CRYPTOCHROME, CIRCADIAN CYCLE, CELL CYCLE CHECKPOINTS, AND CANCER

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ABSTRACT

MICHELE ANN GAUGER: Cryptochrome, Circadian Cycle, Cell Cycle Checkpoints, and Cancer
(Under the direction of Aziz Sancar)

The mammalian circadian clock is a global regulatory system that controls many aspects of physiology, including behavior, metabolism, cell cycle progression, and overall fitness. CRYPTOCHROMES (CRYs) are core elements of the mammalian circadian clock, and loss of CRY expression leads to arrythmicity. Although much work has been done analyzing the mammalian clock, the molecular mechanisms underlying the clock and resulting from its disruption are still largely unknown. There is growing evidence that circadian rhythm disruption in both humans and rodents leads to predisposition to cancer and poor prognosis; however, it has not been determined if cancer predisposition is a hallmark of all types of clock disruption. Here I present evidence that arrhythmic Cry1-/-Cry2-/- mice possess an intact DNA damage checkpoint and repair system and are not predisposed to ionizing radiation-induced cancers relative to wild-type mice. In addition, experiments were conducted to determine the direct effect, if any, that CRY1 exerts on CLOCK-BMAL1 heterodimer DNA-binding. I find that CRY1 neither inhibits nor modifies the DNA binding of a heterodimer consisting of BMAL1 and a 342-amino acid fragment of CLOCK (CLOCK342) *in vitro*. However, this does not rule out the possibility that CRY1 could have an effect on a heterodimer of BMAL1 and full-length CLOCK *in vitro*.

For my family, who have always supported me without question

and

For Aziz, from whom I learned much more than just science.

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LIST OF ABBREVIATIONS AND SYMBOLS

♀	female
♂	male
α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
°C	degrees Celsius
μg	microgram
μl	microliter
aa	amino acid
ACTR	Nuclear receptor coactivator protein
ATR	Ataxia telangiactasia related
ATP	adenosine triphosphate
bHLH	basic helip-loop-helix
BMAL1	Brain-muscle-Arndt-like-protein-1
CBP	Creb-binding protein
CCG	clock-controlled gene
CDC2	cell division cycle 2
CHK1	Checkpoint kinase 1
CK1δ	Casein kinase 1 delta

CK1ε	Casein kinase 1 epsilon
CLOCK	Circadian-locomoter-output-cycles-protein-kaput
cm	centimeter
CO	carbon monoxide
CO_2	carbon dioxide
CRY	Cryptochrome
CYC	Cycle
DBT	Doubletime
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FRQ	Frequency
g	gauge
Gy	Gray
h	hours
HAT	histone acetyltransferase
HU	hydroxyurea
IPTG	isopropyl β-D-1-thiogalactopyranoside
IR	ionizing radiation

J/m^2s	Joules per meter squared-second
KCl	potassium chloride
KO	knockout
L	liter
LB	Luria-Bertani
LD	light:dark
M	molar
mg	milligram
min	minute
ml	milliliters
mm	millimeter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
MTHF	5,10-methenyltetrahydrofolate
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
nm	nanometer
nM	nanomolar
NP-40	Nonidet P-40
NPAS2	Neuronal PAS domain protein 2
^{32}P	phosphorous 32
PAS	Per-Arnt-Sim

PBS	phosphate buffered saline
P/CAF	p300/Creb associated factor
PCC	premature chromatin condensation
PER	Period
PHR	photolyase homology region
PP5	Protein phosphatase 5
REV-ERBα	V-erbα-related protein
rd	retinal degeneration
RNA	ribonucleic acid
RORα	Retinoic acid receptor alpha
rpm	revolutions per minute
SAD	seasonal affective disorder
SCN	suprachiasmatic nucleus
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
TBS	Tris-buffered saline
TIM	Timeless
Tris	Tris(hydroxymethyl)aminomethane
TTFL	transcription-translation feedback loop
UV	ultraviolet
v/v	volume-to-volume ratio
WCC	White-Collar complex
ZT	zeitgeiber time

CHAPTER 1

CRYPTOCHROME AND THE MAMMALIAN CIRCADIAN CLOCK

Introduction

The solar cycle is perhaps the most pervasive environmental stimulus for organisms on Earth. Adaptation to this stimulus and ability to perceive changes in light conditions confers a selective advantage. The earliest application of this adaptation is thought to have evolved for the purpose of protecting replicating DNA from ultraviolet light during the daytime hours. Since then, organisms ranging from cyanobacteria to humans have evolved mechanisms to synchronize physiological processes to the 24-hour solar day, allowing organisms to predict and prepare for constantly changing environmental conditions. These mechanisms comprise an organism's circadian (circa-24 hours) clock and regulate such processes as behavior, metabolism, cell division, sleep-wake cycles, body temperature, heart rate, and hormone production; indeed, most physiological functions undergo circadian oscillation (Rutter et al., 2002).

The circadian clock exerts its control over physiological processes through finely tuned control of gene expression; it is estimated that up to 10% of cellular genes in mammals undergo circadian oscillation in expression (Panda et al., 2002). Although the endogenous circadian clock maintains synchronization under constant environmental conditions, disturbance in clock synchronization by the solar cycle can result in physiological disorders

in humans. Under conditions of jet lag, a rapid change of local time causes the internal clock to not fully synchronize with the local time; this can result in fatigue, irritability, and has been shown to affect fitness and tumor growth in mouse models (Filipski et al., 2005; Filipski et al., 2006). Rotating shift work is another example of environmental clock disruption that can lead to fatigue, insomnia, and has been associated with a higher risk of breast cancer in women (Hansen, 2000). Molecular disruption of circadian rhythms in humans has been associated with seasonal affective disorder (SAD), familial advanced phase sleep syndrome, delayed sleep phase syndrome, cancer, and generally decreased fitness (Sancar, 2000; Stevens, 2005). Although we have thus far gleaned much information on the mammalian clock, evidence towards the biochemical mechanisms underlying the clock are generally lacking.

The Mammalian Circadian Clock

Most eukaryotic organisms possess an intrinsic circadian rhythm with a periodicity of approximately, but not exactly, 24 hours (24.2 hours in humans (Czeisler et al., 1999), 23.5 hours in mice). These rhythms exist in the absence of external stimuli (i.e. in constant darkness) and are self-sustained in every eukaryotic cell, including cells cultured *ex vivo* (Nagoshi et al., 2004).

Endogenous circadian rhythms in mammalian peripheral tissues are entrained to the exact 24-hour solar day through signals from a central circadian pacemaker organ. In mammals, the central circadian pacemaker is located in the suprachiasmatic nucleus (SCN) in the hypothalamus (Hastings and Maywood, 2000). The SCN consists of a cluster of neurons that exhibits robust light-induced changes in gene expression, with light signals

being received through visual photoreceptors and photoreceptive retinal ganglion cells. The response of the SCN to light-induced changes is rapid, with complete entrainment to a shift in daily light-dark cycle occurring within one day of the change (Yamazaki et al, 2000). Photoentrainment of the SCN does not require vision (rod and cone photoreceptors in the outer retina); as such, blindness in humans and mice does not destroy the ability to synchronize the circadian clock (Sancar, 2000). Rather, the non-visual photoreceptors in the inner retina are sufficient for complete photoentrainment. The SCN is critical for maintenance of circadian rhythmicity (Ralph et al., 1990; Sakamoto et al., 1998; Rusak and Zucker, 1979). Lesions of the SCN cause complete ablation of the circadian clock in rats (Klein et al., 1991). The circadian rhythm machinery in the SCN is similar to that in all mammalian cells with the exception of photoreceptive capability. This allows the SCN, through an uncharacterized signaling pathway, to reset an organism's circadian rhythm phase according to the solar cycle and other environmental stimuli, called *zeitgeibers* (literally, "time-givers"). Information from the SCN is passed onto the pineal gland, causing oscillations in secretion of the hormone melatonin. Consequently, these signals are passed onto peripheral tissue clocks to synchronize and regulate transcriptional activity throughout the day.

To date, there is a great deal of genetic evidence for the mechanisms underlying the mammalian circadian clock; however, biochemical characterization of the clock is lacking. Genetic studies have led to the formulation of the current model for the mammalian circadian clock, in which the clock is engendered mainly by the interplay of positive and negative transcriptional feedback loops (Figure 1.1; Gauger and Sancar, 2005). Similar mechanisms have been indicated for all organisms whose circadian rhythms have been extensively studied

(Rutter et al., 2002). At the center of these loops are the core clock proteins, whose gene products are necessary to maintain circadian rhythmicity in individual cells (Takahashi, 2004). The core clock proteins participating in this loop will be described in detail in Section 1.3. Four core clock proteins, BMAL1, CLOCK, PERIOD (PER), and CRYPTOCHROME (CRY), are particularly important in this model. A heterodimer of BMAL1 and CLOCK participate in the positive segment of the transcriptional feedback loop, in which the BMAL1-CLOCK complex activates transcription of genes whose promoters contain E-box elements (CACGTG). These genes include clock-controlled genes (CCGs) whose products are not directly involved in the circadian clock machinery, and also the core clock genes *period* and *cryptochrome*. PER and CRY proteins, after transcription and translation, form the negative segment of the transcriptional feedback loop by inhibiting the BMAL1-CLOCK heterodimer through an unknown mechanism. In this way, PER and CRY negatively regulate their own transcription. In a second, minor feedback loop, the circadian proteins REV-ERBα and RORα regulate transcription of Bmal1 by repression and activation, respectively (Sancar, 2000; Wijnen and Young, 2006). *Rev-Erbα* and *Rorα* are both targets of CLOCK-BMAL1-mediated transcription, thereby regulating their own expression. In addition to the positive and negative transcriptional feedback loops themselves, other elements are believed to contribute to the 24-hour periodicity of oscillation of gene expression in the circadian clock. These elements likely include regulation of protein accumulation in the cytoplasm/nucleus and/or posttranslational modifications; however, these regulatory elements have not been clearly elucidated yet.

The canonical transcription-translation feedback loop (TTFL) model of the mammalian circadian clock has been challenged of late. For some time it has been known

that in addition to contributions from the TTFL model, post-translational modification of the clock proteins occurs and is likely a component of their regulation (Lee et al., 2001). In the clock system of the cyanobacterium *Synechococcus*, in which a similar TTFL had been implicated for many years, it has been recently shown that circadian rhythmicity can be reconstituted using purified proteins in a closed *in vitro* system (Nakajima et al., 2005; Kageyama et al., 2006). This data indicates that, in at least one system, a circadian rhythm can persist in the absence of transcription or translation and is prompting serious re-evaluation of the orthodox mammalian circadian clock model (reviewed in Lakin-Thomas, 2006).

The Core Circadian Clock Proteins

In the mammalian circadian clock, the four main core clock components are the *Clock, Bmal1, Period (Per; Per1, Per2,* and *Per3),* and *Cryptochrome (Cry; Cry1* and *Cry2)* genes and their gene products, CLOCK, BMAL1, PER (PER; PER1, PER2, and PER3), and CRY (CRY; CRY1 and CRY2), respectively. These four proteins comprise the positive and negative arms of the transcriptional feedback loops that engender the mammalian clock. Mutations in the core clock components result in the most dramatic clock phenotypes. I will provide a detailed description of these components here.

CLOCK

Clock was the first mammalian clock gene to be identified (King et al., 1997; Antoch et al., 1997). Homologues of mammalian CLOCK have been found in *Drosophila* (dCLOCK) (Allada et al., 1998) and zebrafish (zCLOCK) (Ishikawa et al., 2002) and a complex orthologous to CLOCK-BMAL1 is formed in Neurospora (the White-Collar

Complex) (Wijnen and Young, 2006). The mammalian *Clock* gene encodes a basic helix-loop-helix (b-HLH) PAS protein that acts as a transcription factor when in complex with another core clock protein, BMAL1 (King et al., 1997; Antoch et al., 1997). The CLOCK-BMAL1 heterodimer binds to E-box elements in gene promoters, most notably the promoters of the *Period* (*Per*) and *Cryptochrome* (*Cry*) core circadian genes, along with circadian gene *Rev-Erbα* and many clock-controlled genes that do not participate in the clock itself. CLOCK has been shown to interact physically with not only BMAL1 but also the CRY and PER proteins (Lee et al., 2001). *Clock* is somewhat unique among the four main clock component genes in that its expression does not oscillate and is not under circadian control; CLOCK protein levels likewise are constant throughout the circadian cycle (Lee et al., 2001). Mice homozygous for a mutation of *Clock* exhibit an abnormally long circadian period, becoming arrhythmic in constant darkness (Vitaterna et al., 1994). CLOCK protein resulting from this mutation lacks residues encoded for in exon 19 of the *Clock* gene. In heterozygous mutant animals, the mutant protein can compete with wild-type CLOCK protein (King et al., 1997); in homozygous mutant animals, the mutant protein results in functionally defective CLOCK:BMAL1 heterodimers (Gekakis et al., 1998; Jin et al., 1999). Interestingly, it was recently reported that CLOCK is not an absolutely essential circadian clock component (DeBruyne et al., 2006). CLOCK-null mice demonstrate altered responses to light and alterations in circadian clock gene expression; however, they still display robust circadian rhythms as measured by locomotor activity. The discrepancy between results obtained with CLOCK-mutant mice and CLOCK-null mice is hypothesized to be due to the dominant-negative effect of CLOCK-mutant protein. A CLOCK-redundant protein has been proposed

to heterodimerize with BMAL1 in the absence of CLOCK to maintain general circadian clock function; NPAS2 is a likely candidate for this.

In addition to DNA-binding and transcriptional activating activities, CLOCK has also been recently shown to have histone acetyltransferase (HAT) activity. The carboxy-terminus of CLOCK shows significant structural similarity to the carboxy-terminus of the HAT protein ACTR (Chen et al., 1997) and CLOCK exhibits HAT activity in experiments using purified histones as substrates (Doi et al., 2006). CLOCK's HAT activity appears to be essential for its function in circadian regulation, as ectopic expression of a HAT-deficient CLOCK mutant protein in a homozygous *Clock* mutant background does not restore circadian transactivation of the *Per* and *Dbp* genes (Doi et al., 2006). However, it is unknown what the effect of ectopic expression of HAT-deficient CLOCK in a true CLOCK-null background will be. The HAT activity of CLOCK indicates that regulation of histone acetylation may be involved in circadian gene expression and specifically CLOCK:BMAL1 function. This hypothesis supports reports that CLOCK interacts with histone acetylases (Etchegaray et al., 2003) and that histone modification displays circadian variation (Ripperger and Schibler, 2006).

BMAL1

BMAL1 (sometimes referred to as MOP3) was originally found as a protein with high expression in mammalian brain and muscle (Ikeda and Nomura, 1997; Hogenesch et al., 1997). BMAL1 has homologues in *Drosophila* (CYCLE) (Rutila et al., 1998) and zebrafish (zBMAL1) (Ishikawa et al., 2002) and a complex orthologous to CLOCK-BMAL1 is formed in Neurospora (the White-Collar Complex) (Wijnen and Young, 2006). Like CLOCK, mammalian BMAL1 is a bHLH-PAS transcription factor. BMAL1 heterodimerizes with

CLOCK to activate transcription off of E-box-containing promoters, notably those promoters of the *Per* and *Cry* genes in addition to other CCGs. While CLOCK-BMAL1 does not control transcription of *Bmal1*, this gene is still under circadian clock control as the orphan nuclear receptor RORα activates its transcription (Sato et al., 2004) and REV-ERBα, another clock protein, negatively regulates its transcription (Reppert and Weaver, 2002). PER2 has also been shown to stimulate transcription of *Bmal1*, through an ill-defined mechanism (Hogenesch et al., 2003; Shearman et al., 2000). BMAL1 protein levels oscillate with circadian periodicity (Fu et al., 2002). Loss of expression of *mBmal1* causes complete loss of circadian clock function and therefore *Bmal1*$^{-/-}$ mice are arrhythmic at both the behavioral and molecular levels (Bunger et al., 2000). Additionally, a variety of other physiological phenotypes have been seen in *Bmal1*$^{-/-}$ mice, including decreased activity levels and body weight, joint disease, and shortened life span (Bunger et al., 2000; Rudic et al., 2004; Bunger et al., 2005; Laposky et al., 2005; Shimba et al., 2005; Kondratov et al., 2006; Sun et al., 2006). This suggests that BMAL1 may play tissue-specific roles in addition to its circadian clock function; this theory has been supported by a recent report (McDearmon et al., 2006).

BMAL1 undergoes post-translational modifications that are thought to contribute to its regulation. Phosphorylation of BMAL1, and of CLOCK, correlates with CLOCK-BMAL1's transcriptional activity (Lee et al., 2001). Also, BMAL1 has been shown to be sumoylated in a CLOCK-dependent manner (Cardone et al., 2005). The C-terminus of BMAL1 (specifically the section containing the last 43 amino acids) has been recently demonstrated to be required for its transcriptional activation activity as well as for its interaction with CRY1; this process is also dependent on the presence of CLOCK (Kiyohara et al., 2006). This portion of BMAL1 is not necessary, however, for association with PER2.

BMAL1 has been found to interact physically with CLOCK, CRY, and PER proteins in what may be a large clock-protein complex (Lee et al., 2001).

BMAL1 has been shown to be important in the regulation of the CLOCK-BMAL1 heterodimer. Nuclear accumulation of CLOCK protein and its subsequent degradation is largely dependent on BMAL1, through a poorly-understood mechanism (Kondratov et al., 2003). In support of this, Kwon and colleagues have recently reported that nucleocytoplasmic shuttling of BMAL1 is necessary for the transactivation and degradation of CLOCK-BMAL1 (Kwon et al., 2006).

PERIOD

The Drosophila *period* gene was first cloned in 1984, spawning a search for its mammalian homologue. Eventually three mammalian homologues were identified (Per1, Per2, Per3) and cloned (Albrecht et al., 1997; Zylka et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997). All three mammalian *Per* genes display homology to Drosophila *period* and oscillation in both the SCN and the retina (Whitmore et al., 1998). PERs are PAS-domain proteins with widespread expression patterns, localizing in the SCN as well as many peripheral tissues (Tei et al., 1997; Albrecht et al., 1997; Shearman et al., 1997; Zylka et al., 1998; Shigeyoshi et al., 1997). Mice deficient in *mPer1* have a persistent circadian rhythm with a shortened period; these mice also show impaired ability to maintain precision and stability of the circadian period (Zheng et al., 2001). Mice carrying a homozygous mutation in *mPer2* display a similarly short period, with complete loss of circadian rhythmicity in constant darkness (Zheng et al., 1999). In contrast, mice deficient in *mPer3* display an essentially normal circadian clock with a slightly shortened period (Shearman et al., 2000a; Reppert and Weaver, 2002). Taken together, these studies suggest

that PER2 is the most critical clock component of the PER proteins, while PER3 is considered to be outside the core circadian clockwork (Bae et al., 2001). A genetic variant in human *Per2* was the first mutation identified as a cause of familial advanced sleep-phase syndrome (Toh et al., 2001). Expression of PER oscillates with the highest amplitude of the core clock proteins.

PER proteins have been shown to function in the nucleus but do not directly bind to DNA (Fu et al., 2002). In *Drosophila*, dPER is one of the two main negative regulators of dCLOCK-CYC (the other being dTIM) (Wijnen and Young, 2006; Lee et al., 1999). Although mammalian PER has been reported to associate with CRY to negatively regulate CLOCK-BMAL1, it is widely accepted that CRY is the more potent circadian repressor in mammals (Reppert and Weaver 2002; Yu and Hardin, 2006). PER2 can suppress transcription by NPAS2-BMAL1 (a circadian transcriptional activator complex similar to CLOCK-BMAL1) as shown by reporter gene assay (Fu et al., 2002). Studies suggest that, in addition to its repressor function, PER2 also acts as a positive regulator, stimulating transcription of BMAL1 (Shearman et al., 2000). The NONO and WDR5 proteins, which associate with PER1, have been shown to modulate PER function: NONO likely functions as an antagonist to PER, while WDR5 appears to assist PER function (Brown et al., 2005). Stability of PER2 appears to be dependent, either directly or indirectly, on mCRY, as mPER2 protein levels in the SCN are markedly reduced in $Cry1^{-/-}Cry2^{-/-}$ animals (Shearman et al., 2000).

PER1 and PER2 may also function in the cell cycle. $mPer2^{m/m}$ mice were found to be hypersensitive to ionizing radiation, and thymocytes from these mice fail to undergo cell cycle arrest or apoptosis after exposure to IR. Additionally, IR was shown to cause an

increase in transcription of *mPer1*, *mPer2*, *Clock*, *Cry1*, and *Bmal1* (Fu et al., 2002). Overexpression of *mPer2* induces apoptosis in cancer cell lines and alters the expression of apoptosis-related genes (Hua et al., 2006). PER1 has also been linked to the cell cycle through interaction with checkpoint proteins ATM and Chk2. Overexpression of *Per1* sensitizes cancer cells to apoptosis after exposure to IR. In contrast, inhibition of *Per1* expression by siRNA causes a marked decrease in IR-induced apoptosis (Gery et al., 2006). Together, these data suggest that the PER family of proteins plays a role in the cell cycle, though it is unknown whether or not this function is independent of PER's circadian clock role.

CRYPTOCHROME

CRYPTOCHROME was originally identified as a plant blue-light photoreceptor which was especially important in cryptogamic plants and possessed a "cryptic" nature (Ahmad and Cashmore, 1993; Sancar, 2000). The first *Cryptochrome* gene isolated was from the mustard plant and was at first thought to be a photolyase (Batschauer, 1993). CRYs belong to the photolyase/cryptochrome family of structurally analogous flavoproteins, having high structural and sequence similarity to DNA photolyase. DNA photolyase, present in prokaryotes and lower eukaryotes but missing in humans, is a repair enzyme which utilizes blue light to repair UV-damaged DNA (Rupert et al., 1958; Sancar, 1994). CRYPTOCHROMEs, while having high similarity to DNA photolyase, by definition exhibit no repair activity (Sancar, 2000). Rather, plant CRYs use blue light to regulate growth and adaptation to the environment, while animal CRYs entrain the circadian clock to the daily light-dark cycle. CRY possesses a region of very high structural homology to photolyase, called the photolyase homology region (PHR) and most CRYs, especially those found in

eukaryotes, have a C-terminal extension whose sequence varies widely (Sancar, 2000; Partch and Sancar, 2005). This C-terminal extension is largely unstructured and is thought to be important in regulating CRY activity through a signal transduction cascade. In humans, CRY1 and CRY2 share a high amount of homology (80%) but have highly divergent C-terminal extensions. CRY1 also contains a well-conserved nuclear localization signal in its C-terminal extension whose deletion abrogates its translocation into the nucleus (Chaves et al., 2006). Both photolyases and CRYs contain two covalently-bound chromophores; most commonly, these are 5,10-methenyltetrahydrofolate (MTHF) and flavin adenine dinucleotide (FAD) (Sancar, 2003). MTHF acts as a photoantenna, absorbing a photon of blue light (Sancar, 2003; Johnson et al., 1988) while FAD is the catalytic chromophore, facilitating electron transfer (Kao et al. 2005; Saxena et al., 2005). Mammalian CRY uses its bound chromophores to function as a circadian photoreceptor in the SCN, absorbing blue light to entrain the circadian clock. CRY may also, as in plants, act as a mammalian blue-light photoreceptor (Sancar, 2000).

Role of CRYPTOCHROME in the Circadian Clock

The CRY proteins are essential to the mammalian circadian clock (Sancar, 2000; Kume et al., 1999; van der Horst et al., 1999). *Cry* mutant animals show seemingly normal circadian behavior under a 12 hour light:12 hour dark (LD 12:12) regimen; however, this behavior is masking caused by visual light perception (Sancar, 2000). Under constant darkness, normal animals should exhibit their endogenous circadian period; it is under these conditions that the phenotype of circadian mutant animals is most apparent. Deletion of *mCry1* results in a shortened circadian period in constant darkness (van der Horst et al., 1999). In contrast, deletion of *mCry2* lengthens the circadian period (Thresher et al., 1998;

van der Horst et al., 1999). mCRY1 and mCRY2, therefore, are necessary to maintain normal circadian periodicity. Mice deficient in both *mCry1* and *mCry2* show complete loss of circadian rhythmicity in constant darkness (van der Horst et al., 1999; Sancar, 2000). With regard to clock gene expression, induction of *Per1* expression is severely blunted in both $Cry1^{-/-}$ and $Cry2^{-/-}$ mice; however, in the double $Cry1^{-/-}Cry2^{-/-}$ knockout *Per1* is expressed at high, non-oscillating levels with no induction after light pulses (Sancar, 2000). Interestingly, in both $Cry1^{-/-}$ and $Cry1^{-/-}Cry2^{-/-}$ mice *Per2* continues to oscillate, with a higher amplitude than that seen in wild-type mice (Vitaterna et al., 1999), with overall high levels of gene expression. Therefore there appears to be a CRY-independent pathway for *Per2* expression. Although *Per* transcript levels are high, PER protein levels in *Cry* mutant animals are low, suggesting that CRY plays a role in PER protein stability. In constant darkness, neither *Per1* nor *Per2* expression are oscillatory in $Cry1^{-/-}Cry2^{-/-}$ mice (Sancar, 2000), consistent with their arrhythmic behavior under such conditions. The status of *Per1* and *Per2* expression in the *Cry* mutant animals is consistent with a role of CRY in negative regulation of *Per* expression.

Indeed, CRY has been determined to be the main negative regulator of the mammalian CLOCK-BMAL1 transcriptional activator complex, effectively "closing" the clock feedback loop (Kume et al., 1999; Sancar, 2000). After CLOCK-BMAL1-mediated transcription of the *Per* and *Cry* genes, PER and CRY proteins are translated in the cytoplasm and form complexes which translocate into the nucleus and downregulate CLOCK-BMAL1-driven transcription (Sancar, 2000). Both CRY1 and CRY2 have been shown to strongly inhibit CLOCK-BMAL1-mediated transcription (Griffin et al., 1999;

Kume et al., 1999; Okamura et al., 1999). In this way, CRY proteins negatively regulate their own transcription, along with that of *Per* and other CCGs.

Interactions of CRYPTOCHROME with other Clock Proteins

Mammalian CRY has been shown to interact with a number of proteins. Yeast two-hybrid screens and co-immunoprecipitation experiments have shown interactions of CRY with BMAL1, CLOCK, PER1, PER2, PER3, and TIM (Griffin et al., 1999, Shearman et al., 1999, Sancar, 2000). CRY's interaction with CLOCK and BMAL1 is likely important for its repressor function; recently CLOCK and BMAL1 mutants were identified which are resistant to CRY-mediated repression (Sato et al., 2006). These mutants also showed decreased interaction with CRY and, to a lesser extent, PER. CRY's interaction with PER is thought to be important for PER protein stability and translocation into the nucleus (Kume et al., 1999). Clock proteins have been found to form large, time-specific multimeric complexes (Lee et al., 2001) including CLOCK, BMAL1, PER1, PER2, CRY1, CRY2, and CK1ε. A yeast two-hybrid screen of CRY2 detected protein phosphatase 5 (PP5) as an interacting protein (Zhao and Sancar, 1997). This protein is a serine/threonine kinase which was subsequently shown to regulate activity of CK1ε; this regulation is modulated by CRY (Partch et al., 2006).

Evolution of CRYPTOCHROME in Animals

Other animal systems with proteins homologous to mammalian CRY include *Drosophila*, *Xenopus*, zebrafish, monarch butterfly, honeybee, bumblebee, red flour beetle, and mosquito (Yuan et al., 2007; Reppert 2006). By phylogenetic analysis, *Cryptochrome* evolution appears to have given rise to two *Cry* genes in insects, now thought to represent the basal animal *Cry* lineage. This is a novel viewpoint because until recently the *Drosophila*

circadian system, about which the most is currently known, was thought to represent the basal evolutionary lineage. *Drosophila* contains only one *Cry* gene (*dCry*) which is a known photoreceptor to entrain the circadian clock; however, it is not required for circadian behavioral rhythms (Stanewsky et al., 1998; Helfrich-Forster et al., 2001). dCRY has traditionally not been considered a transcriptional repressor; however, recent work has shown that it can indeed repress CLOCK-CYCLE in cell culture (Collins et al., 2006). One important feature of dCRY is that it is degraded by light (Lin et al., 2001). Although *Drosophila* expresses only one CRY protein, other insects including the mosquito and monarch butterfly express two CRY proteins, one with homology to dCRY and one with homology to mammalian-like CRYs (Zhu et al., 2005). Monarch butterfly CRY1, similar to dCRY1, is degraded by light; monarch butterfly CRY2, similar to mCRYs, can act as a circadian transcriptional repressor (Zhu et al., 2005). Other insects, including the honey bee and red flour beetle, contain only a mammalian-like CRY (Zhu et al., 2005; Rubin et al., 2006). A new model of CRY evolution has emerged, suggesting that the mosquito and butterfly and those organisms having both types of CRY represent the ancestral insect lineage, and *Drosophila* diverged to retain only one CRY primarily acting as a photoreceptor and other insects diverged with only a mammalian-like CRY acting as a transcriptional repressor (Yuan et al., 2007).

Other Circadian Clock Proteins

In addition to the core clock components, there are other proteins that participate in the mammalian circadian mechanism. These are Casein Kinase 1ε (CK1ε), REV-ERBα, RORα, NPAS2, Protein Phosphatase 5 (PP5), and Timeless (TIM). I will provide a brief description of these proteins here.

Casein Kinase 1ε (CK1ε)

CK1ε is a kinase homologous to the DOUBLETIME kinase in Drosophila. CK1ε has been shown to phosphorylate PER proteins, which results in their ubiquitin-dependent degradation (Akashi et al., 2002). CK1ε can also phosphorylate CRY1, CRY2, and BMAL1 *in vitro* (Eide et al., 2002; Akashi et al., 2002). Although cellular levels of CK1ε do not appear to oscillate, its subcellular localization does appear to be circadian-dependent (Lee et al., 2001). Additionally, it has been found in a large complex of clock proteins whose formation is time-dependent. As post-translational modifications are becoming increasingly important components of the mammalian clock model, CK1ε's circadian function is of great interest. A second related kinase, CK1δ, with high homology to CK1ε has been shown to display similar properties and is likely also a modulator of clock proteins. Activity of both CK1ε and CK1δ is tightly regulated by inhibitory autophosphorylation (Gietzen and Virshup, 1999) and the protein phosphatase PP5 has been indicated as having a role in this process (Partch et al., 2006).

REV-ERBα and RORα

REV-ERBα and RORα are both orphan nuclear receptor proteins. These proteins both participate in regulation of expression of *Bmal1*, in a second mammalian feedback loop. Both the *Rev-Erbα* and *Rorα* genes are targets of CLOCK-BMAL1 transcriptional activation; their gene products REV-ERBα and RORα subsequently regulate *Bmal1* expression by repression (REV-ERBα) or activation (RORα) (Preitner et al., 2002; Ueda et al., 2002).

NPAS2

The *Npas2* gene encodes a functional analog of CLOCK which can heterodimerize with BMAL1 and activate clock gene expression off of E-box promoters (Reick et al., 2001;

Hogenesch et al., 1998). However, the expression profile of *Npas2* differs in that it is most highly expressed in the brain. NPAS2 has been shown to contain a bound heme cofactor which renders the DNA-binding activity of NPAS2-BMAL1 sensitive to CO gas; in this way, NPAS2 may couple the circadian clock to the heme metabolic cycle (Dioum et al., 2002). NPAS2 may also couple the circadian cycle to cellular redox rhythms. NPAS2 is thought to bind nicotinamide adenine dinucleotide (NAD) through its bHLH domain (Wijnen and Young, 2006) and the DNA-binding of NPAS2-BMAL1 can be modulated by NAD *in vitro* (Rutter et al., 2001). This could be especially important in circadian entrainment through feeding, which induces changes in cellular NAD concentrations (Damiola et al., 2000). NPAS2 has also been proposed as a candidate redundant protein to CLOCK which, in the absence of CLOCK, helps to maintain circadian rhythmicity in the SCN. *Clock*$^{-/-}$ mice were recently found to maintain circadian cycling, suggesting a redundant factor; however *Npas2* RNA is undetectable in the SCN (DeBruyne et al., 2006). The ability of NPAS2 to compensate in the absence of CLOCK is, therefore, of great interest.

Protein Phosphatase 5 (PP5)

PP5 is a serine-threonine kinase that is expressed in both the SCN and peripheral tissues in a non-oscillating fashion (Partch et al., 2006). Outside of the circadian clock, PP5 has been implicated in cell cycle by interaction with and regulation of DNA-PKcs (Wechsler et al., 2004), regulation of p53 function (Zuo et al., 1998), and dephosphorylation of the proapoptotic protein ASK1 (Morita et al., 2001). PP5 interacts with CRY1 and CRY2 and this interaction inhibits PP5 activity (Zhao and Sancar, 1997). PP5 can also interact with CK1ε both in vitro and in vivo, regulating its activity in both cases (Partch et al., 2006); CRY noncompetitively modulates this process. PP5 is emerging as an important component of the

mammalian circadian clock, as a result of its interactions with clock proteins and the finding that knockdown of PP5 by shRNA impairs circadian cycling in cultured cells (Partch et al., 2006).

Timeless (TIM)

The inclusion of mammalian TIM in the cadre of circadian clock proteins has been controversial (Barnes et al., 2003; Albrecht, 2002). Deletion of *mTim* results in an embryonic lethal phenotype (Gotter et al., 2000); as this has not been observed for any other bona fide clock protein, many took this as evidence that *mTim*, although partially homologous to *Drosophila* clock gene *dTim*, does not belong to the group of mammalian core clock genes. However, more recent data contradicts this finding, reporting that conditional knockdown of *mTim* expression in the SCN disrupts activity rhythms and alters the expression levels of clock proteins (Barnes et al., 2003). In this report, *mTim* expression was found to oscillate with 24-hour periodicity. In addition to interacting with CRY2, TIM has been shown to interact with the DNA damage checkpoint proteins CHK1 and ATR-ATRIP, and that its expression oscillates also with the cell cycle (Unsal-Kacmaz et al., 2005). Knockdown of mTim expression by siRNA abrogates CHK1 phosphorylation after hydroxyurea treatment and results in premature chromatin condensation (PCC). These data have led to the proposal of TIM as one of the components that couples the cell cycle to the circadian cycle; this relationship is of great interest, especially with regard to the involvement of circadian rhythm disruption with cancer predisposition (Gauger and Sancar, 2005; discussed in detail in Chapter 2).

Circadian Clocks in Other Organisms

Other than mammals, the circadian clock systems of cyanobacteria, *Neurospora crassa*, *Arabidopsis thaliana*, and *Drosophila melanogaster* have been the best characterized. I will provide a brief summary of these systems here.

Cyanobacteria

The cyanobacterium *Synechococcus elongatus* displays a clear circadian rhythm that profoundly affects its cellular metabolism (Liu et al., 1995). The cyanobacterial core oscillator consists of a complex of the KaiA, KaiB, and KaiC proteins (reviewed in Iwasaki and Kondo, 2004). KaiC protein can autophosphorylate; this action is enhanced by KaiA and antagonized by KaiB. The cyanobacteria clock was at first thought to consist of a transcription-translation-derived oscillatory feedback loop, similar to that which has been proposed in the mammalian clock, which does not regulate a specific set of clock-controlled genes but rather regulates genome-wide gene expression (Nakahira et al., 2004). In this model, the core loop revolves around the expression of the *kaiBC* operon (Wijnen and Young, 2006). KaiA enhances expression of *kaiBC*, forming the positive segment of the feedback loop. KaiC represses its own (*kaiBC*) expression, forming the negative segment of the feedback loop. Kai proteins exhibit robust circadian patterns of accumulation, synthesis, degradation, localization, and phosphorylation (Kageyama et al., 2006). Phosphorylation in particular is integral to the cyanobacterial clock, as mutation of KaiC's phosphorylation sites abolishes circadian rhythmicity (Nishiwaki et al., 2004). The canonical cyanobacterial clock TTFL model would require transcription and translation for oscillation to occur. However, recently clock oscillation has been reconstituted with the Kai proteins in a closed system, in which cyclic phosphorylation of KaiC occurred in vitro after mixing the three Kai proteins

with ATP (Kageyama et al., 2006). Because this oscillation occurred in the absence of transcription/translation, the canonical clock model in cyanobacteria has been reevaluated. A new model was proposed in which, over time, KaiA associates with KaiC, resulting in KaiC phosphorylation; KaiB then associates with phosphorylated KaiC, inactivating KaiA and resulting in KaiC dephosphorylation; KaiB subsequently dissociates from KaiC and the cycle begins again (Kageyama et al., 2006). These findings support the theory that in many systems, the TTFL is not solely responsible for circadian cycling and many of the well-characterized clock models are now undergoing a reevaluation.

Neurospora crassa

Similar to other systems, a TTFL is at the core of the filamentous fungus *Neurospora crassa* circadian clock mechanism. The positive arm of the feedback loop in this system is engendered by the transcription factor White-Collar Complex (WCC), consisting of PAS proteins White Collar-1 (WC-1) and White Collar-2 (WC-2) proteins. The negative arm of the feedback loop involves the Frequency (FRQ) protein, which negatively regulates WCC activity. The Frequency gene, *frq*, is a transcriptional target of WCC; therefore FRQ regulates its own expression (Wijnen and Young 2006). FRQ also participates in the positive arm of the feedback loop, helping to assemble the WCC complex in the cytoplasm (Cheng et al., 2001). Post-translational modification, especially phosphorylation, is also important in this system. Hypophosphorylated nuclear FRQ stimulates phosphorylation of WCC, inhibiting WCC's transcriptional activity. Hyperphosphorylated FRQ accumulates in the cytoplasm over the course of the day, which promotes WCC complex assembly. Phosphorylation of FRQ also leads to its degradation (Wijnen and Young 2006). Light

response in Neurospora is mediated by the WC-1 protein, which is a blue-light photoreceptor (Froehlich et al., 2002).

Arabidopsis thaliana

In plants, circadian rhythms regulate many processes including photomorphogenic growth, chloroplast localization, hypocotyls growth, stomata opening, and flowering time. The most well-characterized plant circadian clock is in the model organism *Arabidopsis thaliana*. Again, a set of transcriptional feedback loops governs this clock mechanism. The DNA-binding proteins LHY, CCA1, and LUX participate in the positive feedback loop, activating transcription of a number of genes including the *PRR5/7/9* genes (activated by LHY and CCA1), *LHY*, and *CCA1* (both activated by LUX). Also involved in positive regulation of transcription are the ELF4, GI, and TOC1 proteins. In turn, PRR5/7/9 and ELF3 repress transcription of the *LHY* and *CCA1* genes. The CK2 protein regulates CCA1 activity through phosphorylation. In this way, CK2 inhibits transcription of not only *PRR5/7/9* but also the *ELF3*, *GI*, *LUX*, *ELF4*, and *TOC1* genes. Photic input in the *Arabidopsis* system is signaled by the ZTL and CRY proteins, which absorb blue light, and PHY, which absorbs red light. CRY does not participate in negative regulation in this organism; rather, it acts exclusively as a blue light photoreceptor. (reviewed in Wijnen and Young, 2006)

Drosophila melanogaster

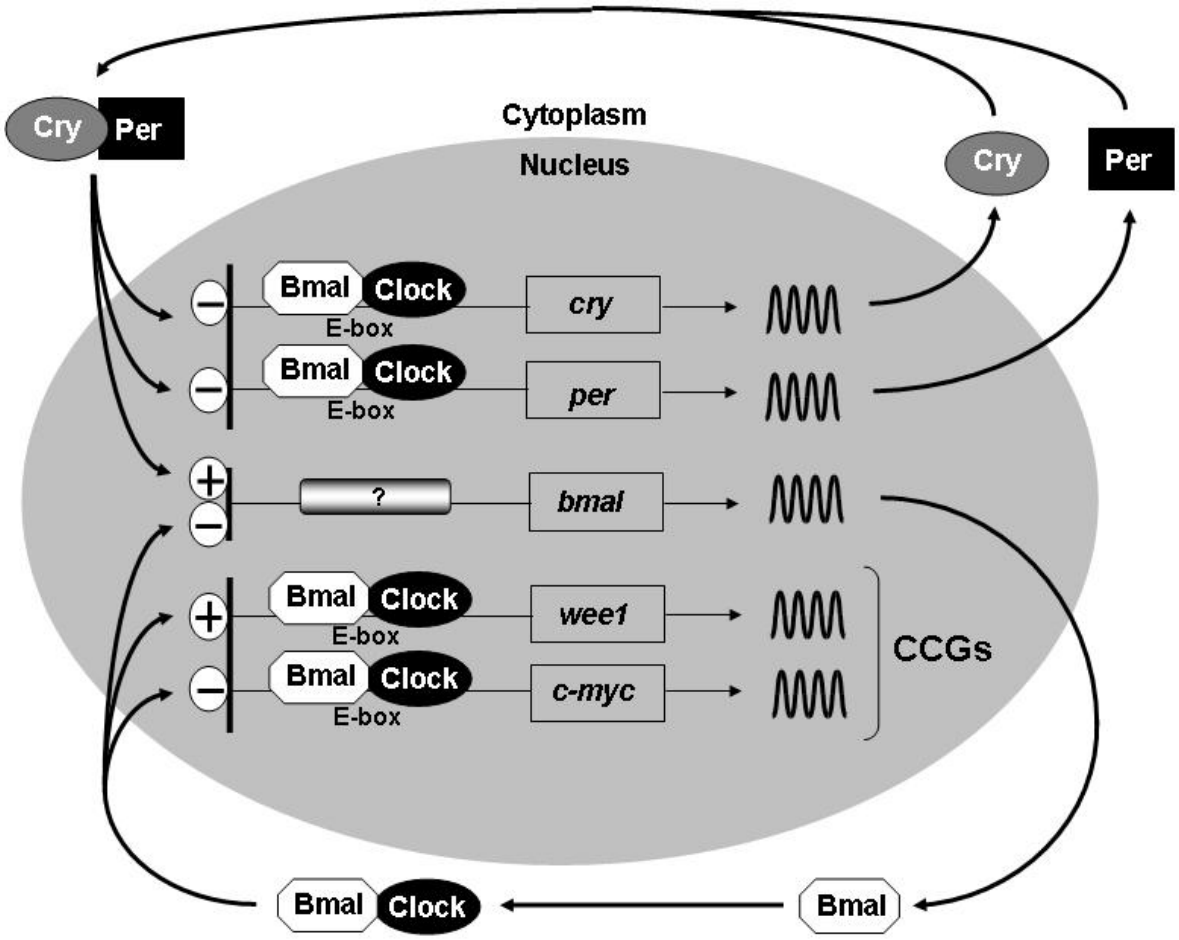
The best-studied insect circadian system is that of *Drosophila melanogaster*, although with the discovery of both insect-like and animal-like CRY proteins in other insect systems such as the monarch butterfly (Yuan et al., 2007), it is likely that we will soon know much more about the clock mechanisms of other insects. The *Drosophila* clock is thought to be

engendered by two feedback loops systems. The first loop is somewhat similar to that of mammals: a transcriptional activation complex comprised of the bHLH-PAS proteins CLOCK and CYCLE (CYC) induces rhythmic transcription of target genes including clock negative regulators *dPer* and *dTim* (Wijnen and Young, 2006). PER and TIM, in turn, act in complex as the main repressors of CLOCK-CYC in the *Drosophila* system, inhibiting their own transcription. The DOUBLETIME (DBT) protein (homologous to mammalian CK1ε) modulates subcellular localization of PER, resulting in its degradation. In the second feedback loop of this system, the CLOCK-CYC heterodimer activates transcription of the *Pdp1* and *Vri* genes, whose gene products PDP1 and VRI act as positive (PDP1) and negative (VRI) regulators, respectively, of expression of the *Clock* and *Cry* genes (Wijnen and Young, 2006). The major difference between the mammalian and Drosophila circadian systems is the function of CRY (Collins et al., 2006). While mammalian CRYs are core clock components that repress gene transcription and are required for circadian rhythmicity (van der Horst et al., 1999; Sancar, 2000), dCRY is not required for maintenance of behavioral circadian rhythms in Drosophila (Stanewsky et al., 1998). Rather, the function of dCRY has been suggested to be primarily as a blue-light photoreceptor that entrains the circadian clock and targets TIM for light-dependent degradation (Wijnen and Young, 2006). However, a recent report showed that dCRY can repress CLOCK-CYC activity in cell culture, appearing to be confined to peripheral clocks (Collins et al., 2006). This suggests that part of dCRY's function is similar to that of mCRY.

Conclusion

Although much work has been done to elucidate the mechanisms underlying the mammalian circadian clock, many questions still remain. Microarray studies indicate that a large number of cellular genes are potentially under circadian control, and the effect of this extensive gene regulation on cellular processes is likely to be far-reaching. In particular, recent studies demonstrating the involvement of circadian genes with cancer development is a field of growing importance. In Chapter 2, I will describe my endeavors to determine the effect of *Cryptochrome* loss on tumor predisposition and DNA damage checkpoint status in mice. Another, more basic, question is the mechanism by which CRY negatively regulates CLOCK-BMAL1. Genetic evidence shows that CRY can inhibit CLOCK-BMAL1, as do reporter gene assays; however, without biochemical characterization the molecular mechanism by which this occurs cannot be determined. In Chapter 3, I will describe experiments done to determine the validity of one theory of how CRY inhibits CLOCK-BMAL1. Together, the experiments described here give important information to increase our knowledge of the physiological role(s) of mammalian CRYPTOCHROME.

Figure 1.1. Model for Circadian Clock in Mammals. CLOCK and BMAL1 are transcriptional activators that make up the CLOCK-BMAL1 complex, which activates the transcription of *Cry*, *Per*, and *Bmal1* genes. The CRY (CRY1 and CRY2) and PER (PER1 and PER2) proteins are transcriptional repressors that interfere with the activity of CLOCK-BMAL1 and downregulate transcription of genes controlled by these factors. In addition, PER stimulates the transcription of *Bmal1* by an ill-defined mechanism which is regulated by the orphan nuclear receptors RORα and REV-ERBα. Clock-controlled genes (CCGs), which include *Wee1* and *c- myc*, are regulated by the clock proteins but do not affect the activity of the clock proteins or the transcription of the clock genes. *Wee1* expression is positively regulated by CLOCK-BMAL1 and *c-myc* expression is repressed by this complex. Hence, in the absence of CRY or PER, *Wee1* expression is expected to be upregulated due to lack of inhibition of CLOCK-BMAL1 by CRY and PER. It has been reported that *c-myc* transcription is elevated in *Per2* mutant mice because of a reduced level of BMAL1, which represses the *c-myc* promoter.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CHAPTER 2

EFFECT OF CIRCADIAN CLOCK DISRUPTION AND *CRYPTOCHROME* LOSS ON TUMORIGENESIS AND THE DNA DAMAGE CHECKPOINT IN MICE[1]

Summary

It has been reported that disruption of the circadian clock may lead to increased risk of breast cancer in humans and to a high rate of ionizing radiation (IR)-induced tumors and mortality in mice. CRYPTOCHROME1 and 2 proteins are core components of the mammalian circadian clock and mice mutated in both genes are arrhythmic. I tested *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ mice and fibroblasts derived from these mice for radiation-induced cancer and killing, and DNA damage checkpoints and killing, respectively. I find that the mutant mice are indistinguishable from the wild-type controls with respect to radiation-induced morbidity and mortality. Similarly, the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mutant fibroblasts are indistinguishable from the wild-type controls with respect to their sensitivity to IR and ultraviolet (UV) radiation and IR-induced DNA damage checkpoint response. These data suggest that disruption of the circadian clock in itself does not compromise mammalian DNA repair and DNA damage checkpoints and does not predispose mice to spontaneous and IR-induced cancers. I conclude that the effect of circadian clock disruption on cellular response to DNA damage and cancer predisposition in mice may depend on the mechanism by which the clock is disrupted.

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Introduction

The circadian clock and cell cycle are two global regulatory systems in most eukaryotic organisms. It has been known for some time that disruption of the circadian rhythm by genetic or environmental factors causes a variety of disorders in humans, such as sleep disturbances, seasonal affective disorder, and jet lag. Disruption of cell cycle regulation causes cancer. Recent epidemiological studies have raised the possibility that disruption of the circadian clock may also increase cancer risk in humans (Stevens, 2005) and adversely affect prognosis in cancer patients (Mormont and Levi, 1997). In particular, it was reported that women working night-shift exhibited a significant increase in breast cancer risk (Davis et al., 2001; Schernhammer et al., 2001). Similarly, it was reported that cancer patients with altered circadian rhythm had poorer survival relative to patients with normal rhythm (Mormont et al., 2000).

These epidemiological studies were complemented by studies with mouse model systems. In one study, transplantation of an osteosarcoma or a pancreatic adenocarcinoma into mice with ablations to the master circadian clock, the SCN, caused accelerated tumor growth rate relative to animals with intact SCN (Filipski et al., 2002). In a second study, it was found that mice that were rendered arrhythmic by repeat 8-hour advance of the light-dark (LD) cycle every 2 days exhibited faster rates of implanted tumor growth relative to control mice maintained under a LD 12h:12h cycle (Filipski et al., 2004).

Finally, the circadian rhythm was disrupted in mice by targeted mutations of the core clock genes that engender the molecular clock not only in the SCN but in all peripheral organs and the effect of this disruption on cell growth and spontaneous and IR-induced tumor incidence were analyzed. The core clock proteins are CLOCK and BMAL1 that act as

transcriptional activators of the *cryptochrome* (*Cry*), *Period* (*Per*), and *Bmal1* genes; and the CRY1, CRY2, PER1, and PER2 proteins that function as transcriptional repressors of the CLOCK-BMAL1-driven genes (Young and Kay, 2001; Reppert and Weaver, 2002) (Figure 1.1). The effect of these core clock proteins is modulated by additional proteins such as REV-ERBα and CK1ε to generate a rather precise molecular oscillator with approximately 24-hour periodicity. This periodicity is transmitted to the clock-controlled genes (CCGs) that constitute about 10% of the expressed genes in a given tissue to generate rhythmic outputs at the physiological and behavioral levels (Delaunay et al., 2002). The molecular mechanism of the mammalian circadian clock has been elucidated in considerable detail in recent years, making it possible to investigate the interfacing of this global regulatory pathway with other global regulatory systems, such as cell cycle checkpoints, at a mechanistic level. One such study found that in mice with a *Per2* mutation, *c-Myc* transcription was upregulated and p53 was downregulated; as a consequence, these animals had increased incidence of spontaneous and ionizing radiation (IR)-induced lymphomas and an increased rate of mortality after IR (Fu et al., 2002). Another study reported that in CRYPTOCHROMEless mice, WEE1 anti-mitotic kinase was elevated and, as a consequence, liver regeneration in these mice following partial hepatectomy was delayed relative to wild-type controls (Matsuo et al., 2003). Finally, our laboratory has recently found that the mammalian TIMELESS protein, which is considered to be a clock protein according to some studies (Barnes et al., 2003) but not others (Gotter et al., 2000), binds to DNA damage checkpoint proteins ATR and CHK1 and is essential for the DNA damage checkpoint response (Unsal-Kacmaz et al., 2005).

Taken together, the epidemiological data, the data from mouse model systems, and that from cell-based assays have led to an emerging consensus that the circadian cycle and

cell cycle are tightly coupled and that disruption of the circadian cycle by any means would disrupt cell cycle checkpoints as well, causing animals with circadian clock disruption to be more prone to spontaneous and DNA damage-induced cancers (Stevens, 2005; Mormont and Levi, 1997; Davis et al., 2001; Schernhammer et al., 2001). Within this conceptual framework, then, I wished to examine the effect of circadian clock disruption caused by *Cryptochrome* knockout on DNA damage checkpoints and on predisposition to spontaneous and IR-induced cancers. I find that clock disruption by *Cryptochrome* knockout does not measurably affect DNA damage checkpoints and does not cause mice to be more susceptible to cancer. I conclude that circadian clock disruption *per se* does not prime cells to cancerous transformation, presumably due to the presence of homeostatic mechanisms, in addition to the circadian rhythm, that regulate cell cycle and cellular responses to DNA damage.

Experimental Procedures

Mice, IR Treatment, and Survival

Cry1$^{-/-}$*Cry2*$^{-/-}$ mice (Selby et al., 2000; Vitaterna et al., 1999) were backcrossed with C57BL/6J six times to obtain Cry mutant mice in essentially the same genetic background as wild-type control C57BL/6J mice obtained from the Jackson Laboratory. The mice were maintained on an LD 12:12 schedule under ambient room lighting at an ambient temperature of 21-23°C and 50-70% humidity. For irradiation treatment, a cesium-137 radiation source emitting γ-rays at a rate of 0.82 Gy/min was used. 24 wild-type and 27 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice were treated at 8 weeks of age with a single dose of 4 Gy at zeitgeiber time (ZT) 10. (By convention, ZT0 is the time of lights-on and ZT12 is the time of lights-off.) For the experiment described in Figure 2.6, 8 wild-type (4 males, 4 females) and 8 *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (4 males, 4 females) mice were treated at approximately 8 weeks of age with 6 Gy of IR at ZT 10.

Fibroblast Cell Lines, Growth Rate Measurement, and UV and IR Survival

Dermal fibroblast cell lines were isolated as described (Thompson et al., 2004) using skin biopsies from wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. Fibroblasts underwent spontaneous immortalization. Figure 2.1 shows Western blots confirming the loss of CRY1 and CRY2 protein expression in the *Cry1*$^{-/-}$*Cry2*$^{-/-}$ cell line.

Cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gemini) and 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco). Cells were maintained in an incubator at 37°C under 5% CO_2. For growth rate measurements, cells were plated in 150mm plates at low density to ensure

continued proliferation throughout the duration of the experiment. Cells were trypsinized and counted at the indicated timepoints using a hemocytometer.

Cell survival to radiation was determined by clonogenic assay. Wild-type and *Cry1*$^{-/-}$ *Cry2*$^{-/-}$ fibroblasts were plated at low density to ensure the formation of approximately 200 colonies per 100 mm plate in the absence of radiation treatment. Following plating, cells were incubated in growth medium for 10-14 hours and then treated with either ultraviolet (UV) or IR of appropriate doses. UV treatment at the indicated doses was performed using a GE germicidal lamp emitting mainly at 254 nm. Cells were washed with PBS, irradiated with UV at a fluence rate of 0.65 J/m^{2}s in the absence of growth medium, and new growth medium was added after treatment. For IR treatment, cells in growth medium were irradiated from a cesium-137 radiation source at a rate of 0.82 Gy/min. After radiation treatment, cells were incubated for 9-10 days until colonies were readily visible. Cells were fixed for 20 minutes in 3:1 methanol:acetic acid, rinsed with water, and stained with Giemsa stain. Colonies containing 50+ cells were scored.

Flow Cytometry

Fibroblasts were grown in DMEM and plated to achieve a density of 1-2 million cells at the time of experiment. Cells were treated with IR as described above. At the indicated time post-treatment, cells were trypsinized and fixed in 70% ethanol. DNA content analysis was performed using propidium iodide staining and a Beckton-Dickinson FACScan analytical flow cytometer. Data acquisition and representation was done with Cicero Software (Cytomation, Inc.).

Western Blotting

Standard western blotting procedures were used for CRY1, CRY2, WEE1, c-MYC, and BMAL1 proteins from wild-type and *Cry* mutant mouse liver extracts and fibroblast cell lysates. For some of the western blots we used livers from *Cry* mutant mice in an *rd/rd* background because of the ready availability of these animals (Thompson et al., 2004). The *rd* mutation does not affect the molecular clock (Young and Kay, 2001; Reppert and Weaver, 2002). Anti-CRY antibodies produced in our laboratory were used to probe for CRY1 and CRY2 proteins. Anti-WEE1 rabbit polyclonal antibody (H-300, Santa Cruz Biotechnology) and anti-cMYC mouse monoclonal antibody (9E10, Santa Cruz Biotechnology) were used to probe for WEE1 and c-MYC, respectively. Anti-BMAL1 guinea pig polyclonal antibody (Lee et al., 2001) was a kind gift of Dr. Choogon Lee (Florida State University). Anti-IgG rabbit and mouse antibodies (Amersham Pharmaceuticals) and anti-IgG guinea pig antibodies (Jackson Immunoresearch Laboratories) were used for secondary antibody blotting.

Results

Expression of BMAL1, WEE1, and c-MYC in Cryptochromeless Mice and Fibroblasts

In the conventional clock model, CLOCK and BMAL1 constitute the positive regulatory branch while the CRYs and PERs make up the negative branch of the autoregulatory oscillatory circuit (Figure 1.1). In addition, CRYs and PERs appear to stimulate transcription of BMAL1 by an ill-defined mechanism (Young and Kay, 2001; Reppert and Weaver, 2002). As a consequence, *Per* and *Cry* mutants often exhibit similar molecular and behavioral phenotypes. However, it must be noted that there are subtle but significant differences between *Cry* and *Per* mutant mice. Hence, it is not possible to predict the responses of *Cry* or *Per* mutant cells and mice to a certain treatment based on responses observed with their *Per* or *Cry* mutant counterparts, respectively.

Recently, it was reported that in *Cry* mutant mice, the level of anti-mitotic WEE1 kinase was elevated in the liver (Matsuo et al., 2003) and that in *Per2* mutant mice, *c-Myc* transcription was upregulated (Fu et al., 2002). Both genes are CCGs that contain multiple E-boxes in their promoters, which are recognized by the CLOCK-BMAL1 complex. In the case of *wee1*, the binding of CLOCK-BMAL1 to the promoter stimulates transcription; in the case of *c-Myc*, binding of the heterodimer (or of the CLOCK-NPAS2 complex) inhibits transcription. The elevation of WEE1 in the *Cry* mutant was ascribed to the lack of inhibition of CLOCK-BMAL1 by CRY (Matsuo et al., 2003; Oishi et al., 2003). Upregulation of *c-Myc* transcription in the *Per2* mutant was ascribed to the reduced level of BMAL1 because PER2, in addition to its inhibitory effect on the CLOCK-BMAL1 complex, stimulates transcription of the *Bmal1* gene (Fu et al., 2002, Hogenesch et al., 2003, Shearman et al., 2000). Thus, to begin to investigate the effect of cryptochrome knockout on cellular

and organismic response to DNA damage, I wished first to determine the expression of BMAL1, c-MYC, and WEE1 in *Cry* mutant mice and fibroblasts. Loss of expression of both CRY1 and CRY2 proteins in knockout mice and derived fibroblasts was confirmed by Western blotting (Figure 2.1).

BMAL1 is expressed very highly and at comparable levels in wild-type and *Cry* mutant fibroblasts (Figure 2.2 A). However, in comparing BMAL1 expression in the liver, I find that BMAL1 is expressed with a circadian periodicity in wild-type liver, but at a reduced and non-oscillating level in the *Cry* mutant (Figure 2.2 B), in agreement with a previous report (Lee et al., 2001). The level of c-MYC has not been previously analyzed in *Cry* mutant mice. However, it was reported that *c-Myc* transcription is upregulated in *Per2* mutant mice, presumably due to a reduced BMAL1 level (Fu et al., 2002). Since BMAL1 is reduced in the *Cry* mutant, I expected to observe elevated c-MYC levels in *Cry* mutant mice and possibly fibroblasts as well. Figure 2.2 C, D shows that c-MYC is expressed in mutant and wild-type fibroblasts at comparable levels and at statistically indistinguishable and non-oscillating levels in the livers of mutant and wild-type mice. These results differ from those of the previous study, which reported that reduction in BMAL1 levels as a consequence of *Per2* mutation causes a substantial increase in c-MYC activity (Fu et al., 2002). However, in that study the *c-Myc* RNA, but not protein level, was measured. Regardless the cause of the discrepancy between the two studies it appears that a decrease in the BMAL1 transcriptional regulator does not necessarily lead to increased c-MYC protein in the mouse liver or fibroblasts.

Because it has been reported that CRY and PER regulate *Wee1* (Matsuo et al., 2003) and *c-Myc* transcription through their effects on BMAL1 activity and *Bmal1* transcription,

respectively, I reasoned that the reduced BMAL1 in the *Cry* mutant would be accompanied by elevated WEE1. Figure 2.3 A, B shows that WEE1 is indeed elevated in *Cry* mutant fibroblasts and liver, respectively, relative to the wild-type control and the moderately elevated (relative to the low level of WEE1 in wild-type mice) WEE1 in *Cry* mutant liver does not oscillate, in agreement with previous reports (Matsuo et al., 2003; Oishi et al., 2003).

DNA Damage Checkpoints and DNA Repair in CRYPTOCHROMEless Fibroblasts

WEE1 is a cell cycle kinase that plays a key role in the G2/M transition. Ongoing DNA replication or the presence of DNA damage activate WEE1, which then phosphorylates Y14 of the CDC2 mitotic kinase, causing its inactivation and delay of mitosis or arrest of the cell cycle at the G2/M interface (Nyberg et al., 2002; Sancar et al., 2004). It is conceivable that elevated WEE1 in *Cry* mutant mice phosphorylates CDC2 at an increased rate even in non-stressed cells, slowing down the G2/M transition and the overall growth rate. Indeed, it was reported that 3 days after partial hepatectomy the regenerating liver of CRYPTOCHROMEless mice contained fewer mitotic figures and lagged in weight recovery by about 15% relative to the wild-type control (Matsuo et al., 2003). Based on these considerations, then, I expected CRYPTOCHROMEless fibroblasts to grow more slowly than wild-type fibroblasts and to arrest for a longer period at the G2/M boundary after DNA damage, possibly remaining at this interface indefinitely and thus undergoing replicative death. Interestingly, I did not observe either of these two effects. First, CRYPTOCHROMEless fibroblasts grew at a rate indistinguishable from wild-type fibroblasts under standard growth conditions (Figure 2.3 C). Second, and more importantly, after 8 Gy of IR the CRYPTOCHROMEless fibroblasts appear to arrest at the G2-M and to

recover from this arrest at the same rate as wild-type fibroblasts (Table 2.1). It is likely that multiple factors contribute to the difference between the reported effect of CRY on liver regeneration rate and our results with tissue culture. It is also possible that the tissue culture conditions do not possess the requisite sensitivity to detect a 15% difference in growth rate. Regardless of these considerations, however, it is safe to conclude that the approximately 3-fold increase in WEE1 level in CRYPTOCHROMEless cells does not cause a comparable decrease in cell growth rate and it has no detectable effect on the DNA damage checkpoint response.

As a further test of DNA damage checkpoint response in the absence of a functional clock, I determined the survival of CRYPTOCHROMEless fibroblasts to ultraviolet and ionizing radiation. Figure 2.4 shows that the *Cry* mutant is indistinguishable from wild-type in its response to both genotoxicants. As most checkpoint defects increase cellular sensitivity to genotoxicants (Nyberg et al., 2002; Sancar et al., 2004) these results further confirm the conclusion based on Table 1 that, in the absence of CRY, DNA damage checkpoints are normal. Of equal significance, the data in Figure 4 also reveals that nucleotide excision repair (as measured by UV resistance) and double-strand break repair (as measured by resistance to IR) are unaffected by loss of the clock. Thus, I conclude that, in the absence of a circadian clock, both DNA damage checkpoints and the two major DNA repair pathways for repairing base damage and backbone breaks operate essentially normally.

IR-induced Morbidity and Mortality in CRYPTOCHROMEless/Clockless Mice

Per2 mutant mice lack circadian rhythm and were reported to be more sensitive to the acute and chronic effects of ionizing radiation relative to wild-type controls (Fu et al., 2002). The acute effects included hair graying, hair loss, and skin ulcerations. Chronic effects were

an approximately 10-fold increase in incidence of lymphomas relative to wild-type controls and a comparable increase in mortality within 70 weeks following treatment with 4 Gy of IR. The increased incidence of lymphomas and higher mortality rate were ascribed in part to the elevated *c-Myc* and in part to a general dysregulation of cell cycle genes as a result of clock disruption.

To determine whether or not clock disruption by any means has similar effects on IR-induced morbidity and mortality, I irradiated 8-week old $Cry1^{-/-}Cry2^{-/-}$ mice and wild-type controls with 4 Gy of IR at ZT10 and followed their survival for 90 weeks. The results obtained differed from those obtained with *Per2* mutant mice. First, I did not observe a difference in the timing and intensity of hair graying and loss between *Cry* mutant and wild-type mice (data not shown). Second, and most significantly, over the 90-week observation period there was no significant difference in the mortality of irradiated *Cry* mutant mice and wild-type mice (Figure 2.5). Moreover, in contrast to the similarly treated *Per2* mutant mice, I did not detect overt lymphomas in irradiated *Cry* mutant animals. The irradiated mice died from a variety of causes including genitourinary prolapses and infections, paralysis, and seizures that necessitated euthanasia, and in some cases from indeterminable causes. Importantly, however, there was no detectable difference between the causes of death of CRYPTOCHROMEless and wild-type animals. These results suggest that clock disruption *per se* does not make mice hypersensitive to the acute effects of IR, nor does it predispose them to increased incidence of spontaneous of IR-induced cancers or mortality from any other cause. The significance of these findings is discussed in detail in the Discussion section.

In addition to examining the chronic effects of IR on mice, I was also interested in the acute effects of IR in wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ animals. A small cohort of wild-type (8; 4 males and 4 females) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (8; 4 males and 4 females) were irradiated with a higher dose of 6 Gy IR at 8 weeks of age at ZT 10. Interestingly, I found that there was a difference in the acute effects of IR between wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice (Figure 2.6). Both male and female wild-type mice showed a strong acute response to IR, displaying a high amount of hair graying and/or hair loss. However, while male *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice showed a similarly strong acute IR response by hair graying and loss (Figure 2.6 A), female *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice were largely unaffected, exhibiting very little if any hair graying or hair loss (Figure 2.6 B). These results, though representative of only a small sample of individuals, would indicate that loss of Cry expression could protect against acute response to DNA-damaging agents, and that hormonal regulation, which is a primary physiological difference between females and males, may play an important role in this function. This data is supported by a recent report by Gorbacheva and colleagues (Gorbacheva et al., 2005), showing that *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice exhibit less morbidity relative to wild-type mice after treatment with cyclophosphamide, a chemotherapeutic drug which causes DNA damage by alkylation.

Discussion

Circadian Cycle and Cell Cycle

The circadian cycle and cell cycle are two global regulatory mechanisms that directly or indirectly influence all biochemical reactions in cells. Hence, it is logical to assume that disruption of one would cause deregulation of the other with adverse consequences for the cell (Bjarnason et al., 2000; Nagoshi et al., 2004; Welsh et al., 2004). Recent advances in understanding the circadian clock at the molecular level have provided the opportunity to approach the circadian cycle-cell cycle connection from a mechanistic perspective. Thus, it was reported that the core circadian clock machinery affects cell cycle progression in proliferating cells by controlling the expression of WEE1, a kinase that regulates the activity of CDC2 and hence the G2/M transition (Matsuo et al., 2003). Another study concluded that *Per2* mutant mice had constitutively elevated levels of the cell growth/proliferation gene *c-Myc* and reduced expression of p53, which plays a critical role in the G1/S checkpoint (Fu et al., 2002). Finally, our laboratory has recently found that the mammalian TIM plays a direct role in cell cycle checkpoints including the intra-S and replication checkpoints (Unsal-Kacmaz et al., 2005). Although the role of the mammalian TIM in the circadian clock is a matter of some debate (Barnes et al., 2003; Gotter et al., 2000), these studies in aggregate have given credence to the general view that circadian disruption by whatever means may lead to a failure of cell cycle checkpoints (Fu and Lee, 2003). However, this view needs to be critically tested experimentally. In particular, it remains to be determined if proteins that have been presumed to be exclusively clock proteins such as CLOCK and BMAL1, which work as partners in the positive branch of the clock, and CRY and PER, which work as partners in the negative branch, also perform functions outside of the clock and unique to

each protein. In the case of mammalian TIM, it is clear that the protein participates in cell cycle control independent of its role in the clock (Unsal-Kacmaz et al., 2005). If the so-called canonical clock proteins CLOCK, BMAL1, CRY, and PER have unique cellular functions outside of the clock machinery, then the mutants of these proteins are expected to exhibit some unique features as well. An additional possibility is that different clock mutants disturb the circadian cycle in different ways, leaving a different pattern of circadian genes "on" and "off" depending on when the clock stops. Although the molecular clock does not "stop" at a given time but rather falls into a stable equilibrium that terminates the cycles in the abundance of its components, one can imagine that leaving certain circadian genes "on" or "off" might cause a susceptibility to cancer or not, without necessarily making cancer susceptibility caused by clock disruption specific to a particular clock gene. Moreover, cell cycle and carcinogenic transformation are also regulated by homeostatic mechanisms and therefore a potentially cell cycle-disruptive effect of a clock gene mutation might be mitigated or completely alleviated by such compensatory mechanisms. As a consequence, *a priori* it cannot be known whether all circadian disruptions will have the same effect on cell cycle and DNA damage checkpoints and whether their potential cell cycle-disruptive effects will necessarily lead to actual disruption.

Cell Cycle Checkpoints in the Absence of Circadian Rhythm

In this study I analyzed the growth properties, cell cycle checkpoints, and DNA repair capacity of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ fibroblasts and the susceptibility to IR-induced cancer and mortality of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. Based on published reports of *Cry* and *Per* mutations on cellular growth (Matsuo et al., 2003) and damage response (Fu et al., 2002), I was expecting the *Cry*

mutants to be defective in DNA damage checkpoints and to exhibit increased IR-induced morbidity and mortality for the reasons outlined below.

First, current clock models presume that CRY and PER function as heterodimers and since it has been reported that in *Per2* mutant mice, *c-Myc* is upregulated, p53 is downregulated, and there is a general cell cycle dysregulation (Fu et al., 2002), I expected that the *Cry* mutant fibroblasts would exhibit some cell cycle checkpoint defects and that the *Cry* mutant mice, like the *Per2* mutants, would be cancer-prone. I find that in *Cry* mutant livers, BMAL1 expression, as in the case of the *Per2* mutant, is reduced. However, in an apparent contrast to the *Per2* mutant mice, the c-MYC level is not elevated in either *Cry* fibroblasts or mice, indicating that PERs and CRYs affect *c-Myc* expression differently. It must be noted, however, that in the *Per2* mutant mice the *c-Myc* RNA, but not the protein level, was measured. It is conceivable that even in the *Per2* mutant mice the elevated level of *c-Myc* mRNA is not accompanied by elevated c-MYC protein and that the increased incidence of spontaneous and IR-induced lymphomas reported in these animals was caused by an unknown effect of PER2 on cell growth and proliferation.

Second, it was reported (Matsuo et al., 2003) that WEE1 kinase, which inhibits the G2/M transition, is elevated in *Cry* mutant mice and evidence was presented suggesting that after partial hepatectomy, the liver of *Cry* mutant mice regenerates more slowly than that of wild-type controls, presumably because of inhibition of mitosis by elevated WEE1. In agreement with previous reports (Matsuo et al., 2003; Oishi et al., 2003), I find that WEE1 is elevated in *Cry* mutant fibroblasts and liver and other tissues of *Cry* mutant mice. However, despite this elevation in WEE1 level the *Cry* mutant fibroblasts grow at a rate indistinguishable from the wild-type controls. It appears that exponentially growing cultures

of mutant fibroblasts had fewer mitotic figures than wild-type (data not shown); I assume that a slight delay in mitotic entry was compensated by faster progression through other phases of the cell cycle such that there was no change in overall growth rate relative to the control. Importantly, the mutant cells did not exhibit an amplified checkpoint response to DNA damage and, as a consequence, their kinetics of checkpoint-induced inhibition of cell cycle progression through G2/M was indistinguishable from wild-type controls. This again indicates the presence of compensatory mechanisms that ensure normal checkpoint response even in the presence of elevated WEE1. These results appear to be contradictory to the report indicating slower recovery of liver mass in *Cry* mutant mice after partial hepatectomy (Matsuo et al., 2003). However, it is possible that the apparent discrepancy may stem from differences in stress responses induced by DNA damaging agents, as opposed to partial hepatectomy, and the nature of the cell types analyzed in the two studies.

Circadian Disruption and Cancer Predisposition

Epidemiological studies have suggested that circadian disruption may contribute to cancer incidence (Stevens, 2005) and adversely affect the course of the disease (Mormont and Levi, 1997). A prospective study with *Per2* mutant mice appears to have provided a molecular explanation for the connection between circadian rhythm disruption and cancer predisposition. My work indicates that disruption of the clock does not necessarily predispose mice to cancer. The cancer predisposition of *Per2* mutant mice was ascribed, in large part, to decreased BMAL1 expression and the consequent increase in *c-Myc* expression. BMAL1 expression is reduced in both *Per2* and *Cry* mutant mice (Fu et al., 2002; Lee et al., 2001; this work) and hence it remains to be proven that the increased *c-Myc* transcription reported in *Per2* mutant mice is a direct consequence of BMAL1 reduction, which, in the

form of either BMAL1-NPAS2 or BMAL1-CLOCK heterodimer, represses *c-Myc* transcription (Fu et al., 2002). Whatever the cause of elevation of *c-Myc* transcription in *Per2* mutants, I do not observe a measurable change in the c-MYC protein level in *Cry* mutant mice and hence it is possible that the absence of PER2 makes mice cancer-prone not by overexpression of *c-Myc* but through an unknown mechanism. It must be noted, however, that the IR-induced mortality of the wild-type mice in our study was the same as that of the *Cry* mutant and, importantly, it was significantly higher than that of the wild-type control mice used in the *Per2* mutant mouse study. It is possible that the genetic background (C57BL/6J in our study and C57/SV129 in the *Per2* study) affects the susceptibility of even "wild-type" mice to both IR-induced cancers and IR-induced mortality. In my study both the *Cry* mutant and the wild-type control mice were in C57BL/6J background and therefore we suggest that the lack of difference in morbidity and mortality between the wild-type and *Cry* mutant mice is most likely because circadian clock disruption by eliminating *Cry* does not affect cell cycle checkpoints, DNA repair, or apoptosis in a way that would result in increased mutations, reduced apoptosis, and eventually cancer.

It should be of interest to find out how mutations in other clock genes, in particular *Clock* and *Bmal1*, affect the incidence of spontaneous and IR-induced cancers. A report was recently published demonstrating that the sensitivity of mice to the acute effects (weight loss and death) of high doses of cyclophosphamide, an alkylating anti-cancer drug, was strongly dependent on the circadian time of drug delivery (Gorbacheva et al., 2005) and that the *Cry* mutant used in my study was resistant to the acute effects of cyclophosphamide at all times of the day. Clearly, further studies are needed to explain the apparent resistance of *Cry* mutant mice to the acute effects of cyclophosphamide and, if reproducible, to the acute

effects of IR as well as shown by my studies. Regardless the precise mechanism of the resistance, the results of the study on the acute effects of a DNA damaging agent and our study on the long-term effects of IR are, in general, in agreement in demonstrating that clock disruption per se does not make mice more susceptible to the acute or chronic effects of DNA damaging agents.

Figure 2.1. Confirmation of loss of CRY1 and CRY2 proteins in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. The figure shows Western blots of (A) cellular lysate from WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (KO) fibroblast cell lines and (B) liver extracts from WT and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (KO) mice. Protein expression was observed using immunoblotting with anti-CRY1 and anti-CRY2 monoclonal antibodies. * denotes the CRY-reactive species.

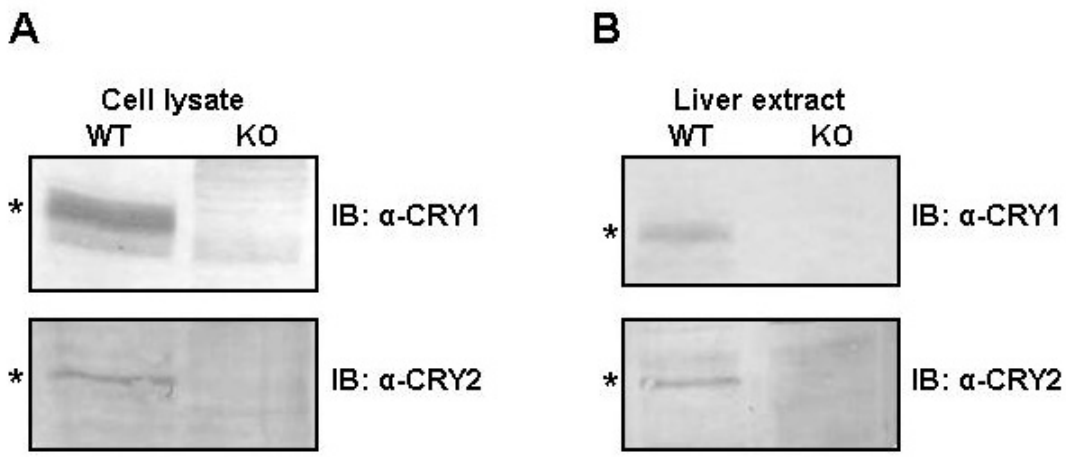

A
Cell lysate
WT
KO
*
IB: α-CRY1
*
IB: α-CRY2
B
Liver extract
WT
KO
*
IB: α-CRY1
*
IB: α-CRY2

Figure 2.2. Effect of CRYPTOCHROME on BMAL1 and c-MYC Expression in Mouse Fibroblasts and Mouse Liver. (A and B) BMAL1 expression analysis. (A) (200 μg) from wild-type (WT) or *Cry* mutant fibroblasts were analyzed for BMAL1 expression by Western blotting using actin as a loading control. (B) Liver extracts from wild-type (WT) and *Cry* mutant mice were prepared at the indicated zeitgeiber times (ZT0 = lights on, ZT12 = lights off) and analyzed for BMAL1 expression using actin as a loading control. It has been reported that in the liver of WT mice, BMAL1 levels are lowest at ZT6 and highest at ZT18. (C and D) c-MYC expression analysis. Extracts (200 μg) from fibroblasts (A) or mouse liver (B) were probed for c-MYC and actin by Western blotting. *Top,* western blot; *bottom,* quantitative analysis of Western blot. Averages of 3 independent experiments, including the one shown in top panel. Bars indicate standard deviation (n=3).

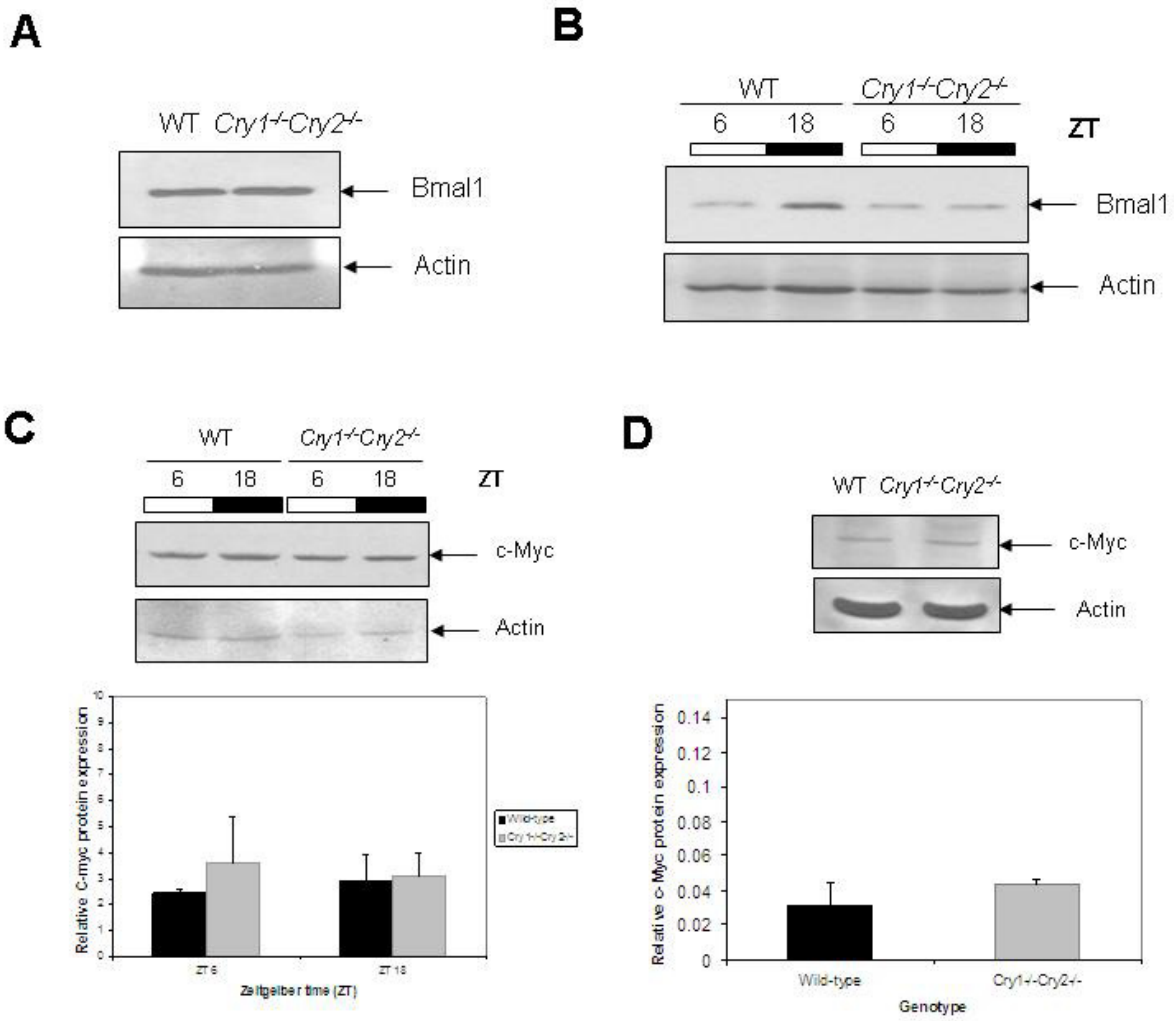

A
WT Cry1-/-Cry2-/-
Bmal1
Actin
B
WT
Cry1-/-Cry2-/-
6
18
6
18
ZT
Bmal1
Actin
C
WT
Cry1-/-Cry2-/-
6
18
6
18
ZT
c-Myc
Actin
Relative C-myc protein expression
Wild-type
Cry1-/-Cry2-/-
ZT 6
ZT 18
Zeitgeber time (ZT)
D
WT Cry1-/-Cry2-/-
c-Myc
Actin
Relative c-Myc protein expression
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Wild-type
Cry1-/-Cry2-/-
Genotype

Figure 2.3. Effect of CRY on WEE1 Expression and Growth Rate of Mouse Fibroblasts. (A and B) WEE1 expression analysis. Extracts (200 μg) from fibroblasts (A) or mouse liver (B) were probed for WEE1 and actin by Western blotting. Liver extracts were prepared at the indicated zeitgeiber times. (C) Growth kinetics of immortalized fibroblasts from wild-type (circle) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (triangle) are shown. Cells were plated at densities of approximately 10^4 cells/dish and cells were counted at the indicated timepoints. Error bars indicate standard deviation (n=3).

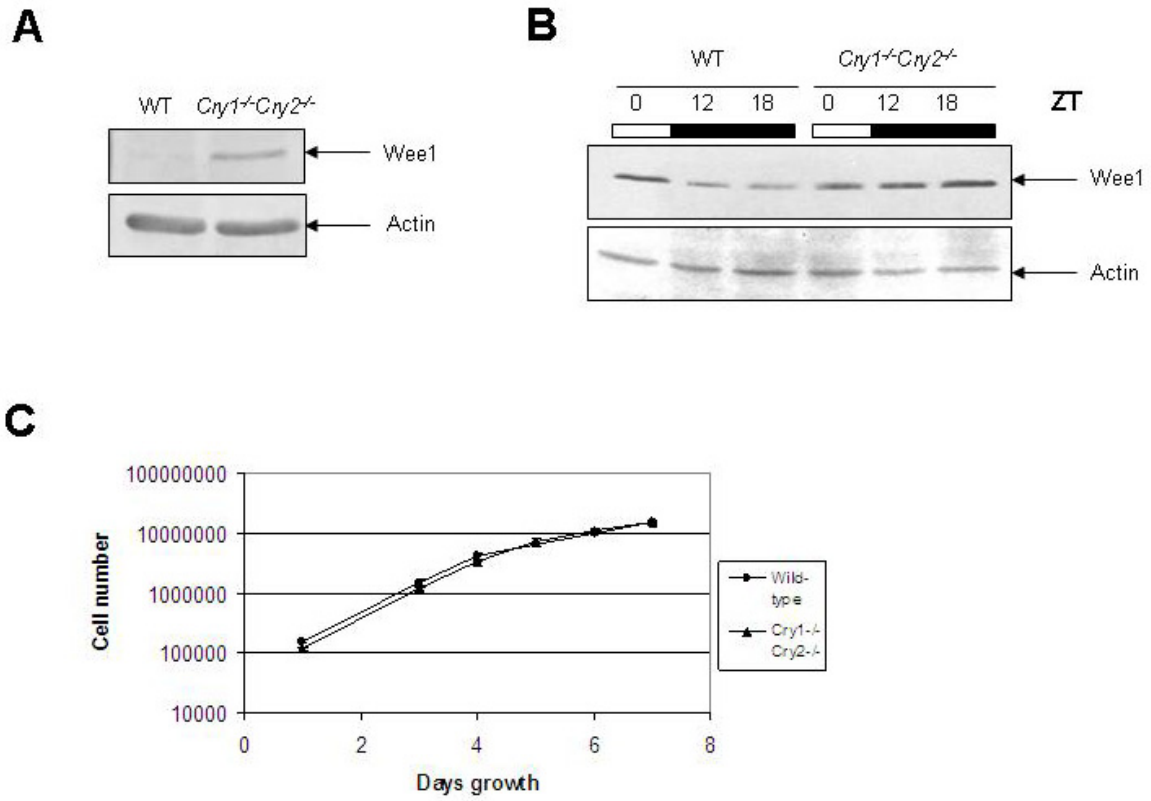
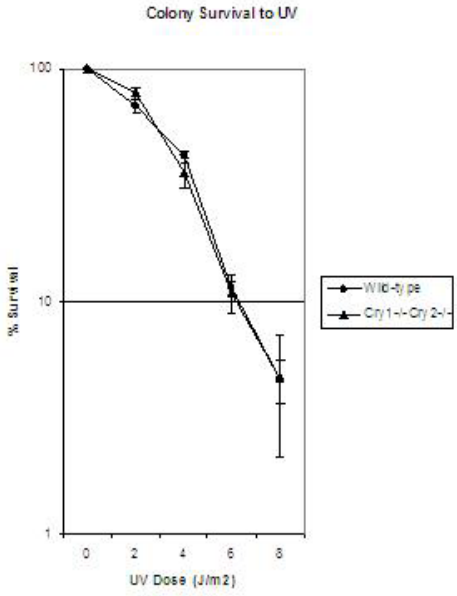

A
WT
Cry1-/-Cry2-/-
Wee1
Actin
B
WT
Cry1-/-Cry2-/-
0
12
18
0
12
18
ZT
Wee1
Actin
C
100000000
10000000
1000000
100000
10000
Cell number
0
2
4
6
8
Days growth
Wild-type
Cry1-/-Cry2-/-

Figure 2.4. Radiation Survival of Wild-type and *Cryptochrome* Mutant Fibroblasts. (A) UV survival; (B) IR survival. Circle, wild-type; triangle, *Cry* mutant. Data points are averages of 3 independent experiments and the error bars indicate standard deviation (n=3).

A


Colony Survival to UV
100
10
1
% Survival
0
2
4
6
8
UV Dose (J/m2)
Wild-type
Cry1-/-Cry2-/-

B

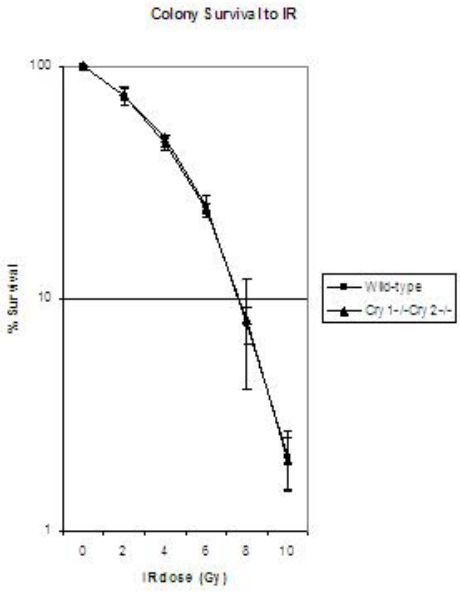

Colony Survival to IR
100
10
1
% Survival
0
2
4
6
8
10
IR dose (Gy)
Wild-type
Cry1-/-Cry2-/-

Figure 2.5. IR survival of wild-type (solid line) and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ (broken line) mice. 8-week-old mice were exposed to 4 Gy at ZT10 and were observed for 90 weeks. Survival is plotted according to the Kaplan-Meier method.

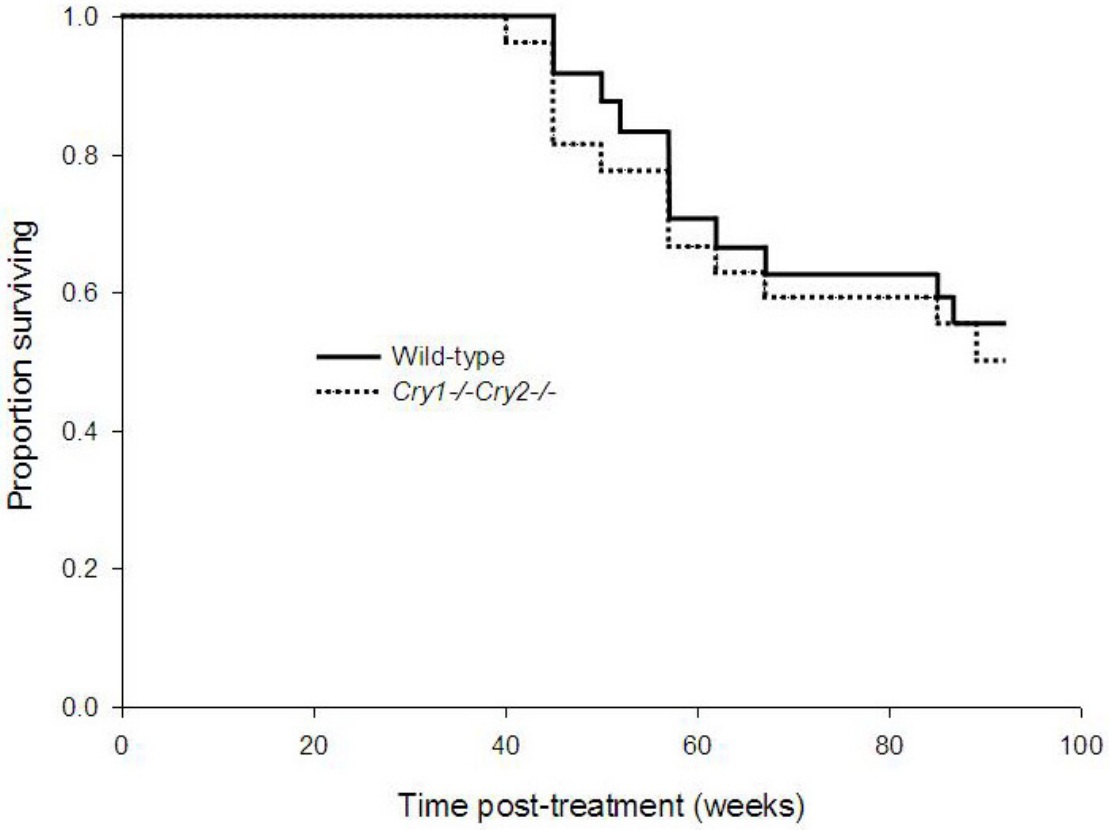

1.0
0.8
0.6
0.4
0.2
0.0
Proportion surviving
Wild-type
Cry1-/-Cry2-/-
0
20
40
60
80
100
Time post-treatment (weeks)

Figure 2.6. Acute Effect of IR on Wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice. 8-week-old mice were exposed to 6 Gy at ZT10 and were observed for 20 weeks (males) or 12 weeks (females). (A) Male wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice; photographs were taken 20 weeks post-treatment. (B) Female wild-type and *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice; photographs were taken 12 weeks post-treatment.

A

WT ♂

Cry1$^{-/-}$*Cry2*$^{-/-}$ ♂

B

WT ♀

Cry1$^{-/-}$*Cry2*$^{-/-}$ ♀

Table 2.1. G_2-M checkpoint in wild-type and Cry mutant fibroblasts after ionizing radiation treatments. Exponentially growing cells were irradiated and at the indicated times were fixed, stained with propidium iodide, and analyzed by flow cytometry for DNA content. The percentage of cells in G_2 and M at 0, 8, and 24 h are shown in bold to highlight the G_2-M arrest and subsequent recovery. Percentages are average values from 3 individual experiments.

Genotype	Time (h) post-IR (8 Gy)	% cell population		
		G_1	S	G_2 and M
Wild-type	0	45.16	30.28	**25.98**
	4	31.53	36.24	34.08
	8	14.25	31.41	**56.06**
	12	18.09	16.85	65.72
	24	39.71	27.95	**33.68**
Cry1$^{-/-}$*Cry2*$^{-/-}$	0	41.20	34.34	**26.35**
	4	24.17	42.43	36.37
	8	10.64	36.63	**56.50**
	12	21.04	9.91	68.05
	24	39.08	26.88	**32.94**

CHAPTER 3

NEGATIVE REGULATION OF CLOCK-BMAL1 BY CRYPTOCHROME

Summary

Although it is known that mammalian CRYPTOCHROME represses transcription by CLOCK-BMAL1, the biochemical mechanism by which this occurs is unknown. *In vitro* experiments were performed to determine the direct effect of CRY1 on DNA binding of the CLOCK-BMAL1 heterodimer. Assays were performed with purified mammalian BMAL1, CRY1, CLOCK, and CLOCK342 (a 342-aa truncation of CLOCK). I find that, although experiments with full-length CLOCK are inconclusive, CRY1 has no observable effect on the binding of CLOCK342-BMAL1 to an E-box DNA substrate. Additionally, I investigated the induction of CLOCK342-BMAL1 DNA binding by two agents, NADH and DTT in response to conflicting reports that NADH or DTT can increase the DNA binding of the heterodimer complex. I find that under one set of reaction conditions, NADH can increase the DNA binding of the CLOCK342-BMAL1 heterodimer while DTT cannot, and under a slightly different set of conditions DTT can increase heterodimer DNA binding while NADH cannot. The physiological relevance of these findings is in question, as the NADH concentrations used are likely out of the range of physiological levels and DTT is not a physiologically relevant agent.

Introduction

The current model of the mammalian molecular clock depicts a network of positive and negative transcriptional feedback loops (Figure 1.1). In this model, the CLOCK-BMAL1 heterodimer drives the positive arm of the feedback loop, activating transcription of the clock genes *Period* and *Cryptochrome* (along with other CCGs). In the negative arm of the feedback loop, PER and CRY proteins negatively regulate their own expression by inhibiting CLOCK-BMAL1. There is much genetic evidence and molecular evidence to this model: *Cry1*$^{-/-}$*Cry2*$^{-/-}$ knockout mice show elevated levels of *Per1* transcript, while *Per* mutant animals show elevated levels of *Cry* transcript (Sancar, 2000; Fu et al., 2002); numerous reports have shown that CRY can inhibit activity of CLOCK-BMAL1 in luciferase reporter gene assays (Sato et al., 2006). While both CRY and PER proteins repress CLOCK-BMAL1, CRY1 and CRY2 have been shown to inhibit the complex more strongly than PER proteins (Maemura et al., 2000; Shearman et al., 2000; Gotter et al., 2000).

Although it is known that CRY functions in the mammalian circadian clock as a repressor of CLOCK-BMAL1, the molecular mechanism by which this occurs is unknown. Recent studies in the mammalian system along with *Drosophila* and zebrafish have postulated numerous possible mechanisms. The mammalian CRY proteins have been found to interact with CLOCK and BMAL1, by both yeast two-hybrid (Griffin et al., 1999) and immunoprecipitation assays (Lee et al., 2001). It is likely that this interaction is important for CRY's negative regulation of CLOCK-BMAL1, either through direct or indirect effects on the complex, since it correlates with the time of highest CRY repression (Lee et al., 2001).

One possible mechanism by which CRY represses CLOCK-BMAL1 is through modulation of post-translational modifications. In *Drosophila*, phosphorylation of dCLOCK

has been shown to be dependent on PER, perhaps through PER-dependent entry of DOUBLE-TIME kinase into the CLOCK-CYCLE complex (Yu et al., 2006). In the *Neurospora* circadian clock, posttranslational modification of not the White-Collar Complex (analogous to mammalian CLOCK-BMAL1, the transcriptional activator) but its regulator FRQ changes FRQ from a repressor to an activator. In the mammalian system, transcriptional activation of CLOCK-BMAL1 correlates with phosphorylation of both proteins, nuclear translocation, and subsequent degradation (Kondratov et al., 2003). CRY may be involved in one or more of these posttranslational modifications, possibly through recruitment of a kinase or other protein-modifying enzyme. It is important to note that phosphorylation of CLOCK and/or BMAL1 is not necessary for their heterodimerization; however, phosphorylation may be important for interaction with the negative regulators CRY and PER, as it is mainly hyperphosphorylated CLOCK that immunoprecipitates with these proteins (Lee et al., 2001).

Chromatin modification is involved in the regulation of expression of many mammalian genes, and has been proposed as a key regulator of CLOCK-BMAL1-activated transcription. Histone modification through both aceytlation and methylation has been linked to circadian clock gene expression. Rhythms in histone H3 aceytlation and trimethylation have been correlated with circadian gene expression (Etchegaray et al., 2003; Ripperger and Schibler, 2006; Naruse et al., 2004). In addition to CLOCK's recently-identified histone acetyltransferase (HAT) activity (Doi et al., 2006), the HAT p300 has been shown to immoprecipitate with CLOCK in a time-dependent fashion; additionally, CRY inhibits a p300-induced increase in CLOCK-BMAL1-activated transcription (Etchegaray et al., 2003; Ripperger and Schibler, 2006). Overexpression of p300 can overcome CRY2-mediated repression of NPAS2-BMAL1 (Curtis et al., 2004). The PCAF and ACTR HAT proteins

also associate with CLOCK and its ortholog NPAS2 (Curtis et al., 2004). WDR5, a component of a histone methyltransferase complex, has been found to associate with PER1 (Brown et al., 2005). Finally, the polycomb group enzyme EZH2, which catalyzes methylation of histone H3, associates with both CLOCK and BMAL1 (Etchegaray et al., 2006). Taken together, it is likely that histone and chromatin modification plays at least some part in regulation of clock gene expression.

Due to the physical interaction of CRY with CLOCK and BMAL1, it is possible that CRY directly affects the CLOCK-BMAL1 complex's DNA binding properties. This could occur in a number of ways (Figure 3.1). CRY could prevent CLOCK-BMAL1 from binding to E-box DNA, perhaps through formation of a trimeric complex or causing conformational change in the CLOCK-BMAL1 heterodimer (Figure 3.1, #1). Such a mechanism has been proposed in the *Drosophila* circadian clock, where the negative regulators PER and TIM inhibit the DNA binding of CLOCK-CYC without appearing to disrupt the heterodimer itself (Lee et al., 1999). Alternatively, CRY could form a trimeric complex with CLOCK-BMAL1 that, while still binding to E-box DNA, is no longer transcriptionally active (Figure 3.1, #2). This has been implicated in the zebrafish circadian system, in which zCRY1a forms a large complex on DNA with zBMAL and zCLOCK (Ishikawa et al., 2002). A third possibility is that CRY disrupts the CLOCK-BMAL1 heterodimer by replacing one of the components (Figure 3.1, #3). Although the aforementioned studies suggest mechanisms in the *Drosophila* and zebrafish clock systems, both studies are missing important experimental controls which are necessary for conclusions to be drawn. The DNA-binding status of CLOCK-BMAL1 throughout the circadian cycle is somewhat controversial. It has been reported that the CLOCK-BMAl1 heterodimer is constitutively bound to DNA throughout

the circadian cycle (Lee et al., 2001). However, a recent report by Ripperger and Schibler contradicts this somewhat, showing that CLOCK-BMAL1 is rhythmically bound to E-box DNA and this binding is correlated with changes in chromatin modification and expression of the mouse clock-controlled gene *Dbp* (Ripperger and Schibler, 2006).

In light of the conflicting evidence concerning the mechanism of CRY's negative regulation of CLOCK-BMAL1, we examined the effect of CRY on direct DNA-binding of CLOCK-BMAL1 in *in vitro* DNA-binding assays. We find that CRY has no effect on the DNA-binding of a heterodimer consisting of BMAL1 and CLOCK342, a 342-amino acid fragment of CLOCK. Future studies must be done with purified full-length CLOCK to determine what the effect of CRY is on what is thought to be a more physiologically relevant protein.

Experimental Procedures

Expression and Purification of Recombinant mBMAL1 and hCLOCK from SF-21 cells

mBMAL1 and hCLOCK proteins were expressed and purified from SF-21 cells as described in the Bac-to-Bac Baculovirus standard protocol (Invitrogen). Baculovirus for expression of Flag-His-mBMAL1 was created by cloning into the pFastBac expression vector by Dr. Laura Lindsey-Boltz. Flag-His-mBMAL1 has a single Flag and a single 6xHis tag N-terminal to mBMAL1. Baculovirus for expression of Flag-hCLOCK was created by cloning into the pFastBac expression vector (by Dr. Christopher Selby) and the virus was prepared by Dr. Sezgin Ozgur. Flag-hCLOCK has a single Flag tag N-terminal to hCLOCK.

For expression and purification of Flag-His-mBMAL1 and Flag-hCLOCK, 300 ml of SF-21 cells at a density of 1 x 10^6 cells/ml were inoculated with the appropriate virus (MOI = 30-100). Cells were incubated at 27° C for 48 hours after virus inoculation. The cells were then harvested by centrifugation at 3000 rpm for 10 minutes and washed once with 1X phosphate-buffered saline (PBS). The cell pellets were frozen and kept at -80° C until purification. The cell pellets were subjected to three freeze-thaw cycles before lysis. Cells were lysed in NP-40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol (v/v), 1% Tween-20 (v/v), 0.1% NP-40 (v/v)) by sonication (8 cycles of 30 seconds), Dounce homogenization (10 rounds), and, in the case of Flag-hCLOCK, shearing through a 22g needle; this led to 50-80% protein solubility in both cases. Soluble cell lysate was prepared by centrifuging the cell lysis mixtures at 15000 rpm for 45 minutes at 4°C. The supernatant was incubated overnight with Flag-agarose (Sigma; 150-300 µl packed resin) at 4°C. The resin was then centrifuged at 4000 rpm for 5 minutes and flow-through removed and discarded. The resin was washed with lysis buffer, tris-buffered saline (TBS) with 1M NaCl,

TBS, and TBS with 10% glycerol (15 ml each wash). Bound protein was eluted in 200-400 μl of elution buffer (TBS with 10% glycerol and 0.1 mg/ml Flag-peptide) and purification products were visualized using silver staining. Protein concentration was determined using the Bio-Rad protein assay and absorbance at 595 nm.

Expression and purification of His-mCLOCK342 in E. coli

His-*mClock342* had been previously cloned into the pet15b bacterial expression vector (Novagen) by Dr. Christopher Selby. The expression construct was transformed into and expressed in BL-21 Gold (Invitrogen) bacterial cells. His-mCLOCK342 expression was induced in 1 L of cells at an O.D. of 0.45 using 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and shaking at room temperature for 8 hours. Bacteria were harvested by centrifugation at 4000 rpm for 10 minutes and washed once with 1X PBS. Expression using this system resulted in nearly 100% insoluble His-CLOCK342 which was subsequently purified by extraction and salvation from inclusion bodies. After washing with PBS, the cell pellet was resuspended in 20 ml of resuspension buffer (10 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA) and sonicated (5 cycles of 30 seconds). The mixture was centrifuged for 10 minutes at 10000 rpm and the supernatant discarded. The pellet was then resuspended in 20 ml of homogenizing buffer (50 mM Hepes pH 7.9, 2 mM EDTA, 0.1 mM DTT, 0.05% deoxycholate, 1% Triton X-100, 5% glycerol) and subjected to 10 cycles of Dounce homogenization. Inclusion bodies were collected by centrifugation for 10 minutes at 10000 rpm. Inclusion bodies were then stirred overnight at 4°C in 40 ml of denaturing buffer (20 mM Hepes pH 7.9, 500 mM KCl, 0.2 mM EDTA, 2 mM DTT, 8M urea). The resolubilized mixture was centrifuged to remove precipitate and the supernatant containing the solubilized His-CLOCK342 was dialyzed against storage buffer (20 mM Hepes pH 7.9,

100 mM KCl, 0.2 mM EDTA, 2 mM DTT). Dialyzed protein was centrifuged to remove precipitate and soluble His-CLOCK342 was visualized using Coomassie staining. Protein concentration was determined using the Bio-Rad protein assay and absorbance at 595 nm.

Expression and purification of Flag-Myc-His-hCRY1 in HEK293T cells

Flag-Myc-His-*hCry1* had been previously cloned into the pcDNA4 mammalian expression vector (Invitrogen) by Dr. Carrie Partch. The construct included a single Flag, a single Myc, and a single 6xHis tag at the N-terminus of *hCry1*. This construct was transfected into HEK293T cells (20 150-cm plates; approximately 70% confluence) using standard calcium chloride transfection protocol. After 48 hours, cells were harvested and washed once with 1X PBS. Cells were lysed in NP-40 lysis buffer on ice for 20 minutes and then sonicated (5 cycles of 10 seconds); soluble cell lysate was prepared by centrifugation of the lysed cells at 15000 rpm for 45 minutes at 4°C. The supernatant was incubated overnight with Flag-agarose (Sigma; 200 µl packed resin) at 4°C. The resin was then centrifuged at 4000 rpm for 5 minutes and flow-through removed and discarded. The resin was washed with lysis buffer, tris-buffered saline (TBS) with 1M NaCl, TBS, and TBS with 10% glycerol (15 ml each wash). Bound protein was eluted in 300 µl of elution buffer (TBS with 10% glycerol and 0.1 mg/ml Flag-peptide) and purification products were visualized using silver staining. Protein concentration was determined using the Bio-Rad protein assay and absorbance at 595 nm.

Electrophoretic Mobility Shift Assays (EMSAs)

EMSA was used to investigate the effect of hCRY1 on the DNA binding properties of the CLOCK-BMAl1 heterodimer. Either a double-stranded 14mer containing a single E-box element (M34) or a double-stranded 58mer containing a single E-box element (E58), as

indicated in the figure legends, was used as the DNA probe in reactions at a concentration of approximately 1 nM. Figure 3.3, panel A shows the sequences of the M34 and E58 substrates. The DNA probes were 5'-radiolabeled with ^{32}P using a standard radiolabeling protocol. Flag-His-mBMAL1, His-mCLOCK342, Flag-hCLOCK, and/or Flag-Myc-His-hCRY1 were incubated at the indicated concentrations with 1 nM DNA probe in 25 µl reactions containing (unless otherwise indicated) 50 mM Tris pH 8.0, 100 mM KCl, 100 µg/ml BSA, 1 mM EDTA, 8 mM DTT, 1 µg poly dI/dC, and 10% (v/v) glycerol for 20 minutes at room temperature and 10 minutes at 4° C. Reactions were loaded onto a pre-run 4% nondenaturing polyacrylamide gel in 0.5X TBE (25 mM Tris-borate, pH 7.9, 0.6 mM EDTA) and electrophoresis was carried out at 4° C for approximately 3 hours. After electrophoresis, the gel was dried and exposed to a PhosphorImager screen and analyzed by autoradiography using ImageQuant software.

Results

Expression and Purification of Mammalian Circadian Clock Proteins

Although genetic evidence shows that CRY inhibits transcriptional activation by CLOCK-BMAL1, there is little biochemical data indicating the mechanism by which this occurs. For this reason, I decided to use a biochemical approach to answer this question, using electrophoretic mobility shift assays (EMSA) to directly assess the effect of CRY on CLOCK-BMAL1 DNA binding in an *in vitro* system. Bacterial, baculovirus, and mammalian cell expression systems were used to purify CLOCK342, BMAL1, CLOCK, and CRY1 proteins (Figure 3.2). The proteins were fused to small peptide tags to enable purification and to minimize any interference of the tags with protein folding or DNA binding. Full-length hCRY1 (with N-terminal Flag, Myc, and 6xHis tags; Lane 2) was affinity-purified after transient transfection in HEK293T cells. Flag-Myc-His-CRY1, of a molecular weight of ~65 kDa, was obtained in high purity using this system. Full-length mBMAL1 (with N-terminal Flag and 6xHis tags; Lane 3) and hCLOCK (with a N-terminal Flag tag; Lane 5) were affinity-purified from SF-21 insect cells using the baculovirus expression system. Flag-His-BMAL1, of a molecular weight of ~85 kDa, was obtained in high purity using this system. Flag-CLOCK, of a molecular weight of ~105 kDa, consistently co-purified with a ~70 kDa contaminant protein. This is likely Hsp70, perhaps associating with CLOCK due to its C-terminal poly-Q region.

Previously, a truncated from of mCLOCK containing the N-terminal 342 amino acids of the protein, which includes the bHLH and PAS domains, has been used successfully in EMSAs (Rutter et al., 2001). A construct for bacterial expression of this protein (referred to here as CLOCK342) was obtained from Dr. Christopher Selby (UNC Chapel Hill).

CLOCK342 (with a N-terminal 6xHis tag; Lane 4) was expressed in and purified from E. coli; expression using this system generated exclusively insoluble protein that was subsequently purified from inclusion bodies.

Effect of CRY on DNA-Binding of CLOCK-BMAL1 Heterodimers

Once the clock proteins were purified, their DNA-binding activity had to be determined. This was done using electrophoretic mobility shift assays (EMSAs). CLOCK-BMAL1 activates transcription off of E-box DNA promoter elements (Gekakis et al., 1998) and has been shown to bind to E-box DNA elements specifically; therefore, as DNA substrates I used either a ds 14mer containing a single E-box (referred to as M34; Figure 3.3 A) or a ds 58mer containing a single E-box (referred to as E58; Figure 3.3 B). Under standard conditions, reactions containing full-length CLOCK alone result in a smear pattern, while CLOCK342 alone binds to DNA with extremely low affinity and again results in a slight smearing of the DNA (Figure 3.3 C, lanes 2 and 3). BMAL1 has been shown to bind to DNA in the absence of CLOCK, presumably as a homodimer (Rutter, Reick, McKnight, Science 2001); I was able to reproduce this under standard conditions (Figure 3.3 C, lane 4). Formation of the CLOCK342-BMAL1 heterodimer was readily observable (Figure 3.3 C, lane 6), and was heavily favored over formation of the BMAL1 homodimer (compare lane 4 to lane 6). In comparison, when full-length CLOCK and BMAL1 were incubated together, although formation of the BMAL1 homodimer on DNA was inhibited, I was unable to observe discrete formation of CLOCK-BMAL1 heterodimer (Figure 3.3 C, lane 5). For this reason, further experiments to determine the effect of CRY1 on heterodimer DNA-binding were performed using both the CLOCK-BMAL1 heterodimer and the CLOCK342-BMAL1 heterodimer.

Although CRY proteins share high homology with DNA photolyases, they lack any DNA repair activity (Sancar, 2000). However, the similarity between the proteins suggests that CRY, like DNA photolyase, may also bind DNA *in vivo*. Human CRY2 has been shown to bind to DNA, with a much higher affinity to single-stranded DNA than to double-stranded DNA; this binding was indifferent to DNA sequence but displayed a preference for DNA containing a (6-4) UV photoproduct (Ozgur and Sancar, 2003). Human CRY1 also displayed DNA-binding activity on a double-stranded substrate with moderate-to-high affinity, qualitatively (Figure 3.4). The previous work with hCRY2 suggests that hCRY1's DNA-binding activity is likely to be nonspecific, though this has yet to be conclusively determined.

I performed titration experiments with hCRY1 to determine the effect of increasing concentrations of the protein on the DNA-binding activity of the CLOCK-BMAL1 and CLOCK342-BMAL1 heterodimers. Although I was unable to detect discrete formation of the CLOCK-BMAL1 heterodimer on DNA, I did observe a slow-migrating smear pattern which was unaffected by the addition of increasing concentrations of CRY1 (Figure 3.4). I performed similar experiments using the CLOCK342-BMAL1 heterodimer, which formed a discrete species on DNA and led to more conclusive results. These experiments were performed under conditions where CRY1 bound to DNA with both high affinity (Figure 3.5, left) and low affinity (Figure 3.4, right) to lessen the possibility that any inhibitory effect seen of CRY1 would be due to nonspecific competition. In both cases, increasing concentrations of CRY1 had no observable effect on the DNA-binding intensity of CLOCK342-BMAL1. These data would indicate that CRY1 does not affect the heterodimer by inhibiting its DNA-binding activity. However, there are a number of caveats to this set of experiments. First, it is possible that the type of assay used, EMSA, cannot observably

capture any inhibitory effect of CRY1 on the heterodimer. Second, the truncated CLOCK342 protein may not be a physiologically relevant protein to use in this experiment. It may display an increased ability, relative to full-length CLOCK, to bind to DNA in the CLOCK342-BMAL1 heterodimer form, which could abrogate CRY1's inhibitory effect. Also, as CLOCK342 is not full-length it may be missing a residue/motif necessary for repression and/or interaction with CRY1; this will be discussed further in the Discussion section.

It was also of interest to determine whether CRY1 can affect the binding of the individual clock proteins to DNA. This is a relevant question due to the recent finding that CRY's interaction with CLOCK and BMAL1 is increased when both CLOCK and BMAL1 proteins are overexpressed, relative to interaction levels when only one of the proteins is overexpressed (Kiyohara et al., 2006). Although a discrete DNA-binding species can only be seen in the case of BMAL1, it appears that CRY1 does not affect either discrete binding of BMAL1 homodimer to DNA or the lower-affinity binding of either CLOCK342 or CLOCK to DNA (Figure 3.6).

Effect of NADH and DTT on CLOCK342-BMAL1 DNA-Binding Activity

It has been suggested that modulation of cellular redox state is the mechanism by which feeding entrains the circadian clock (Rutter et al., 2001). Rutter and colleagues showed that addition of reduced NAD intermediates (NADH, NADPH), which change as a function of expression of lactate dehydrogenase (LDHA) during cellular respiration, increase DNA-binding activity of both NPAS2-BMAL1 and CLOCK-BMAL1 on a ds E-box DNA substrate. However, experiments using homologous zebrafish clock proteins zfCLOCK2 and zfBMAL3 showed no NAD/NADH dependence, although increased concentration of DTT

resulted in increased DNA-binding activity of the heterodimer (Ishikawa et al., 2002). Additionally, previous experiments in our laboratory showed DTT-dependent enhanced DNA-binding of CLOCK-BMAL1 but no NAD/NADH dependence (data not shown). It was therefore of interest to resolve this discrepancy. Using standard conditions, identical to those used in previous experiments in our laboratory, addition of 10 mM NADH did not induce CLOCK342-BMAL1 DNA-binding; however, addition of 10 mM DTT resulted in a marked increase in heterodimer DNA-binding (Figure 3.7). However, different results were obtained when conditions were adjusted to reflect those used by Rutter and colleagues (Rutter et al., 2001). These conditions, though still within physiological range, are slightly less stringent than the standard conditions used in my other experiments. Using these reaction conditions, addition of NADH up to 10 mM dramatically increased the DNA-binding of CLOCK342-BMAL1, while addition of DTT to 10 mM had no effect (Figure 3.8). Because both the NAD/NADH effect and the DTT effect on heterodimer binding is very sensitive to slight changes in reaction conditions, it is difficult to assess the physiological relevance of either effect. This will be discussed in more detail in the Discussion section.

Discussion

Repression of CLOCK-BMAL1 by CRY1

Despite much genetic evidence which led to proposal of the current mammalian clock model, biochemical evidence indicating the molecular mechanisms underlying the clock is generally lacking. This is especially true in the case of mammalian CRYPTOCHROME, a protein whose *in vivo* biochemical function is still unclear.

Determining the mechanism by which CRY inhibits transcriptional activation by CLOCK-BMAL1 will fill in a long-standing gap in the current clock model. Data from previously published reports has shed some light on how the negative circadian feedback loop works in other model systems. In zebrafish, zCRY1a forms a large complex with zfCLOCK2 and zfBMAL3 on DNA which is presumably transcriptionally inactive (Ishikawa et al., 2002). A different mechanism is indicated in *Drosophila*, where negative regulators dPER and dTIM eliminate the binding of dCLOCK-CYC to DNA (Lee et al., 1999). While distinct mechanisms are indicated in both zebrafish and Drosophila, in both systems negative regulation occurs through direct modulation of CLOCK-BMAL1 (or CLOCK-CYC) DNA-binding. Therefore, in the present study I investigated the possibility of direct modulation of CLOCK-BMAL1 DNA binding by CRY in the mammalian system. Here I present evidence that, under the conditions of the experimental system used, mammalian CRY1 does not inhibit or otherwise affect the DNA-binding of the CLOCK-BMAL1 heterodimer.

Although the data presented here indicates no effect of CRY1 on CLOCK-BMAL1, there is an important caveat to consider. The experiments described used a truncated form of CLOCK containing only the first 342 N-terminal amino acids of the protein (CLOCK342). This protein has been shown to efficiently form a heterodimer with BMAL1 on E-box DNA

(Rutter et al., 2001; unpublished results from our laboratory). However, the DNA-binding activity of this protein may not be identical to that of full-length CLOCK. If CLOCK342 binds to DNA in the heterodimer form with a much higher affinity compared to that of CLOCK, it is possible that the concentrations of CRY1 used are insufficient to affect CLOCK342-BMAL1 DNA binding. An additional consideration to these experiments was brought to light in a recent report which identified CLOCK and BMAL1 mutations that eliminate repression by CRY. A single mutation in residue 360 of CLOCK resulted in abrogation of repression of CLOCK-BMAL1 by CRY1 (Sato et al., 2006); this residue is notable because it is outside of the 342-aa fragment of CLOCK342. This would again indicate that CLOCK342 may not be a physiologically relevant, as it lacks a residue that is necessary for CRY's repression function. There exists the possibility that the reason I saw no effect of CRY on CLOCK342-BMAL1's DNA binding in my EMSA experiments is the absence of this key residue. Taken together, these considerations illustrate the need to do these experiments with full-length mammalian CLOCK protein in order to ensure relevant activity levels and that all necessary residues are present in the proteins being used. If the results of those experiments mirror those described here and again show no effect of CRY1 on CLOCK-BMAL1 DNA-binding, it would suggest that repression by CRY1 is not achieved through modulation of DNA binding state, and would support the proposals that modification of chromatin and/or CLOCK-BMAL1 post-translational changes are more likely candidates for the pathway involved.

Effect of NADH and DTT on CLOCK-BMAL1 DNA Binding

Identification of the physiological factors that, in addition to light, can entrain the circadian cycle is crucial to fully understand the mechanisms underlying the clock. It is

known that feeding cycles can entrain the mammalian clock, and one proposal for how this occurs is through inducing rhythmic changes in the redox state of nicotinamide adenine dinucleotide cofactors. Rutter and colleagues demonstrated by EMSA that increasing concentrations of NADPH and NADH induce DNA binding of NPAS2-BMAL1 and CLOCK342-BMAL1 (Rutter et al., 2001); they surmised from these data that changes in cellular NAD redox state, which would give rise to NADPH and NADH, could be the mechanism by which feeding entrains the clock.

However, there are questions as to the physiological relevance of the aforementioned data. This effect was not seen with homologous zebrafish clock proteins (Ishikawa et al., 2002), although an increase in CLOCK-BMAL1 heterodimer DNA binding was seen upon addition of increasing concentrations of dithiothreitol. In my experiments, I was able to reproduce induction of CLOCK342-BMAL1 DNA binding by DTT; after slight changes in reaction conditions, I was also able to replicate induction by NADH. Because both sets of reaction conditions were within physiological ranges (with the exception of NADH and/or DTT concentration), the relevance of either effect is suspect. Additionally, the concentrations of NAD cofactors used by Rutter and colleagues are likely much higher than mammalian cellular concentrations reach, by at least a factor of ten, perhaps much more (Bergmeyer, 1974; Tischler et al., 1977; O'Donnell and Kuhn, 1997).

In conclusion, there is a clear need for further experiments both to determine the relevance of induction of CLOCK-BMAL1 DNA binding by NADH and/or DTT and to fully elucidate the mechanism by which CRY represses clock-activated gene transcription. Biochemical experiments similar to those described here will be crucial to understand the

molecular mechanisms underlying the mammalian clock. This will be especially useful in the current re-evaluation of the TTFL mammalian circadian clock model.

Figure 3.1. Possible Mechanisms for CRY's Negative Regulation of CLOCK-BMAL1 by Directly Affecting DNA Binding. CRY may directly affect CLOCK-BMAL1 by (1) changing the conformation of CLOCK-BMAL1, possibly by forming a trimeric complex, such that the complex no longer binds to DNA; (2) forming a trimeric complex on DNA which no longer is transcriptionally active; or (3) replacing one of the components of the CLOCK-BMAL1 heterodimer to form a transcriptionally inactive complex on DNA.

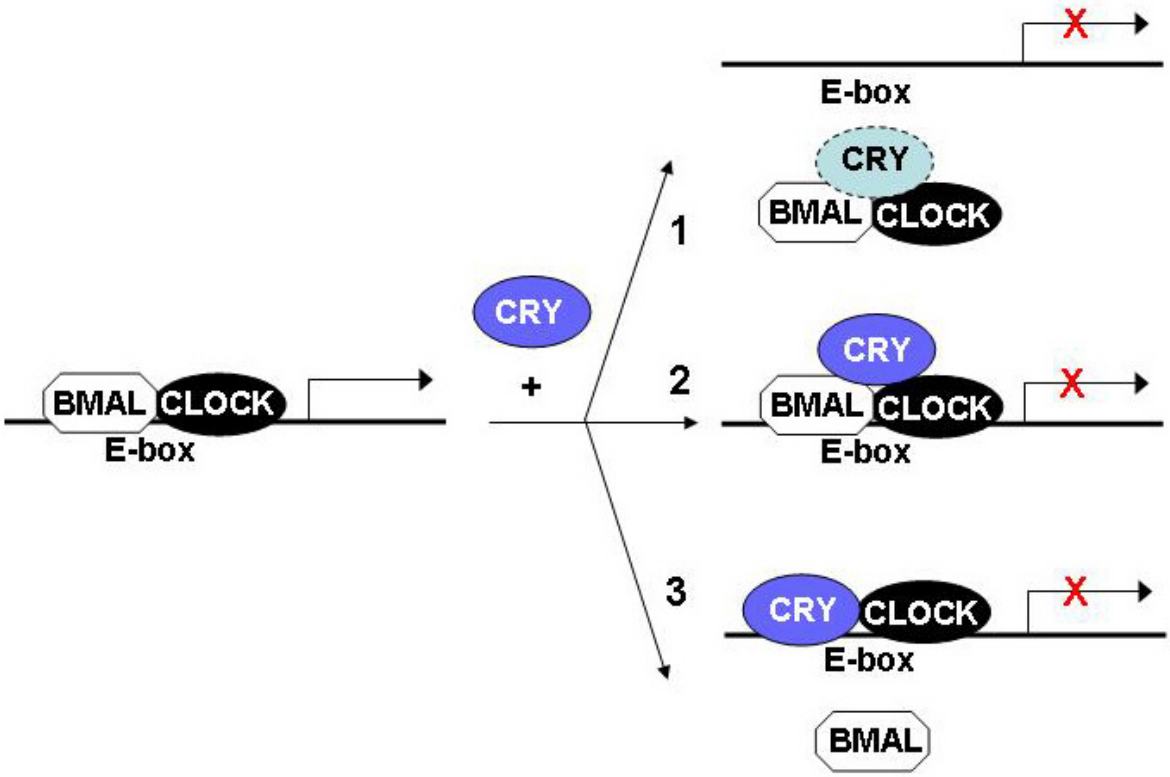

X
E-box
CRY
BMAL
CLOCK
1
CRY
+
2
CRY
BMAL
CLOCK
X
BMAL
CLOCK
E-box
E-box
3
CRY
CLOCK
X
E-box
BMAL

Figure 3.2. Purified hCRY1, mBMAL1, mCLOCK342, and hCLOCK proteins. (Lane 1) Molecular weight marker. (Lane 2) Flag-Myc-His-hCRY1, purified from transient transfection of 293T cells. Protein was purified to a concentration of approximately 314 nM. (Lane 3) Flag-His-mBMAL1, purified from baculovirus infection of SF-21 insect cells. Protein was purified to a concentration of approximately 312 nM. (Lane 4) His-CLOCK342, purified from inclusion bodies in *E. coli*. Protein was purified to a concentration of approximately 2 μM. (Lane 5) Flag-hCLOCK, purified from baculovirus infection of SF-21 insect cells. Protein was purified to a concentration of approximately 115 nM. * denotes a contaminant, likely heat-shock protein. Proteins were visualized by silver-staining.

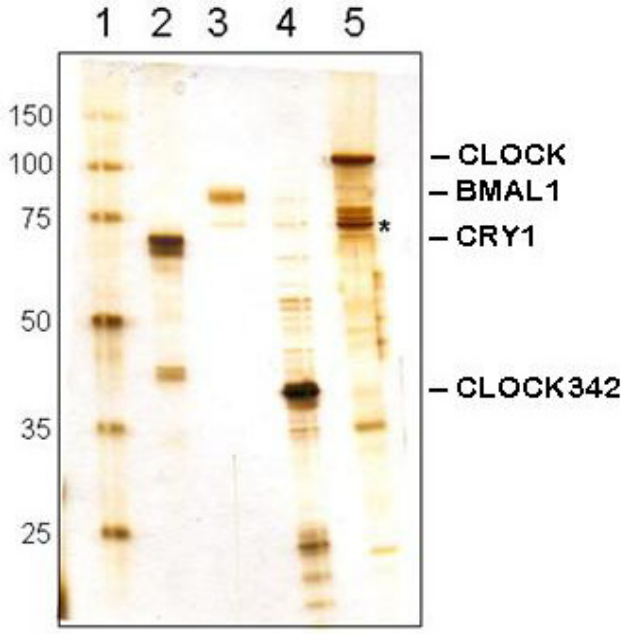

1 2 3 4 5
150
100
75
50
35
25
– CLOCK
– BMAL1
– CRY1
*
– CLOCK342

Figure 3.3. Formation of CLOCK342-BMAL1 and (possible) CLOCK-BMAL1 Heterodimer on E-box DNA. (A) M34 DNA substrate sequence. **(B)** E58 DNA substrate sequence. **(C)** EMSAs were carried out as described using E58 ds radiolabeled DNA. (Lane 1) DNA alone. (Lane 2) DNA + CLOCK. (Lane 3) DNA + CLOCK342. (Lane 4) DNA + BMAL1. (Lane 5) DNA + CLOCK + BMAL1. (Lane 6) DNA + CLOCK342 + BMAL1. Arrows denote DNA-protein complexes. *Protein concentrations used:* CLOCK (20 nm), BMAL1 (50 nm), CLOCK342 (80 nm).

A

```
5′    GGGACACGTGACCC    3′
3′    CCCTGTGCACTGGG    5′
```

B

```
5′    CATCACCCACTCACCCCTTACTACACGTGGGCCCTCAATTGCCCTTCTCCAGGATCTG    3′
3′    GTCGTGGGTGAGTGGGGAATGATGTGCACCCGGGAGTTAACGGGAAGAGGTCCTAGAC    5′
```

C

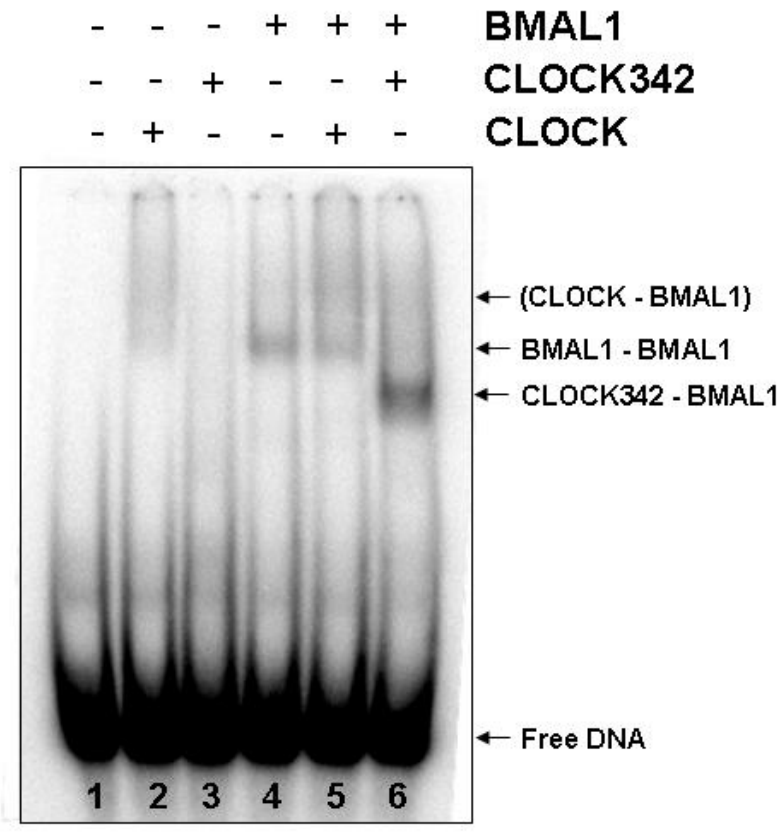


Figure 3.4. Effect of CRY1 on Binding of CLOCK-BMAL1 to E-box DNA. EMSAs were carried out as described using E58 ds radiolabeled DNA. Arrows indicate the binding of BMAL1-BMAL1 homodimer, CLOCK-BMAL1 heterodimer, or CRY1 to DNA. *Protein concentrations used:* BMAL1 (50 nm), CLOCK (20 nm), CRY1 (14-100 nm).

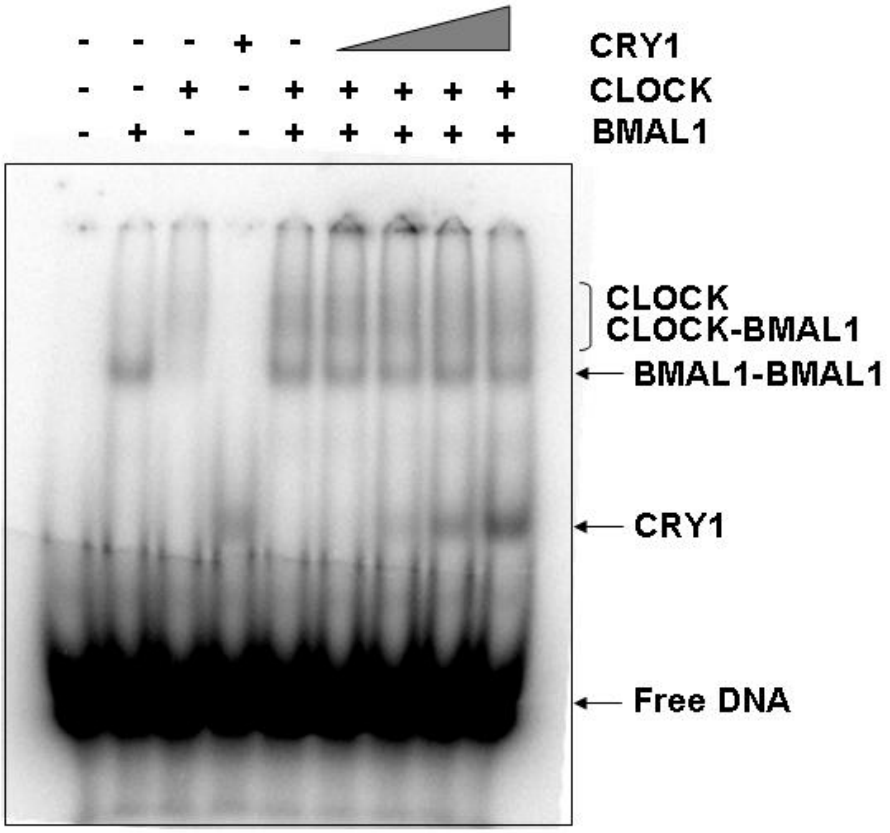

- - - + -
CRY1
- - + - + + + + +
CLOCK
- + - - + + + + +
BMAL1
CLOCK
CLOCK-BMAL1
BMAL1-BMAL1
CRY1
Free DNA

Figure 3.5. Effect of CRY1 on Binding of CLOCK342-BMAL1 to E-box DNA. EMSAs were carried out as described using E58 ds radiolabeled DNA under conditions of either high CRY1 DBA-binding affinity (left panel) or low CRY1 DNA-binding affinity (right panel). Arrows indicate the binding of BMAL1-BMAL1 homodimer, CLOCK342-BMAL1 heterodimer, or CRY1 to DNA. *Protein concentrations used:* BMAL1 (50 nm), CLOCK342 (80 nm), CRY1 (14-100 nm).

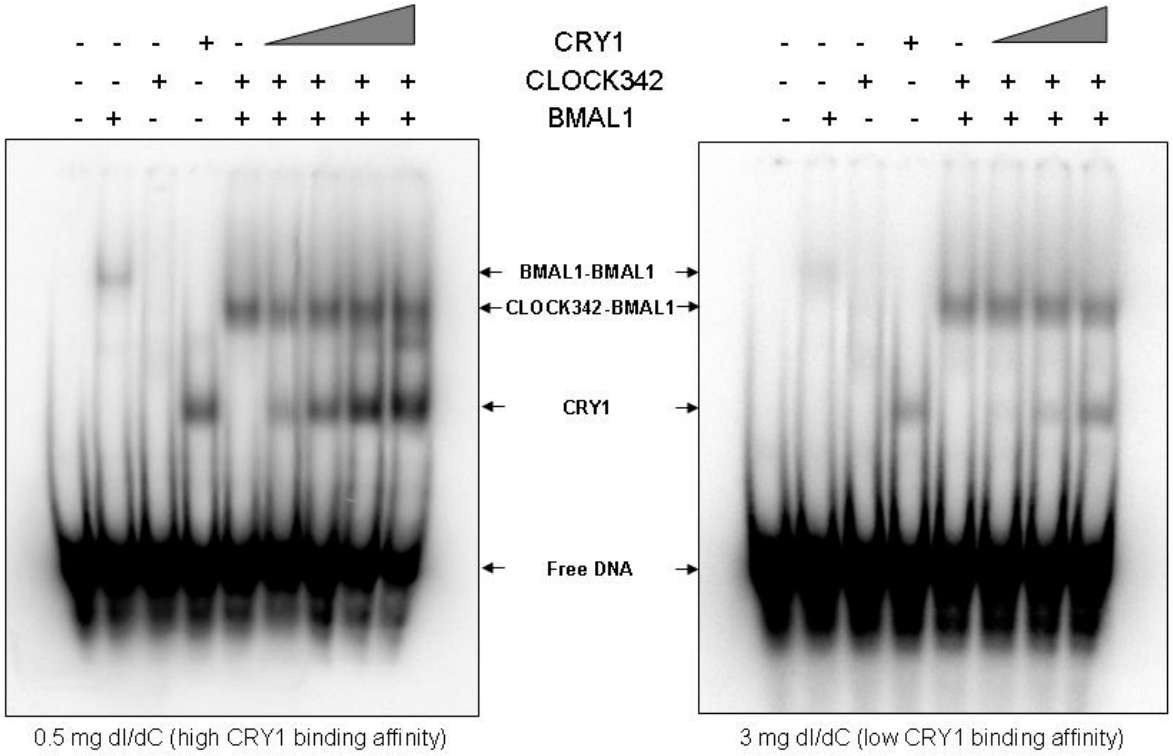

CRY1
CLOCK342
BMAL1
BMAL1-BMAL1
CLOCK342-BMAL1
CRY1
Free DNA
0.5 mg dI/dC (high CRY1 binding affinity)
3 mg dI/dC (low CRY1 binding affinity)

Figure 3.6. Effect of CRY on DNA-Binding of BMAL1, CLOCK342, and CLOCK Individual Proteins. EMSAs were carried out as described using E58 ds radiolabeled DNA under standard conditions. Arrows indicate the binding of BMAL1-BMAL1 homodimer, CLOCK342-BMAL1 heterodimer, CLOCK342, CLOCK, or CRY1 to DNA. *Protein concentrations used:* CLOCK (20 nm), BMAL1 (50 nm), CLOCK342 (80 nm), CRY1 (10-40 nm).

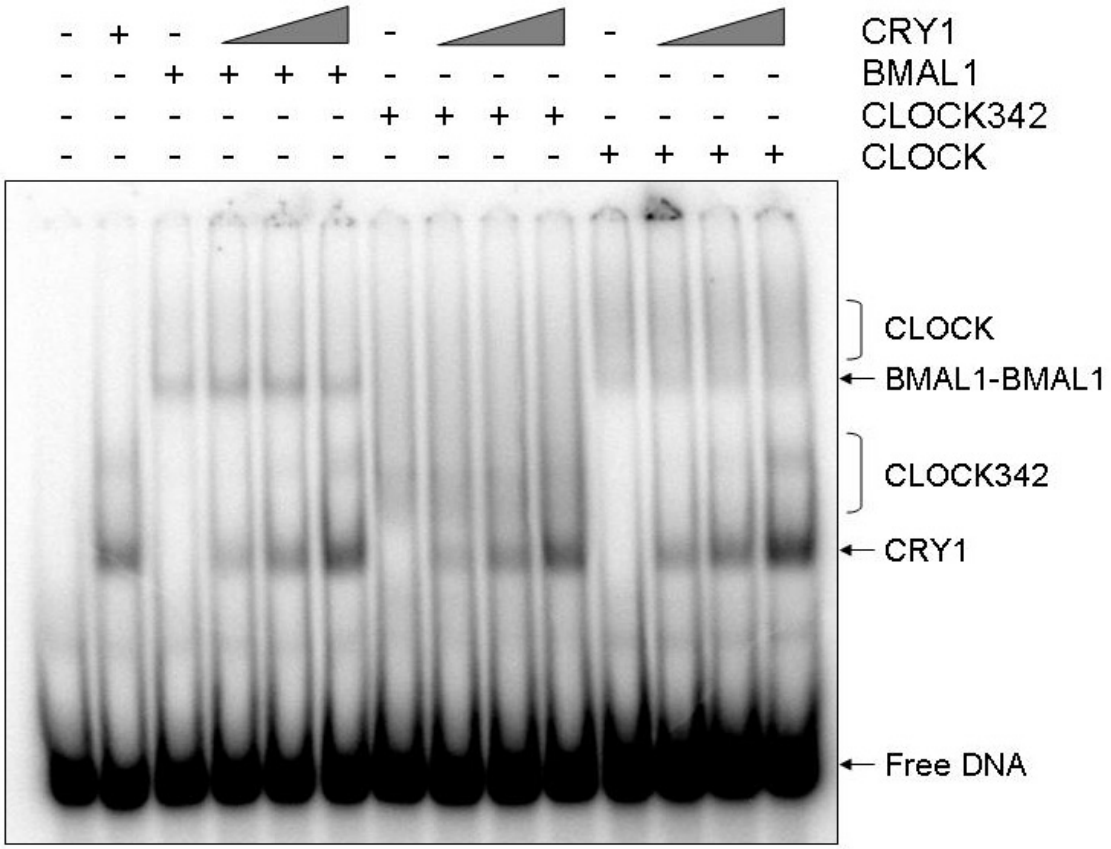

- + - - - + + + + + + + + + + CRY1
- - + + + + - - - - - - - - - BMAL1
- - - - - - + + + + - - - - CLOCK342
- - - - - - - - - - + + + + CLOCK
CLOCK
BMAL1-BMAL1
CLOCK342
CRY1
Free DNA

Figure 3.7. Effect of NADH and DTT on DNA-Binding of CLOCK342-BMAL1 heterodimer under standard conditions. EMSAs were carried out using the M34 DNA probe under standard conditions without DTT: 50 mM Tris pH 8.0, 100 mM KCl, 100 µg/ml BSA, 1 mM EDTA, 0.5 µg poly dI/dC, and 10% (v/v) glycerol for 20 minutes at room temperature and 10 minutes at 4° C. 10 mM DTT or 10 mM NADH was included in the reaction as indicated. *Protein concentrations used:* BMAL1 (20 nm), CLOCK342 (50 nm).

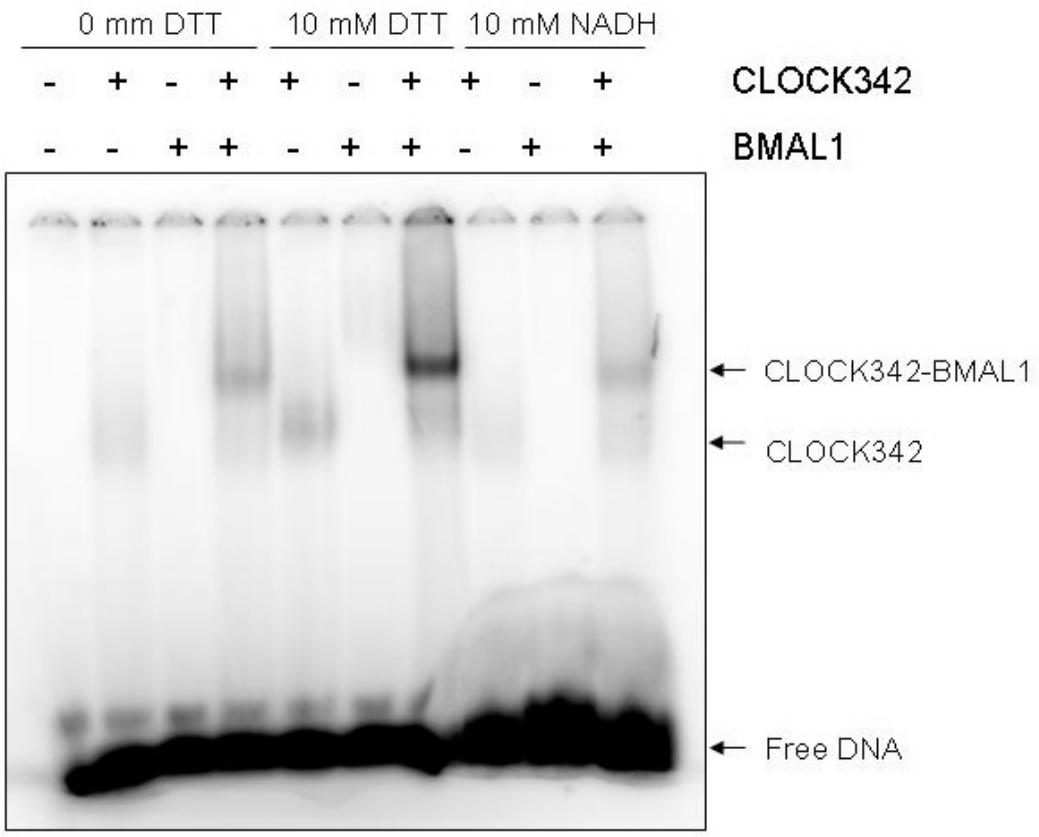
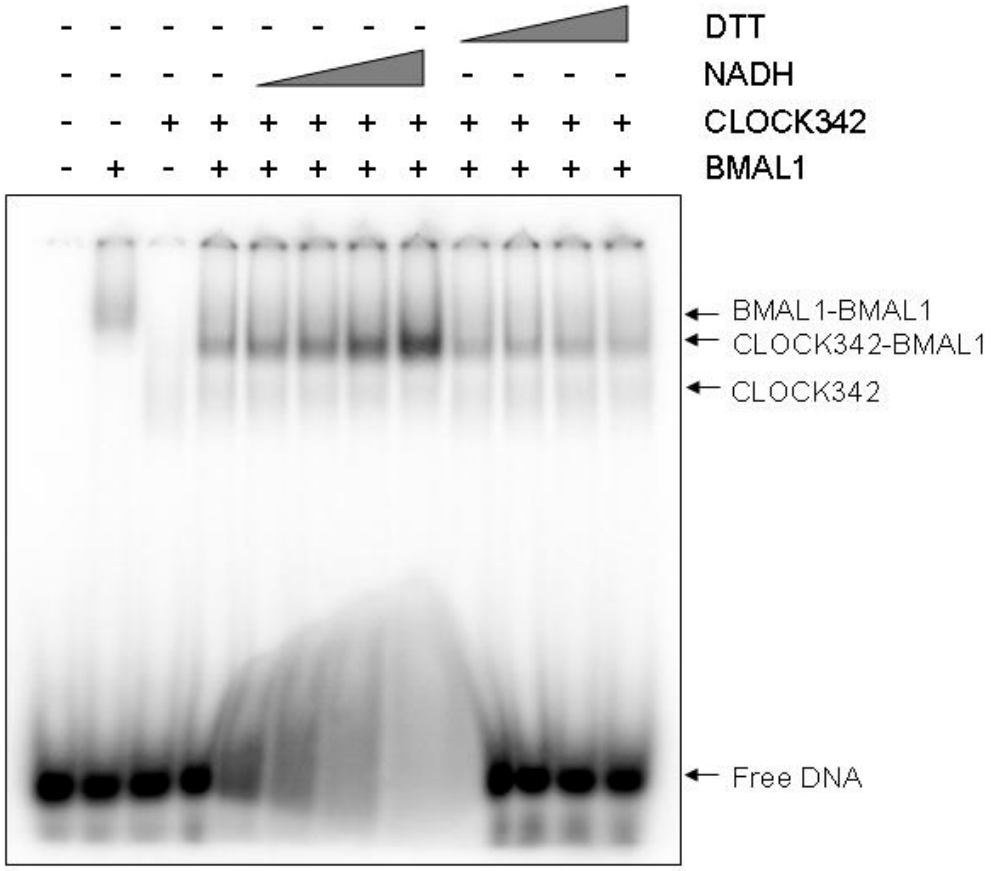

0 mm DTT
10 mM DTT
10 mM NADH
- + - + + - + + - +
CLOCK342
- - + + - + + - + +
BMAL1
CLOCK342-BMAL1
CLOCK342
Free DNA

Figure 3.8. Effect of NADH and DTT on DNA-Binding of CLOCK342-BMAL1 heterodimer under low-stringency conditions. EMSAs were carried out using the M34 DNA probe under low-stringency conditions: 10 mM Tris pH 7.5, 50 mM KCl, 1 mg/ml BSA, 1 ug poly dI/dC, and 10% glycerol for 30 minutes at room temperature. DTT or NADH was included in the reaction as indicated; concentrations ranged from 1.25 mM (lowest concentration) to 10 mM (highest concentration). *Protein concentrations used:* BMAL1 (50 nm), CLOCK342 (50 nm).


- - - - - - - - DTT
- - - - - - - - NADH
- - + + + + + + + + + + CLOCK342
- + - + + + + + + + + + BMAL1
BMAL1-BMAL1
CLOCK342-BMAL1
CLOCK342
Free DNA

CHAPTER 4

FINAL DISCUSSION AND FUTURE DIRECTIONS

The development of life on Earth would not have been possible without the sun's energy. Although this is a well-known concept, the extent to which the sun has shaped evolution of Earth organisms is even more remarkable. The first advent of adaptation to the daily solar cycle is thought to have been necessary to protect replicating genetic material from damage by ultraviolet light during Precambrian times before the protective ozone layer developed. The absence of the ozone layer resulted in heavy daily exposure to ultraviolet radiation for Earth organisms, forcing them to adapt in order to survive. To avoid UV, which can penetrate 25 meters of clear water, metazoans descended deep into the oceans during the daytime and migrated closer to the surface at night. Blue light is the only wavelength in the visual spectrum that can penetrate deep ocean water. Consequently, blue light became the cue for the organisms' internal timekeeping mechanism (Gehring and Rosbash, 2003).

The evolutionary adaptation demonstrated by the metazoans has extended into an extremely complex set of pathways in organisms ranging from cyanobacteria to humans. These pathways, called circadian clocks, synchronize behavioral processes, metabolism, hormone production, cell division, and a host of other important cellular processes to the daily solar cycle. Although organisms possess endogenous ~24 hour rhythms, entrainment by light-time signals synchronizes endogenous clocks to the precise 24 hour cycle of the

Earth's rotation. Again, blue light is likely the most important wavelength of light in this process, evidenced by the fact that the human eye is most sensitive to blue light.

Clearly, the effect of light on Earth organisms is extensive, and expanding our understanding of how we adapt to light-time signals will be a great scientific step. Perhaps the newly-discovered model system of *Danaus plexippus*, the monarch butterfly, will be especially helpful in achieving greater understanding of circadian clock mechanisms in animals. A sophisticated clock exists in the monarch butterfly, indicated by their complex yearly migration pattern which uses light to signal the fall and early spring journeys, for long-range navigation, and to cause seasonal variations in reproduction. While the more characterized *Drosophila* system has only one CRY (insect-like) and the mammalian system has two similar CRYs which are somewhat divergent from *Drosophila*, *Danaus* possesses both an insect-like CRY and a mammalian-like CRY (Reppert, 2006). This discovery has led to *Danaus*'s emergence as a new model system for the animal circadian clock and study of this system will greatly increase our understanding of how CRY functions and, more generally, of the mechanisms underlying the clock.

Here I describe experiments to further characterize the role of CRY in cancer predisposition and as a negative regulator of CLOCK-BMAL1. Various reports link circadian clock disruption to increased risk of breast cancer in humans (Hansen, 2000; Stevens, 2005), and mice with SCN ablation exhibit a faster rate of growth of transplanted tumors (Filipski et al., 2002). Furthermore, mice with a mutant form of *Per2* are hypersensitive to IR-induced mortality and have an abrogated DNA damage checkpoint (Fu et al., 2002). Although circadian disruption is indicated as a possible cancer risk factor, the mechanism through which this occurs is unknown. My experiments with *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice

show no predisposition to cancer or abrogation of DNA damage checkpoints relative to wild-type, indicating that circadian disruption *per se* does not increase cancer risk. This does not rule out involvement of circadian proteins with cell cycle; indeed, reports suggest that a function of the PER family of proteins, possibly outside of their clock role, is the connection between circadian rhythms and cancer (Fu et al., 2002; Gery et al., 2006; Hua et al., 2006). Further characterization of circadian control of the cell cycle will be key to both the possible establishment of clock disruption as a cancer risk factor and the use of chronotherapy, which seeks to maximize efficacy of chemotherapy while minimizing side effects through modulation of time of treatment (Hrushesky, 1995).

The mechanism by which CRY represses CLOCK-BMAL1 activity has been as "cryptic" as the protein's name suggests. Work with Drosophila and zebrafish proteins has indicated that in both organisms, negative regulation of CLOCK-BMAL1 by its repressors is achieved through effects on the DNA binding of the complex (Lee et al., 1999; Ishikawa et al., 2002). If one of these mechanisms was preserved in mammals, it would logically follow that CLOCK-BMAL1's binding to DNA would be rhythmic. However, reports on this are conflicting: there is evidence that the complex is constitutively bound to DNA (Lee et al., 2001), and a recent report claims rhythmic binding (Ripperger and Schibler, 2006). In order to clarify these conflicting reports, I used purified proteins to examine the effect of CRY1 on CLOCK-BMAL1 (using a 342-aa fragment of CLOCK, CLOCK342) in *in vitro* DNA binding assays. I show here that CRY1 does not change the DNA binding of CLOCK342-BMAL1. This is a notable finding as it would indicate that mammalian CRY represses CLOCK-BMAL1 by a mechanism distinct from that in other animal systems. However, it is possible that CLOCK342 is not a physiologically relevant protein for use in these assays as it

is missing a residue recently discovered to be crucial to repression of the heterodimer by CRY (Sato et al., 2006). Therefore, to conclusively determine if CRY directly affects the DNA binding of CLOCK-BMAL1, these experiments must be repeated with full-length CLOCK. Additionally, I investigated the effect of both NADH and DTT on induction of CLOCK-BMAL1 DNA binding in response to previous reports (Rutter et al., 2001; Ishikawa et al., 2002). This phenomenon is of interest because modulation of cellular redox state has been proposed as the pathway by which feeding entrains the circadian clock (Rutter et al., 2001). I find that in conditions where NADH induces heterodimer DNA binding, DTT does not; and in conditions where DTT induces heterodimer DNA binding, NADH does not. Based on these data, the physiological relevance of the effect of either NADH or DTT is in doubt.

Overall, these experiments contribute to our overall understanding of the mammalian circadian clock. However, ever more biochemical evidence is needed in order for us to fully comprehend this complex mechanism.

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