

mCRY1 and mCRY2 Are Essential Components of the Negative Limb of the Circadian Clock Feedback Loop

Kazuhiko Kume,*||# Mark J. Zylka,*†||
Sathyanarayanan Sriram,*|| Lauren P. Shearman,*||
David R. Weaver,* Xiaowei Jin,*
Elizabeth S. Maywood,‡ Michael H. Hastings,‡
and Steven M. Reppert*†§

*Laboratory of Developmental Chronobiology
Pediatric Service
Massachusetts General Hospital and
Harvard Medical School
Boston, Massachusetts 02114

†Program in Neuroscience
Harvard Medical School
Boston, Massachusetts 02115

‡Department of Anatomy
University of Cambridge
Downing Street
Cambridge CB2 3DY
United Kingdom

Summary

We determined that two mouse cryptochrome genes, *mCry1* and *mCry2*, act in the negative limb of the clock feedback loop. In cell lines, mPER proteins (alone or in combination) have modest effects on their cellular location and ability to inhibit CLOCK:BMAL1-mediated transcription. This suggested cryptochrome involvement in the negative limb of the feedback loop. Indeed, *mCry1* and *mCry2* RNA levels are reduced in the central and peripheral clocks of *Clock/Clock* mutant mice. mCRY1 and mCRY2 are nuclear proteins that interact with each of the mPER proteins, translocate each mPER protein from cytoplasm to nucleus, and are rhythmically expressed in the suprachiasmatic circadian clock. Luciferase reporter gene assays show that mCRY1 or mCRY2 alone abrogates CLOCK:BMAL1-E box-mediated transcription. The mPER and mCRY proteins appear to inhibit the transcriptional complex differentially.

Introduction

Circadian rhythms in mammals are regulated by a master clock located in the suprachiasmatic nucleus (SCN) of the brain (Klein et al., 1991; Reppert and Weaver, 1997). Environmental light–dark cycles entrain the SCN clock to the 24 hr day via direct and indirect retinal projections. The timekeeping capability of the SCN is expressed at the level of single neurons (Welsh et al., 1995). Synchronization among SCN clock neurons leads

to coordinated circadian outputs from the nucleus, ultimately regulating rhythms in physiology and behavior (Welsh et al., 1995; Liu et al., 1997; Herzog et al., 1998). Circadian clocks also appear to exist in several peripheral tissues of mammals that are synchronized by the SCN (Balsalobre et al., 1998; Sakamoto et al., 1998; Zylka et al., 1998a).

A number of mammalian genes have been cloned recently that resemble the well-studied circadian clock genes from the fruit fly *Drosophila melanogaster* (reviewed in Dunlap, 1999). Based on these cross-species parallels, the mammalian genes were postulated to be components of an intracellular transcriptional/translational feedback loop (reviewed in Reppert, 1998; Dunlap, 1999).

Clock was the first clock gene identified in mammals. A mutation of *Clock* causes abnormally long circadian periods in behavior, and homozygous mutant mice become arrhythmic in constant darkness (Vitaterna et al., 1994). The *Clock* gene encodes a basic helix-loop-helix (bHLH)-PAS transcription factor (King et al., 1997) and dimerizes with another bHLH-PAS transcription factor named BMAL1 (also known as MOP3, JAP3, or ARNT3) to effect transcriptional activation (Gekakis et al., 1998; Hogenesch et al., 1998; Takahata et al., 1998).

The importance of CLOCK and BMAL1 homologs in a central feedback loop mechanism has been most convincingly demonstrated in *Drosophila*. Genetic and biochemical analyses in the fly show that the transcriptional activation of the rhythmically regulated clock genes *period* (*per*) and *timeless* (*tim*) is controlled by dCLOCK: dBMAL1 heterodimers that bind to CACGTG E box enhancers in the promoters of *per* and *tim* (Hao et al., 1997; Allada et al., 1998; Darlington et al., 1998; Rutilla et al., 1998). Rhythms in intracellular PER and TIM protein levels begin with their accumulation in the cytoplasm, phosphorylation, and heterodimerization (reviewed in Dunlap, 1999). PER–TIM heterodimers then translocate to the nucleus, where they negatively regulate their own transcription (Saez and Young, 1996; Darlington et al., 1998). Negative transcriptional regulation appears to involve interference with dCLOCK:dBMAL1-mediated transcription by direct interaction of PER and TIM with dCLOCK (Lee et al., 1998). The temporal phosphorylation of PER provides at least part of the time delay between transcription and PER–TIM negative feedback necessary to sustain a 24 hr molecular oscillation (Price et al., 1998).

Per1 was the first mammalian gene cloned with sequence similarity to *Drosophila per* (Sun et al., 1997; Tei et al., 1997). Database searches quickly revealed that *Per1* is a member of a family of three distinct *Per* genes (mouse genes designated *mPer1*, *mPer2*, and *mPer3*) (Albrecht et al., 1997; Shearman et al., 1997; Takumi et al., 1998a, 1998b; Zylka et al., 1998a). The mPER proteins contain PAS domains (but no bHLH domains), and the RNA abundance and protein levels of each *mPer* gene display circadian rhythms in the SCN (Zylka et al., 1998a; Hastings et al., 1999; unpublished data).

§ To whom correspondence should be addressed (e-mail: reppert@helix.mgh.harvard.edu).

|| These authors contributed equally to this work.

On sabbatical leave from the Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo, Tokyo, 113-0033 Japan.

Similar to the situation in the fly, Gekakis and coworkers (1998) identified CACGTG E box sequences within the promoter region of the *mPer1* gene. Luciferase reporter gene assays show that coexpression of CLOCK and BMAL1 in cell culture can positively regulate transcription through the *mPer1* E boxes (Gekakis et al., 1998). Consistent with the luciferase reporter gene studies in cell lines, the three *mPer* RNA rhythms within the SCN are all reduced in homozygous *Clock/Clock* mutant mice, indicating CLOCK and its partner(s) regulate *mPer* gene expression in vivo (Gekakis et al., 1998; Jin et al., 1999). Thus, the positive limb of a core feedback loop for the mammalian clock appears to consist of CLOCK:BMAL1-driven transcription of several genes, including the *mPers*.

Database searches have also identified a mammalian homolog of *Drosophila tim*. The mouse *Timeless* gene (*mTIM*) is expressed in the SCN, but neither its RNA nor protein levels oscillate in the nuclei under constant conditions (Koike et al., 1998; Sangoram et al., 1998; Zylka et al., 1998b; Hastings et al., 1999; Takumi et al., 1999). Mammalian TIM does not interact with the mPERs in yeast (Zylka et al., 1998b), yet it has been reported to interact with mPER1 in COS7 cells (Takumi et al., 1999) and with *Drosophila* PER in vitro (Sangoram et al., 1998). The three mPER proteins and mTIM appear to participate in the negative limb of the mammalian clock feedback loop, since each is able to inhibit CLOCK:BMAL1-induced transcription in NIH3T3 cells (Sangoram et al., 1998; Jin et al., 1999). Interestingly, transcriptional inhibition in the cell line is only partial and does not appear to require coexpression of the mPER and mTIM proteins. Thus, the mammalian circadian feedback loop appears to differ mechanistically from the *Drosophila* clock loop. In mammals, factors other than the mPER and mTIM proteins appear to be important for the negative limb of the feedback loop.

A striking finding concerning mammalian clock genes is the recent discovery that two mouse cryptochrome genes, *mCry1* and *mCry2*, are essential components of a central clock mechanism (van der Horst et al., 1999). Cryptochromes are pterin/flavin-containing proteins, first identified in plants (reviewed in Cashmore et al., 1999). These molecules are structural homologs of the DNA repair enzyme DNA photolyase, but they lack DNA repair activity. The two cryptochrome proteins in plants are involved in blue light-dependent entrainment of circadian functions (Somers et al., 1998; Cashmore et al., 1999). A CRY homolog has been cloned in *Drosophila*, and molecular and genetic evidence strongly suggests that the fly homolog is an important component of the photoreceptive pathway for clock entrainment (Emery et al., 1998; Stanewsky et al., 1998). In addition, the fly homolog appears to be necessary for normal circadian function, independent of its effects on light entrainment. Thus, *Drosophila* CRY may also have a critical role in a core clockwork by mechanisms that remain to be defined.

The mouse cryptochrome genes are both expressed in the SCN, but only *mCry1* RNA levels exhibit a circadian oscillation there (Miyamoto and Sancar, 1998). *mCry1* and *mCry2* are also expressed in ganglion cells and the inner nuclear layer of the retina, suggesting that they may function in circadian photoreception (Miyamoto

and Sancar, 1998). Importantly, targeted deletion of *mCry1* shortens circadian period (van der Horst et al., 1999), whereas the deletion of *mCry2* lengthens circadian period and modestly alters photic entrainment of circadian rhythms (Thresher et al., 1998; van der Horst et al., 1999). Amazingly, mice lacking both mCRY1 and mCRY2 exhibit a complete loss of circadian rhythmicity in wheel-running behavior (van der Horst et al., 1999). The two *mCry* genes thus appear to be necessary for clock function. The ways in which the mCRY molecules participate in a central clock mechanism have been a mystery, however.

The studies in this paper provide insights into a core clock mechanism in mammals, focusing on the functions of mCRY1 and mCRY2 in the CLOCK:BMAL1-driven feedback loop. In contrast to the mPER proteins, which have modest effects on each other's intracellular distribution and on the inhibition of CLOCK:BMAL1-induced transcription in cell lines, mCRY1 and mCRY2 have profound effects on these activities. The data thus indicate that mCRY1 and mCRY2 effectively "close" the mammalian clock feedback loop. Each mCRY protein is a redundant, but essential, component of the negative limb of the central clock loop, explaining the strong loss-of-function phenotype of *mCry1^{-/-}mCry2^{-/-}* knockout animals.

Results and Discussion

mPER Proteins Interact in Mammalian Cells

We set out by examining the importance of mPER:mPER interactions in the negative limb of the clock feedback loop. This became an initial focal point because previous studies using the yeast two-hybrid assay showed that all of the mPERs interact with one another and that mPER1 and mPER2 can homodimerize (Zylka et al., 1998b). No interactions were detectable between mTIM and any of the mPER proteins in yeast. We thus expected that mPER:mPER interactions would serve a function comparable to PER:TIM interactions in *Drosophila*. We first extended the results in yeast to mammalian cells by performing coimmunoprecipitation experiments using epitope-tagged proteins expressed in COS7 cells.

Expression plasmids were constructed that encode full-length coding regions for each mPER protein and mTIM with either a hemagglutinin (HA) or a V5 epitope tag at the carboxyl terminus. COS7 cells were transiently cotransfected with expression plasmids encoding mPER3-HA and either mPER1-V5, mPER2-V5, mPER3-V5, or mTIM-V5. Cell lysates were immunoprecipitated with anti-HA antibody, and the immunoprecipitated material was blotted and probed with anti-V5 antibodies to assess interactions.

Western blotting of cell lysates prior to immunoprecipitation showed that all four proteins tagged with the V5 epitope were expressed at detectable levels (Figure 1A, top). The coimmunoprecipitation data showed that mPER3 homodimerized and heterodimerized with mPER1 and mPER2 but did not interact at detectable levels with mTIM (Figure 1A, middle). When the blot was stripped and reprobed with the anti-HA antibody, similar amounts of mPER3-HA were precipitated in each sample (Figure

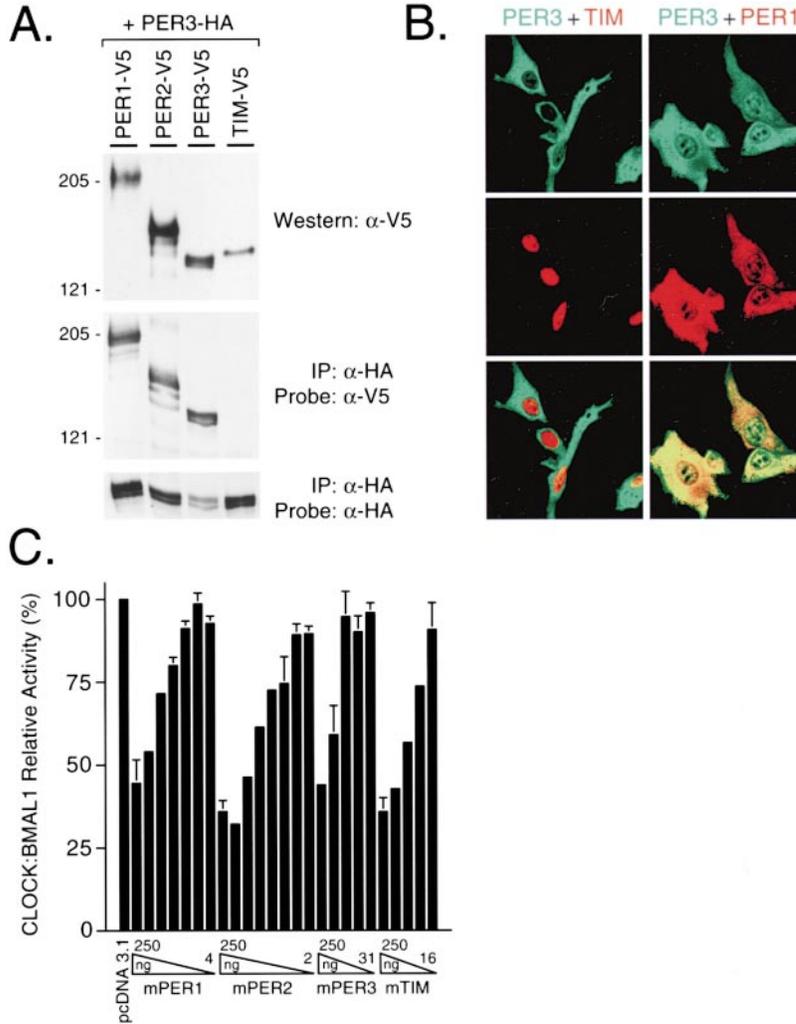


Figure 1. mPER:mPER Interactions Have Modest Effects on Their Cellular Location and Ability to Inhibit CLOCK:BMAL1-Induced Transcription

(A) Coimmunoprecipitation experiment showing mPER:mPER interactions in COS7 cells. (Upper panel) Equivalent amounts of the lysates from cells cotransfected with mPER3-HA, and the indicated V5-tagged clones were Western blotted prior to immunoprecipitation and probed with anti-V5 antibodies to confirm that the indicated proteins were present in the lysates. (Middle panel) Lysates were immunoprecipitated with anti-HA antibodies, and precipitated proteins were Western blotted and detected with anti-V5 antibodies. (Lower panel) The blot was stripped and re-probed with anti-HA antibodies to confirm that similar amounts of mPER3 were present in each sample. Locations and molecular masses (in kDa) of the protein standards are listed to the left. Similar results were found in a replicate experiment.

(B) Subcellular location of mPER and mTIM proteins expressed in NIH3T3 cells and examined by confocal microscopy. (Left column) The localization of mPER3 and mTIM was unaffected in cotransfected cells, with mPER3 (green; upper panel) remaining in the cytoplasm and mTIM (red, center panel) localized in the nucleus. The lower panel shows the merged image. (Right column) When mPER3 (green, top panel) and mPER1 (red, center panel) were cotransfected, the location of mPER3 was altered. Both proteins were expressed in both the cytoplasm and nucleus upon cotransfection, evident in the merged image in the lower panel.

(C) Dose-response studies of inhibition of CLOCK:BMAL1-induced transcription by the mPER proteins and mTIM. Data from 16 transcription assays were combined by normalizing the relative luciferase activity values in

each experiment to the activity from CLOCK:BMAL1 alone (set at 100%). The amounts of the mPER or mTIM expression constructs transfected are listed (in nanograms) at the extremes of the triangles. Individual experiments were done in duplicate or triplicate. Values are plotted as the mean \pm SEM when three or more experiments were performed with a given amount of expression construct. All other values represent averages from two experiments.

1A, bottom). Thus, the lack of detection of an mPER3:mTIM interaction was not due to a transfection or expression artifact. A similar pattern of interactions was obtained when the coimmunoprecipitation experiments were performed using mPER1-HA in place of mPER3-HA (i.e., coimmunoprecipitation of the mPER proteins but not mTIM) (data not shown).

These results in mammalian cells confirm the findings in yeast: each mPER can homodimerize with itself or heterodimerize with another mPER but does not detectably interact with mTIM. Our results do not rule out the possibility of biologically relevant mPER:mTIM interactions in the mammalian clockwork. But the data do suggest that such mPER:mTIM interactions must be much weaker than the strong mPER:mPER interactions found in both yeast and mammalian cells.

Subcellular Location of mPER3 Changes in the Presence of mPER1 or mPER2

In *Drosophila*, the heterodimerization of PER and TIM is necessary for their transport to the nucleus and subsequent inhibition of transcription (Saez and Young, 1996;

Darlington et al., 1998). mPER1 expression is primarily nuclear in the SCN when the circadian oscillation in *mPer1* RNA levels is at its nadir (Hastings et al., 1999). Thus, mPER:mPER interactions may be important for the nuclear translocation of the mPERs and their subsequent negative feedback on transcription. We therefore examined the functional relevance of mPER:mPER interactions by first evaluating the subcellular location of the HA epitope- and V5 epitope-tagged constructs when transfected into NIH3T3 and COS7 cells. Immunofluorescence of epitope-tagged proteins was used to observe protein location within cells. The cellular location was scored as one of three categories: both cytoplasm and nucleus, cytoplasm only, or nucleus only.

When expressed singly in NIH3T3 cells, mPER1 and mPER2 were each found predominantly in both cytoplasm and nucleus (78% and 61% of transfected cells, respectively; $n = 3$ experiments), but they were also detected in the nucleus only (15% and 29%, respectively). In contrast, mPER3 was found in mostly cytoplasm only (95% of transfected cells), and mTIM was mostly found in the nucleus only (89%).

To determine whether coexpression promotes nuclear entry of the proteins, all possible pairwise combinations of the mPER and mTIM plasmids were cotransfected. mTIM coexpressed with any of the mPER proteins did not affect subcellular location of mTIM or the mPER proteins ($p > 0.05$). The most obvious example of this was observed when mPER3 and mTIM were coexpressed: mPER3 remained cytoplasmic, and mTIM remained nuclear (Figure 1B). The inability of mTIM to influence subcellular location of the mPER proteins provides further evidence that mTIM does not interact functionally with the mPER proteins in a manner analogous to the interactions of PER and TIM in *Drosophila*.

When mPER3 was coexpressed with either mPER1 or mPER2, mPER3 was dramatically redistributed from cytoplasm only to both cytoplasm and nucleus (Figure 1B; $p < 0.01$, $n = 3$ experiments). mPER1 was more effective than mPER2 in promoting nuclear entry of mPER3; that is, nucleus-only location was found in 3× more cells with mPER1 cotransfections, compared with mPER2. The same redistribution profile was observed when the amounts of the mPER1 and mPER3 plasmids transfected were decreased by 75% (from 500 ng to 125 ng; data not shown). All of the subcellular localization experiments described above in NIH3T3 cells were also performed in COS7 cells with similar results (data not shown).

Despite trying all possible combinations of mPER proteins with mTIM, including adding all four proteins at once (data not shown), we were unable to induce a nucleus-only location of mPER1 or mPER2 in >30% of NIH3T3 cells. This differs dramatically from the *in vivo* situation in which both mPER1 and mPER2 are entirely nuclear in SCN cells when detectable (Hastings et al., 1999; unpublished data). Thus, it would appear that we have not completely reconstituted mPER function in NIH3T3 cells. This suggested that there are other clock-relevant factors important for the nuclear translocation of the mPER proteins.

mPER:mPER Interactions Do Not Augment Inhibition of CLOCK:BMAL1-Induced Transcription

We next determined whether mPER1/2:mPER3 interactions, which promote the nuclear entry of mPER3, augment the inhibition of CLOCK:BMAL1-induced transcription. For these studies, a luciferase reporter gene assay in NIH3T3 cells was used. The reporter construct utilizes a 200 bp fragment of the promoter region of the mouse arginine vasopressin (preproressophysin) gene containing a CACGTG E box, as previously described (Jin et al., 1999). This reporter gene construct is activated by CLOCK and BMAL1 acting together on the E box enhancer (Jin et al., 1999).

CLOCK:BMAL1-induced transcription was maximally inhibited by transfection of 250 ng of each of the *mPer* and *mTim* constructs (Figure 1C). Maximal inhibition reached 55%–70% for each construct and was not substantially augmented by any pairwise transfection of the *mPer* and *mTim* constructs (at 250 ng each; data not shown). As the amounts of each expression plasmid transfected were decreased, there was decreasing inhibition of CLOCK:BMAL1 transcription (Figure 1C). From the dose–response curves, we were able to identify

amounts of each expression construct that were at the threshold of causing transcriptional inhibition.

Using threshold amounts of each expression construct, all possible pairwise mPER–mPER and mPER–mTIM combinations were next examined to look for synergistic or additive interactions. In no instance, however, were we able to find a consistent augmentation of transcriptional inhibition with low-dose, pairwise combinations of mPER expression constructs or mPER plus mTIM expression constructs ($n = 4$ experiments). Coexpression experiments with low doses of mPER1 and mPER3 did show a consistent trend toward inhibition of CLOCK:BMAL1-induced transcription, but the effects were only significant ($p < 0.05$) in one of three experiments.

The data hint that mPER1:mPER3 heterodimers may be functionally relevant for transcriptional inhibition. The endogenous expression of the *mPer1*, *mPer2*, *mPer3*, and *mTim* genes in NIH3T3 cells may obscure finding a more robust inhibitory effect on transcription. On the basis of the modest effects of mPER:mPER interactions on nuclear localization and transcriptional inhibition, however, it seemed more likely that there are other factors necessary for nuclear translocation and/or retention of the mPER proteins and for their subsequent inhibition of CLOCK:BMAL1-induced transcription.

mCry1 and *mCry2* RNA Levels in the SCN and in Peripheral Clocks Are Regulated by CLOCK

Since mice lacking both mCRY1 and mCRY2 show a complete loss of circadian rhythmicity (van der Horst et al., 1999), these proteins became prime candidates for the “missing factors” involved in the negative limb of the mammalian clock feedback loop. We thus determined whether the cryptochromes are involved in the CLOCK:BMAL1-driven *mPer* feedback loop. We first examined *mCry1* and *mCry2* gene expression in wild-type and homozygous *Clock/Clock* mutant mice, because a decrease in gene expression in *Clock/Clock* mice would place the cryptochrome genes within the CLOCK-driven feedback loop.

mCry1 RNA levels exhibited a prominent circadian rhythm in the SCN of wild-type animals (ANOVA, $p < 0.05$; Figure 2A), similar to that described by others (Miyamoto and Sancar, 1998). The phase of the *mCry1* RNA rhythm was most similar to the phase of the *mPer2* RNA oscillation in the SCN (Shearman et al., 1997; Jin et al., 1999). In sharp contrast to wild-type mice, no *mCry1* RNA rhythm was apparent in the SCN of *Clock/Clock* mice (ANOVA, $p > 0.05$; Figure 2A). Thus, the *mCry1* RNA rhythm is dependent on a functional CLOCK protein. These results are similar to the finding that the amplitude of RNA rhythms for each of the three *mPer* genes is markedly reduced in *Clock/Clock* mice (Jin et al., 1999).

mCry2 RNA levels in the SCN of wild-type animals did not show a circadian rhythm (Figure 2A; $p > 0.05$), consistent with a previous study (Miyamoto and Sancar, 1998). Interestingly, mean steady-state *mCry2* RNA levels were nonetheless significantly lower in *Clock/Clock* mice, compared to those in wild-type controls (ANOVA, $p < 0.005$). This finding suggests that *mCry2* transcription is also at least partially dependent on a functional

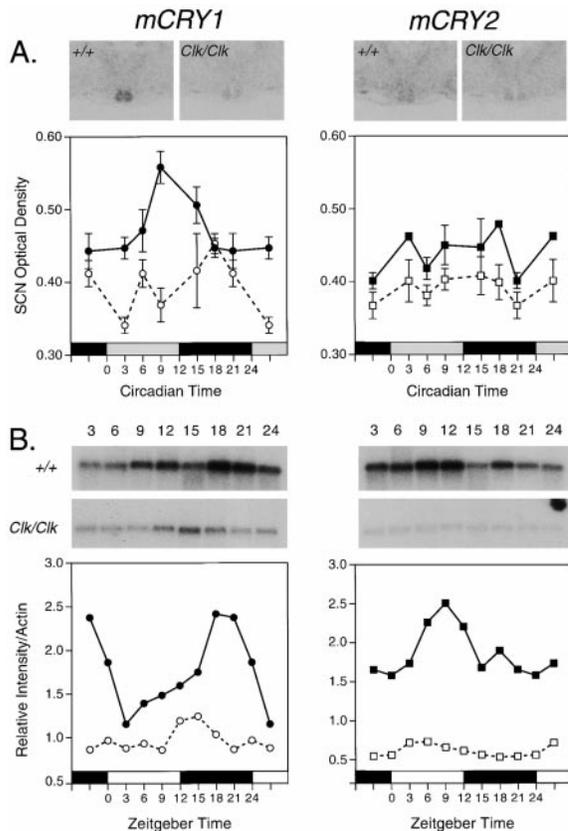


Figure 2. *mCry1* and *mCry2* RNA Levels Are Reduced in the SCN and Skeletal Muscle of *Clock/Clock* Mice

(A) *mCry* RNA levels in SCN. Panels depict the temporal profiles of *mCry1* RNA levels (left) and *mCry2* RNA levels (right) in the SCN of wild-type mice (solid lines) and *Clock/Clock* mice (dashed lines). Each value is the mean \pm SEM of four animals. The horizontal bar at the bottom of the panels represents lighting cycle prior to placement in DD; stippled areas represent subjective day, and the filled areas represent subjective night. The photomicrographs (upper) show representative autoradiographs of *mCry1* and *mCry2* gene expression from coronal brain sections (15 μ m) at the level of the SCN from wild-type (+/+) and *Clock/Clock* (*Clk/Clk*) mice at CT9. The brain sections were examined by in situ hybridization using cRNA probes.

(B) *mCry* RNA levels in skeletal muscle. Autoradiograms (upper panels) illustrate Northern blots of *mCry1* (3.0 kb transcript, left) and *mCry2* (4.4 kb transcript, right) RNA levels at each of eight time points in 12L:12D, with lights on from Zeitgeber times (ZT) 0–12. The lower panels depict quantitative assessment of *mCry1* and *mCry2* RNA levels in skeletal muscle of wild-type (solid lines) and *Clock/Clock* (*Clk/Clk*) mice (dashed lines). The values are the average relative intensity of two replicate blots with each probe. Data were normalized and expressed relative to hybridization intensity of actin control probe (see Experimental Procedures). Data at ZT21, ZT0/24, and ZT3 are double plotted.

CLOCK protein. It is worth noting that of five genes studied whose RNA levels do not manifest a circadian rhythm in the SCN, *mCry2* is the only one in which we have found decreased RNA levels in *Clock/Clock* animals (see Jin et al., 1999).

Since circadian clocks also appear to exist in peripheral tissues (Balsalobre et al., 1998; Sakamoto et al., 1998; Zylka et al., 1998a), we further examined the temporal profiles of *mCry1* and *mCry2* RNA level in skeletal

muscle. This tissue was chosen because the three *mPer* genes manifest robust RNA rhythms there (Zylka et al., 1998a). In contrast to the situation in the SCN, both *mCry1* and *mCry2* RNA levels in muscle exhibited a daily rhythm under 12 hr light:12 hr dark (LD) (Figure 2B) and a circadian rhythm under constant darkness (DD) (data not shown). The peak of the *mCry2* rhythm preceded that of *mCry1* by 6 to 9 hr, and the *mCry1* RNA rhythm was delayed by several hours relative to the phase of its RNA rhythm in the SCN. A phase delay between the SCN and peripheral oscillations is also observed in the RNA rhythms of the three *mPer* genes (Zylka et al., 1998a).

In skeletal muscle of *Clock/Clock* animals, the *mCry1* RNA rhythm was dampened and phase advanced, while the *mCry2* RNA rhythm was abolished (Figure 2B). For both genes, RNA levels were lower in *Clock/Clock* animals at all times, compared to wild-type controls.

Taken together, these data indicate that the transcriptional regulation of *mCry1* and *mCry2* is under CLOCK control in both the SCN and in peripheral clocks. These findings provide strong evidence that the mouse cryptochromes are components of the CLOCK:BMAL1-driven feedback loop. Moreover, the occurrence of a functional CACGTG E box ca 300 bp upstream of the *mCry1* transcription start site (data not shown) suggests that CLOCK directly participates in rhythmic *mCry1* transcription through an E box enhancer in its promoter. We do not yet know whether a CACGTG E box resides in the *mCry2* promoter and/or intronic regions.

mCRY1 and mCRY2 Block CLOCK:BMAL1-Induced Transcription in NIH 3T3 Cells

We directly examined mammalian cryptochrome involvement within the negative limb of the feedback loop by determining whether mCRY1 and/or mCRY2 can inhibit CLOCK:BMAL1-induced transcription. For this phase of study, luciferase reporter gene studies were performed in NIH3T3 cells using either the vasopressin promoter (Jin et al., 1999) or 1.8 kb of the 5' flanking region of the *mPer1* gene subcloned into a promoterless luciferase reporter vector.

When the vasopressin and *mPer1* promoters were used in the reporter vectors, mPER1 caused a maximal inhibition of 61% and 30%, respectively, similar to our previous findings (Figure 3). mCRY1 and mCRY2, on the other hand, inhibited CLOCK:BMAL1-induced transcription by >90% from either reporter (Figure 3). This dramatic effect on transcriptional inhibition was dose dependent for each of the two mCRY proteins (Figure 3).

These results indicate that mCRY1 and mCRY2 are each potent inhibitors of CLOCK:BMAL1-mediated transcription. The mCRY-induced transcriptional inhibition must occur through direct or indirect interaction with the CLOCK:BMAL1-E box complex because this is the only complex common to both the vasopressin and *mPer1* reporters.

Both mCRY1 and mCRY2 Are Nuclear Proteins

For the mCRY proteins to interact with the CLOCK:BMAL1-E box complex, they must be present in the nucleus. Previous studies have shown that mCRY2 is indeed a nuclear antigen (Kobayashi et al., 1998;

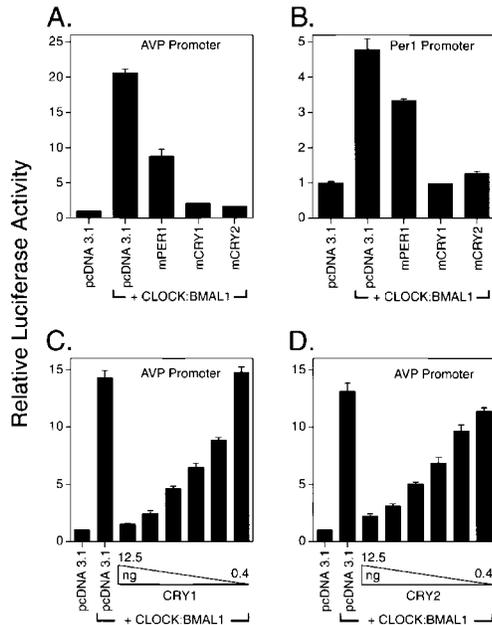


Figure 3. mCRY1 and mCRY2 Potently Inhibit CLOCK:BMAL1-Mediated Transcription

(A and B) Inhibition of CLOCK:BMAL1-mediated transcription from the vasopressin (AVP) promoter (A) or *mPer1* promoter (B) by mPER1, mCRY1, and mCRY2 (250 ng each). Each value is the mean \pm SEM of three replicates from a single assay. The results are representative of three independent experiments.

(C and D) Dose-response curves for mCRY1 (C) or mCRY2 (D) inhibition of CLOCK:BMAL1-mediated transcription from the vasopressin (AVP) promoter. Each value is the mean \pm SEM of three replicates from a single assay. Similar results were found in replicate experiments.

Thresher et al., 1998). The situation with mCRY1 is ambiguous, however, because previous studies of the endogenous protein and green fluorescent protein (GFP)-tagged mCRY1 fragments indicate localization mainly in mitochondria (Kobayashi et al., 1998). We examined this issue by tagging mCRY1 with a number of different epitopes and placing the epitopes at the ends of the protein. The constructs were transfected into NIH3T3 cells, and both their cellular localization (by immunofluorescence) and ability to inhibit CLOCK:BMAL1-induced transcription were assessed.

The results clearly showed that mCRY1 translocates to the nucleus when tagged with either the V5 or HA epitope (Figure 4). This was true when HA was placed at either the N-terminal or C-terminal ends, as well as when epitope tags were placed on both ends of the protein. In each instance, the protein was nuclear and inhibited CLOCK:BMAL1-induced transcription by >90%.

Interestingly, when enhanced (E)GFP was fused to either end of mCRY1, immunofluorescence was found diffusely throughout the cell and there was no transcriptional inhibition. The same diffuse staining and lack of transcriptional inhibition were found with EGFP alone. When EGFP was fused to an N-terminal fragment of mCRY1 containing a putative signal sequence for transport into mitochondria, the cellular location was mainly cytoplasmic and punctate and appeared to be in mitochondria. Using a specific anti-mCRY1 antibody, we

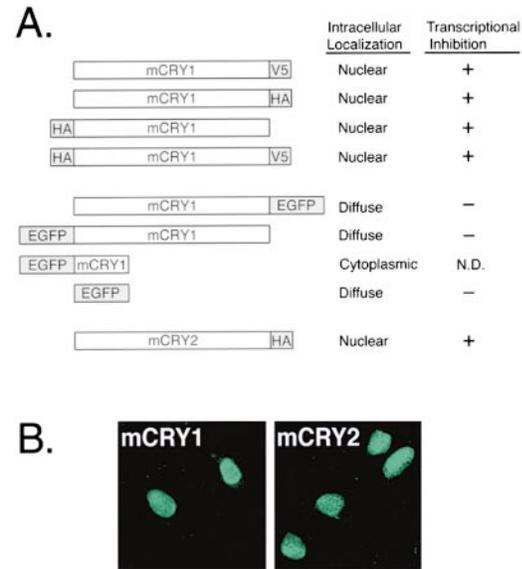


Figure 4. mCRY1 and mCRY2 Are Nuclear Proteins

(A) Epitope-tagged mCRY1 and mCRY2 proteins evaluated for cellular location and inhibition of CLOCK:BMAL1-mediated transcription. EGFP, enhanced green fluorescent protein. N.D., not determined. (B) Confocal images of mCRY1 and mCRY2 immunofluorescence (green) in the nuclei of transfected NIH3T3 cells. The constructs transfected were mCRY1-HA and mCRY2-HA. Nuclear location was confirmed by Hoechst (bisBenzimide) staining in each case. mCRY1 was nucleus-only in 92% of transfected cells; mCRY2 was nucleus-only in 93% of transfected cells.

also showed that the endogenous mCRY1 protein is nuclear in nontransfected NIH3T3 cells (data not shown) and in SCN (see below). We conclude that mCRY1 is normally a nuclear protein and that GFP fusions alter the location of the native protein by changing its conformation.

mCRY2-HA was found in the nucleus, consistent with previous findings (Kobayashi et al., 1998; Thresher et al., 1998), and the tagged protein inhibited CLOCK:BMAL1-induced transcription by >90% (Figure 4).

mCRY1 and mCRY2 Directly Interact with the mPER Proteins and Translocate Them into the Nucleus

In addition to a potential direct inhibitory effect of the mCRY proteins on the CLOCK:BMAL1-E box complex, the cryptochromes could also inhibit transcription by directly interacting with the mPER proteins and translocating them to the nucleus for subsequent transcriptional effects. To evaluate the potential for protein-protein interactions between the mCRY and mPER families, we utilized coimmunoprecipitation using epitope-tagged proteins.

COS7 cells cotransfected with expression plasmids encoding mCRY1-HA and either mPER1-V5, mPER2-V5, mPER3-V5, or mTIM-V5 expressed each V5-tagged protein prior to immunoprecipitation (Figure 5, left top). Immunoprecipitation with the HA antibody and analysis of the immunoprecipitated material with anti-V5 antibodies indicated the presence of heterodimeric interactions between mCRY1 and each of the mPER and mTIM proteins (Figure 5, left center). There was no interaction

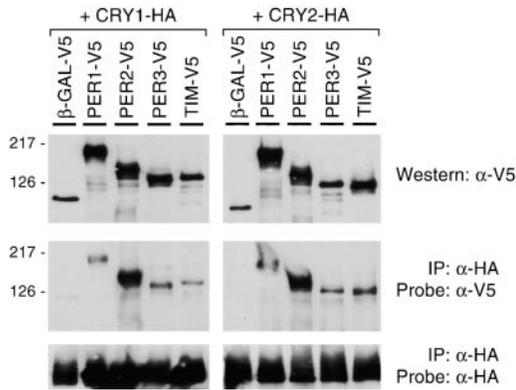


Figure 5. Coimmunoprecipitation Experiments Showing mCRY1 or mCRY2 Interacts with the mPER and mTIM Proteins
(Top panels) Equivalent amounts of lysates from cells cotransfected with mCRY1-HA or mCRY2-HA and the indicated V5-tagged clones were Western blotted prior to immunoprecipitation and probed with anti-V5 antibodies to confirm that the indicated proteins were present in the lysates.
(Middle panels) Lysates were immunoprecipitated (IP) with anti-HA antibodies, and precipitated proteins were Western blotted and detected with anti-V5 antibodies.
(Lower panels) The blots were stripped and reprobed with anti-HA antibodies to confirm that similar amounts of mCRY1 or mCRY2 were present in each sample. Locations and molecular masses (in kDa) of the protein standards are listed to the left. Similar results were found in replicate experiments.

between mCRY1 and β -galactosidase, which served as a specificity control. Coimmunoprecipitation experiments using mCRY2-HA instead of mCRY1-HA similarly showed the presence of heterodimeric interactions between mCRY2 and each of the mPER and mTIM proteins (Figure 5, right).

Having shown that mCRY:mPER heterodimers could exist, we next determined whether such interactions translocate the mPER proteins to the nucleus. In marked contrast to the lack of effect of any pairwise combination of mPER:mPER or mPER:mTIM interactions to translocate mPER1 and mPER2 to the nucleus, each mCRY protein profoundly changed the location of all three mPER proteins in NIH3T3 and COS7 cells. This was most apparent for mPER1 and mPER2, which were almost entirely nuclear after cotransfection with either mCRY1 or mCRY2 (Figure 6). Curiously, each mCRY protein changed mPER3 from mainly cytoplasm only (>80%) to both cytoplasm and nucleus (>70%) to a degree similar to that induced by cotransfection of mPER3 with mPER1 (compare Figure 6 right column with Figure 1B). When mPER3 was cotransfected with mPER1 and either mCRY1 or mCRY2, however, each of the three protein combinations changed mPER3's location from 13%–20% nucleus-only to predominantly nucleus-only (54%–68% of transfected cells). Cotransfection of either mCRY1 or mCRY2 with mTIM did not change the predominantly nucleus-only location (>90% of transfected cells) of any of the three proteins.

These data indicate that the mCRY proteins can heterodimerize with the mPER proteins and mTIM and that mCRY:mPER interactions mimic the *in vivo* situation—the almost complete translocation of mPER1 and

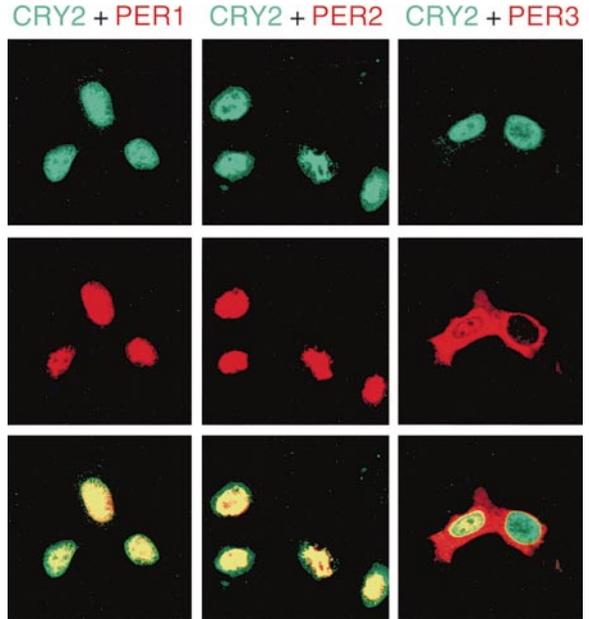


Figure 6. Confocal Images of Epitope-Tagged Proteins Showing that mCRY2 Translocates the mPER Proteins to the Nucleus in NIH3T3 Cells

When mCRY2 (green) was cotransfected with mPER1 (red; in the left column) or with mPER2 (red; in the middle column), >90% of transfected cells were nucleus-only for the cotransfected proteins (merged image is yellow; lower panels). This contrasts with the <30% nucleus-only location when mPER1 or mPER2 was singly transfected (data not shown). When mCRY2 was cotransfected with mPER3 (red; right column), mCRY2 remained nucleus-only (92% of transfected cells), and mPER3 was changed from >80% cytoplasm-only (see Figure 1B) to 70% in both cytoplasm and nucleus. mCRY2 cotransfection did not change the cellular location of β -galactosidase, which remained 100% in both cytoplasm and nucleus (data not shown). Nuclear location was confirmed by Hoechst staining. The same subcellular pattern for all tagged proteins was found for cotransfection of mCRY1 with mPER1, mPER2, or mPER3. Similar results for both mCRY1 and mCRY2 cotransfections with the mPER proteins were found in two other experiments. Results were identical between NIH3T3 cells and COS7 cells.

mPER2 to the nucleus. Moreover, trimeric interactions among the mPER and mCRY proteins appear necessary for complete nuclear translocation of mPER3. The data also suggest that the nuclear translocation of the mPER proteins is dependent on mCRY1 and mCRY2. The mCRY proteins, however, appear to be able to translocate to the nucleus independent of the mPERs. Even with massive overexpression of mCRY proteins in cell culture, they are always >90% nuclear. If a PER partner were required for CRY nuclear translocation, a high CRY:PER ratio should result in cytoplasmic trapping of CRY. This was not observed.

mCRY1 and mCRY2 Levels Express Synchronous Circadian Rhythms in the SCN

mPER1 and mPER2 immunoreactivities exhibit striking circadian oscillations in the nuclei of SCN neurons (Hastings et al., 1999; unpublished data). During the peak time of expression (circadian time [CT] 12), the nucleus of most SCN neurons stains for mPER1 and mPER2.

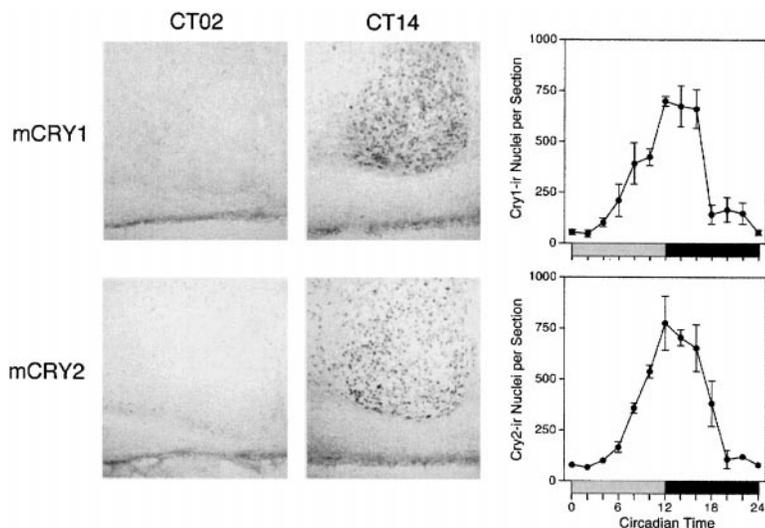


Figure 7. Circadian Rhythms of mCRY1 and mCRY2 Immunoreactivity in the SCN

The photomicrographs show immunoreactivity at CT2 (left) and CT14 (center). In these coronal sections, the unilateral SCN is shown with the third ventricle to the right in each image. Cell counts of SCN nuclei staining for the two proteins are shown in the right panels. For each antigen, values shown are mean \pm SEM of three animals at 2 hr intervals throughout the circadian cycle.

Thus, if nuclear entry of mPER1 and mPER2 is dependent on the mCRY proteins, as suggested by our cell culture experiments, then similarly synchronous circadian oscillations of endogenous mCRY1 and mCRY2 levels in the nuclei of SCN neurons might be expected.

Immunocytochemical analysis of mCRY1 and mCRY2 in the brains of mice sampled at Zeitgeber time (ZT) 15 (3 hr after lights off) identified them both as nuclear antigens in the SCN and elsewhere, including piriform cortex (mCRY2) and hippocampus (mCRY1, mCRY2, data not shown). The majority of SCN neurons appeared to be immunoreactive for the antigen tested, and the immunoreactivities were specific, being blocked by preincubation with the peptide (10 μ g/ml) used to raise the respective serum (data not shown). In contrast, the SCN from animals sampled at ZT3 contained very few mCRY1- or mCRY2-immunoreactive nuclei, and those which were evident were located in a dorsolateral position comparable to that reported for mPER1 immunoreactive nuclei at this phase (Hastings et al., 1999). Rhythmic expression of mCRY1 and mCRY2 was sustained under free-running conditions, with low levels at CT2 and high expression throughout the SCN at CT14 (Figure 7). Quantitative analysis of the number of immunoreactive nuclei in the SCN sampled at 2 hr intervals over 24 hr in DD showed a clear circadian variation (Figure 7). The abundance of both proteins was low in the early subjective day, rising in later subjective day to peak at CT12–CT16. There was a progressive decline during subjective night to basal counts at CT24. This temporal profile of mCRY1 and mCRY2 immunoreactivity in the SCN is directly comparable with that observed for mPER1 (Hastings et al. 1999) and mPER2 (unpublished data), indicative of a synchronous nuclear accumulation of these proteins in the SCN. In contrast, expression of mCRY1 and mCRY2 immunoreactivity in other areas did not exhibit appreciable circadian variation, consistent with the constitutive expression of mPER proteins in brain sites outside the SCN.

These in vivo data, in conjunction with our cell culture data, strongly suggest that the mCRY proteins are the dominant movers of the mPER1 and mPER2 proteins

from cytoplasm to nucleus. We do not yet know the temporal pattern of mPER3 immunoreactivity in the SCN, but we have no reason to believe it will be any different from that found for mPER1 and mPER2.

Dissociation between the Inhibitory Effects of the mPER Proteins and the mCRY Proteins on Transcription

By varying the amounts of mPER and mCRY plasmids in cotransfection experiments, we have observed at best additive effects of pairwise combinations of mPER with mCRY proteins on the inhibition of CLOCK:BMAL1-mediated transcription (data not shown). Although these studies in cell culture are confounded by the endogenous expression of the *mPer1*, *mPer2*, *mPer3*, *mTim*, *mCry1*, and *mCry2* genes in the cell lines used (data not shown), the lack of synergism of pairwise combinations on transcriptional inhibition suggested that the mPER and mCRY proteins have independent effects on the transcriptional machinery. To examine this in more detail, we exploited the fact that MOP4:BMAL1 heterodimers also activate transcription via a CACGTG E box (Hogenesch et al., 1998).

We first tested CLOCK, MOP4, and BMAL1 alone or in pairwise combinations for transcriptional activation (Figure 8A). Significant transcriptional activation was seen only when CLOCK and BMAL1 (10-fold increase) or MOP4 and BMAL1 (37-fold increase) were coexpressed. Transcriptional activation was dependent on the E box, because no transcriptional activation was detected when the vasopressin promoter with a mutated E box was used (data not shown). The greater levels of transcriptional activation with MOP4:BMAL1 than with CLOCK:BMAL1 appeared due to much higher levels of MOP4 protein expression compared with CLOCK based on Western blot analysis of epitope-tagged proteins (data not shown).

We next examined whether each mPER alone, mTIM, or each mCRY alone could inhibit MOP4:BMAL1-induced transcription. Even though each mPER protein can inhibit CLOCK:BMAL1-induced transcription (see Figure 1C), the mPER proteins (500 ng of each plasmid)

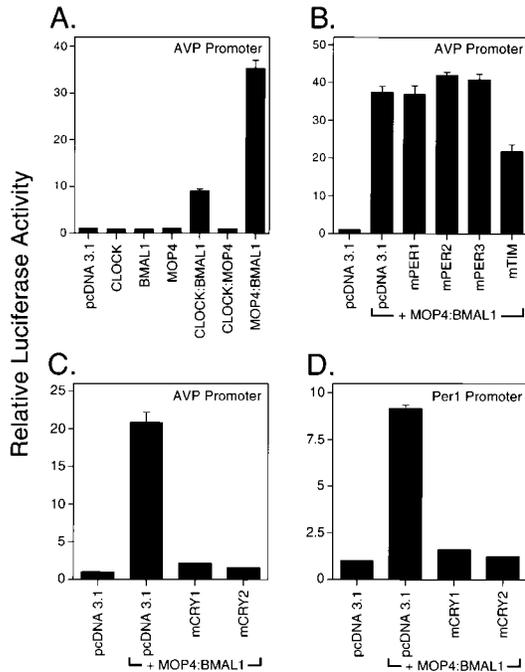


Figure 8. The mPER and mCRY Proteins Have Different Specificity in Inhibiting Transcription

(A) MOP4:BMAL1 activates transcription from the vasopressin (AVP) promoter in NIH3T3 cells. Combinations of CLOCK, BMAL1, and MOP4 expression constructs (250 ng each) were evaluated for transcriptional activation. Each value is the mean \pm SEM of three replicates for a single assay.

(B) The mPER proteins do not inhibit MOP4:BMAL1-mediated vasopressin (AVP) transcription. Five hundred nanograms of each mPER plasmid was transfected. mTIM (500 ng of plasmid transfected) inhibited MOP4:BMAL1-mediated transcription ($p < 0.01$; Student's t-test). Each value is the mean \pm SEM of three replicates for a single assay.

(C and D) The mCRY proteins (250 ng of each plasmid) potently inhibit MOP4:BMAL1-mediated transcription from the vasopressin promoter (C) and *mPer1* promoter (D). Each value is the mean \pm SEM of three replicates for a single assay. Similar results were found in a replicate assay.

did not affect MOP4:BMAL1-induced transcription (Figure 8B). When the amount of MOP4 was reduced so that the relative luciferase values were equal to those seen with CLOCK and BMAL1 activation, the mPER expression plasmids were still unable to inhibit transcription (data not shown). In contrast to the lack of inhibition of the mPER proteins, mTIM (at 500 ng) was able to inhibit MOP4:BMAL1-induced transcription by about 40% (Figure 8; $p < 0.01$). Combinations of each mPER and the mTIM expression plasmids, or pairwise combinations of mPER expression plasmids did not inhibit more effectively than when the mTIM plasmid was transfected alone (data not shown). Remarkably, each mCRY protein (250 ng each) abrogated MOP4:BMAL1-mediated transcription (Figures 8C and 8D), just like the inhibition found for CLOCK:BMAL1-induced transcription (see Figure 3).

These data suggest that the mPER proteins have their action on CLOCK, perhaps as mPER:mCRY heterodimers, while the mCRY proteins appear capable of interacting directly with either BMAL1 or the CACGTG E box.

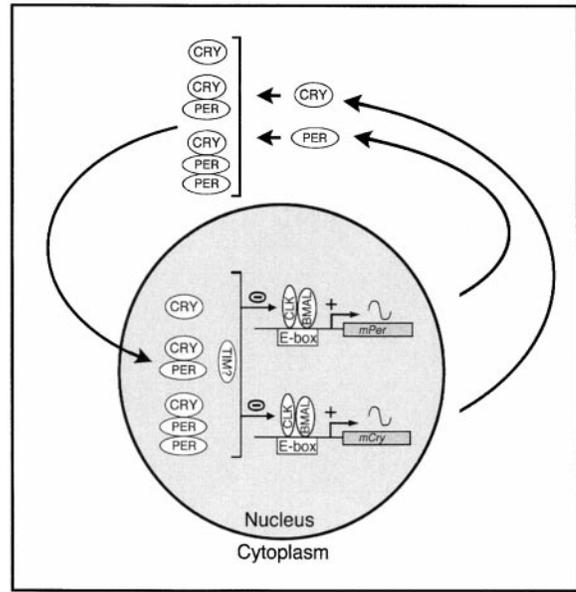


Figure 9. Model of Mammalian Clock Feedback Loop in the SCN Incorporating the Proposed Functions of the mCRY, mPER, and mTIM Proteins

mCry1 and *mPer1* are known to have CACGTG E boxes in their promoters. *mPer2* and *mPer3* RNA levels exhibit similar rhythms in the SCN in vivo, but it is not clear that E box-mediated transcription is involved.

It is worth noting that MOP4 does not appear to play a major role in circadian function, as its RNA is not detectably expressed in the SCN of either wild-type (Shearman et al., 1999) or *Clock* mutant mice (unpublished data).

Scheme of mCRY1 and mCRY2 Function in the Mammalian Clockwork

Our discovery of the functions of mCRY1 and mCRY2 within the clock feedback loop provides a sharper view of the molecular working of the mammalian clockwork. The cloning of a family of three *mPer* genes over the past 2 years has added to our understanding of the negative limb of a mammalian clock feedback loop (see Reppert, 1998; Dunlap, 1999). But close examination of these putative clock elements and *mTim* has shown that they alone cannot fully explain the negative limb of the feedback loop (Figure 1). It thus seemed likely that other factors were involved. Our data now show that mCRY1 and mCRY2 are major players in the negative limb of the clock feedback loop (Figure 9). These data also explain the strong loss-of-function phenotype of *mCry1*^{-/-} *mCry2*^{-/-} mice.

Our cell culture data show that the mCRY proteins function as dimeric and potentially trimeric partners for the mPER proteins and that these interactions lead to the nuclear translocation and/or retention of the mPER proteins. This is in marked contrast to the inability of mTIM to translocate the three mPER proteins to the nucleus in cell culture and the invariant nature of endogenous mTIM levels in the nuclei of SCN neurons; mTIM immunoreactivity is present in the nucleus of most SCN

neurons at all times throughout the circadian cycle (Hastings et al., 1999). Thus, the mCRY proteins appear to function as nuclear translocators of the mPERs. In addition, mCRY nuclear translocation does not appear to be dependent on mPER:mCRY interactions. This is different from the situation in the fly in which PER:TIM heterodimers appear essential for the translocation of both PER and TIM to the nucleus (Saez and Young, 1996).

The role of mTIM in the mammalian clockwork remains enigmatic. Even though mTIM does not appear to be important for the nuclear translocation of the mPER proteins, mTIM is localized to the nucleus *in vivo*, and it does cause a modest inhibition of CLOCK:BMAL1- and MOP4:BMAL1-mediated transcription in cell culture (Sangoram et al., 1998; Hastings et al., 1999; Jin et al., 1999). In addition, mCRY1 and mCRY2 each appear capable of forming heterodimeric complexes with mTIM. Once in the nucleus mTIM could therefore still have a role in modulating negative feedback of the mPER and/or mCRY1 rhythms.

Another feature to arise from our studies is the finding that the *mCry1* gene forms its own interacting loop within the collective mammalian clock feedback mechanism. Evidence for this contention is substantial. *mCry1* RNA and protein levels exhibit a circadian rhythm in the SCN, the RNA rhythm is dependent on a functional CLOCK protein, and the *mCry1* promoter region contains a functional CACGTG E box. In fact, it is entirely possible that the *mCry1* rhythm is the dominant oscillation in the mammalian clock feedback loop. This might explain the dominant circadian function of the *mCry1* gene over *mCry2*, whose RNA levels do not oscillate. One normal *mCry1* allele sustains normal circadian rhythms in behavior, while one *mCry2* allele leads to arrhythmicity with increasing time in DD (van der Horst et al., 1999).

We do not yet know precisely how the mPER and mCRY proteins inhibit CLOCK:BMAL1-mediated transcription, but our data suggest differential sites of action (Figure 9). In the fly, multimeric complexes involving PER, TIM, and CLOCK appear to be important (Lee et al., 1998). It is thus possible that the mPER proteins, mTIM, and the mCRY proteins are all complexed with CLOCK. In addition, mCRY1 and mCRY2 appear to be capable of inhibiting E box-mediated transcription independent of CLOCK. This suggests that the mammalian cryptochromes also interact directly with either BMAL1 or the E box itself. Indeed, mCRY1 can bind tightly to dsDNA Sepharose (Kobayashi et al., 1998).

Even though the major components of the loop have been identified, we have not begun to elucidate the way in which a 24 hr time constant is incorporated into the mammalian clock loop. Based on studies in *Drosophila*, posttranslational processes such as phosphorylation, proteosomal proteolysis, and gated nuclear entry are likely to contribute to the time delay. We do not yet know which component(s) of the loop is affected by these processes.

In summary, the data show that mCRY1 and mCRY2 are redundant, but essential components of the negative limb of the clock feedback loop. The redundant function of these proteins explains the maintenance of circadian rhythmicity when either gene is deleted and explains the strong arrhythmic phenotype of double knockout

mice discovered by van der Horst and colleagues (1999). The different direction of period change in *mCry1*^{-/-} versus *mCry2*^{-/-} mice may result from differing affinities of these proteins for the mPER proteins or other clock components, and/or different levels of protein expression. We predict that the SCN of *mCry1*^{-/-}*mCry2*^{-/-} animals will show disrupted *mPer* RNA and protein rhythms with the mPER proteins stuck in the cytoplasm and *mPer* RNA levels at constant high values because of the absence of negative feedback. Placing the mammalian cryptochromes in the negative limb of the clock feedback loop sets forth a number of new hypotheses that can now be tested.

Experimental Procedures

Cloning Studies

The coding regions of human MOP4 (U51625), mPER1 (AF022992), mPER2 (AF035830), mPER3 (AF050182), mTIM (AF071506), and mCRY1 (AB000777) were ligated into the pcDNA 3.1 V5-His expression vector (Invitrogen). In some cases, clones were also ligated into pcDNA 3.1 containing either an N-terminal or C-terminal HA tag. Full-length coding regions were amplified with *Pfu* Turbo (Stratagene) from plasmid DNA (hMOP4 and mPER1) or from cDNA. For mCRY2, the nucleotide sequence encoding the amino-terminal portion of the coding region was not available in GenBank (partial clone accession no. AB003433). The 5' end of the mCRY2 coding region was thus cloned by 5' rapid amplification of cDNA ends. The full-length coding region was then amplified as described above, sequenced, and deposited in GenBank (accession number AF156987).

Correct orientation of each construct was verified by sequence analysis. Clones were also transcribed and translated *in vitro* using TnT T7 Quick (Promega) to confirm that a protein of the correct size was produced. Moreover, clones were transiently transfected into NIH3T3 cells and into COS7 cells. Crude cell extracts were prepared, Western blotted, and probed with anti-V5 or anti-HA antibodies to detect full-length, epitope-tagged proteins (see below).

In Situ Hybridization

A breeding colony of mice carrying the *Clock* mutation was established on a BALB/c background. For studies, both male and female mice 5–15 weeks of age were used. Mice were housed in LD, except as noted. Animals were killed by decapitation. Genotypes were determined using a PCR mutagenesis method, as previously described (Jin et al., 1999). Animal studies at Massachusetts General Hospital were approved by the Subcommittee on Research Animal Care.

Antisense and sense cRNA probes were generated from each plasmid by *in vitro* transcription in the presence of ³⁵S-UTP (1200 Ci/mmol), as previously described (Weaver, 1993). The probe for *mCry1* (AB000777) was from nucleotides 1081–1793, and the probe for *mCry2* (AB003433) was from nucleotides 1060–1664. Probe quality and size were confirmed by determining ³⁵S incorporation into TCA-precipitable material and by gel electrophoresis and subsequent autoradiography of the gel.

Prehybridization, hybridization, and wash procedures have been previously described in detail (Weaver, 1993). Probe (50 μ l at 10⁷ cpm/ml) was applied to each slide. Coverslipped slides were then incubated in humidified chambers overnight at 55°C. Following completion of the wash steps, slides were air dried and apposed to Kodak BioMax MR film for 8 days.

Densitometric analysis of hybridization intensity was accomplished using NIH Image software on a Macintosh computer; data are expressed as absolute optical density values as determined by calibration with Kodak photographic step tablet 3. ¹⁴C standards (American Radiolabeled Chemicals) included in each cassette were used to verify that the optical density values measured were within the linear response range of the film.

Northern Analysis

Total RNA was extracted from tissues using the Ultraspec RNA Isolation Reagent (Biotecx Labs). Polyadenylated (poly[A]⁺) RNA was

prepared using oligotex poly dT spin columns (Qiagen). Poly(A)⁺ RNA was separated by electrophoresis through a 1% agarose-formaldehyde gel, blotted onto GenScreen (New England Nuclear), and hybridized with random prime-labeled probe (S. A. = 2×10^6 cpm/ml). The blots were hybridized with Express Hybridization Solution (Clontech) and washed following the manufacturer's protocol. Probes used were *mCry1* (nt 1081–1793 of accession number AB000777) and *mCry2* (nt 1060–1664 of accession number AB003433). Probe for actin was from human β -actin, purchased from Clontech. Blots were exposed at -80°C to BioMax film with two intensifying screens.

Four blots were prepared from the RNA samples, with each blot consisting of the eight time points from one genotype and a standard lane. One microgram of poly(A)⁺ RNA was loaded per lane for each genotype. Each blot was probed, stripped, then reprobed to detect *mCry1*, *mCry2*, and actin. To calculate relative RNA abundance, optical densities of *mCry1* and *mCry2* hybridization were divided by densities from actin hybridization to the same blot. Normalized values were then averaged for the two replicate blots prepared from a single set of RNA samples. Comparison across blots probed and exposed under similar conditions suggested that the absolute level of expression of the *mCry* genes was lower in *Clock/Clock* mice than in wild types. This difference in absolute expression level was confirmed using two additional blots that included selected (peak-trough) RNA samples from the two genotypes side by side, and they were probed for *mCry1*, *mCry2*, and actin.

Tissue Culture and Transient Transfections

NIH3T3 and COS7 cells were grown at 37°C and 5% CO_2 . NIH3T3 were grown in Dulbecco's modified Eagle's medium (DMEM, MGH Cell Culture Core Facility) supplemented with 10% calf serum. COS7 cells were grown in DMEM supplemented with 10% fetal calf serum, penicillin, streptomycin, and fungisone. For transfections, Lipofectamine Plus (GIBCO) was used according to the manufacturer's protocol.

Transcriptional Assay

Luciferase reporter gene assays were performed in NIH3T3 cells as previously described (Gekakis et al., 1998; Jin et al., 1999). Cells (3×10^5) were seeded in six-well plates and transfected the following day. Each transfection contained the vasopressin promoter (10 ng) or 1.8 kb of the 5' flanking region of the *mPer1* gene each cloned into pGL3 Basic (Promega) (10 ng) and CMV β -galactosidase (25 ng). Mouse CLOCK, hamster BMAL1, and human MOP4, each subcloned into pcDNA3.1-V5, were each used at 250 ng per transfection. Amounts of the mPER and mTIM constructs transfected varied depending on the experiment (see figure legends for details). The total amount of DNA per well was adjusted to 1 μg by adding pcDNA 3.1 vector as carrier. Forty-eight hours after transfection, cells were harvested to determine β -galactosidase activity (Galacton Plus, Tropix) and luciferase activity (Promega) by luminometry.

Immunocytochemistry

All experimental manipulations were conducted under license by the Home Office (UK), in accordance with the Animals (Scientific Procedures) Act 1986, and the University of Cambridge code of practice for scientific procedures on animals. Housing, perfusion, and sampling of brain tissue from adult male CD1(ICR) mice (Harlan Olac, UK) were as described previously (Hastings et al., 1999). Briefly, mice entrained to a schedule of 12L:12D were transferred to constant dim red light. CT was initially defined relative to predicted lights off (CT12), and on the day of sampling it was confirmed by the coincident onset of group activity, as monitored by passive infrared movement detectors. After 20 (CT8) to 42 (CT6) hr in constant dim red light, mice were killed with an anesthetic overdose and perfused (4% paraformaldehyde). Brains were removed, postfixed, transferred to cryoprotectant-buffered sucrose solution (20%), and then sectioned on a freezing microtome. Alternate free-floating sections (40 μm) were incubated with affinity-purified anti-mCRY1 or anti-mCRY2 (both at 0.5 $\mu\text{g/ml}$) primary sera (Alpha Diagnostic International). The sera were raised against synthetic peptides corresponding to specific sequences close to the C terminals of the mCRY1 (26 amino acids) and mCRY2 (22 amino acids) proteins. To

test for specificity of the sera, some SCN sections were incubated with affinity-purified sera to which synthetic peptide (10 $\mu\text{g/ml}$) had been added. Immunoreaction was visualized by avidin-biotin/peroxidase in conjunction with diaminobenzidine chromogen (Vector Labs, Peterborough, UK). Counts of the number of immunoreactive nuclear profiles in the SCN were made using an image analysis system as described previously (Hastings et al., 1999).

Immunofluorescence

Cells (3×10^5) were seeded on glass coverslips in 6-well dishes and transfected the following day as described above with 1 μg of total DNA per well. Forty-eight hours after transfection, cells adherent to the coverslip were washed twice with phosphate-buffered saline (PBS), fixed with -20°C methanol (10 min), washed, and blocked in 5% normal goat serum/0.1% Triton X-100 in PBS (1 hr). Mouse anti-V5 IgG (1:500; Invitrogen) or rabbit anti-HA IgG (1:100; Santa Cruz Biotechnology) was applied for 1.5 hr. Cells were washed and then incubated in the dark (1 hr) with secondary antibodies. These consisted of either goat anti-rabbit IgG conjugated to Cy2 (1:200) or goat anti-mouse IgG conjugated to Cy3 (1:200; Jackson ImmunoResearch). Cells were washed, and the nuclei were stained with bisBenzimide and then mounted for fluorescence microscopy. A random population of 30–60 cells from each coverslip was examined by epifluorescence microscopy, and the subcellular distributions of the transfected proteins were recorded without knowledge of the treatment. At least three independently transfected coverslips were analyzed.

Coimmunoprecipitations

Coimmunoprecipitations were performed as described in Lee and colleagues (1998) with the following modifications. COS7 cells (5×10^6) were seeded in 10 cm dishes and transfected the following day with the expression plasmids described above. Forty-eight hours posttransfection, the cells were washed twice with PBS, homogenized in binding buffer (20 mM HEPES [pH 7.5], 100 mM KCl, 2.5 mM EDTA, 5 mM DTT, 2.5 mM PMSF, 0.05% Triton X-100, 10% glycerol, 10 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ aprotinin), and clarified by centrifugation. Protein concentrations were determined by the Bradford method according to the manufacturer's instructions (Pierce). Total protein (30 μg) from the clarified supernatant was combined with 15 μl of protein A/G agarose beads (Santa Cruz Biotechnology) and incubated for 1 hr at 4°C to remove nonspecific interactions. The samples were centrifuged and the supernatant was incubated for 3 hr at 4°C with anti-HA mouse monoclonal antibodies (Babco, 1:50 dilution) and 15 μl of protein A/G agarose beads. Subsequently, beads were washed four times (400 μl binding buffer for 10 min per wash), mixed with 5 μl of 4 \times sodium dodecyl sulfate (SDS) gel loading buffer, boiled, and centrifuged. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotted as described below.

Western Blot Analysis

Total protein (5 μg) from COS7 cells was extracted as described above, separated by SDS-PAGE, and transferred to a nitrocellulose membrane using a semidry blotting apparatus. Membranes were blocked with 5% nonfat milk. Blots were incubated with either the mouse anti-HA antibody (1:10,000) or the mouse anti-V5 antibody (1:5,000) 1 hr at 4°C . A goat anti-mouse horseradish peroxidase secondary antibody (1:10,000) was used in combination with enhanced chemiluminescence (NEN) to detect proteins.

Following detection of epitope-tagged proteins with one antibody, the blots were stripped in stripping buffer (62.5 mM Tris-HCl [pH 6.7], 100 mM 2-mercaptoethanol, 2% SDS) at 50°C for 30 min. The membrane was washed extensively (20 mM Tris, [pH 7.6], 137 mM NaCl, 0.05% Tween-20), then blocked again and processed for detection of the second epitope-tagged protein.

During the course of these experiments, we noticed the mPER and mTIM proteins migrate by SDS-PAGE with apparent molecular masses that are 50 to 75 kDa larger than their calculated masses (Figure 1A). Curiously, in vitro translated proteins also migrate at roughly the same sizes as the overexpressed proteins from COS7 or NIH3T3 cell lysates (data not shown). This size discrepancy is unlikely due to posttranslational modifications alone. Instead, it is

probably due to skewed charge:mass ratios for these proteins in SDS polyacrylamide gels. It is worth mentioning that bacterially expressed *Drosophila* PER also migrates in SDS polyacrylamide gels at a size larger than that predicted from the primary sequence (Edery et al., 1994; Lee et al., 1998).

Acknowledgments

We thank Kurtis Gray and Austin Lemieux for technical assistance; Martha H. Vitaterna and Joseph S. Takahashi for supplying us with founder *Clock* mice; John B. Hogenesch and Christopher A. Bradfield for providing numerous plasmids including human MOP4 used in this study; Charles J. Weitz for providing hamster BMAL1 and mouse CLOCK expression plasmids; and Joel D. Levine for useful discussions.

This work was supported by R37 HD14427 and S09882. K. K. was supported by the Uehara Memorial Foundation, M. J. Z. was supported in part by National Research Service Award MH11547, and X. J. was supported by National Research Service Award MH12067.

Received June 2, 1999; revised June 28, 1999.

References

- 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.
- Allada, R., White, N.E., So, W.V., Hall, J.C., and Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of period and timeless. *Cell* **93**, 791–804.
- Balsalobre, A., Damiola, F., and Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93**, 929–937.
- Cashmore, A.R., Jarillo, J.A., Wu, Y.-J., and Liu, D. (1999). Cryptochromes: blue light receptors for plants and animals. *Science* **284**, 760–765.
- Darlington, T.K., Wagner-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S., and Kay, S.A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors *per* and *tim*. *Science* **280**, 1599–1603.
- Dunlap, J.C. (1999). Molecular bases for circadian clocks. *Cell* **96**, 271–290.
- Edery, I., Zwiebel, L.J., Dembinska, M.E., and Rosbash, M. (1994). Temporal phosphorylation of the *Drosophila period* protein. *Proc. Natl. Acad. Sci. USA* **91**, 2260–2264.
- Emery, P., So, W.V., Kaneko, M., Hall, J.C., and Rosbash, M. (1998). CRY, a *Drosophila* clock and light-regulated cryptochrome, is a major contributor to circadian rhythm resetting and photosensitivity. *Cell* **95**, 669–679.
- Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280**, 1564–1569.
- Hao, H., Allen, D.L., and Hardin, P.E. (1997). A circadian enhancer mediates PER-dependent mRNA cycling in *Drosophila melanogaster*. *Mol. Cell Biol.* **17**, 3687–3693.
- Hastings, M.H., Field, M.D., Maywood, E.S., Weaver, D.R., and Reppert, S.M. (1999). Differential regulation of mPER1 and mTIM proteins in the mouse suprachiasmatic nuclei: new insights into a core clock mechanism. *J. Neurosci.* **19**, RC11, 1–7.
- Herzog, E.D., Takahashi, J.S., and Block, G.D. (1998). *Clock* controls circadian period in isolated suprachiasmatic nucleus neurons. *Nat. Neurosci.* **1**, 708–713.
- Hogenesch, J.B., Gu, Y.-Z., Jain, S., and Bradfield, C.A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. *Proc. Natl. Acad. Sci. USA* **95**, 5474–5479.
- Jin, X., Shearman, L.P., Weaver, D.R., Zylka, M.J., De Vries, G.J., and Reppert, S.M. (1999). A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* **96**, 57–68.
- King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D.L., Vitaterna, M.H., Kornhauser, J.M., et al. (1997). Positional cloning of the mouse circadian *Clock* gene. *Cell* **89**, 641–653.
- Klein, D.C., Moore, R.Y., and Reppert, S.M. (1991). *Suprachiasmatic Nucleus: the Mind's Clock* (New York: Oxford University Press).
- Kobayashi, K., Kanno, S.-I., Smit, B., van der Horst, G.T.J., Takao, M., and Yasui, A. (1998). Characterization of photolyase/blue-light receptor homologs in mouse and human cells. *Nucleic Acids Res.* **26**, 5086–5092.
- Koike, N., Hida, A., Numano, R., Hirose, M., Sakaki, Y., and Tei, H. (1998). Identification of the mammalian homologs of the *Drosophila timeless* gene, *Timeless1*. *FEBS Lett.* **441**, 427–431.
- Lee, C., Bae, K., and Edery, I. (1998). The *Drosophila* CLOCK protein undergoes daily rhythms in abundance, phosphorylation, and interactions with the PER-TIM complex. *Neuron* **21**, 857–867.
- Liu, C., Weaver, D.R., Strogatz, S.H., and Reppert, S.M. (1997). Cellular construction of a circadian clock: period determination in the suprachiasmatic nuclei. *Cell* **91**, 855–860.
- Miyamoto, Y., and Sancar, Y. (1998). Vitamin B₂-based blue-light photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. *Proc. Natl. Acad. Sci. USA* **95**, 6097–6102.
- Price, J.L., Blau, J., Rothenfluh, A., Adodeely, M., Kloss, B., and Young, M.W. (1998). *double-time* is a new *Drosophila* clock gene that regulates PERIOD accumulation. *Cell* **94**, 83–95.
- Reppert, S.M. (1998). A clockwork explosion! *Neuron* **21**, 1–4.
- Reppert, S.M., and Weaver, D.R. (1997). Forward genetic approach strikes gold: cloning of a mammalian *Clock* gene. *Cell* **89**, 487–490.
- Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., and Hall, J.C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. *Cell* **93**, 805–814.
- Saez, L., and Young, M.W. (1996). Regulation of nuclear entry of the *Drosophila* clock proteins Period and Timeless. *Neuron* **17**, 911–920.
- Sakamoto, K., Nagase, T., Fukui, H., Horikawa, K., Okada, T., Tanaka, H., Sato, K., Miyake, Y., Ohara, O., Kako, K., and Ishida, N. (1998). Multitissue circadian expression of rat *period* homolog (*rPer2*) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. *J. Biol. Chem.* **273**, 27039–27042.
- Sangoram, A.M., Saez, L., Antoch, M.P., Gekakis, N., Staknis, D., Whiteley, A., Fruechte, E.M., Vitaterna, M.H., Shimomura, K., King, D.P., et al. (1998). Mammalian circadian autoregulatory loop: a *Timeless* ortholog and *mPer1* interact and negatively regulate CLOCK-BMAL1-induced transcription. *Neuron* **21**, 1101–1113.
- Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F., Jr., and Reppert, S.M. (1997). Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron* **19**, 1261–1269.
- Shearman, L.P., Zylka, M.J., Reppert, S.M., and Weaver, D.R. (1999). Expression of basic helix-loop-helix/PAS genes in the mouse suprachiasmatic nucleus. *Neuroscience* **89**, 387–397.
- Somers, D.E., Devlin, P.F., and Kay, S.A. (1998). Phytochromes and cryptochromes in the entrainment of the *Arabidopsis* circadian clock. *Science* **282**, 1488–1490.
- Stanewsky, R., Kaneko, M., Emery, P., Beretta, B., Wager-Smith, K., Kay, S.A., Rosbash, M., and Hall, J.C. (1998). The *cry²* mutation identifies cryptochrome as a circadian photoreceptor in *Drosophila*. *Cell* **95**, 681–692.
- Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., and Lee, C.C. (1997). RIGUI, a putative mammalian ortholog of the *Drosophila period* gene. *Cell* **90**, 1003–1011.
- Takahata, S., Sogawa, K., Kobayashi, A., Ema, M., Mimura, J., Ozaki, N., and Fujii-Kuriyama, Y. (1998). Transcriptionally active heterodimer formation of an Arnt-like PAS protein, Arnt3, with HIF-1 α , HLF, and Clock. *Biochem. Biophys. Res. Commun.* **248**, 789–794.
- Takumi, T., Matsubara, C., Shigeyoshi, Y., Taguchi, K., Yagita, K.,

- Maebayashi, Y., Sakakida, Y., Okumura, K., Takashima, N., and Okamura, H. (1998a). A new mammalian *period* gene predominantly expressed in the suprachiasmatic nucleus. *Genes Cells* 3, 167–176.
- Takumi, T., Taguchi, K., Miyake, S., Sakakida, Y., Takashima, N., Matsubara, C., Maebayashi, Y., Okumura, K., Takekida, S., Yamamoto, S., et al. (1998b). A light-independent oscillatory gene *mPer3* in mouse SCN and OVLT. *EMBO J.* 17, 4753–4759.
- Takumi, T., Nagamine, Y., Miyake, S., Matsubara, C., Taguchi, K., Takekida, S., Sakakida, Y., Nishikawa, K., Kishimoto, T., Niwa, S., et al. (1999). A mammalian ortholog of *Drosophila timeless*, highly expressed in SCN and retina, forms a complex with mPER1. *Genes Cells* 4, 67–75.
- Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M., and Sakaki, Y. (1997). Circadian oscillation of a mammalian homolog of the *Drosophila period* gene. *Nature* 389, 512–516.
- 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.
- van der Horst, G.T.J., Muijtjens, M., Kobayashi, K., Takano, R., Kanno, S.-I., Takao, M., De Wit, J., Verkerk, A., Eker, A.P.M., Leenen, D.V., et al. (1999). Mammalian *Cry1* and *Cry2* are essential for maintenance of circadian rhythms. *Nature* 398, 627–630.
- 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.
- Weaver, D.R. (1993). A2a adenosine receptor gene expression in developing rat brain. *Mol. Brain Res.* 20, 313–327.
- Welsh, D.K., Logothetis, D.E., Meister, M., and Reppert, S.M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. *Neuron* 14, 697–706.
- Zylka, M.J., Shearman, L.P., Weaver, D.R., and Reppert, S.M. (1998a). Three *period* homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron* 20, 1103–1110.
- Zylka, M.J., Shearman, L.P., Levine, J.D., Jin, X., Weaver, D.R., and Reppert, S.M. (1998b). Molecular analysis of mammalian *Timeless*. *Neuron* 21, 1115–1122.

GenBank Accession Number

The sequence reported in this paper has been deposited with the accession number AF156987.