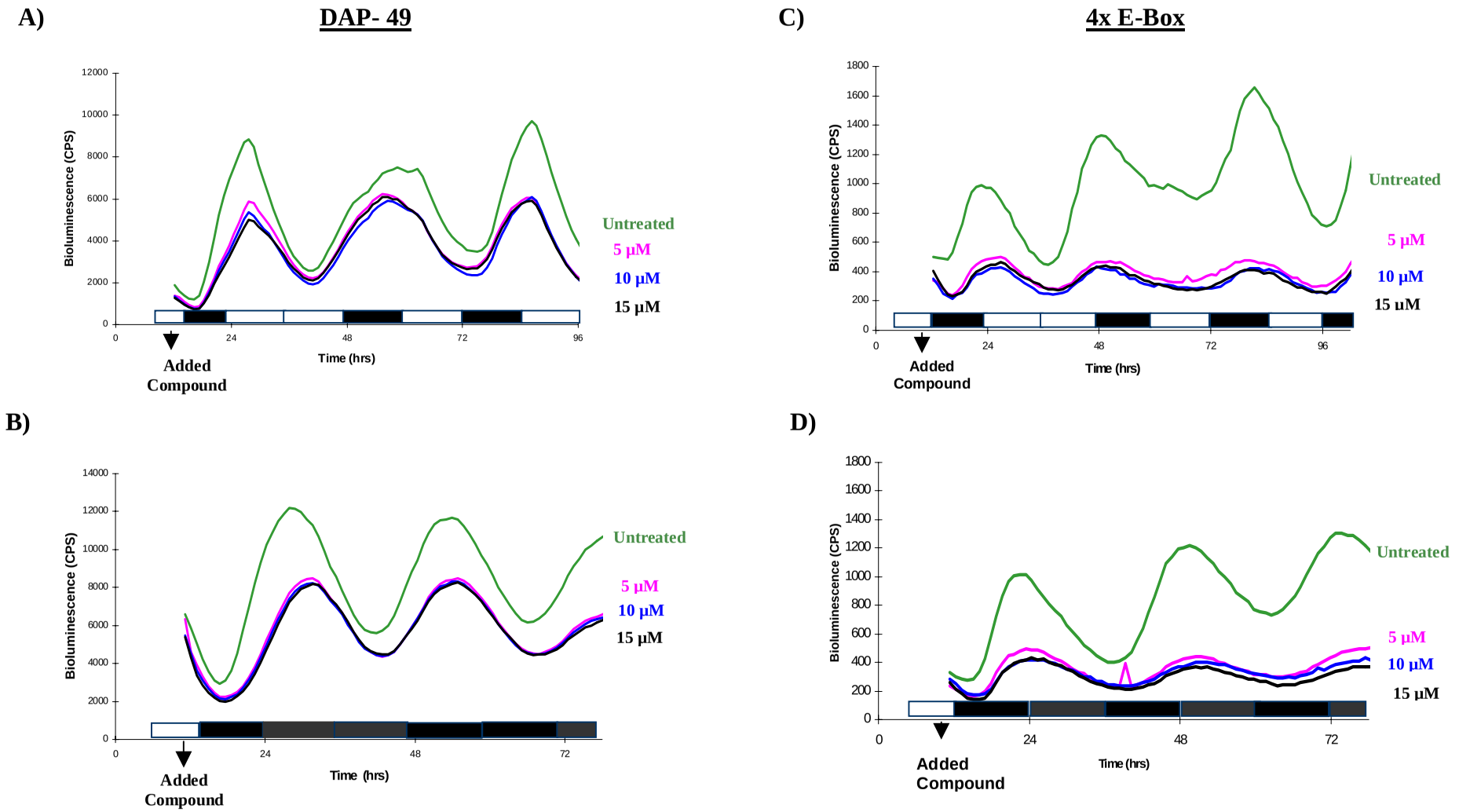


Figure.19

Influence of forskolin on circadian clock regulated gene expression. On the 'y' axis, bioluminescence is plotted as counts per second (cps), and on the 'x' axis is represented time in hours. Cells were entrained initially for two days under Light Dark (LD) cycles represented by the black and white bars below the traces. Black (subjective night) and grey (subjective day) bars represent periods of constant darkness (DD). Forskolin was added on the second day just one hour before entering the dark period, represented by the arrow mark at the beginning of each bar. Under regime 1 they were then subjected to one LD cycle, and by subsequent exposure to a 24 hours light period, the LD cycle was reversed (panels A, C) and the cells exposed to 3 cycles of LD conditions. Under regime 2, following addition of forskolin they were subsequently exposed to DD conditions for 59 hours. Green traces represent the data from the untreated cells, pink, blue and black traces are traces when treated with 5, 10 and 15µM forskolin respectively. A and B shows the effect of forskolin on the DAP-49 cell clone whereas, C and D shows the effect on the 4x E-Box clones.

We also tested the involvement of the cAMP pathway using an alternative activator, Di-Butyryl cAMP (dbcAMP). This is a more stable analogue of cAMP that strongly activates cAMP-dependent protein kinase A. Treatment of both the DAP-49 (Figure.20-A, B) and 4x E-Box clones (Figure.20-C, D) under the two lighting regimes lead to a very similar profile to that already observed following treatment with forskolin. This result supports the notion that the cAMP pathway is involved in the control of the amplitude of the clock and light-regulated rhythmic gene expression for both the *Period4* promoter and the E-box elements.

3.10.4 The PKC pathway is involved in regulating the phase of the rhythm and maintaining circadian rhythmicity under free running conditions.

We next tested the involvement of the PKC pathway by first treating with the PKC activator, the phorbol ester 4-α-Phorbol 12-Myristate 13-Acetate (PMA). Following addition of PMA at three different concentrations: (0.5, 1, 3μM), expression of the DAP-49 clone under regime 1 was significantly down regulated during the first 24hrs. Then subsequently expression increased and started to oscillate in-phase with the untreated cell controls, although with higher peak and trough values (Figure.21-A). Under regime 2 following addition of PMA, there was a strong phase shift of the rhythm evident during the first 24 hrs (Figure.21-B). In the second cycle the peak and trough values were again significantly elevated (Figure.21-B) and a 9 hr phase delay was observed relative to the un-treated controls. In the case of the 4x E-Box clones under regime 1 and 2, during the first 36 hrs there was a sustained increase in expression relative to non-treated controls and for the first cycle, a slight delay in the phase of the rhythm. Subsequently under regime 1 for the next two cycles, the phase of the rhythm in treated cells was equivalent to that in un-treated controls. Under regime 2 the phase difference persisted (Figure.21-C, D). Thus, these results suggest treatment with PMA influences the phase of the

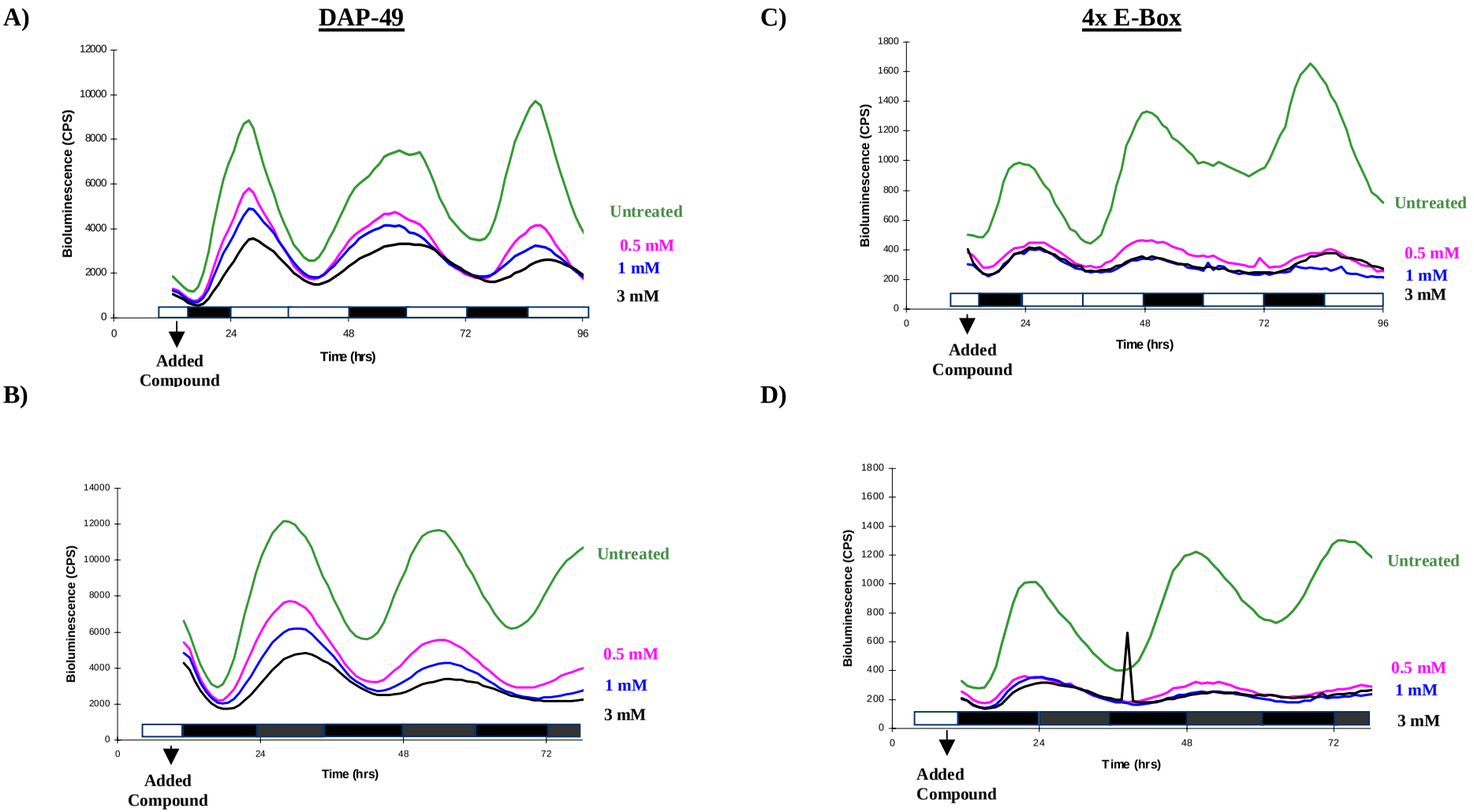


Figure.20

Effects of Di-butyrylcAMP (dbcAMP) on luciferase reporter gene expression. Treatment of the cells and entrainment conditions are similar to those described in Figure.19. Green traces represent the untreated cells, while pink, blue and black traces are those of *Period4-luc* expression when treated with 0.5, 1 and 3mM dbcAMP respectively. A and B shows the effect of dbcAMP on the DAP-49 cell line whereas, C and D shows the effect on 4x E-Box clones.

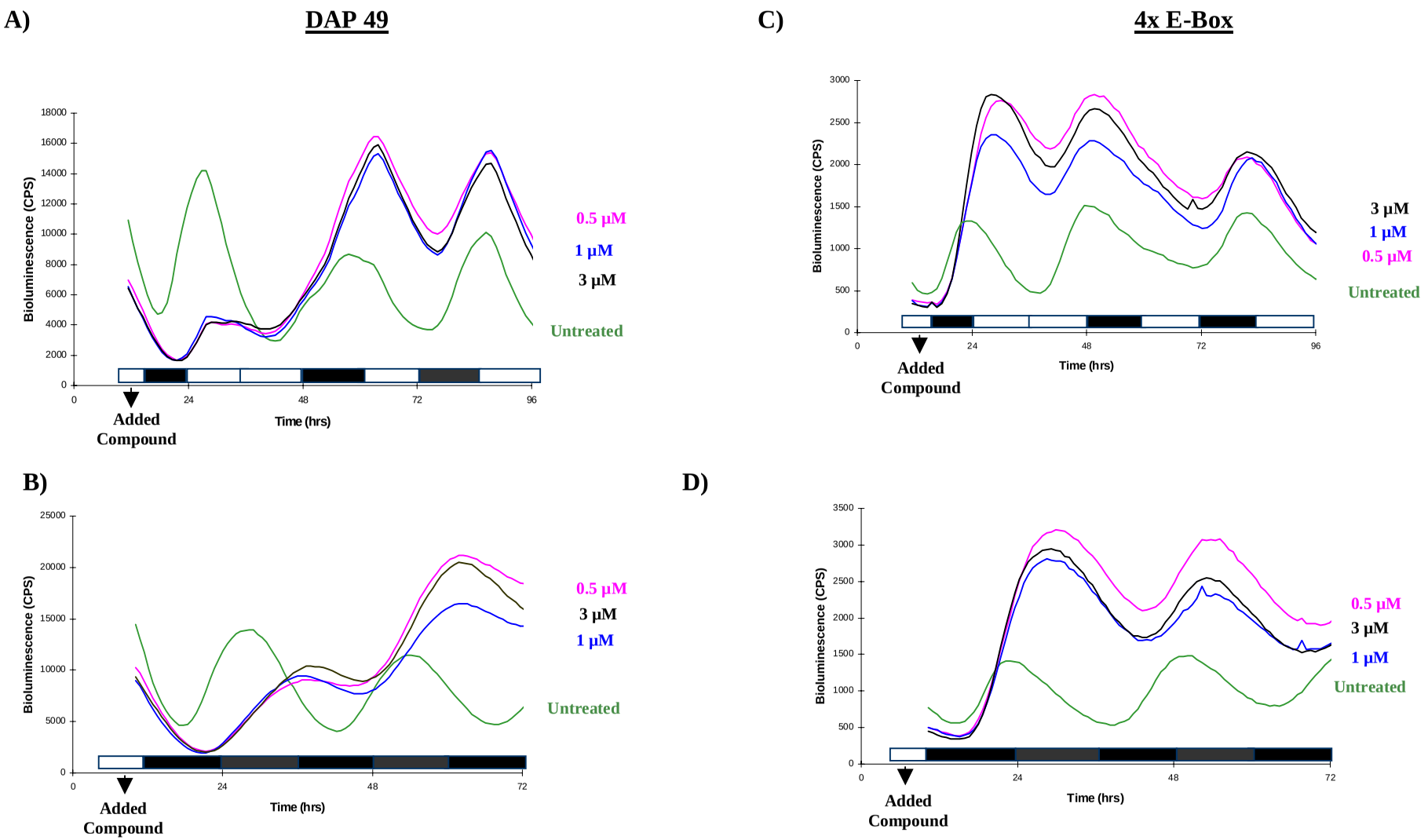


Figure.21

Effect of Phorbol 12-myrstate 13-Acetate (PMA) on circadian gene expression. The entrainment and the treatment conditions are identical to those explained in Figure.19. Green traces represent the untreated cells, while pink, blue and black traces are those cells treated with 0.5, 1, 3µM PMA respectively. Panel A and B shows the effect of PMA on the DAP-49 cell clone whereas, panel C and D shows the effect on 4x E-Box clones.

rhythm under free running conditions but do not interfere with the entrainment by LD cycles.

Treatment with the PKC inhibitor Ro-31-8220 resulted in a very strong effect on rhythmic expression of both reporters. In the case of DAP 49 clones under both regime 1 and 2, during the first 24 hrs there is initially a strong down regulation followed by an increase in expression. Subsequently under regime 1, the reporter starts to oscillate with a phase perfectly matching the reverse LD cycle (Figure.22-A). Indeed the rhythm appears to have entrained more rapidly than the untreated controls. In sharp contrast under regime 2, in constant darkness there is no rhythmic expression in reporter activity (Figure.22-B). In order to confirm the continued viability of the cells in the regime 2 experiment, after 71 hrs in constant darkness we removed the plate from the counter and replaced the culture medium with fresh medium lacking the compound. The plate was then returned to the counter and they were initially entrained again under a LD cycle for 1 day and then returned to DD conditions. This analysis confirmed the viability of cells since, under LD and DD conditions rhythmic expression was immediately restored (Figure.22-B). Our analysis of 4x E-box clones revealed under both regime 1 and 2 during the first 24 hrs had a profile comparable to that seen in DAP 49 clones (Compare figure.22-A, B with C, D). Subsequently under regime 1 a very rapidly dampening rhythm of expression was established with the phase matching that of the untreated controls (Figure.22-C), while under regime 2 as in the DAP 49 cells, no rhythmic expression was detected under constant darkness (Figure.22-D). Together these results indicate that light driven expression of the *period4* promoter in DAP 49 clones is unaffected by the PKC inhibitor. However transcriptional regulation by the circadian clock via the E-boxes is severely impaired, consistent with the Ro-31-8220 inhibitor “stopping” the clock.

Our results have revealed that the disruption of the PKC signaling pathway strongly influences the activity of the circadian clock. In mammals, nearly 10-15 isoforms of PKC have been identified and the activity of these PKC isoforms is under the control of three distinct phosphorylation events. A

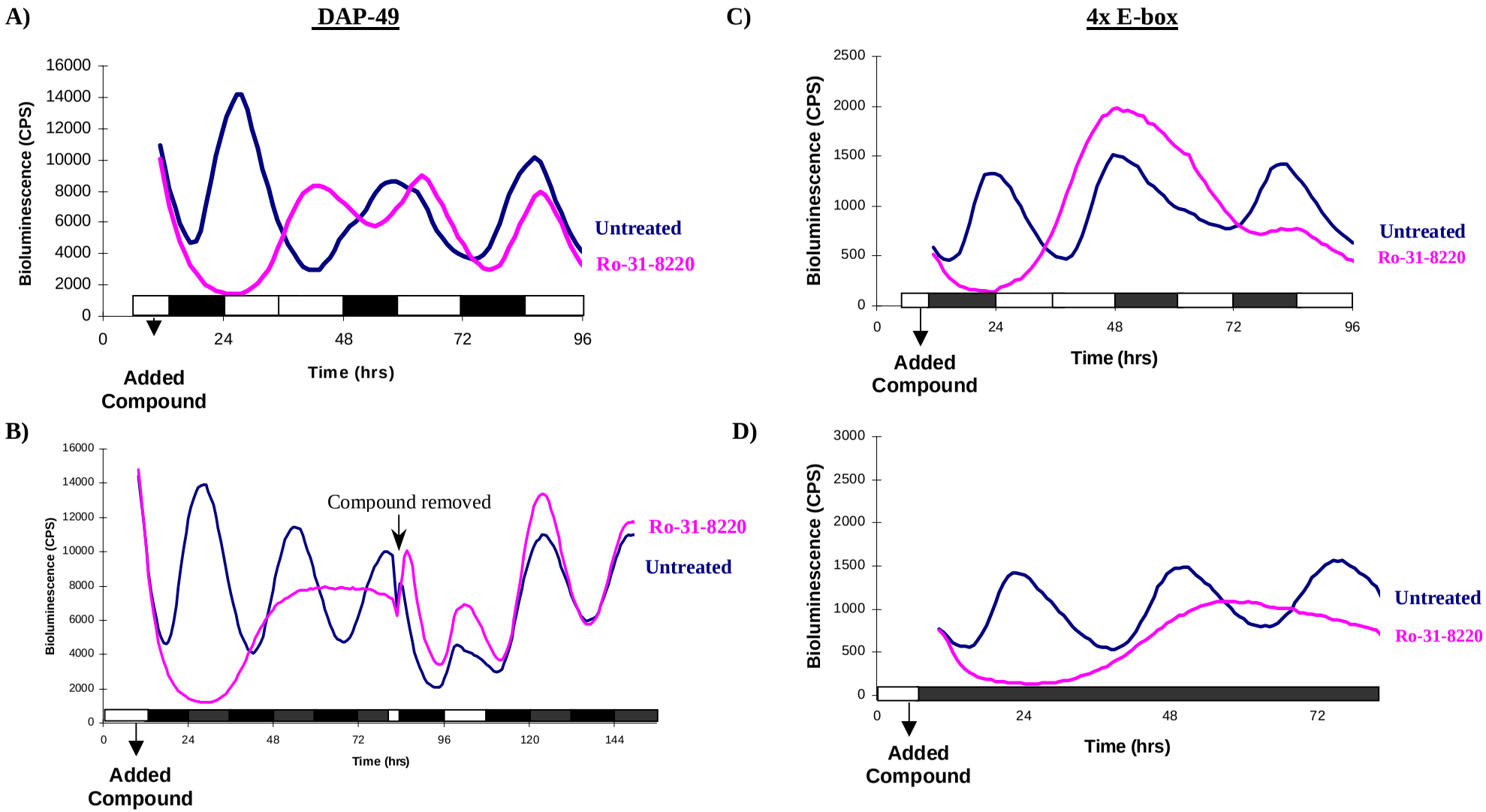


Figure.22

Effect of the PKC inhibitor Ro-31-8220 (2µM) on DAP-49 and 4x E-box gene expression. Treatment of the cells and entrainment conditions are the same as those explained in Figure.19 with the exception of the DAP-49 clones under regime 2 (panel B). In this case, the cells were entrained for 59 hrs in DD before washing away the compound and then returning them to an LD cycle for 24hrs followed by 36 hrs in DD conditions. Blue traces represent the untreated cells while the pink trace is that of the inhibitor treated cells. Panel A and B are traces from DAP-49 clones and panel C and D are traces from 4x E-box clones.

phospho-PKC (pan) antibody has been generated that specifically recognizes all mammalian PKC isoforms when they are phosphorylated at a Serine residue within a highly conserved protein domain at the carboxy terminus (Godbout et al., 2002). Phosphorylation at this site is linked with induction of the PKC kinase activity. We wished to understand whether treatment with the R0-31-8220 inhibited the activity of all or only a subset of PKC isoforms. We therefore attempted to use the phospho PKC (pan) antibody to visualize active PKC isozymes in the zebrafish PAC-2 cell line upon a time course of treatment with R0-31-8220. Following addition of 2μM R0-31-8220 to the medium, cells were harvested over the course of four days each 12 hours and protein extracts were prepared for western blot analysis (Figure.23). Control, non-treated cells were analyzed in parallel. Under constant dark conditions, in the untreated cells multiple bands of the predicted size for the mammalian PKC isozymes were visible (Figure.23). However, in the presence of R0-31-8220, bands corresponding to phosphorylation of a subset of PKC isozymes were absent through the entire time course (Figure 23). An identical profile was observed in equivalent experiments performed under LD cycle conditions (data not shown). Furthermore, no significant circadian oscillation of the phospho-PKC specific bands was detected in higher resolution analyses (data not shown). Thus the various PKC isozymes may be differentially involved in regulating the circadian clock.

3.10.5 The MAP Kinase pathway affects the rhythm amplitude of *zfperiod4* expression

Binding of the growth factor EGF to its high affinity receptor leads to activation of the MAP kinase signaling cascade. In order to test the involvement of this signaling pathway, we therefore treated our clones with EGF at 3 different concentrations (30,50,80 ng/ml). Under lighting regime 1, for both DAP49 and 4xE-box clones a similar effect was observed (See Figure.24-A and C). While the phase of rhythmic expression matched that of the untreated controls, the levels of expression increased progressively during

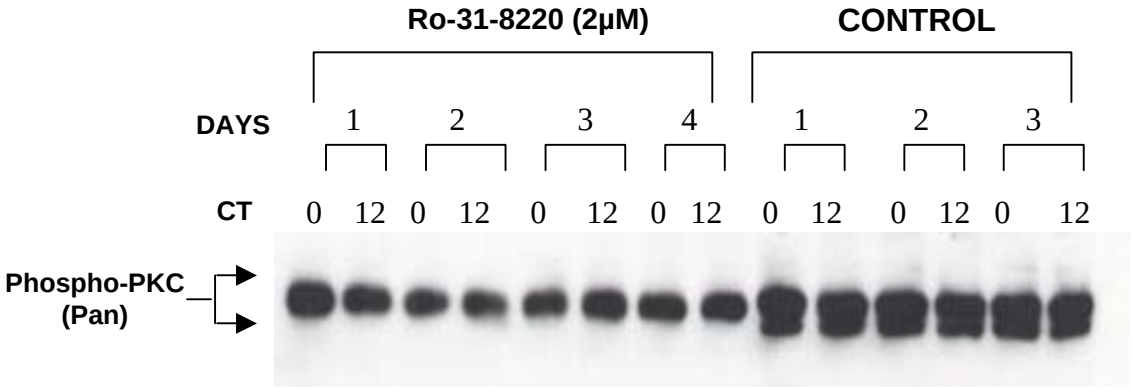


Figure.23

Western blot analysis of the phosphorylation levels of endogenous PKC proteins. Sets of PAC-2 cells were entrained under DD conditions with or without treatment with 2μM Ro-31-8220. Samples were collected from day-1 to day-4 at circadian times (CT) 0 and 12 each day. Incubation of the membrane with phospho PKC (Pan) antibody revealed at least three distinct bands in the control samples, whereas only one distinct band was observed with the inhibitor-treated cell extracts indicated by an arrowhead.

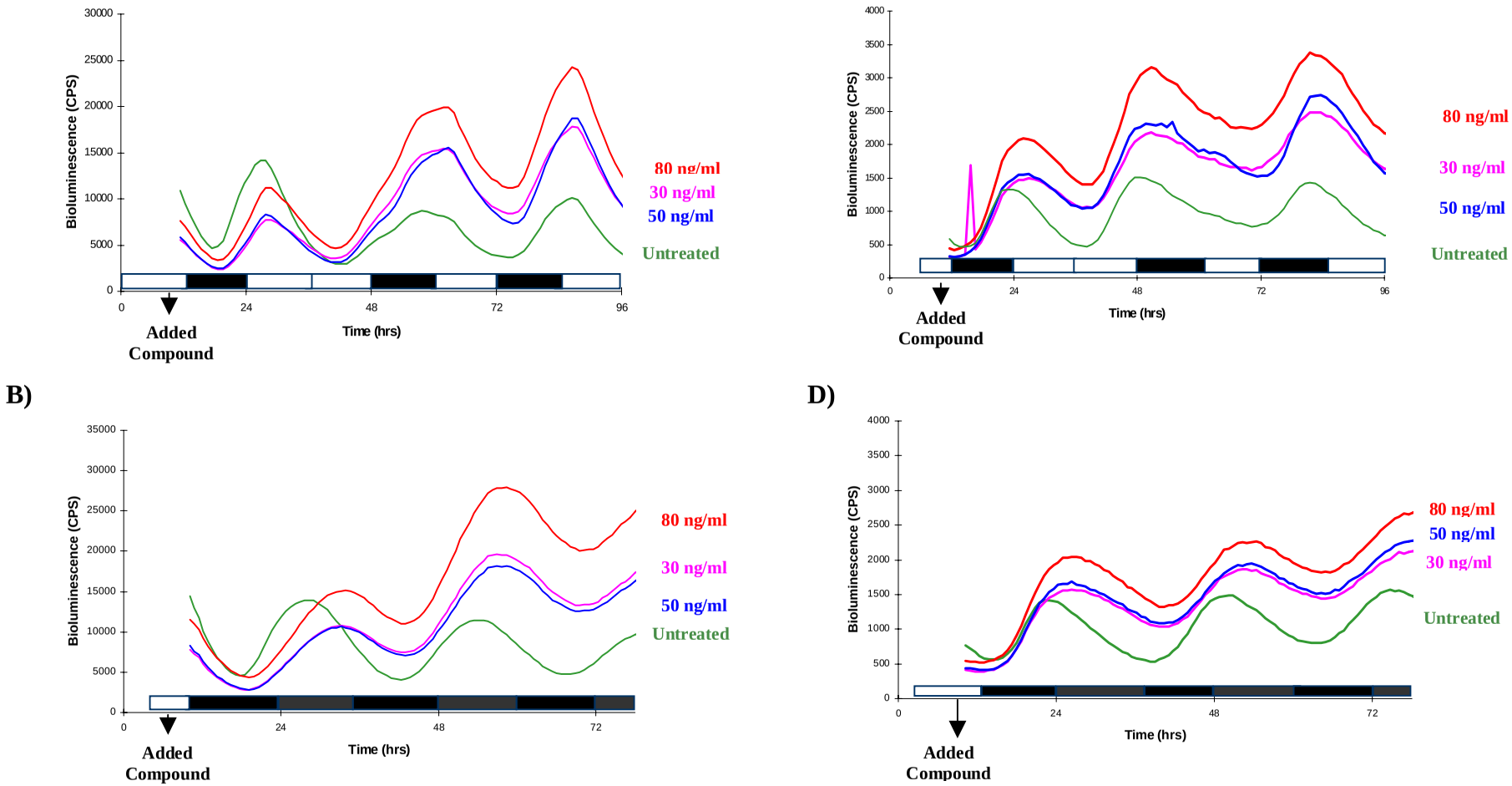


Figure.24

Effect of Epidermal growth factor (EGF) on *zfperiod4* and 4x E-box luciferase reporter gene expression. Treatment of the cells and entrainment conditions are equivalent to those described in Figure.19. Green represents the trace of the untreated cells, pink, blue and red traces are those treated with 30, 50 and 80ng/ml EGF. Panel A, B shows the effect of EGF on the DAP-49 cell clone whereas, panel C and D shows the effect on 4x E-Box clones.

the experiment (Figure.24-A & C). However, under regime 2, both clones show a characteristic 7 hours phase delay of the free running rhythm in addition to the general increase in expression observed under regime 1 (Figure.24-C & D).

Addition of 10, 20 and 40 μM U0126, which is a specific inhibitor of MEK1/2, to the two cell clones under both regime 1 and 2 lead to a significant, dose-dependent reduction in rhythm amplitude (Figure.25). The highest concentration resulted in the strongest reduction in amplitude. However for both cell lines in both lighting regimes, the phase of the rhythms were identical to those of the untreated control cells (Figure. 25). Together, these findings suggest a role for the MAPK pathway in determining the amplitude of *zfperiod4* and the E-box directed rhythmic expression. Also, the results obtained following EGF treatment imply some phase shifting role.

Given the clear dose-dependent reduction in rhythm amplitude observed following treatment with U0126, we decided to verify that treatment with this compound lead to a specific reduction in the activity of the MAPK pathway. Activation of the MEK1 and 2 kinases results in a specific phosphorylation of p44/42 MAPK proteins at a highly conserved Thr 202/Tyr204 residue. These phosphorylated residues can be detected using a phospho-specific antibody. We treated PAC-2 cells with the three different concentrations of U0126 and after 12 hours prepared protein extracts from these and control non-treated cells (Figure.26-A). Western blotting analysis using the phospho-MAPK specific antibody confirmed that increasing the concentration of U0126 lead to a corresponding decrease in the levels of phosphorylated MAPK (Figure.26-A). Given its involvement in setting the amplitude of the circadian rhythm, we wondered whether the activity of the MAPK pathway might vary through the circadian cycle. We therefore used the same phospho-MAPK antibody to analyse a high resolution series of PAC-2 protein extracts prepared from cells maintained under forward and reversed LD cycles (Figure.26-B). Our results demonstrate the presence of elevated, non-oscillating levels of MAPK phosphorylation under both lighting regimes.

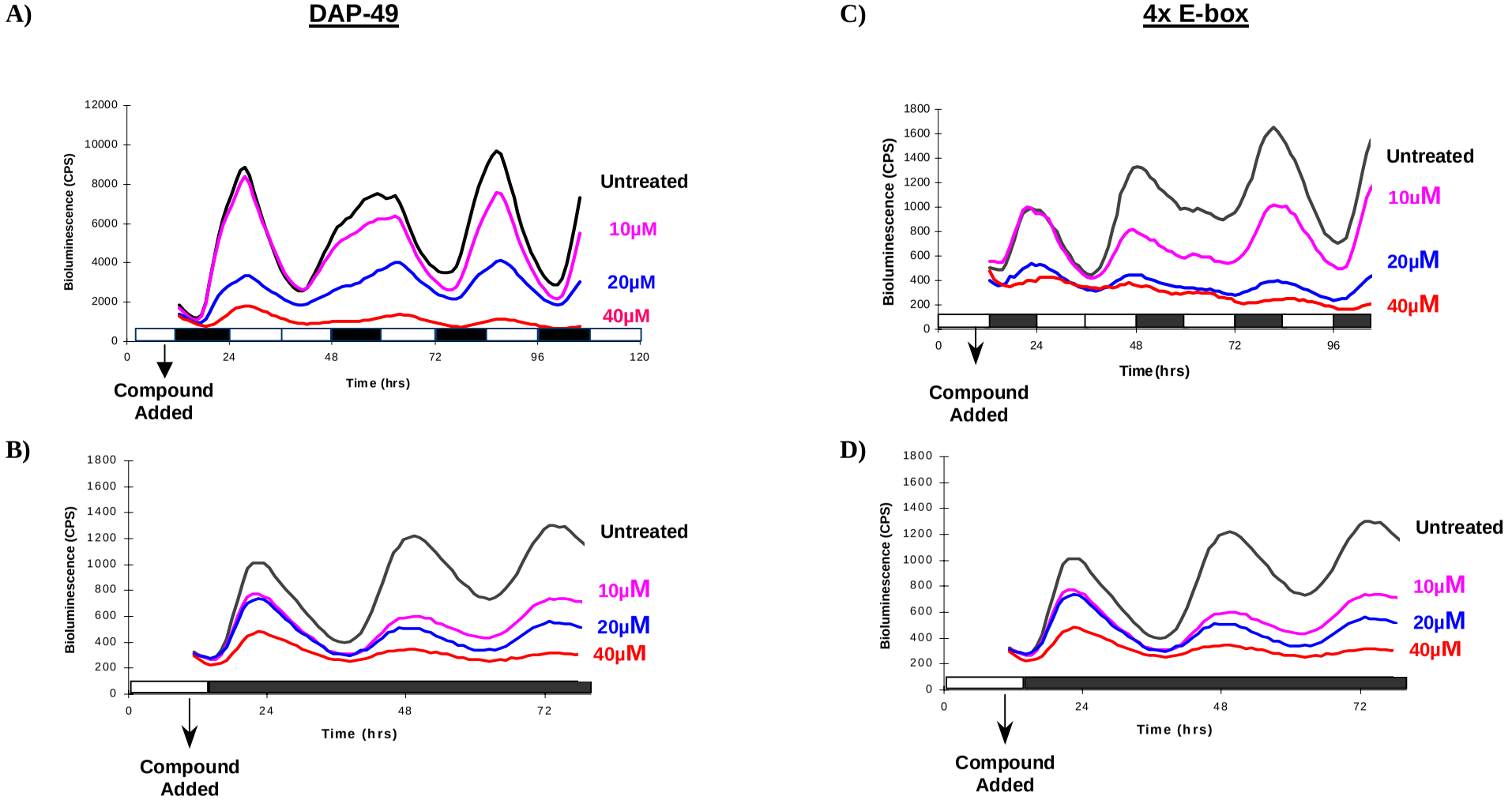
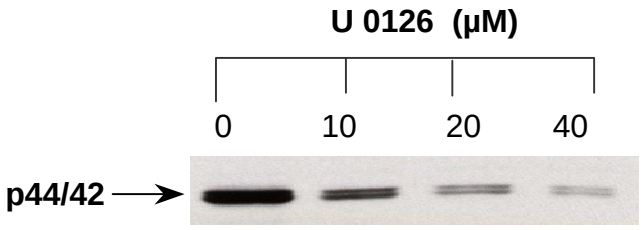


Figure.25

Effect of the MAPK inhibitor U0126 on *zfPeriod4* and 4x E-box luciferase gene expression. Treatment of the cells and entrainment conditions are equivalent to those in Figure.19. In panels A, B, C and D, the black trace represents untreated cells and pink, blue and red traces, cells treated with 10,20 and 40µM U0126. Panel A and B are traces from DAP-49 clones whereas panel C and D shows the traces of the 4x-E-box clones.

A)



B)

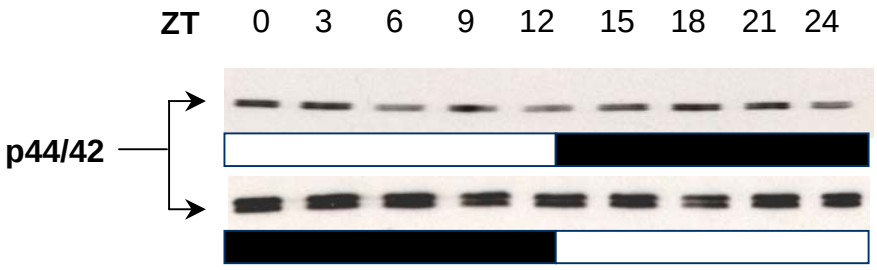


Figure.26

Western blot analysis of phosphorylation of the endogenous MAPK protein p44/42. A) Wild type PAC-2 cells in 25cm^2 flasks were treated with 10, 20 and 40 μM U0126 with one flask serving as an untreated control. Protein extracts were then collected after 12 hours. A dose dependent reduction in phosphorylation of p44/42MAPK was observed. B) High resolution experiment to test for a circadian rhythm of phosphorylation of p44/42 MAPK protein. Sets of wild type PAC-2 cells in 25cm^2 flasks were entrained under LD and DL cycles and protein extracts were prepared each three hours between ZT0 and ZT24. Incubating the resulting membrane with anti-p44/42 phospho-specific antibody revealed a constant level of phosphorylation in both sets of samples, with two distinct bands visible. The gel for the analysis in the top panel was run for a shorter distance than that in the bottom panel. For this reason the separation of the two bands is greater in the lower panel than in the upper panel.

4. Discussion

4.1 The advantages of a luciferase reporter system for zebrafish cell lines

The work presented in this thesis documents how a luciferase reporter assay was developed as a key tool to study the circadian clock in zebrafish cell lines. The ability to monitor clock gene expression in real time with a non-invasive assay greatly adds to the value of these cells since until now, the only way to monitor the clock in this system was by RNA and protein analysis (Cermakian and Sassone-Corsi, 2002; Pando et al., 2001; Whitmore et al., 2000). One major advantage of using a bioluminescent reporter assay in photosensitive cells, is that it avoids the need to shine light on the cells in order to visualize the protein, as would be required to quantify green fluorescent protein (GFP) expression. However, one potential complication is that the bioluminescence from the reporter might directly influence the clock. We were able to demonstrate that "autoentrainment" was unlikely in our cell lines since the addition of luciferin under constant darkness did not result in the activation of light inducible clock gene expression.

One of the most striking general observations from our data is the high reproducibility and smoothness of the bioluminescence traces compared with those reported for other circadian clock model systems such as adult, transgenic *Drosophila* (Brandes et al., 1996). The high quality of our bioluminescence data may be explained by the emission of light from a static monolayer of uniformly expressing cells. Bioluminescence is more problematic to accurately quantify in *Drosophila* since normally the fly moves around inside the plate well and the efficiency of photon detection is not uniform at different positions within the well. Furthermore, the observation that the quality of the bioluminescence traces does not deteriorate even during three weeks assays following a single addition of luciferin suggests this compound is quite stable in the culture medium. Another striking property of the cells is their excellent viability even during culture for long periods at confluence in a 96 well plate. The ability to monitor high amplitude bioluminescence rhythms over long time

periods under light dark cycles contrasts with the transient, dampening rhythms described for mammalian cell lines transfected with equivalent clock gene promoter – luciferase reporter constructs (see Figure.27) (Izumo et al., 2003) . This result stems from the direct light entrainability of the clock in the zebrafish cells, a property not shared by mammalian cells (Whitmore and Pando, unpublished observations). Furthermore, most mammalian cell lines have a reduced viability once they arrive at 100% confluence. Mammalian cells require transient treatment with high concentrations of serum or activators of various signaling pathways in order to induce only rapidly dampening circadian rhythms of gene expression. For all these reasons, the luciferase reporter zebrafish cell lines offer unique possibilities as a cell culture model to study the circadian clock.

4.2 Light entrainment of a cell culture clock.

An examination of the changes in luciferase activity that accompany alterations in the light dark cycle suggests that light represents a potent entraining signal for the PAC-2 cell clock. We have confirmed that this is the case by performing the first phase response curve analysis to be reported for a cell line. We demonstrate that the PAC-2 clock shows typical high amplitude PRC (Johnson, 1999). Maximum phase shifts are obtained with light pulses delivered during the early subjective night. In addition, at the beginning of the early subjective day only small phase delays are observed representing the so-called “dead zone” (Johnson, 1999). We have further confirmed the importance of light for setting the clock by showing that exposure of the cells to light dark cycles shorter (20 hours) and longer (30 hours) than the normal 24 hours cycle leads to a corresponding shortening or lengthening of the clock rhythm period length (Vallone et al., 2004). Following transfer of the cells to constant darkness, the cells re-establish a circadian (25.19 +/- 0.21 hours) free running rhythm period (Vallone et al., 2004). Therefore, one may predict that direct

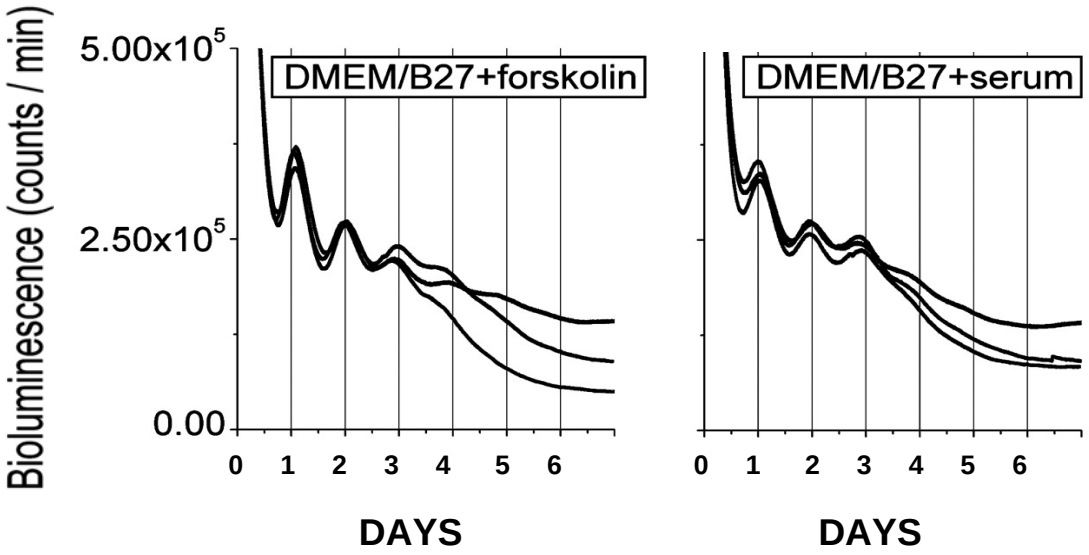


Figure.27

Rat-1 fibroblast bioluminescence rhythms generated by a stably transfected rat *period1* promoter linked to the luciferase reporter. The fibroblast cells were stimulated using transient treatments with forskolin or serum. The traces show rapid dampening of the rhythms within 3-4 days. Figure adapted from Izumo et.al. (2003).

light exposure plays a key role in the entrainment of peripheral clocks in the zebrafish circadian timing system.

4.3 Dampening rhythms in constant darkness

Following entrainment by light dark cycles and transfer to constant dark conditions, one of the most characteristic features of the bioluminescence rhythms, is their progressive dampening to intermediate levels relative to the previous LD cycles. Ueli Schibler and colleagues originally discussed the issue of the dampening of clock rhythms in the context of cell cultures (Balsalobre et al., 1998). They proposed two alternative models to explain the phenomenon. In one, following transfer to free-running conditions, individual cell clocks continue to oscillate normally, but the phase of the rhythms start to drift apart until the cells are oscillating completely asynchronously. As a result, when measured as a population, the rhythm amplitude gradually dampens. In the alternative hypothesis, the cell clocks remain synchronized with each other, but the amplitude of the rhythm gradually decreases with each cycle in the absence of exposure to the original zeitgeber. In essence, in this scenario the zeitgeber is required to drive the clock and maintain its rhythm (Balsalobre et al., 1998) amplitude. The most direct way to test these two hypotheses is to monitor how clock activity at the single cell level responds in free running conditions. However, single cell imaging of clock gene activity over long time-periods has proved to be technically challenging. Single cell measurements of electrical activity of SCN neurons (a clock output) has been performed in dissociated neurons plated on a multi-electrode grid as well as in the context of brain slices (Schaap et al., 2003; Welsh et al., 1995). However, to date this has not been possible in peripheral tissues and derived cell lines. In one report, changes in the bandwidth of dampening luciferase activity rhythms of transfected Rat 1 fibroblasts held at different temperatures has been used as evidence for the second hypothesis: the presence of dampening of the pacemaker and not desynchronization (Izumo et al., 2003).

Our luciferase reporter cells have enabled a direct test of the two hypotheses. As part of collaboration, David Whitmore (UCL, London), has performed single cell imaging of the luciferase reporter PAC-2 cells, during 3 or 4 days following transfer to constant darkness. His results show that during the experiment, individual cell clocks continue to oscillate although with slightly different period lengths. Consequently, phase differences between the individual cell clocks increase with increasing time in DD. An analysis was also performed with cultures that have been maintained for 3 months in constant darkness. This reveals that individual cells continue to oscillate but completely asynchronously with their neighboring cells. Together, these results would tend to support the first hypothesis that dampening results from progressive desynchronization. Thus, exposure to a light dark cycle is essential to maintain synchronization of individual cell clocks but not for driving the clock mechanism. It is therefore possible that a different mechanism accounts for dampening clock rhythms in mammalian and zebrafish peripheral tissues.

4.4 The nature of the PAC-2 cell photopigment

One important issue is the nature of the photoreceptor within the PAC-2 cells. Early characterization of the PAC-2 cells failed to detect expression of any of the major rod and cone opsin photoreceptors expressed in the eye (Moutsaki et al., 2003). Current favorite candidates for this function include novel opsins such as tmt opsin as well as cryptochromes that are widely expressed in many zebrafish tissues and the PAC-2 cell line (Cermakian et al., 2002; Moutsaki et al., 2003). Important clues as to the nature of this photopigment can come from measuring the efficiency of monochromatic light of different wavelengths to entrain the clock, a so-called action spectrum. By a low-resolution action spectrum analysis, Cermakian et al., (2002) have predicted that a blue light photoreceptor, possibly a cryptochrome, is responsible for direct entrainment of the clock by light (Cermakian et al., 2002). This work used an RPA assay to quantify levels of the light inducible *zfperiod2* transcript following illumination with monochromatic light. The scale of such an analysis is limited by the

number of RNA samples that can reasonably be analysed as well as the limits of the accuracy of quantification of a gel based assay. In addition, this analysis makes the assumption that *zfperiod2* induction is directly linked with clock entrainment. In the absence of a specific test of the function of *zfperiod2* in the zebrafish circadian clock, this assumption may not be reasonable. A more direct assay that accurately quantifies stable phase shifting of a free running clock rhythm at a higher resolution of wavelengths is required to better test the involvement of opsins or cryptochromes. David Whitmore's group is currently using the *zfperiod4* luciferase reporter cell lines to quantify stable phase shifts in the free running bioluminescence rhythms induced by short light pulses of different wavelengths. The possibility to include up to 96 samples per light pulsed plate and the ability to assay up to 20 plates simultaneously should permit a high resolution action spectrum analysis with a high degree of statistical certainty.

4.5 Light entrainment of the clock during early zebrafish development.

We have documented the expression profile of the *zfperiod4* gene during zebrafish embryonic development. The presence of a shallow oscillation on day 1 points to light responsiveness maturing very early during development. Indeed a recent report has documented light inducible gene expression already during gastrulation – well before any specialized photoreceptor cells or structures have differentiated (Tamai et al., 2004). Another important observation is that under a LD cycle, the amplitude of rhythmic expression steadily increases with increasing age of the embryo, while under constant darkness this early shallow rhythmic expression dampens rapidly so that by day 6, no significant rhythm is detectable. The presence of some shallow rhythmicity in DD conditions during the first 2 days of the time course may be explained by the exposure of the embryos to 4 or 5 hours of light during their collection, cleaning and sorting before they were aliquoted into flasks and placed in the dark (see Figure.8). Therefore, we conclude that the presence of a LD cycle is essential to establish a robust circadian clock function during early

development. These results disagree with an earlier report claiming that a robust functional circadian clock was maternally inherited in zebrafish (Delaunay et al., 2000). In this regard, our results are in agreement with many subsequent reports that fail to detect circadian rhythms in several circadian clock outputs, including the daily timing of increased locomotor activity and cell proliferation in larvae raised under DD conditions (Cahill, 2002; Dekens et al., 2003b).

4.6 Promoter analysis.

We have analysed the *zfperiod4* promoter by stably transfecting luciferase promoter reporter constructs into PAC-2 cells and then testing for regulation by light and the endogenous clock. This approach has the major advantage that regulation by physiological levels of endogenous factors is tested. Performing promoter analysis with transgenic animals offers similar advantages but has the draw back of being far more time consuming. Many previous studies exploring transcriptional regulation in the clock have been based upon transient transfection assays. However, such studies may be misleading since over-expression of a candidate regulator may drive physiologically non-relevant interactions. Here we demonstrate for the first time in a vertebrate cell culture model, that a functional circadian clock drives rhythmic expression via E-box elements in the context of a minimal heterologous promoter. This result is consistent with current models for the circadian clock however; these rhythms are 6 hours phase advanced compared with the *zfperiod4* promoter. Our observations that the phase of the E-box heterologous promoter is unaffected when the number and spacing of the E-boxes are changed tends to support the notion that this phase difference directly reflects the function of the E-box and is not an artefact of multimerization. It is therefore clear that E-boxes can direct rhythmic expression with significant differences in phase depending on their promoter context.

Mutational analysis has demonstrated that the E-boxes contribute to maintaining a low level of promoter expression. This is surprising since they would be predicted to bind CLK/BMAL and so function as enhancers. Indeed mutation of circadian E boxes has previously been documented to reduce expression levels *in vivo* (Hao et al., 1997; McDonald and Rosbash, 2001). We have also implicated these elements in robust rhythmic expression and down regulation by light. Interestingly, in the context of a heterologous promoter, these E-box elements direct rhythmic expression that is not repressed by light pulses implying that the local promoter environment might determine their function. It is noteworthy that none of the 4 E-boxes correspond to the optimal binding sites for mammalian CLK/BMAL (Hogenesch et al., 1998). It will be interesting to test whether the additional zebrafish CLK and BMAL homologs bind differentially to these elements and thereby confer light-responsive, rhythmic expression with low basal levels (Ishikawa et al., 2002).

The repression of *zfperiod4* expression following exposure to light occurs only after a delay of 4 hours. This suggests earlier induction of a repressor factor. Expression of the *zfperiod2* gene is induced within the first 2 hours following light exposure (Cermakian et al., 2002; Pando et al., 2001). Given the role of period proteins in the circadian timing mechanism, it is tempting to speculate that light-induced *zfperiod2* may down-regulate *zfperiod4* expression via the E-box elements. Period proteins appear to function in combination with Cryptochromes to repress CLK:BMAL heterodimer activation. In the chicken pineal, light has been shown to acutely induce cryptochrome expression (Yamamoto et al., 2001). However, the lack of repression of the 4xE-box heterologous promoter constructs by light would tend to argue against this. Alternatively, light may induce expression of other transcriptional repressors which bind to distinct enhancer elements and then interact with E-box bound factors in the context of the promoter (Doi et al., 2001).

An important result from our promoter analysis is that heterologous promoter constructs containing multimerized transcription factor binding sites linked to a minimal promoter, are expressed efficiently once stably transfected into the

PAC-2 cells. Thus, theoretically using the same approach, gene expression directed by other enhancer elements in response to other signaling pathways can be visualized in real time. This could potentially be a useful tool in studies of other signaling pathways and target enhancer elements in zebrafish.

4.7 Pharmacological analysis.

We have treated our cell lines with various soluble activators or inhibitors of the cAMP, PKC and MAPK signaling pathways in an attempt to explore the contribution of these pathways to regulating the circadian clock and its entrainment by light. Our results represent preliminary findings that provide the basis for future studies. We chose to use a more simple chronic treatment protocol to avoid the complication of short-term treatments. One major limitation of our studies is that the half-lives of the compounds in the culture medium and therefore the durations of their activity are unknown. Ideally, in order to test for phase shifting effects, the drugs should be applied under constant darkness for only a short period and then washed away before monitoring the phase of the free running rhythm. This poses a significant technical challenge to manipulate the 96 well cultures in conditions of constant darkness, avoiding temperature changes. Using “night-vision” goggles may be one solution to performing cell culture in constant darkness.

We have implicated the PKA pathway in determining the amplitude of circadian rhythms of clock gene expression. Previous reports have documented diurnal rhythms of cAMP and PKA levels in the SCN of syrian hamsters (Ferreyra and Golombek, 2000). Entrainment of the *xenopus* photoreceptor circadian oscillator by light has been linked with decreases in cAMP levels (Hasegawa and Cahill, 1998), Also phase shifts in the clock have been reported following treatment of the rat SCN with PKA activators and inhibitors (Lee et al., 1999b; Prosser and Gillette, 1989).

Our results point to a strong effect of the PKC pathway in phase shifting and sustaining the activity of the circadian clock in free running conditions. In particular, the absence of rhythmic expression in both the *zfperiod4* and 4xE-

box luciferase reporters under DD conditions in the presence of a PKC inhibitor suggests that this treatment may even "stop" the clock. Light driven expression of the *zfperiod4* promoter in PAC-2 cells seems however to be unaffected by the same inhibitors. Several reports have demonstrated the phase shifting effects of PKC activators and inhibitors on the SCN clock (Biello et al., 1997; McArthur et al., 1997; Schak and Harrington, 1999). Interestingly, the PKC pathway has also been implicated in the signal transduction pathways linking the high affinity, G protein-coupled melatonin receptor with the circadian clock mechanism in the SCN (McArthur et al., 1997). Transient treatment with phorbol esters can induce circadian rhythms of gene expression in mammalian cell lines (Balsalobre et al., 2000b). However, this induction of rhythmic gene expression was shown to be inhibited by a MEK inhibitor, U0126 implying that the MAPK pathway may mediate some of the effects of PKC activation on the circadian clock (Akashi and Nishida, 2000).

Our results predict a role for the MAPK pathway in both phase shifting and setting the amplitude of circadian rhythms of gene expression in the PAC-2 cells. Many studies point to a link between the MAPK pathway and the circadian clock, in particular its entrainment by light. Thus, brief exposure to light during the subjective night, but not during the subjective day, has been reported to activate the p44/42 MAPK signaling cascade in the SCN (Obrietan et al., 1998). Inhibition of the MAPK pathway also disrupts light-induced phase shifting of the circadian clock in the mouse SCN (Butcher et al., 2002). A transient inhibition of MAPK induces a phase shift of the oscillator in the chick pineal gland (Hayashi et al., 2001; Sanada et al., 2000) and bull frog retina (Harada et al., 2000). In the chick pineal gland, the MAPK pathway seems to be regulated via the Ras-Raf-MEK pathway and forms part of an interconnecting regulatory feedback loop associated with the core, clock feedback loop (Butcher et al., 2003; Sanada et al., 2000). Furthermore, activated MAPK has recently been shown to phosphorylate and negatively regulate the transcription factor BMAL1 (Sanada et al., 2002). Additional direct evidence showing the involvement of the MAPK pathway in the

zebrafish clock comes from studies involving zebrafish cell lines where light induced expression of *zfperiod2* is blocked by MAPK pathway inhibitors (Cermakian and Sassone-Corsi, 2002).

4.8 Future Prospects

The luciferase reporter cell lines that have been established as well as the development of a reliable protocol for stable and transient transfection of the PAC-2 cells provide a wide range of possibilities for future studies.

We have already embarked upon a comparable promoter analysis of another clock gene: zebrafish cryptochrome *cry1A* using a stable transfection assay. We have shown that this clock gene is acutely induced by light. The aim of this on-going study will be to identify the promoter elements that mediate the response to light, as a first step towards unraveling the signaling pathways that lead from the circadian photoreceptor. The results of this study should complement the findings reported in this thesis and further improve our understanding of the transcriptional control systems of the circadian clock in these cells.

Another line of investigation is to use the transient transfection protocol to test the effects of over expression of wild type and dominant negative versions of zebrafish clock gene products upon the regulation of the *zfperiod4* promoter by the endogenous, light entrainable circadian clock mechanism. Avoiding the time consuming step of selecting for neomycin resistant stably transfected clones, this should constitute a rapid route to explore the role of the members of the various clock gene families. Indeed, given the links between the circadian clock and the timing of the cell cycle, the establishment of cell lines stably over expressing clock gene products may be difficult if cell proliferation is impaired.

We are also using the *zfperiod4*-luciferase reporter cell lines to study how temperature regulates the zebrafish circadian clock. Our group has recently shown that temperature cycles entrain the zebrafish clock as efficiently as light - dark cycles. Also, expression of the *zfperiod4* gene is acutely regulated in response to temperature changes. We will therefore test how expression of the

various promoter deletion – reporter constructs responds to temperature as a step to identify which promoter elements mediate regulation of clock gene expression by temperature.

The culture properties of the PAC-2 cells make these clock-luciferase reporter cell lines potentially ideal for high throughput screening. One possibility is to use our cell culture assay to screen small compound libraries to identify compounds that either directly influence the clock or its entrainment by light or temperature. Such compounds might subsequently prove valuable tools for biochemical analysis and potentially may even represent starting points for developing therapeutic drugs that might be more specific regulators of the circadian clock.

Finally, our results have validated the function of various zebrafish promoters that are now being used to generate stable transgenic lines of fish. Such clock reporter bioluminescent fish can be anticipated to constitute valuable tools for mutant screening. The opportunity to assay clock function non-invasively by monitoring luciferase activity in individual embryos in a 96 well plate assay under various lighting and temperature conditions should prove invaluable to identify mutations affecting for example light and temperature entrainment.

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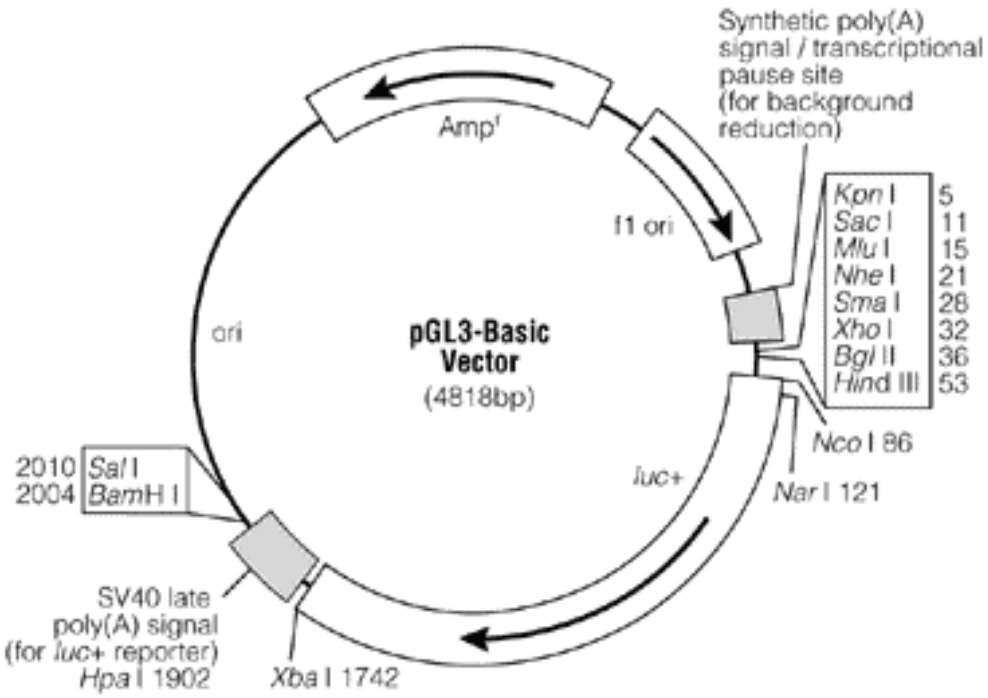
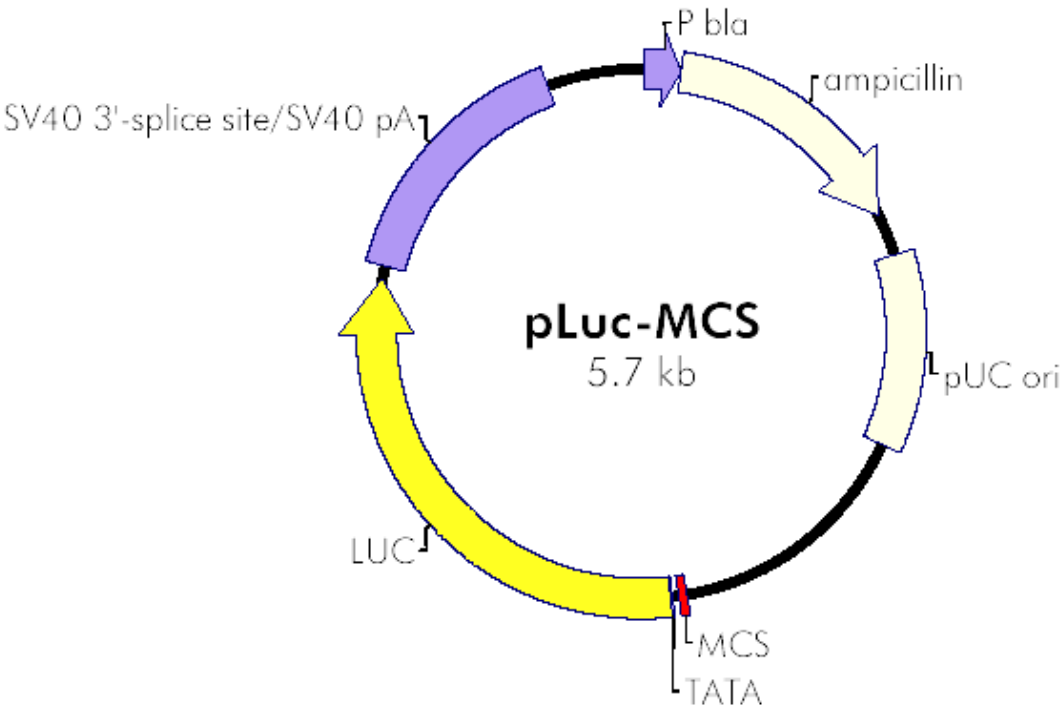
6. Appendix

6.1 pGL3 basic vector map



The pGL3 basic vector used to clone all *zfperiod4* promoter fragments. Fragments were inserted between the KpnI or XhoI (5') and HindIII (3') sites in the multiple cloning site, located at the 5'end of the luciferase gene (Luc+). 'Ori' is the origin of replication of the plasmid derived from the E.coli plasmid vector pBR322. The SV40 poly(A) signal serves to ensure transcription termination and polyadenylation of the luciferase transcript. The synthetic polyA signal upstream of the MCS serves to reduce background by terminating and polyadenylating any transcripts initiated non-specifically upstream of the MCS and thereby prevent them incorporating the luciferase coding sequence. Map adapted from Promega catalog.

6.2 pLuc MCS vector map



The pLuc MCS is a heterologous promoter vector, which was used to clone all the E-box sequences. It contains a luciferase reporter gene driven by a basic promoter element (TATA box). A multiple cloning site (MCS) is located upstream of the TATA element and allows the insertion of any cis-acting enhancer elements. The SV40 poly(A) signal serves to ensure transcription termination and polyadenylation of the luciferase transcript. pUC ori is the the origin of replication and an ampicillin resistance gene is included.

6.3 Pharmacological analysis

Activators	Company	Solvent	Stock concentration (1000x)
PMA (PKC activator)	Sigma	DMSO	3mM
Calcimycin (Calcium ionophore)	Sigma	DMSO	3mg/ml
EGF (RTK-MAPK activator)	Sigma	H_2O	200µg
Di Butyryl cAMP (cAMP dependent PKA activator)	Sigma	H_2O	3M
Forskolin (Adenylate cyclase activator)	Sigma	DMSO	10µM

Inhibitors	Company	Solvent	Stock concentration (1000x)
U0126 (MEK1/2 Inhibitor)	Cell Signalling	DMSO	40mM
Ro-31-8220 (PKC Inhibitor)	Calbiochem	DMSO	8mM
H-89 (PKA inhibitor)	Calbiochem	DMSO	30mM

The above table summarizes the different compounds used in our pharmacological analysis. The commercial supplier, the solvent for dissolving each compound and the stock concentration used are all indicated.

Lebenslauf

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