# [31] Oligodeoxynucleotide Methods for Analyzing the Circadian Clock in the Suprachiasmatic Nucleus

*By* SHELLEY A. TISCHKAU and MARTHA U. GILLETTE

## Abstract

The recent identification of specific genes responsible for the generation of endogenous circadian rhythmicity in the suprachiasmatic nucleus presents a new level of investigation into endogenous rhythmicity and mechanisms of synchronization of this circadian clock with the environmental light/dark cycle. This article describes techniques that employ antisense and decoy oligodeoxynucleotides (ODN) to determine the roles of specific molecular substrates both in endogenous rhythmicity and in regulating the effects of light on the mammalian circadian clock. Application of antisense ODN technology has revealed a role for *timeless* (*Tim*) in the core clock mechanism and established that induction of *period1* (*Per1*) is required for light responsiveness. Likewise, a decoy ODN designed to sequester activated CREB protein definitively demonstrated a requirement for CRE-mediated transcription in light signaling. Experiments designed with these molecular tools offer new insights on the interaction of cellular processes and signaling with the molecular clockworks.

## Introduction

The rotation of the earth on its axis, and the resulting daily alternation of light and darkness, imparts arguably the single-most persistent, stable environmental factor influencing the evolution of life on this planet. The importance of the ability to measure time on a daily scale is reflected by the genomic incorporation of circadian rhythmicity, or near 24-h patterns of physiology and behavior driven by gene expression, into nearly all eukaryotic organisms. This internal clock can generate and maintain rhythms in the absence of external stimuli, but it also retains the ability to respond to specific cues to allow synchronization with the solar cycle.

There is an emerging consensus that individual cells in virtually all tissues contain clocks; however, in mammals, organismic rhythmicity remains an emergent property of the suprachiasmatic nucleus (SCN). Strategically positioned in the basal hypothalamus adjacent to the optic chiasm, the SCN navigates a recurrent sequence of dynamic cellular events, driven by the coordinated function of a core group of clock genes and organized into discrete time domains. Progression through this daily cycle



0076-6879/05 $35.00

is characterized by waxing and waning sensitivities of the SCN to resetting stimuli, such as to light extending inappropriately into the night. This circadian oscillation in sensitive periods is underscored by clock-controlled adjustments in available molecular and biochemical substrates and activation of signal transduction pathways (Gillette and Mitchell, 2002).

The circadian clockwork is embedded in alternating transcription–translation loops of a limited number of clock genes and their products that together generate near 24-h periodicity (King and Takahashi, 2000). Clock genes, by convention, are those essential to circadian timekeeping. Several mammalian clock genes appear to be highly conserved elements of circadian clocks across phyla (Okamura *et al*., 2002). In some cases, they have assumed additional roles, as regulators of developmental pattern formation (Li *et al*., 2000; Xiao *et al*., 2003) or the cell cycle (Matsuo *et al*., 2003), which has complicated analysis of a specific gene's timekeeping role in the context of a central brain clock of mammals. Furthermore, the SCN is composed of paired clusters of ~10,000 cells. Whereas circadian clocks are properties of single cells, how timekeeping is organized within the SCN tissue is unclear. Nevertheless, insights into the positive and negative regulation of clock gene transcription and the roles of clock effector proteins and their regulation within the SCN are emerging. Studies *in vivo* and *in vitro* are defining essential elements and critical regulators. This article reviews methods for analyzing SCN rhythmicity *in vivo* and *in vitro* with new techniques that can probe the contribution of specific molecular elements to the mammalian clockwork, the regulatory pathways that intersect it, and clock-controlled outputs.

## Analysis of SCN Rhythmicity *In Vivo* and *In Vitro*

Changes in rhythmic locomotor activity in freely behaving rodents have long been considered the ''gold standard'' for analysis of function in mammal models of circadian timekeeping. These studies take advantage of the fact that locomotor activity cycles readily entrain to light:dark cycles and persist when the animal is placed in constant environmental lighting conditions, albeit with a somewhat altered period (*tau*, $\tau$) under these free-running conditions. Over the years, a plethora of substances have been tested by injection, either systemically or by stereotaxic direction into the ventricular system, into the SCN or other brain regions of interest. Typically substances are tested for their direct effects on the circadian timing system or for their impact on well-characterized photic or nonphotic phase shifts in behavioral rhythms. Advantages of this model system include the opportunity to examine overt effects on the entire circadian system and, more recently, the ability to directly couple changes in the

expression of specific genes to alterations in animal behavior. Disadvantages include the fact that results may not reflect only what is happening in the SCN, but also within multiple layers of integration at other brain and body sites because the SCN is intricately connected to multiple input and output systems.

Direct measures of SCN activity *in vivo* have revealed an oscillatory pattern of a high activity in the daytime and low activity at night. This pattern extends from spontaneous neural activity (Inouye and Kawamura, 1982) to energy utilization (2-deoxyglucose) (Schwartz *et al*., 1980, 1983) to mRNA expression of certain clock genes (*Per*, *Tim*). High daytime activity is consistent across diverse mammals, including both nocturnal (Burgoon *et al*., 2004; Inouye and Kawamura, 1979; Kurumiya and Kawamura, 1988; Yamazaki *et al*., 1998) and diurnal rodents (Sato and Kawamura, 1984). Other brain regions also show circadian oscillations in neural activity (Inouye and Kawamura, 1982; Szymusiak *et al*., 1998), but only in the presence of the SCN. Thus, spontaneous oscillation in the SCN drives circadian rhythms at other brain sites. The selective pressures that have caused universal high daytime activity in the SCN are unknown; however, it follows that the signals that pattern the range of circadian behaviors, such as when sleep/arousal occur relative to the day/night cycle, must be regulated outside of the SCN.

Hypothalamic brain slices containing the paired SCN provide a mechanism for direct probing of SCN function with minimal interference from extra-SCN sources. Despite surgical deafferentation, separation of rostral and caudal components, and lack of endocrine stimulation, the cultured SCN retains its spontaneous, nearly 24-h oscillations in gene expression (Yamazaki *et al*., 2000), neuronal firing rate (Prosser and Gillette, 1989), and peptide secretion (Shinohara *et al*., 1995). Neuronal activity of SCN slices shows a distinct rhythm of electrical activity with a peak in midsubjective day (Green and Gillette, 1982) like those observed *in vivo* (Inouye and Kawamura, 1979). The time of the peak in electrical activity predictably displays circadian periodicity that persists up to 3 days *ex vivo* (Prosser and Gillette, 1989). Thus, this circadian rhythm in neuronal firing rate has been used extensively as a bioassay to study mechanisms of phase resetting in the SCN (Gillette and Mitchell, 2002).

The SCN expresses specific temporal domains, or windows of sensitivity, defined by continuously changing access to intrinsic signaling molecules. These sensitivities are easily probed in the SCN slice by applying stimuli to receptive regions and observing the effects on the timing of the peak oscillation of the electrical activity rhythm. Pharmacological approaches using the SCN slice preparation have identified specific cellular substrates that underlie phase resetting in response to a variety of stimuli.

Importantly, the validity of the SCN brain slice preparation has been demonstrated repeatedly by experiments performed in whole animal models using circadian wheel-running activity as a measure of rhythmicity. In most instances, results from pharmacological approaches using SCN slices *in vitro* are consistent with *in vivo* studies, particularly when the test substance is injected directly onto the SCN. For example, inhibition of nitric oxide synthase blocks light-induced phase resetting *in vivo* (Weber *et al*., 1995a), and glutamate, the neurochemical messenger of light, stimulates the light-activated signaling pathways and phase resetting *in vitro* (Ding *et al*., 1994). Furthermore, inhibition of protein kinase G blocks only light- or glutamate-induced phase advances, with no effect on phase delays (Ding *et al*., 1998; Mathur *et al*., 1996; Weber *et al*., 1995b). The major difference between *in vivo* and *in vitro* approaches to phase resetting lies in the magnitude of the phase shift. Phase shifts *in vitro* are generally larger in amplitude than those observed *in vivo*. In the rat, light-induced phase shifts *in vivo* are commonly in the range of 1 h, whereas glutamate-induced phase shifts in this same animal model are usually around 3 h (Ding *et al*., 1994). Similarly, we observed 3.5-h phase delays induced by PKG inhibition *in vitro*, but the same treatment into the SCN *in vivo* caused phase delays of less than 1 h (Tischkau *et al*., 2003b).

Behavioral wheel-running activity and SCN electrical activity have been valuable tools to assess cellular mechanisms that underlie circadian rhythmicity. The discovery of circadian clock genes has provided another level of depth of inquiry into circadian clock function. Behavioral wheel running and SCN electrical activity rhythms remain essential bioassays for exploring gene function and coupling signaling pathways to circadian gene function within the clock. This article provides insight into new techniques that enable exploration of the significance of clock genes, as well as other molecular elements that activate the genome, to circadian behavior.

## Targeted Deletion of Clock Genes

The generation of animals bearing genetic deficiency in a specific protein product is often considered the definitive test for establishing the physiological role of any gene. The same is true for those genes tentatively defined as circadian clock genes. Whereas these techniques are relatively inexpensive and expeditious in several model circadian systems, such as *Drosophila* and *Neurospora*, the development of mammalian models deficient in one or more clock genes has required considerable effort. Animals are subjected to customary testing, typically wheel-running behavior under constant conditions and responsiveness to nocturnal light, to assess rhythmicity. These animals have been important for establishing the

relative importance of several core clock genes, including *Clock* (King *et al.*, 1997; Vitaterna *et al.*, 1994), *bmal1* (Bunger *et al.*, 2000), *Per 1–3* (Bae *et al.*, 2001; Cermakian *et al.*, 2001; Shearman *et al.*, 2000; Zheng *et al.*, 1999, 2001), and *Cry1–2* (Okamura *et al.*, 1999; Thresher *et al.*, 1998; van der Horst *et al.*, 1999) for circadian rhythmicity. In addition, mutant animals have also established roles for an ever-expanding number of regulatory elements, such as casein kinase Iε (Lowrey *et al.*, 2000) and REV ERBα (Preitner *et al.*, 2002), and clock output molecules, such as prokineticin 2 (Cheng *et al.*, 2002), phospholipase C β4 (Park *et al.*, 2003) and D-binding protein (DBP) (Lopez-Molina *et al.*, 1997).

Experience indicates that analysis of data from these animal models is not always straightforward and must be interpreted with caution. *Bmal1*$^{-/-}$ mice exhibit the expected immediate disruption of circadian wheel-running activity under constant environmental conditions (Bunger *et al.*, 2000). In contrast, animals bearing an ENU-induced mutation of the transactivation domain of mCLK exhibit a lengthened free-running period followed by a gradual loss of rhythmicity (Antoch *et al.*, 1997; King *et al.*, 1997; Vitaterna *et al.*, 1994). Moreover, evolutionary duplication of the *Per* and *Cry* genes adds additional complexity. Deletion of *Per3* has no effect on rhythmicity (Shearman *et al.*, 2000). Whereas some discrepancies have been noted among mice with targeted deletion of either *Per1* or *Per2*, the consensus is that these animals have a partially functional clock. Most animals display significantly reduced free-running periodicity (Bae *et al.*, 2001; Cermakian *et al.*, 2001; Zheng *et al.*, 1999, 2001); animals generated by one laboratory also exhibit disrupted rhythms after prolonged exposure to constant darkness (Bae *et al.*, 2001). Likewise, mutations in either *Cry1* or *Cry2* result in altered periodicity, but retention of rhythmicity (Okamura *et al.*, 1999; Thresher *et al.*, 1998). Simultaneous disruption of *Per1* and *Per2* or of *Cry1* and *Cry2* results in animals whose rhythms are immediately disrupted when they are placed in constant darkness (van der Horst *et al.*, 1999; Zheng *et al.*, 2001).

Inconsistencies with studies in mutant animals likely stem from the developmental nature of the model, where other genes may compensate for the missing gene, or from differing genetic backgrounds of mouse strains, thereby providing misleading or false-negative results. For example, pharmacological studies have implicated a role for nitric oxide in light-induced phase resetting (Ding *et al.*, 1994; Melo *et al.*, 1997; Weber *et al.*, 1995b). However, mice deficient in either nNOS or eNOS show normal responses to nocturnal light (Kriegsfield *et al.*, 1999a,b, 2001). Furthermore, animals with a targeted deletion of protein kinase G-II display abnormal phase delays and normal phase advances, despite pharmacological data that implicate a role for PKG restricted to the late night, when light causes

phase advance of rhythmicity (Ding *et al*., 1998; Mathur *et al*., 1996; Oster *et al*., 2003; Weber *et al*., 1995b). Additionally, the embryonic lethality of $mTim^{-/-}$ mice led to premature exclusion of this core gene from the mammalian clockwork (Gotter *et al*., 2000).

## Antisense Oligodeoxynucleotides and Small Interfering RNA as Tools to Investigate Gene Function in Circadian Timekeeping

Whereas generation of tissue-specific, conditional deletion or induction of target genes will likely overcome many of the problems associated with traditional approaches to genetic deletion of clock genes, this technology remains in its infancy. Thus, antisense oligodeoxynucleotide ($\alpha$ODN) and, more recently, small interfering RNA (siRNA) approaches have been used as inexpensive models devoid of the developmental problems inherent to the whole animal targeted deletion paradigms (Estibeiro and Godfray, 2001). The distinct advantage of this approach in circadian biology is the ability to reversibly downregulate expression of the gene of interest in an adult animal in a time- and tissue-specific manner. In the brain, $\alpha$ODN and siRNA can be directed to the site of interest by stereotaxic cannula placement. Furthermore, $\alpha$ODN technology is widely adaptable for use in numerous species, whereas traditional genetic approaches are restricted primarily to mice.

Watson–Crick base pair formation dictates that $\alpha$ODN will bind to the sense stand of the RNA or DNA of interest with a high degree of specificity and thus block the formation of gene products. Typically, the $\alpha$ODN is designed to target a specific mRNA sequence. Criteria that must be considered when designing an $\alpha$ODN include (1) the uniqueness of the target sequence, (2) the length and sequence of the $\alpha$ODN, (3) modifications of the nucleotides, and (4) appropriate controls (Kashikara *et al*., 1998). Although there is currently no way to predict the best target sequence to yield the maximal effect, two commonly successful targets include the 3′-untranslated region and the AUG translation initiation codon. Use of the latter predicts a mechanism of action through blockage of translation by preventing binding of initiation factors. The length of the $\alpha$ODN must be considered: it must be long enough to confer specificity to the sequence of interest, yet short enough to allow uptake into the cell. In our hands, 15- to 21-mer $\alpha$ODNs are highly effective (Barnes *et al*., 2003; Tischkau *et al*., 2003a,b). The $\alpha$ODN sequence should always be evaluated to avoid those that will form strong secondary structures because they are self-complementary.

Modifications can be introduced to increase the half-life of the $\alpha$ODN within the cell. Common modifications include phosphorothioate, methyl phosphate, phosphoroamidate, and methyl phosphate derivatives

(Cho-Chung, 2003). The unmodified αODN is readily degraded by endo- or exonucleases so it has a relatively short half-life of an estimated 20 min (Kashikara *et al*., 1998). In contrast, phosphorothioate modifications confer nuclease resistance and are still present 8–16 h after injection into hypothalamic sites (Ogawa *et al*., 1995). However, this same modification can impair cellular uptake of the αODN, decrease the specificity of binding to the RNA of interest (Kashikara *et al*., 1998), exert nonspecific effects by binding to proteins and small molecules (Perez *et al*., 1994; Yakubov *et al*., 1993) and, in some cases, can be toxic (Hebb and Robertson, 1997).

Because of the potential for nonspecific effects, design and use of appropriate controls are critical for interpretation of data obtained in αODN experiments. Sense ODN that is complementary to the αODN is a commonly used control. However, sense ODN has the potential to bind DNA for the gene of interest and thereby inhibit transcription (Kashikara *et al*., 1998). Therefore, a scrambled ODN with the same base composition as the αODN but in random order is a better control. Additionally, replacing one or more nucleotides in the αODN with a different base can disrupt complementary binding. We have found that as little as a single base pair mismatch in a 15-mer is enough to disrupt the effectiveness of the αODN (Barnes *et al*., 2003; Tischkau *et al*., 2003a,b).

### *Effects of Antisense Oligodeoxynucleotides in Circadian Clock Responses to Light*

αODN technology has been employed to explore the function of clock gene proteins and other regulatory elements in the molecular events leading to phase resetting in response to nocturnal light *in vivo* or its messenger, glutamate, *in vitro*. A rapid, transient increase in *Per1* mRNA is a hallmark of the molecular response to nocturnal light (Albrecht *et al*., 1997; Miyake *et al*., 2000; Shearman *et al*., 1997; Shigeyoshi *et al*., 1997; Takumi *et al*., 1998). An unmodified αODN designed to target the region surrounding the initiation codon of *Per1* mRNA (5′-taggggaccactcatgtct-3′) blocks glutamate-induced phase resetting of the SCN electrical activity rhythm *in vitro* and light-induced phase delays *in vivo* (Akiyama *et al*., 1999; Tischkau *et al*., 2003a); control sequences with 1- to 3-bp changes are ineffective. These results demonstrate definitively that induction of *Per1* mRNA is required for phase resetting in response to nocturnal light. It follows that the PER1 protein contributes critically to molecular changes that mediate clock resetting.

The αODN technique has also been used to examine the roles of other proteins in the light/Glu signaling pathway. The core of the hamster SCN contains a densely packed population of calbindin-containing cells

that receive direct retinal input and express *Fos* in response to light (Bryant *et al*., 2000; Hamada *et al*., 2003; Silver *et al*., 1996). Intracerebroventricular injection of phosphothionate-modified αODN against calbindin (5′-aggtgcgattctgccatgg-3′) significantly reduced calbindin mRNA and protein in the SCN core and attenuated both the light-induced increase in *Per1* and phase advances in response to light (Hamada *et al*., 2003). These data implicate $Ca^{2+}$ buffering via calbindin in the light response and further the idea that $Ca^{2+}$ is critical for light-induced signaling in the SCN. Interestingly, simultaneous inhibition of *JunB* and *cFos* with αODN also blocked light-induced phase resetting (Schlingensiepen *et al*., 1994).

The cGMP/protein kinase G (PKG) signal transduction cascade has also been implicated in light-induced phase shifting. Activation of cGMP/PKG occurs only in response to light that signals advance clock phase in the last half of subjective night. Pharmacological inhibition of PKG blocks light- and glutamate-induced phase advances, but not the phase-delaying effect of light/Glu in early night (Ding *et al*., 1998; Mathur *et al*., 1996; Weber *et al*., 1995b). Two major isoforms of PKG have been described and a different αODN can be designed to the start site of each. Preliminary studies suggest that this αODN approach is a powerful tool for differentiating isoform-specific function of PKG in the SCN (S. A. Tischkau and M. U. Gillette, unpublished observations).

### *Effects of Antisense Oligodeoxynucleotides on Circadian Clock Rhythmicity*

Because short-term inhibition using αODN technology is effective for identifying the roles of specific proteins in light/Glu-induced phase resetting, we hypothesized that long-term inhibition might be used to identify molecular components that are required for the expression of overt rhythmicity in the SCN. Initially, we utilized this approach to examine whether mammalian *timeless (Tim)* is required for the generation of circadian rhythmicity. Traditional approaches using genetic deletion of *Tim* had been unsuccessful because the $Tim^{-/-}$ mutation yields an embryonic lethal phenotype (Gotter *et al*., 2000). We circumvented this developmental problem by examining electrical activity rhythms in SCN-containing brain slices incubated continuously with *Tim* αODN (acagtccatacacc). SCN slices treated with *Tim* αODN expressed ∼ 40% of the TIM protein levels in controls and were completely arrhythmic (Barnes *et al*., 2003). Moreover, short-term application of *Tim* αODN over the course of a 24-h cycle revealed a specific sensitive period where downregulation of *Tim* led to phase resetting of the circadian clock (Barnes *et al*., 2003). Furthermore, introduction of siRNA targeting *Tim* demonstrated that *Tim* knockdown

alters expression of other clock genes. Together, these data demonstrate a specific requirement for *Tim* in SCN rhythmicity and allow restoration of *Tim* as a core mammalian circadian clock element.

The success of these experiments led us to explore the role of additional elements in SCN rhythmicity. Previously, we had demonstrated a circadian rhythm of cGMP levels and PKG activity inherent to the SCN. Pharmacological disruption of this endogenous rise in PKG activity caused significant phase delay of the circadian clock *in vitro* and *in vivo* specifically at the dawn-to-dusk transition (Tischkau *et al*., 2003b). These data led to the hypothesis that increased PKG activity at the end of subjective night is required for circadian clock progression. A corollary of this hypothesis is that continuous inhibition of PKG would lead to arrhythmicity. Utilizing isoform-specific αODNs, we can discriminate the isoform required for PKG mediation of clock function at the dawn transition (Tischkau *et al*., 2003b). These studies demonstrate the power of αODN technology in defining a role for specific proteins in circadian clock function.

### *Use of Antisense Oligodeoxynucleotides in Defining Circadian Clock Output*

The αODN approach has also been used to examine the roles of proteins predicted to be components of one of the numerous SCN output programs. Vasoactive intestinal peptide (VIP), which is secreted from the SCN in a diurnal fashion (reviewed in van Esseveldt *et al*., 2000), is an important regulator of the estrogen-induced luteinizing hormone and prolactin surges (van der Beek *et al*., 1999). Intracerebroventricular or intra-SCN injection of a VIP αODN attenuates and/or phase delays rhythms of circulating corticosterone, luteinizing hormone, and prolactin (Harney *et al*., 1996; Scarbrough *et al*., 1996; van der Beek *et al*., 1999). The latter is likely through an effect on neuroendocrine dopaminergic neurons in the hypothalamic arcuate nucleus and periventricular nucleus (Gerhold *et al*., 2002). Together, these results highlight the potential of αODN technology in exploring the specific roles of the array of output networks connected to the SCN.

## Decoy Oligodeoxynucleotides as Tools to Investigate Transcriptional Control in Circadian Timekeeping

Using the same methods as for αODN, ectopic enhancer oligodeoxynucleotides sequences can be introduced in excess into cells or brain tissue. These supernumerary enhancer sequences act as *decoys*, competing with native *cis* elements for binding of specific transactivating DNA-binding proteins and thereby diminishing activity at intrinsic transcriptional regulatory

Transcriptional activation
A
Transcription factors
Transcription and Expression
RNA Polymerase
TGACGTCA
TGACGTCA
TGACGTCA
ACCTACTATTGATGTTGCTTTTAGC...
B
Decoy ODN Mechanism
Palindromic CRE sequences self-hybridize, forming hairpins that bind CREB.
TGACGTCA TGACGTCA TGACGTCA
TGACGTCA TGAC
ACTGCACT ACTG
No RNA Polymerase
(No Expression)
TGACGTCA TGACGTCA TGACGTCA
ACCTACTATTGATGTTGCTTTTAGC...

sites (Cho-Chung, 2003). Thus, a single-stranded 24-mer comprising three repeats of the consensus CRE sequence (trioctamer of 5′-TGACGTCA-3′) can be introduced to inhibit CRE-mediated transcription. The palindromic nature of this CRE decoy allows it to self-hybridize upon entering the cell, forming a hairpin structure that effectively binds CREB, which results in loss of the ability to activate native CRE (Park *et al*., 1999). Previously, this ODN has been used to interfere with CRE-mediated transcription and thus potently inhibit growth in cancer cells (Park *et al*., 1999). In primary cultures of neonatal hippocampal neurons, the CRE decoy was employed to demonstrate the importance of CRE-mediated transcription in providing protection against glutamate-induced cell death (Mabuchi *et al*., 2001).

### *Effects of Decoy Oligodeoxynucleotides in Circadian Clock Response to Light*

CRE-mediated transcription has been implicated in the molecular events leading to light/glutamate-induced phase resetting of the circadian clock. Throughout the night, stimuli associated with light-induced phase resetting cause increased phosphorylation of CREB (Ding *et al*., 1997; Ginty *et al*., 1993) and activation of CRE-mediated transcription (Obrietan *et al*., 1999). Furthermore, CREB may play a role in the stimulation of *Per1* (Travnickova-Bendova *et al*., 2002; Yamaguchi *et al*., 2000), which is required for light-induced phase resetting (Akiyama *et al*., 1999; Tischkau *et al*., 2003a). However, those studies fell short of establishing a requirement for CRE-mediated transcription for the molecular events leading to phase resetting in response to light.

We introduced the CRE decoy to block CRE-mediated transcription in the presence of stimuli known to mediate nocturnal light-induced phase

FIG. 1. Mechanism by which decoy ODN inhibits transcription. (A) Normal expression is regulated by transcription factors binding to specific sequences in the promoter region of a gene. These transcription factors provide a scaffold for RNA polymerase, which creates an RNA copy of the coding sequence and leads to expression of the gene product. (B) Decoy ODN technology is based on providing a tissue excess of short ODN recognition sequences for a specific transcription factor, such as the CRE sequence (CACGTG) in the model. Decoy ODN are constructed of the same sequence as a specific promoter element, but these ectopic-binding sites are present in excess. This causes transcription factor binding to recognition sequences in intrinsic promoter elements (e.g., CREB binding to CREs) to be outcompeted by binding to the decoy. The target transcription factors will be unavailable to bind the promoter and will fail to form the scaffold for tethering RNA polymerase at the endogenous gene target. If these transcription factors are necessary for transcription of the gene, then normal expression is interrupted.

resetting in the SCN. Our synthetic CRE decoy included one important distinction compared with previous studies in other cell systems. The CRE decoy employed in those studies was composed of phosphorothioate-modified ODN to provide nuclease resistance and increase stability within the cell (Agrawal *et al*., 1997; Zon, 1988). Because CREB is likely to regulate many genes (Panda *et al*., 2002), not solely those activated during light-induced transcription, we reasoned that the long-term presence of the CRE decoy could disrupt other functions and complicate interpretation of data. To restrict inhibition of CRE-mediated transcription to a narrow window, our CRE decoy was synthesized with less stable, unmodified nucleotides. This CRE decoy successfully blocked CRE-CREB binding and CRE-mediated transcription in SCN2.2 cells (Tischkau *et al*., 2003a). When applied to SCN brain slices, the CRE decoy blocked glutamate-stimulated phase resetting of SCN electrical activity rhythms and induction of *Per1*. *In vivo*, when injected unilaterally into the SCN, the CRE decoy blocked light-induced phase resetting of behavioral wheel-running rhythms (Tischkau *et al*., 2003a). This was the first study to combine use of this technology in cells, brain slices, and *in vivo*. In a subsequent study, intracerebroventricular injection of the CRE decoy effectively blocked CREB-DNA binding in the gerbil hippocampus, confirming the efficacy of this technique for use *in vivo* (Hara *et al*., 2003; Fig. 1).

## Conclusions

Antisense and decoy oligodeoxynucleotides provide an inexpensive, effective, and complementary alternative to molecular genetics for analyzing the function of specific gene products in the circadian timekeeping system. Whereas these approaches may be limited somewhat by cell permeability, degradation, and inability to totally knock out the RNA or protein of interest (Scarbrough, 2000), the capacity to locally antagonize the expression of a single gene product for a discrete, controlled time renders this technology highly useful. Moreover, the reversibility of $\alpha$ODN effects is particularly attractive for physiological studies. When coupled with monitoring electrical activity rhythms *in vitro* in SCN brain slices or with locomotor activity rhythms in freely behaving animals, this technology provides an efficient means to screen candidate gene products for a role in the generation or regulation of rhythmicity.

## Acknowledgments

We thank the members of the Gillette laboratory who contributed conceptually and experimentally to developing these methods, P. T. Lindberg for developing the model, and S. C. Baker for manuscript preparation. Supported by Public Health Service Grants NS22155,

NS35859, and HL67007 (MUG) and by a grant from the UIUC Governor's Venture Technology Fund/Molecular and Endocrine Pharmacology Program (SAT). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the National Institutes of Neurological Diseases and Stroke, Heart, Lung, and Blood, or General Medicine.

## References

Agrawal, S., Jiang, Z., Zhao, Q., Shaw, D., Cai, Q., Roskey, A., Channavajjala, L., Saxinger, C., and Zhang, R. (1997). Mixed-backbone oligonucleotides as second generation antisense oligonucleotides: *In vitro* and *in vivo* studies. *Proc. Natl. Acad. Sci. USA* **94,** 2620–2625.

Akiyama, M., Kouzu, Y., Takahashi, S., Wakamatsu, H., Moriya, T., Maetani, M., Watanabe, S., Tei, H., Sakaki, Y., and Shibata, S. (1999). Inhibition of light- or glutamate-induced *mPer1* expression represses the phase shifts of the mouse circadian locomoter and suprachiasmatic firing rate rhythms. *J. Neurosci.* **19,** 1115–1121.

Albrecht, U., Sun, Z. S., Eichele, G., and Lee, C. C. (1997). A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light. *Cell* **91,** 1055–1064.

Antoch, M. P., Song, E. J., Chang, A. M., Vitaterna, M. H., Zhao, Y., Wilsbacher, L. D., Sangoram, A. M., King, D. P., Pinto, L. H., and Takahashi, J. S. (1997). Functional identification of the mouse circadian Clock gene by transgenic BAC rescue. *Cell* **89,** 655–667.

Bae, K., Jin, X., Maywood, E. S., Hastings, M. H., Reppert, S. M., and Weaver, D. R. (2001). Differential functions of *mPer1*, *mPer2*, and *mPer3* in the SCN circadian clock. *Neuron* **30,** 525–536.

Barnes, J. W., Tischkau, S. A., Barnes, J. A., Mitchell, J. W., Burgoon, P. W., Hickok, J. R., and Gillette, M. U. (2003). Requirement of mammalian *Timeless* for circadian rhythmicity. *Science* **302,** 439–442.

Bryant, D. N., LeSauter, J., Silver, R., and Romero, M. T. (2000). Retinal innervation of calbindin-D28K cells in the hamster suprachiasmatic nucleus: Ultrastructural characterization. *J. Biol. Rhythms* **15,** 103–111.

Bunger, M. K., Wilsbacher, L. D., Moran, S. M., Clendenin, C., Radcliffe, L. A., Hogenesch, J. B., Simon, M. C., Takahashi, J. S., and Bradfield, C. A. (2000). Mop3 is an essential component of the master circadian pacemaker in mammals. *Cell* **103,** 1009–1017.

Burgoon, P. W., Lindberg, P. T., and Gillette, M. U. (2004). Different patterns of circadian oscillation in the suprachiasmatic nucleus of hamster, mouse and rat. *J. Comp. Physiol. A.* **190,** 167–171.

Cermakian, N., Monaco, L., Pando, M., Dierich, A., and Sassone-Corsi, P. (2001). Altered behavioral rhythms and clock gene expression in mice with a targeted mutation in the *period1* gene. *EMBO J.* **20,** 3967–3974.

Cheng, M. Y., Bullock, C. M., Li, C., Lee, A. G., Bermak, J. C., Belluzzi, J., Weaver, D. R., Leslie, F. M., and Zhou, Q. Y. (2002). Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* **417,** 405–410.

Cho-Chung, Y. S. (2003). CRE-enhancer DNA decoy: A tumor target-based genetic tool. *Ann. N. Y. Acad. Sci.* **1002,** 124–133.

Ding, J. M., Buchanan, G. F., Tischkau, S. A., Chen, D., Kuriashkina, L., Faiman, L. E., Alster, J. M., McPherson, P. S., Campbell, K. P., and Gillette, M. U. (1998). A neuronal ryanodine receptor mediates light-induced phase delays of the circadian clock. *Nature* **394,** 381–384.

Ding, J. M., Chen, D., Weber, E. T., Faiman, L. E., Rea, M. A., and Gillette, M. U. (1994). Resetting the biological clock: Mediation of nocturnal circadian shifts by glutamate and NO. *Science* **266,** 1713–1717.

Ding, J. M., Faiman, L. E., Hurst, W. J., Kuriashkina, L. R., and Gillette, M. U. (1997). Resetting the biological clock: Mediation of nocturnal CREB phosphorylation via light glutamate and nitric oxide. *J. Neurosci.* **17,** 667–675.

Estibeiro, P., and Godfray, J. (2001). Antisense as a neuroscience tool and therapeutic agent. *Trends Neurosci.* **24,** S56–62.

Gerhold, L., Sellix, M. T., and Freeman, M. E. (2002). Antagonism of vasoactive intestinal peptide mRNA in the suprachiasmatic nucleus disrupts the rhythm of FRAs expression in neuroendocrine dopaminergic neurons. *J. Comp. Neurol.* **450,** 135–143.

Gillette, M. U., and Mitchell, J. W. (2002). Signaling in the suprachiasmatic nucleus: Selectively responsive and integrative. *Cell Tissue Res.* **309,** 99–107.

Ginty, D. D., Kornhauser, J. M., Thompson, M. A., Bading, H., Mayo, K. E., Takahashi, J. S., and Greenberg, M. E. (1993). Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science* **260,** 238–241.

Gotter, A. L., Manganaro, T., Weaver, D. R., Kolakowski, J., L. F., Possidente, B., Sriram, S., MacLaughlin, D. T., and Reppert, S. M. (2000). A time-less function for mouse timeless. *Nature Neurosci.* **3,** 755–756.

Green, D. J., and Gillette, R. (1982). Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice. *Brain Res.* **245,** 198–200.

Hamada, T., LeSauter, J., Lokshin, M., Romero, M. T., Yan, L., Venuti, J. M., and Silver, R. (2003). Calbindin influences response to photic input in suprachiasmatic nucleus. *J. Neurosci.* **23,** 8820–8826.

Hara, T., Hamada, J., Yano, S., Morioka, M., Kai, Y., and Ushio, Y. (2003). CREB is required for acquisition of ischemic tolerance in gerbil hippocampal CA1 region. *J. Neurochem.* **86,** 805–814.

Harney, J. P., Scarbrough, K., Rosewell, K. L., and Wise, P. M. (1996). *In vivo* antisense antagonism of vasoactive intestinal peptide in the suprachiasmatic nuclei causes aging-like changes in the estradiol-induced luteinizing hormone and prolactin surges. *Endocrinology* **137,** 3696–3701.

Hebb, M. O., and Robertson, H. A. (1997). End-capped antisense oligodeoxynucleotides effectively inhibit gene expression *in vivo* and offer a low-toxicity alternative to fully modified phosphorothioate oligodeoxynucleotides. *Brain Res. Mol. Brain Res.* **47,** 223–228.

Inouye, S. T., and Kawamura, H. (1979). Persistence of circadian rhythmicity in a mammalian hypothalamic ''island'' containing the suprachiasmatic nucleus. *Proc. Natl. Acad. Sci. USA* **76,** 5962–5966.

Inouye, S. T., and Kawamura, H. (1982). Characteristics of a circadian pacemaker in the suprachiasmatic nucleus. *J. Comp. Physiol. A.* **146,** 153–160.

Kashikara, N., Maeshima, Y., and Makino, H. (1998). Antisense oligonucleotides. *Exp. Nephrol.* **6,** 84–88.

King, D. P., and Takahashi, J. S. (2000). Molecular genetics of circadian rhythms in mammals. *Annu. Rev. Neurosci.* **23,** 713–742.

King, D. P., Zhao, Y., Sangoram, A. M., Wilsbacher, L. D., Tanaka, M., Antoch, M. P., Steeves, T. D., Vitaterna, M. H., Kornhauser, J. M., Lowrey, P. L., Turek, F. W., and Takahashi, J. S. (1997). Positional cloning of the mouse circadian clock gene. *Cell* **89,** 641–653.

Kriegsfield, L. J., Demas, G. E., Lee, S. E., Dawson, T. M., Dawson, V. L., and Nelson, R. J. (1999a). Circadian locomotor analysis of male mice lacking the gene for neuronal nitric oxide synthase (nNOS−/−). *J. Biol. Rhythms* **14,** 20–27.

Kriegsfield, L. J., Drazen, D. L., and Nelson, R. J. (2001). Circadian organization in male mice lacking the gene for endothelial nitric oxide synthase (eNOS−/−). *J. Biol. Rhythms* **16,** 142–148.

Kriegsfield, L. J., Eliasson, M. J. L., Demas, G. E., Blackshaw, S., Dawson, T. M., Nelson, R. J., and Snyder, S. J. (1999b). Nocturnal motor coordination deficits in neuronal nitric oxide synthase knock-out mice. *Neuroscience* **89,** 311–315.

Kurumiya, S., and Kawamura, H. (1988). Circadian oscillation of the multiple unit activity in the guinea pig suprachiasmatic nucleus. *J. Comp. Physiol. A.* **162,** 301–308.

Li, Z., Stuart, R. O., Qiao, J., Pavlova, A., Bush, K. T., Pohl, M., Sakurai, H., and Nigam, S. K. (2000). A role for Timeless in epithelial morphogenesis during kidney development. *Proc. Natl. Acad. Sci. USA* **97,** 10038–10043.

Lopez-Molina, L., Conquet, F., Dubois-Dauphin, M., and Schibler, U. (1997). The DBP gene is expressed according to a circadian rhythm in the suprachiasmatic nucleus and influences circadian behavior. *EMBO J.* **16,** 6762–6771.

Lowrey, P. L., Shimomura, K., Antoch, M. P., Yamazaki, S., Zemenides, P. D., Ralph, M. R., Menaker, M., and Takahashi, J. S. (2000). Positional systemic cloning and functional characterization of the mammlain circadian mutation tau. *Science* **288,** 483–492.

Mabuchi, T., Kitagawa, K., Kuwabara, K., Takasawa, K., Ohtsuki, T., Xia, Z., Storm, D. R., Yanagihara, T., Hori, M., and Matsumoto, M. (2001). Phosphorylation of cAMP response element-binding protein in hippocampal neurons as a protective response after exposure to glutamate *in vitro* and ischemia *in vivo*. *J. Neurosci.* **21,** 9204–9213.

Mathur, A., Golombek, D. A., and Ralph, M. R. (1996). cGMP-dependent protein kinase inhibitors block light-induced phase advances of circadian rhythms *in vivo*. *Am. J. Physiol.* **270,** R1031–R1036.

Matsuo, T., Yamaguchi, S., Mitsui, S., Emi, A., Shimoda, F., and Okamura, H. (2003). Control mechanism of the circadian clock for timing of cell division *in vivo*. *Science* **302,** 255–259.

Melo, L., Golombek, D. A., and Ralph, M. R. (1997). Regulation of circadian photic responses by nitric oxide. *J. Biol. Rhythms* **12,** 319–326.

Miyake, S., Sumi, Y., Yan, L., Takekida, S., Fukuyama, T., Ishida, Y., Yamaguchi, S., Yagita, K., and Okamura, H. (2000). Phase-dependent responses of *Per1* and *Per2* genes to a light stimulus in the suprachiasmatic nucleus of the rat. *Neurosci. Lett.* **294,** 41–44.

Obrietan, K., Impey, S., Smith, D., Athos, J., and Storm, D. R. (1999). Circadian regulation of cAMP response element-mediated gene expression in the suprachiasmatic nuclei. *J. Biol. Chem.* **274,** 17748–17756.

Ogawa, S., Brown, S. E., Okana, H. J., and Pfaff, D. W. (1995). Cellular uptake of intracerebrally administered oligodeoxynucleotides in mouse brain. *Reg. Pep.* **59,** 143–149.

Okamura, H., Miyake, S., Sumi, Y., Yamaguchi, S., Yasui, A., Muijtjens, M., Hoeijmakers, J. H., and van der Horst, G. T. (1999). Photic induction of *mPer1* and *mPer2* in cry-deficient mice lacking a biological clock. *Science* **286,** 2531–2534.

Okamura, H., Yamaguchi, S., and Yagita, K. (2002). Molecular machinery of the circadian clock in mammals. *Cell Tissue Res.* **309,** 47–56.

Oster, H., Werner, C., Magnone, M. C., Mayser, H., Feil, R., Seeliger, M. W., Hofmann, F., and Albrecht, U. (2003). cGMP-dependent protein kinase II modulates *mPer1* and *mPer2* gene induction and influences phase shifts of the circadian clock. *Curr. Biol.* **13,** 725–733.

Panda, S., Antoch, M. P., Miller, B. H., Su, A. I., Schook, A. B., Straume, M., Schultz, P. G., Kay, S. A., Takahashi, J. S., and Hogenesch, J. B. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* **109,** 307–320.

Park, D., Lee, S. E., Jun, K., Hong, Y.-M., Kim, D., Kim, Y. I., and Shin, H.-S. (2003). Translation of rhythmicity into neural firing in suprachiasmatic nucleus requires mGluR-PLC$\beta$4 signaling. *Nature Neurosci.* **6,** 337–338.

Park, Y. G., Nesterova, M., Agrawal, S., and Cho-Chung, Y. S. (1999). Dual blockade of cyclic AMP response element-(CRE) and AP-1-directed transcription by CRE transcription factor decoy oligodeoxynucleotide: Gene specific inhibition of tumor growth. *J. Biol. Chem.* **274,** 1573–1580.

Perez, J. R., Li, Y., Stein, C. A., Majumder, S., Oorschot, A. V., and Narayanan, R. (1994). Sequence-independent induction of Sp1 transcription factor activity by phosphorothioate oligodeoxynucleotides. *Proc. Natl. Acad. Sci. USA* **91,** 5957–5961.

Preitner, N., Damiola, F., Lopez-Molina, L., Zakany, J., Duboule, D., Albrecht, U., and Schibler, U. (2002). The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110,** 251–260.

Prosser, R. A., and Gillette, M. U. (1989). The mammalian circadian clock in the suprachiasmatic nuclei is reset *in vitro* by cAMP. *J. Neurosci.* **9,** 1073–1081.

Sato, T., and Kawamura, H. (1984). Circadian rhythms in multiple unit activity inside and outside the suprachiasmatic nucleus in the diurnal chipmunk (*Eutamais sibiricus*). *Neurosci. Res.* **1,** 45–52.

Scarbrough, K. (2000). Use of antisense oligodeoxynucleotides to study biological rhythms. *Methods* **22,** 255–260.

Scarbrough, K., Harney, J. P., Rosewell, K. L., and Wise, P. M. (1996). Acute effects of antisense antagonism of a single peptide neurotransmitter in the circadian clock. *Am. J. Physiol.* **270,** R283–288.

Schlingensiepen, K. H., Wollnik, F., Kunst, M., Schlingensiepen, R., Herdegen, T., and Brysch, W. (1994). The role of Jun transcription factor expression and phosphorylation in neuronal differentiation, neuronal cell death and plastic adaptations *in vivo*. *Cell. Mol. Neurobiol.* **14,** 487–505.

Schwartz, W. J., Davidsen, L. C., and Smith, C. B. (1980). *In vivo* metabolic activity of a putative circadian oscillator, the rat suprachiasmatic nucleus. *J. Comp. Neurol.* **189,** 157–167.

Schwartz, W. J., Reppert, S. M., Eagan, S. M., and Moore-Ede, M. C. (1983). *In vivo* metabolic activity of the suprachiasmatic nuclei: A comparative study. *Brain Res.* **274,** 184–187.

Shearman, L. P., Jin, X., Lee, C., Reppert, S. M., and Weaver, D. R. (2000). Targeted disruption of the *mPer3* gene: Subtle effects on the circadian clock function. *Mol. Cell. Biol.* **20,** 6269–6275.

Shearman, L. P., Zylka, M. J., Weaver, D. R., Kolakowski, J., L. F., and Reppert, S. M. (1997). Two *period* homologs: Circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* **19,** 1261–1269.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J. J., Dunlap, J. C., and Okamura, H. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. *Cell* **91,** 1043–1053.

Shinohara, K., Honma, S., Katsuno, Y., Abe, H., and Honma, K. (1995). Two distinct oscillators in the rat suprachiasmatic nucleus *in vitro*. *Proc. Natl. Acad. Sci. USA* **92,** 7396–7400.

Silver, R., Romero, M. T., Besmer, H. R., Leak, R., Nunez, J. M., and LeSauter, J. (1996). Calbindin-D28K cells in the hamster SCN express light-induced Fos. *Neuroreport* **7,** 1224–1228.

Szymusiak, R., Alam, N., Steininger, T. L., and McGinty, D. (1998). Sleep-waking discharge patterns of ventrolateral preoptic/anterior hypothalamic neurons in rats. *Brain Res.* **803,** 178–188.

Takumi, T., Matsubara, C., Shigeyoshi, Y., Taguchi, K., Yagita, K., Maebayashi, Y., Sakakida, Y., Okumura, K., Takashima, N., and Okamura, H. (1998). A new mammalian period gene predominantly expressed in the suprachiasmatic nucleus. *Genes Cells* **3,** 167–176.

Thresher, R. J., Vitaterna, M. H., Miyamoto, Y., Kazantsev, A., Hsu, D. S., Petit, C., Selby, C. P., Dawut, L., Smithies, O., Takahashi, J. S., and Sancar, A. (1998). Role of mouse cryptochrome blue-light photoreceptor in circadian photoresponses. *Science* **282,** 1490–1494.

Tischkau, S. A., Mitchell, J. W., Tyan, S.-H., Buchanan, G. F., and Gillette, M. U. (2003a). $Ca^{2+}$/cAMP response element-binding protein (CREB)-dependent activation of *Per1* is required for light-induced signaling in the suprachiasmatic nucleus circadian clock. *J. Biol. Chem.* **278,** 718–723.

Tischkau, S. A., Weber, E. T., Abbott, S. M., Mitchell, J. W., and Gillette, M. U. (2003b). Circadian clock-controlled regulation of cGMP/protein kinase G in the nocturnal domain. *J. Neurosci.* **23,** 7543–7550.

Travnickova-Bendova, Z., Cermakian, N., Reppert, S. M., and Sassone-Corsi, P. (2002). Bimodal regulation of *mPeriod* promoters by CREB-dependent signaling and CLOCK/BMAL activity. *Proc. Natl. Acad. Sci. USA* **99,** 7728–7733.

van der Beek, E. M., Swarts, H. J. M., and Weigant, V. M. (1999). Central administration of antiserum to vasoactive intestinal peptide delays and reduces luteinzing hormone and prolactin surges in ovariectomized, estrogen-treated rats. *Neuroendocrinology* **69,** 227–237.

van der Horst, G. T., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S., Takao, M., de Wit, J., Verkerk, A., Eker, A. P., van Leenan, D., Buijs, R., Bootsma, D., Hoeijmakers, J. H., and Yasui, A. (1999). Mammalian *Cry1* and *Cry2* are essential for maintenance of circadian rhythms. *Nature* **398,** 627–630.

van Esseveldt, K. E., Lehman, M. N., and Boer, G. J. (2000). The suprachiasmatic nucleus and the circadian time-keeping system revisited. *Brain Res. Brain Res. Rev.* **33,** 34–77.

Vitaterna, M. H., King, D. P., Chang, A.-M., Kornhauser, J. M., Lowrey, P. L., McDonald, J. D., Dove, W. F., Pinto, L. H., Turek, F. W., and Takahashi, J. S. (1994). Mutagenesis and mapping of a mouse gene, *Clock*, essential for circadian behavior. *Science* **264,** 719–725.

Weber, E. T., Gannon, R. L., Michel, A. M., Gillette, M. U., and Rea, M. A. (1995a). Nitric oxide synthase inhibitor blocks light-induced phase shifts of the circadian activity rhythm, but not *c-fos* expression in the suprachiasmatic nucleus of the Syrian hamster. *Brain Res.* **692,** 137–142.

Weber, E. T., Gannon, R. L., and Rea, M. A. (1995b). cGMP-dependent protein kinase inhibitor blocks light-induced phase advances of circadian rhythms *in vivo*. *Neurosci. Lett.* **197,** 227–230.

Xiao, J., Li, C., Zhu, N.-L., Borok, Z., and Minoo, P. (2003). *Timeless* in lung morphogenesis. *Dev. Dyn.* **228,** 82–94.

Yakubov, L., Khaled, Z., Zhang, L., Truneh, A., Vlassov, V., and Stein, C. A. (1993). Oligonucleotides interact with recombinant CD4 at multiple sites. *J. Biol. Chem.* **268,** 18818–18823.

Yamaguchi, S., Mitsui, S., Miyake, S., Yan, L., Onishi, H., Yagita, K., Suzuki, M., Shibata, S., Kobayashi, K., and Okamura, H. (2000). The 5′ upstream region of *mPer1* gene contains two promoters and is responsible for circadian oscillation. *Curr. Biol.* **10,** 873–876.

Yamazaki, S., Kerbeshian, M. C., Hocker, C. G., Block, G. D., and Menaker, M. (1998). Rhythmic properties of the hamster suprachiasmatic nucleus *in vivo*. *J. Neurosci.* **18,** 10709–10723.

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R.-I., Ueda, M., Block, G. D., Sakaki, Y., Menaker, M., and Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science* **288,** 682–685.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z. S., Eichele, G., Bradley, A., and Lee, C. (2001). Nonredundant roles of the *mPer1* and *mPer2* genes in the mammalian circadian clock. *Cell* **105,** 683–694.

Zheng, B., Larkin, D. W., Albrecht, U., Sun, Z. S., Sage, M., Eichele, G., Lee, C., and Bradley, A. (1999). The *mPer2* gene encodes a functional component of the mammalian circadian clock. *Nature* **400,** 169–173.

Zon, G. (1988). Oligonucleotide analogs as potential chemotherapeutic agents. *Pharm. Res.* **5,** 539–549.

# [32] Assaying the *Drosophila* Negative Feedback Loop with RNA Interference in S2 Cells

*By* PIPAT NAWATHEAN, JEROME S. MENET, and MICHAEL ROSBASH

## Abstract

Transcriptional negative feedback loops play a critical role in the molecular oscillations of circadian genes and contribute to robust behavioral rhythms. In one key *Drosophila* loop, CLOCK and CYCLE (CLK/CYC) positively regulate transcription of *period* (*per*). The *period* protein (PER) then represses this transcriptional activation, giving rise to the molecular oscillations of *per* RNA and protein. There is evidence that links molecular oscillations with behavioral rhythms, suggesting that PER also regulates the expression of downstream genes, ultimately resulting in proper behavior rhythmicity. Phosphorylation of PER has also been shown to be critical for rhythms. DOUBLETIME (DBT) and casein kinase II (CKII) have been implicated in the phosphorylation of PER, which affects its stability as well as nuclear localization. We investigated the role of these kinases on PER transcriptional repression using the *Drosophila* S2 cell line in combination with RNA interference (RNAi) to knock down specific gene expression. This article describes the methods used to study PER repression activity in the S2 cell system as well as to exploit RNAi in this system. We also include protocols for immunocytochemistry and the application of leptomycin to differentiate direct effects on repression from indirect effects on subcellular localization. Finally, we discuss the generation of stable cell lines in the S2 cell system; these will be useful for experiments requiring homogeneous cell populations.

## Introduction

Many eukaryotic and some prokaryotic organisms regulate their metabolism, physiology, and behavior with a circadian (~24-h) period. These



 0076-6879/05 $35.00