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Nonredundant Roles of the *mPer1* and *mPer2* Genes in the Mammalian Circadian Clock

Binhai Zheng,[1,5] Urs Albrecht,[2,7] Krista Kaasik,[1] Marijke Sage,[1] Weiqin Lu,[1] Sukeshi Vaishnav,[1,3] Qiu Li,[1] Zhong Sheng Sun,[1,8] Gregor Eichele,[2,6] Allan Bradley,[1,3,4,9] and Cheng Chi Lee[1,4]

[1] Department of Molecular and Human Genetics
[2] Verna and Marrs McLean Department of Biochemistry
[3] Howard Hughes Medical Institute
Baylor College of Medicine
One Baylor Plaza
Houston, Texas 77030

Summary

Mice carrying a null mutation in the *Period 1* (*mPer1*) gene were generated using embryonic stem cell technology. Homozygous *mPer1* mutants display a shorter circadian period with reduced precision and stability. Mice deficient in both *mPer1* and *mPer2* do not express circadian rhythms. While mPER2 regulates clock gene expression at the transcriptional level, mPER1 is dispensable for the rhythmic RNA expression of *mPer1* and *mPer2* and may instead regulate mPER2 at a posttranscriptional level. Studies of clock-controlled genes (CCGs) reveal a complex pattern of regulation by mPER1 and mPER2, suggesting independent controls by the two proteins over some output pathways. Genes encoding key enzymes in heme biosynthesis are under circadian control and are regulated by mPER1 and mPER2. Together, our studies show that mPER1 and mPER2 have distinct and complementary roles in the mouse clock mechanism.

Introduction

Circadian rhythms are an evolutionarily conserved property of many biological processes in diverse life forms (Dunlap, 1999; Lowrey and Takahashi, 2000). In mammals, a master circadian clock resides in the suprachiasmatic nuclei (SCN) of the hypothalamus. In the absence of environmental cues (such as light), this clock continues to operate (free runs) with remarkable precision, stability, and persistence, with a cycle time that is approximately 24 hr. This internal clock can also be reset (entrained) in response to external time cues. Recent studies have revealed that the clock mechanism is present in many peripheral tissues, suggesting that it has a pervasive role in temporal organization of biochemical and physiological functions (Young, 2000).

The first clock mutants were isolated in *Drosophila* (Konopka and Benzer, 1971) and the corresponding molecular defects were later identified in the *Period* (*per*) gene (Bargiello et al., 1984). Since then, a number of additional clock genes, including *timeless* (*tim*), *clock* (*clk*), *cycle* (*cyc*), *doubletime* (*dbt*), *cryptochrome* (*cry*), and recently *vrille* (*vri*), have been identified in this model organism (Allada et al., 1998; Blau and Young, 1999; Kloss et al., 1998; Myers et al., 1995; Rutila et al., 1998). Mutations in these genes alter the period length and/or lead to behavioral arrhythmicity. At the molecular level, the clock is characterized by the oscillating expression of specific clock genes including *per* and *tim* (Young, 2000). This oscillating expression is driven by an autoregulatory negative feedback loop where CLK and CYC act positively to drive the expression of *per* and *tim*, while PER and TIM act as a complex to regulate their own transcription negatively by inhibiting CLK/CYC (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). The cyclical activity of PER and TIM is also controlled posttranslationally by regulation of phosphorylation that involves the kinase DBT and nuclear entry (Kloss et al., 1998).

Orthologs of most *Drosophila* circadian clock genes have been identified in mammals, highlighting a general conservation in the clock mechanism between insects and mammals. In particular, three mammalian *Period* genes (*mPer1*, *mPer2*, and *mPer3*), two *Cryptochrome* genes (*mCry1* and *mCry2*), as well as *Clock*, *Bmal1* (ortholog to *Cyc*), and *CK1*ϵ (ortholog to *Dbt*) have been identified (Albrecht et al., 1997a; Bunger et al., 2000; King et al., 1997; Lowrey and Takahashi, 2000; Miyamoto and Sancar, 1998; Sun et al., 1997; Tei et al., 1997; van der Horst et al., 1999; Zylka et al., 1998). The mRNA transcripts and protein products for the *mPer* genes are expressed in a circadian manner in the SCN and in peripheral tissues. In vitro studies have shown that the expression of *mPers* is driven by the CLOCK/BMAL1 transcription complex, and it was hypothesized that mPERs, together with mCRYs, serve to regulate the CLOCK/BMAL1 transcription complex negatively (Dunlap, 1999; Gekakis et al., 1998; Griffin et al., 1999; Kume et al., 1999). However, it is not known whether mPERs play a negative feedback role in clock gene expression in vivo.

Among the three *mPer* genes, only *mPer2* and *mPer3* have been tested directly for a role in the circadian clock in vivo. We previously reported that an *mPer2* mutant displays a short circadian period followed by a loss of circadian rhythmicity in constant darkness, revealing a prominent role for mPER2 in the mammalian clock (Zheng et al., 1999). However, RNA expression analysis of the *mPer2* mutant did not support a negative feedback role for mPER2 in the clock mechanism (Zheng et al., 1999). In contrast, *mPer3* knockout mice have an essentially normal clock (Shearman et al., 2000a).

Here we show that *mPer1*-deficient mice display a persistent circadian rhythm, but they have an ~1 hr

[4] Correspondence: abradley@sanger.ac.uk or ching@bcm.tmc.edu
[5] Present address: Department of Anatomy, University of California, 513 Parnassus S1479, San Francisco, California 94143.
[6] Present address: Max Planck Institute for Experimental Endocrinology, Feodor-Lynen-Strasse 7, 30625 Hannover, Germany.
[7] Present address: Institute of Biochemistry, University of Fribourg, Rue du Museé 5, 1700 Fribourg, Switzerland.
[8] Present address: Department of Pediatrics, LC604, Weill Medical College of Cornell University, 1300 York Avenue, New York, New York 10021.
[9] Present address: The Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom.

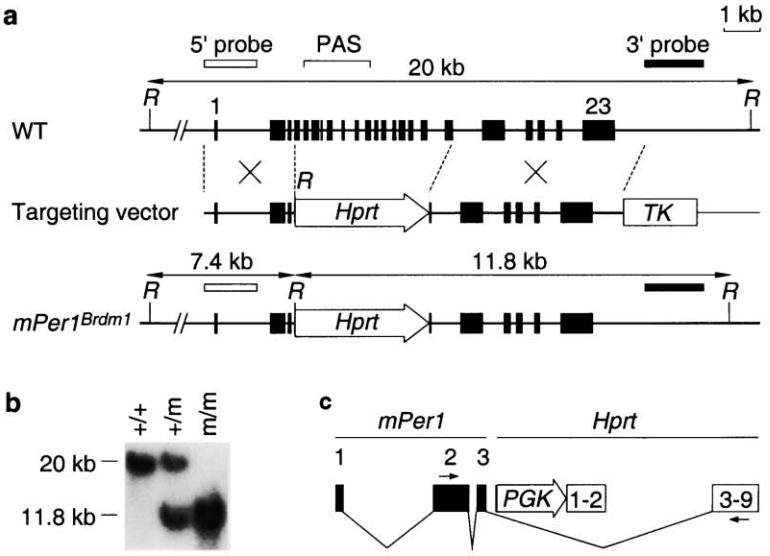

Figure 1. Generation of *mPer1*^{Brdm1} Mutant Mice

(a) Genomic structure of the murine *mPer1* gene, the targeting vector, and the predicted structure of the targeted allele. Exons are indicated by vertical black bars with the first and last exons numbered. WT, wild-type; *R*, EcoRI; *Hprt*, hypoxanthine phosphoribosyltransferase gene; *TK*, *Herpes Simplex Virus thymidine kinase* gene. A 1.6 kb 3′ external probe that detects a 20 kb wild-type EcoRI fragment and an 11.8 kb mutant EcoRI fragment were used to detect targeted ES cell clones and to genotype test mutant mice. A 1.5 kb 5′ internal probe that detects a 20 kb wild-type EcoRI fragment and a 7.4 kb mutant EcoRI fragment were used to confirm correct targeting at the 5′ homology region (data not shown).

(b) Southern analysis of F_2 littermates obtained from intercrosses between (C57BL/6*Tyr*$^{c\text{-}Brd}$ × 129S7) F_1 heterozygous mice using the 3′ probe and EcoRI digestion. +, wild-type allele; m, mutant allele. Southern analysis with a probe within the deleted region confirmed the deletion in homozygous mutants (data not shown).

(c) Structure of a m*Per1-hprt* fusion transcript in the mutants as determined by sequence analysis of an RT-PCR product. *PGK*, phosphoglycerate kinase gene promoter. Arrows indicate the primers used in RT-PCR.

shorter period and their ability to maintain the precision and the stability of the period is impaired. In the absence of mPER1, rhythmic RNA expression of clock genes persists while mPER2 protein levels are elevated. Mice deficient for both *mPer1* and *mPer2* have no circadian rhythms in locomotor activity, clock, or clock-controlled gene expression. The genes encoding the enzymes 5-aminolevulinate synthase 1 and 2 that govern the rate-limiting step of heme biosynthesis are under circadian control, suggesting a mechanism by which the clock controls physiological body rhythms via regulation of heme metabolism. Our data illustrate the central role of mPER1 and mPER2 in the function of the clock, and indicate that they regulate the clock via distinct mechanisms and that they can regulate different output pathways of the clock.

Results

Generation of Mice with a Targeted Mutation in the *mPer1* Gene

A targeting vector was designed to replace a 4.3 kb *mPer1* genomic region encompassing 15 of the 23 exons of the *mPer1* gene with an *Hprt* minigene (Figure 1a). The vector was designed such that if splicing over the deleted region occurred in a mutant transcript (connecting exon 3 to exon 19), a shift in the reading frame would have precluded the translation of the C-terminal region of mPER1. The targeted allele, *mPer1*^{Brdm1}, was obtained in AB2.2 ES cells (derived from an XY 129S7 embryo) (Festing et al., 1999) as described (Ramirez-Solis et al., 1993) and used to generate chimeric mice. Intercrosses between heterozygous (C57BL/6*Tyr*$^{c\text{-}Brd}$ × 129S7) F_1 offspring gave rise to F_2 homozygous mutants (Figure 1b) at the expected Mendelian ratio. Homozygous mutants are viable, fertile, and morphologically indistinguishable from their wild-type littermates. Reverse transcriptase–polymerase chain reaction (RT-PCR) and sequence analysis of RNA from kidney tissues indicated the presence of a fusion transcript consisting of exons 1 to 3 of *mPer1* spliced to exons 3–9 of the *Hprt* minigene (Figure 1c). The fusion transcript, if translated, would generate a protein that contains the first 124 of the 1,291 amino acid residues of mPER1. Because this N-terminal region of mPER1 does not contain any recognizable sequence motif (Sun et al., 1997; Tei et al., 1997), we conclude that the *mPer1*^{Brdm1} mutation is most likely a null allele.

mPer1 Mutant Mice Display a Shorter Circadian Period with Reduced Precision and Stability

We assessed the circadian phenotype of *mPer1* mutant mice by monitoring their wheel-running activity (Figures 2a and 2b). All homozygous and heterozygous mutants entrained to the 12 hr light/12 hr dark (LD 12:12, or LD) cycle, as did the wild-type controls. In constant darkness (DD), the homozygous *mPer1* mutants displayed an average circadian period (22.6 ± 0.2 hr [mean ± SEM], n = 16) that is significantly shorter than that of the wild-type controls (23.7 ± 0.1 hr, n = 16. $P <$ 0.00005, Student's t test) (Figure 2c). The heterozygous *mPer1* mutants displayed an average period (23.5 ± 0.1 hr, n = 8) that is not significantly different from that of the wild-type ($P > 0.05$, Student's t test). The period distribution among homozygous *mPer1* mutants is much more variable than among wild-type mice (Figure 2c). To determine whether genetic background affects expression of the circadian phenotype, we generated *mPer1* mutants in the 129S7 inbred background by crossing the chimera mice to 129S7 strain females. The same highly variable period length circadian phenotype was observed in the *mPer1* mutants. To investigate the variable circadian period in the *mPer1* mutants further, we examined whether individual mutants would display variable periods in repeated experiments. The same group of animals was assessed a second time by re-entrainment followed by a second release into DD. The

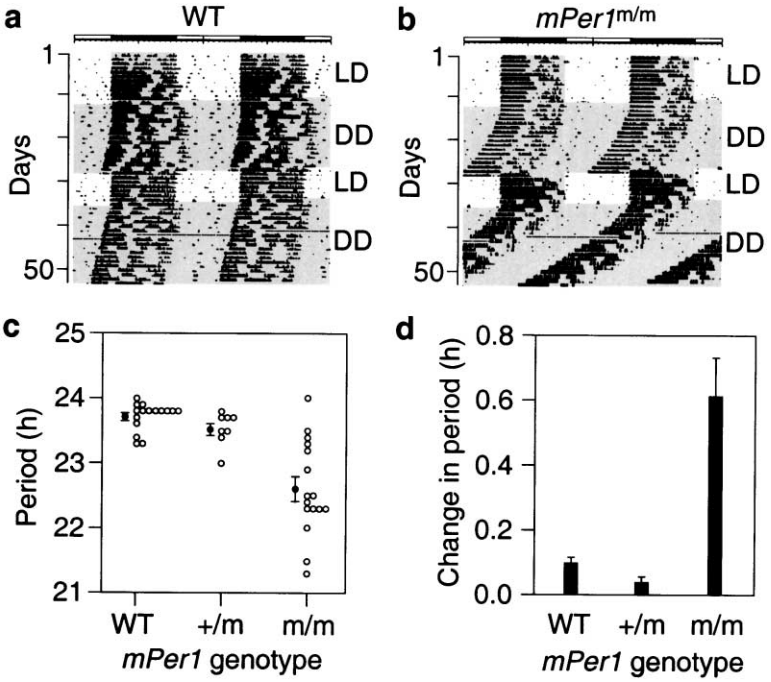

Figure 2. Circadian Period Phenotype of *mPer1* Mutant Mice

Representative locomotor activity records of F_2 wild-type (a) and homozygous *mPer1* mutant mice (b). A gray background indicates the dark periods. LD, 12 hr light/12 hr dark cycle; DD, constant darkness. The bar over the records indicates the LD cycle. Mice were subjected to alternate LD and DD cycles (LD, 10 days; DD, 16 days; LD, 7 days; DD, 18 days) in order to assess their free-running period twice. At day 43, the recording was disrupted for about 17 hr, as indicated by a thin horizontal line. (c) The initial free-running period of F_2 wild-type (WT) heterozygous mutant (+/m) and homozygous mutant (m/m) mice. Open circles indicate individual animals. Closed circles indicate the average period for a genotype. (d) The absolute value of change in period between the two DD periods (as in [a] and [b]), averaged for each genotype. The same group of animals was used as in (c). All error bars indicate SEM.

wild-type animals exhibited no significant differences in period length between the two DD periods. However, many *mPer1* mutants had a different period length on their second assessment. On average, the period lengths varied by 0.6 hr compared with less than 0.1 hr in the wild-type or the heterozygous animals (Figure 2d, also see 2a and 2b). The change in period length between the two assessments in the *mPer1* mutants did not show a trend and the average period remained the same (second assessment, 22.5 $\pm$ 0.2 hr, n = 16; $P = 0.78$, paired Student's t test). These results illustrate that the variability in period length is most likely due to the loss of mPER1. An increased variability in period length between animals reflects a reduced precision of the period, while that between repeated assays on a single animal reflects a reduced stability of the period. A loss of persistent circadian rhythmicity was rarely observed in homozygous *mPer1* mutants—only 1 out of 16 homozygous *mPer1* mutant animals lost its circadian rhythmicity after 4 weeks in DD (data not shown). Taken together, these data provide genetic evidence that *mPer1* is functionally involved in circadian regulation and further indicate that mPER1 functions to regulate the period length and to maintain the precision and stability of the period.

mPer1, *mPer2* Double Mutant Mice Have No Circadian Rhythms

As shown above, mPER1 is not absolutely required for the generation and maintenance of circadian rhythms. The locomotor rhythmicity of the *mPer1* mutants is very different from that of the *mPer2* mutant animals, which lose their circadian rhythms within 3 weeks in constant darkness (Zheng et al., 1999). The majority of the *mPer2* mutant mice do not lose their rhythms immediately upon entry into DD and, upon loss of rhythmicity, a light pulse immediately reestablishes their rhythms (Zheng et al., 1999). This indicates that the *mPer2* mutants have a partially functional clock. Because both the *mPer1* and the *mPer2* mutants have a partially functional clock, we generated *mPer1*, *mPer2* double mutant mice. *mPer1*, *mPer2* double mutants are viable, fertile, and morphologically indistinguishable from their wild-type littermates, indicating that both *mPer1* and *mPer2* are dispensable for normal development. The double mutants appear to have normal rhythms in locomotor activity in LD 12:12 (Figure 3, compare a and b), indicating that their behavior is still under the control of light (see below). However, when the double mutants (n = 30) were placed in DD, they immediately lost their circadian rhythms (Figure 3, compare a and b). Fourier periodogram analysis (Bracewell, 1986) on the first 10 day interval in DD confirmed the loss of the circadian peak in double mutants (data not shown). A light pulse did not reestablish a circadian rhythm in the double mutants (Figure 3b, see arrow), which would be consistent with a complete loss of a functional clock. Abolition of a partially functional clock in *mPer2*-deficient mice by the *mPer1* deletion argues for a role of mPER1 in the core clock mechanism.

Entrainment of *mPer1*, *mPer2* Double Mutants to LD Is Caused by the Masking Effect of Light

The apparently normal entrainment of the *mPer1, 2* double mutants to LD cycle raised the possibility that there is some residual clock function. Alternatively, the entrainment to LD may be due to a masking effect of the light (light inhibition of locomotor activity) on rodents. To distinguish between these two possibilities, we subjected the mice to a very short light/dark cycle, LD 4:4. We hypothesized that true entrainment should be restricted to rhythms in the circadian range and that a loss of clock control would allow the animals to respond passively to external cues. As expected, wild-type ani-

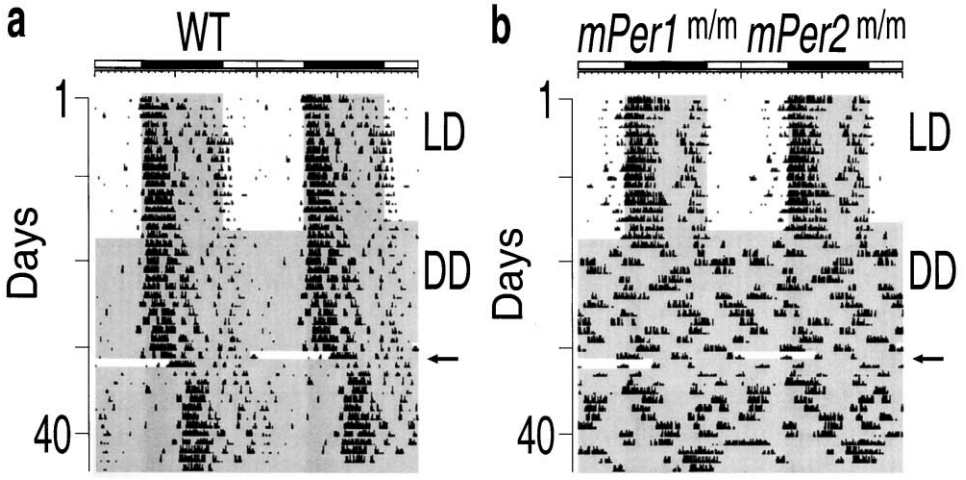

Figure 3. Circadian Phenotypes of Double *mPer* Mutants

Activity records of an F_2 wild-type (a) and an F_2 *mPer1, mPer2* double homozygous mutant mouse (b). Light regime: 17 days in LD followed by 27 days in DD. Half way into DD, a 12 hr light pulse was given, as indicated by an arrow. A 12 hr light pulse was applied to ensure the coverage of the active phase of double homozygous mutants by the light pulse. In separate experiments, a 6 hr light pulse gave similar results (data not shown).

mals continued to show a distinct rhythm in the circadian range in LD 4:4 (Figure 4a). Within the active phase of this circadian rhythm, light had an inhibitory (masking) effect on the locomotor activity. Conversely, during the normal resting phase, a dark period did not induce locomotor activity (Figure 4a). In contrast, the double homozygous mutants synchronized their rhythms to the LD 4:4 cycle, but did not display a circadian rhythm (Figure 4b). The *mPer2* mutants behaved similarly (Figure 4d). We also applied this scheme with a LD 2:2 or LD 6:6 cycle and observed a similar result (data not shown). Thus, the short rhythms are generated by a masking response to light and are under separate control from the clock mechanism. The behavior of the *mPer1* mutants ranged between that of the wild-types and that of the *mPer2* mutants (Figure 4c). These results are

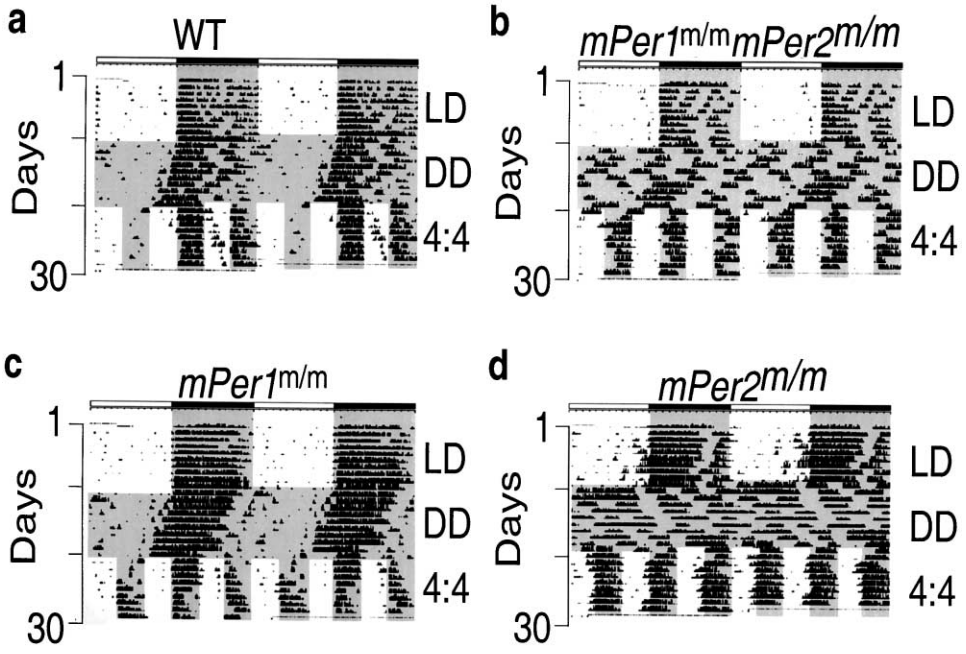

Figure 4. Masking Effect of LD Cycle in Double and Single *mPer* Mutants

Activity records of a wild-type (a), m*Per1, mPer2* double mutant (b), m*Per1* mutant (c), and m*Per2* mutant (d) mice that were subjected first to LD (10 days), and then DD (10 days), followed by a 4 hr light/4 hr dark (4:4) cycle (10 days). Fourier analysis confirmed the presence of a circadian rhythm for (a) but an 8 hr rhythm for (b) and (d) (data not shown). Note the poor synchronization to the short light cycles by this *mPer1* mutant during the first few days in LD 4:4.

consistent with a nonfunctional clock in the double mutants that releases the animals from regulation by the internal clock and enables them to adjust freely to the external cues. The short LD regime presents another instance where the *mPer1*-deficient clock and the wild-type clock can be distinguished. Taken together, these data illustrate further that mPER1 and mPER2 together are essential for a functional clock.

mPER1, but Not mPER2, Is Dispensable for the Rhythmic Expression of *mPer1* or *mPer2*

Both the *mPer1* and *mPer2* genes are thought to be targets of circadian expression regulation (Dunlap, 1999). To determine the effect of these mutations on the clock at the molecular level, we analyzed the expression of *mPer1* and *mPer2* in the SCN of wild-types, single *mPer1* or *mPer2* mutants, and double mutants, as described (Albrecht et al., 1997b; Zheng et al., 1999). This was possible because a mutant transcript is produced from the *mPer1* (see above) or *mPer2* alleles (Zheng et al., 1999). As shown previously (Albrecht et al., 1997a; Sun et al., 1997), wild-type mice exhibited a strong oscillation of both *mPer1* and *mPer2* transcripts in the SCN in DD, with a peak level of *mPer1* transcript at around circadian time (CT) 6 and the *mPer2* transcript peak at CT 9–12 (Figures 5a and 5b). The mutant *mPer1* transcript in the *mPer1* mutants continued to cycle, but exhibited a higher peak than in wild-type controls (Figure 5a). Northern analysis on liver and kidney tissue RNA isolated from the *mPer1* mutants also detected a robust cycling of the m*Per1* mutant transcript of the expected size (data not shown). These results demonstrate that mPER1 is dispensable for the rhythmic expression of its own gene. The expression of *mPer2* in the *mPer1* mutants was not detectably different from that in the wild-type controls in DD (Figure 5b), indicating that *mPer1* is also dispensable for *mPer2* transcript oscillation. In contrast, *mPer1* and *mPer2* expression oscillates at markedly reduced levels in the SCN and peripheral tissues of the *mPer2* mutant mice in DD (Figures 5a and 5b; Zheng et al., 1999). The cycling of the *mPer1* transcript was only marginal in the *mPer2* mutant (Figure 5a). No apparent cycling of *mPer1* or *mPer2* expression was observed in the double mutants (Figures 5a and 5b). These results demonstrate that while mPER2 regulates clock gene expression through a transcriptional control, mPER1 is dispensable for circadian RNA expression of *mPer1* and *mPer2*.

Posttranscriptional Regulation of mPER2 by mPER1

Our current understanding of the molecular clock is based on a negative feedback mechanism in which the clock gene products inhibit their own mRNA expression. The surprising observation that loss of mPER1 does not lead to a loss or dampening of its circadian RNA expression raised the question of the role played by mPER1 in the negative feedback mechanism. To address this, we examined protein expression in the mutant mice using polyclonal antibodies raised against mPER1 and mPER2. We and others have shown that the molecular components of the peripheral clock mirror those of the SCN (Balsalobre et al., 1998; Zheng et al., 1999; Zylka et al., 1998). Therefore, we examined the expression of mPER1 and mPER2 in a peripheral tissue. Western analysis was carried out on kidney tissues from wild-types, *mPer1* mutants, and *mPer2* mutants isolated every 6 hr after the first day into DD. Antibodies to mPER1 detected a protein of about 140 kDa in wild-types, *mPer2* mutants, and in extracts from COS cells transfected with an mPER1 expression construct, but not in *mPer1* mutant animals (Figure 6a). Low level cross-reactivity with mPER2 (which runs at about 135 kDa) was observed in extracts from COS cells transfected with an mPER2 expression construct (Figure 6a), but not with vector alone (data not shown). In wild-type animals, the peak level of mPER1 was observed at CT 18 and the trough level at CT 6. In *mPer2* mutants, mPER1 was observed at moderate levels but apparently did not vary in level at different time points.

Antibodies to mPER2 detected a protein of about 135 KD in wild-types, *mPer1* mutants, and in extracts from COS cells transfected with mPER2, but not mPER1 (Figure 6a). A faint band ~10 kDa smaller than the wild-type protein was observed in *mPer2* mutants, consistent with the in-frame deletion of 87 amino acids. Unlike the robust cycling of mPER2 expression in the wild-type animals (peak at CT 18 and trough at CT 6), the mutant mPER2 level is low and apparently constant with time. This low level of mutant mPER2 is consistent with its significantly dampened mRNA level. In the *mPer1* mutants, the level of mPER2 is significantly elevated at every time point compared with wild-type animals, but a circadian pattern persists. There is no apparent difference in the level of *mPer2* mRNA between wild-types and *mPer1* mutants by Northern analysis, which is consistent with the result from the SCN (Figure 6b). Thus, the elevated level of mPER2 protein in the absence of mPER1 implicates a role for mPER1 in the posttranscriptional regulation of mPER2. These results illustrate the mechanistically distinct involvement of mPER1 and mPER2 in the core clock.

Clock-Controlled Genes Are Differentially Regulated by mPER1 and mPER2

Although mPER1 does not appear to be a major regulator of the RNA expression of clock genes such as *mPer1* and *mPer2*, it may control some output pathways of the clock. To explore this possibility, we screened for clock-controlled genes (CCGs) that are regulated by mPER1 and mPER2 using cDNA micro-array analysis. We used mRNA from liver for these studies because most CCGs are more likely to be expressed in peripheral tissues. Probes generated from liver RNA were compared between wild-type mice and *mPer2* or double mutants at ZT12, or wild-types at ZT0, on a 6K mouse UniGene clone cDNA chip. Out of 198 differentially expressed genes identified on the micro-array, 53 that displayed at least a 2.5-fold difference in expression levels were further subjected to Northern analysis. The Northern blots contain liver RNA samples from six time points in DD from wild-type, *mPer1*, *mPer2*, and double mutant animals. The majority of these genes (37 out of 53) that showed differential expression by micro-array analysis were not substantiated by Northern analysis. The re-

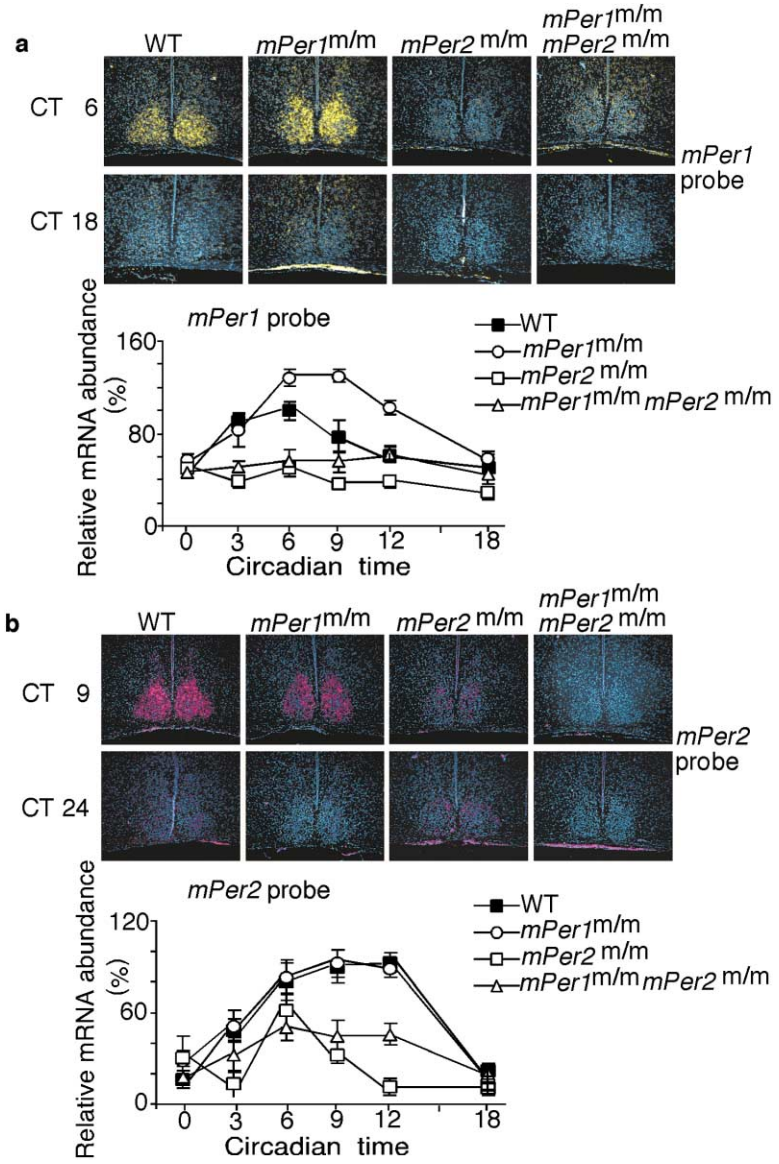

Figure 5. RNA In Situ Hybridization Results on SCN of F_2 Wild-Type, *mPer1* Mutant, *mPer2* Mutant, and *mPer1, mPer2* Double Mutant Mice

CT, Circadian time. (a) *mPer1* probe; (b) *mPer2* probe; quantification was based on at least two independent experiments, each performed on a different group of animals. Error bar = SD.

maining 16 genes displayed circadian expression in wild-type animals. Among the 16 CCGs, we observed four patterns of changes in circadian expression caused by the *mPer1* and *mPer2* mutations. Here, we present data on one example for each of these patterns (Figures 7 and 8). A common feature of all the CCGs is that circadian expression is observed in wild-type, but not in the *mPer1, mPer2* double mutant animals, substantiating a complete loss of clock function in the double mutants as assayed by output pathways other than locomotor function. It is noteworthy that a loss of circadian expression pattern in the CCGs can be reflected either by a flattening of the curve or a random pattern of peaks and troughs (for instance, Figure 7 top panel, *mCrbp1* probe in *mPer1* mutants).

The expression of some CCGs continues to cycle in *mPer2* but not *mPer1* mutants, as represented by the gene encoding the murine cellular retinol binding protein1 (*mCRBP1*), whose function is essential for vitamin A homeostasis (Ghyselinck et al., 1999) (Figures 7 and 8a). In contrast, a second set of CCGs continues to cycle in expression in *mPer1* but not *mPer2* mutants, as represented by a gene named NG27 that was identified as part of the mouse MHC class II locus of unknown physiological function (Figures 7 and 8b). Yet, there are CCGs the expression of which continues to cycle in both *mPer1* and *mPer2* mutants, as represented by the gene encoding the murine 5-aminolevulinate synthase 1 (*mAlas1*) (Figures 7 and 8c). 5-aminolevulinate synthase 1 is the enzyme in the first step of heme biosynthesis and controls the rate of heme synthesis in nonerythroid cells (Riddle et al., 1989). A last set of CCGs does not display a circadian profile in either *mPer1* or *mPer2* mutants, thus requiring both for circadian expression, as represented by the gene encoding the murine 5-aminolevulinate synthase 2 (*mAlas2*) (Figures 7 and 8d). The corresponding enzyme controls the rate of heme synthesis in erythroid cells (Riddle et al., 1989). These results indicate a complex pattern of circadian regulation of CCGs by mPER1 and mPER2.

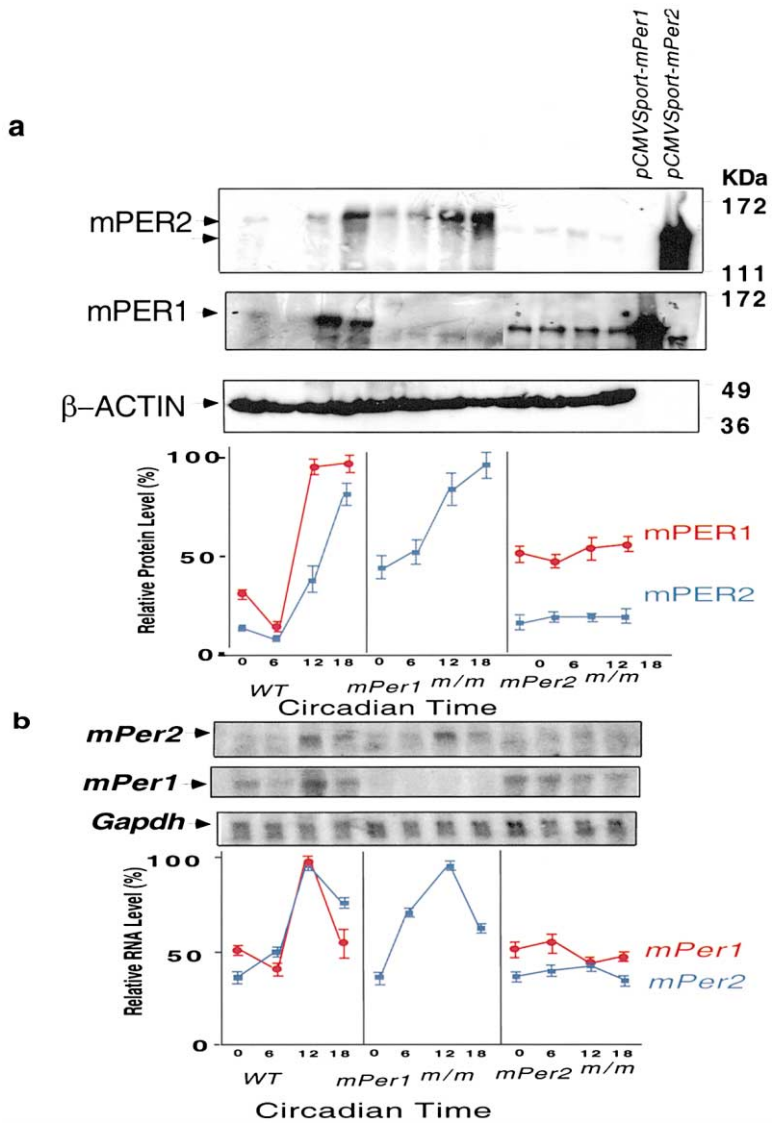

Figure 6. Western Analysis of a Peripheral Clock of *mPer* Mutants

(a) Western blot analysis was carried out on kidney tissues obtained from F_2 wild-type, *mPer1* mutant, and *mPer2* mutant at four circadian times. Transiently transfected COS cells with pCMV-SPORT-*mPer1* or pCMV-SPORT-*mPer2* were used as controls.

(b) Northern analysis on kidney RNA from different genotypes at four circadian times. For protein and RNA quantification, the intensity of each band was determined by scanning densitometry (Alpha Innotech Corp). Intensities of the mPER1 and mPER2 bands were normalized to β-actin levels. Intensities of *mPer1* and *mPer2* bands were normalized to *Gapdh* levels. The highest intensity was assigned a value of 100%. The data shown are representative of three independent studies with standard deviation. The *mPer1* and *mPer2* probes were from nucleotide position 1800–2510 (AF022992) and 1350–2650 (AF036893), respectively. Note that the *mPer1* probe and the mPER1 antigenic region in these experiments are in the region deleted in the *mPer1* mutant.

Discussion

Much of our initial knowledge for understanding the mammalian circadian clockwork is derived from studies of the *Drosophila* clock. In *Drosophila*, a transcription-translation feedback loop involving Per and Tim as negative elements, Clock and Cyc as positive elements, and the kinase Dbt as a critical modulator drives the rhythmic expression of *Per* and *Tim* (Young, 2000). The identification of mammalian clock genes, together with genetic and biochemical analysis of these genes, reveal a general conservation of clock components between mammals and *Drosophila*, yet with each showing distinct molecular features. To date, eight genes have been identified that are thought to be part of the central clock core mechanism of the mammalian circadian clock: *Clock, Bmal1, mPer1, mPer2*, and *mPer3, mCry1, mCry2, CK1ϵ*. Genetic evidence that supports their role in the clock mechanism has been shown for *Clock, mCry1, mCry2, mPer2*, *CK1ϵ*, *Bmal1* (Bunger et al., 2000; King et al., 1997; Lowrey et al., 2000; van der Horst et al., 1999; Zheng et al., 1999) and, in this study, for *mPer1*. It is noteworthy that so far, all mammalian circadian clock mutants are viable, suggesting that most, if not all, clock components are dedicated to behavioral control and are not essential for embryonic development.

There appear to be major differences in the negative control aspects of the mammalian circadian clock compared with the *Drosophila* paradigm. First, mammals have expanded Per to a family of three proteins: mPER1, mPER2, and mPER3, suggesting functional diversification. Second, mammals appear to lack a true ortholog of *Drosophila* Tim, the heterodimerization partner of the *Drosophila* Per (Clayton et al., 2001). This raises the possibility that the Per/Tim complex, the functional negative element in the *Drosophila* clock, has been replaced with other components in the mammalian clock. Third, mammals have expanded Cry to a family of two proteins, mCRY1 and mCRY2, which appear to have an essential negative regulatory role in the clock that is not shared by the *Drosophila* Cry (Kume et al., 1999; van der Horst et al., 1999).

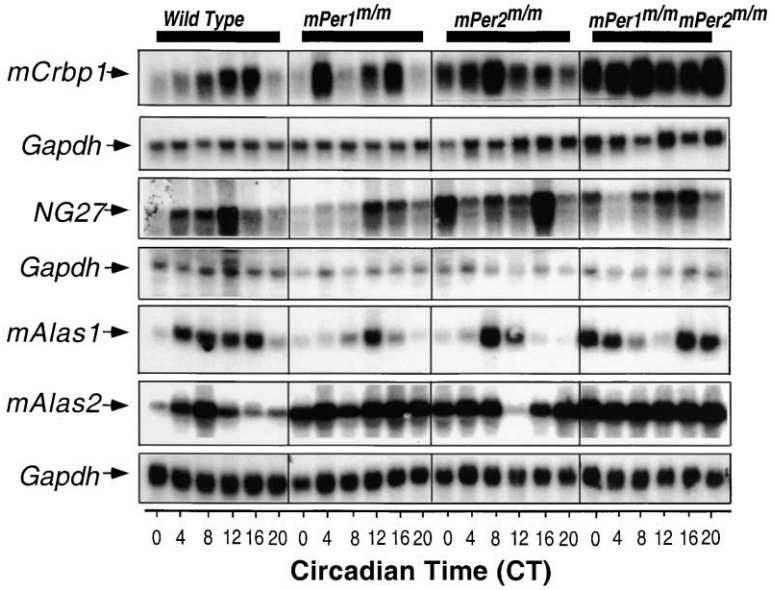

Figure 7. Differential Expression of Clock-Controlled Genes (CCGs) in *mPer1* and *mPer2* Mutants

The top four panels are derived from two blots probed sequentially with *mCRBP1* (~1.0 kb) or *NG27* (~2.0 kb), and *Gapdh* (1.8 kb). The bottom three panels are from another blot probed sequentially with *mAlas1* (~2.1 kb), *mAlas2* (~2.0 kb), and *Gapdh* (1.8 kb). 5′ and 3′ sequence information from the clones for *mCRBP1*, *NG27*, *mAlas1*, and *mAlas2* are identical to accession numbers X60367, AAC97965, W15813, and M63244, respectively. The Northern blots illustrated are from one of at least two duplicate sets of animals sacrificed.

The other parts of the clock appear to be more conserved between flies and mammals, including the positive control aspects of the feedback loop and the casein kinase 1ε (CK1ε) homolog. Homozygous *Clock* mutant mice have a longer period but lose persistence in their circadian rhythm (Vitaterna et al., 1994). Mice carrying a targeted *Bmal1* null mutation lose circadian rhythmicity immediately in DD, indicating an essential role that cannot be compensated by any other component in the clock (Bunger et al., 2000). A mutation in *CK1ε* is responsible for the shortened period displayed by the semidominant hamster *Tau* mutant, possibly by affecting the ability of the kinase to phosphorylate the mPER proteins (Lowrey et al., 2000).

We previously reported a deletion mutation in the *mPer2* gene, $mPer2^{Brdm1}$, that leads to a shortened period but shares a delayed loss of rhythm phenotype with the *Clock* mutation (Zheng et al., 1999). That the $mPer2^{Brdm1}$ mutation is a null mutation is supported by two pieces of evidence. First, the $mPer2^{Brdm1}$ mutant phenotype is recessive. Second, we recently generated a truncation mutation in the *mPer2* gene that disrupts the PAS domain and places all downstream coding sequence out of frame. We found that compound heterozygous mutants between the truncation mutation and $mPer2^{Brdm1}$ show the same phenotype as that of $mPer2^{Brdm1}$ homozygous mutants (C.C.L. et al., unpublished data). These studies support a role for *mPer2* in the core clock mechanism.

Here, we described the phenotypic and molecular analyses of a targeted null mutation of *mPer1*. On average, the *mPer1* mutants display a moderately shorter period (by ~1 hr), similar to the phenotype of a recently described *mPer3* mutant (by ~0.5 hr) (Shearman et al., 2000a). However, the unique feature of the *mPer1* mutant is that the clock has reduced precision and stability, indicating a more profound disruption of the intrinsic properties of the clock as compared with the *mPer3* mutant. Loss of rhythmicity was occasionally observed in *mPer1* mutants, but these are greatly outnumbered by mutants with persistent rhythms. Two pieces of evidence argue against genetic background as the basis for this variability in phenotype. First, inbred 129S7 mutants show the same variability in period. Second, when the same *mPer1* mutant animals were reassessed, their period lengths could vary significantly from the first assessment. Overall, the phenotype of the *mPer1* mutant is mild in contrast with that of the *mPer2* mutant, which exhibits an eventual breakdown of the circadian rhythmicity (Zheng et al., 1999). Nevertheless, *mPer1*, *mPer2* double mutants have no circadian rhythms, assayed either at the behavioral or the molecular level. This result implicates *mPer1*, along with *mPer2*, in the central clock mechanism because an immediate and complete loss of rhythm phenotype in double mutants would not be expected if mPER1 were not involved in the core clock mechanism.

Loss of mPER1 did not disrupt the rhythmic expression of its mutant transcript, indicating that mPER1 is not essential to maintain its own circadian expression. Although the mutant *mPer1* transcript oscillates with a higher peak and an apparent phase delay compared with the wild-type controls (Figure 5a), this may simply reflect an increased stability of the mutant transcript. Consistent with this possibility is our observation that the level of the noncycling *mPer1* transcripts throughout the brain is elevated in the *mPer1* mutant (data not shown). None of the other clock genes, including *mPer2, mCry1*, and *mBmal1*, shows any obvious change in expression levels in the absence of mPER1 (this study and our unpublished observations). These observations deviate from the expected role for mPER1 in the negative feedback that has been demonstrated for dPer in *Drosophila* and inferred from in vitro studies with mammalian cell cultures (Darlington et al., 1998; Kume et al., 1999; Sangoram et al., 1998). Indeed, among the three mPERs, mPER2 is the only one that appears to play a role in the transcriptional control of clock genes (Shearman et al., 2000a; Zheng et al., 1999; and this study). Furthermore, the transcriptional control by mPER2 appears to be in the opposite direction to its *Drosophila* counterpart: loss of mPER2 function results in significantly reduced peak expression of *mPer1*,

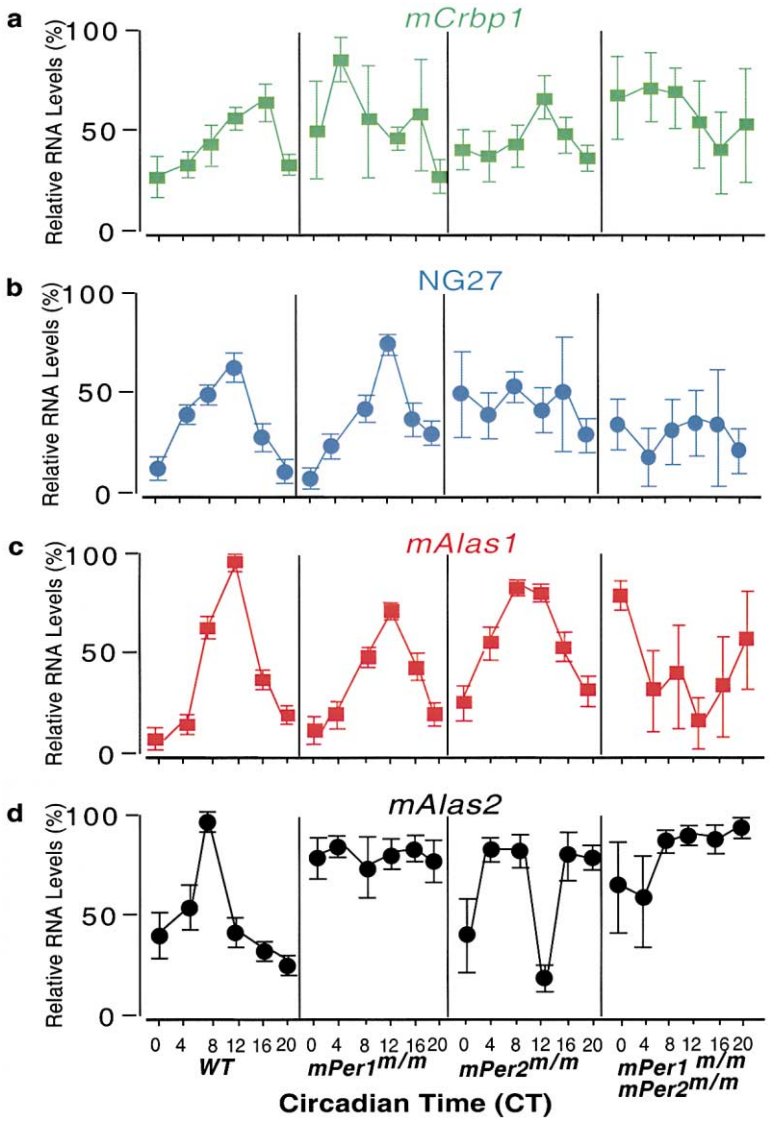

Figure 8. Quantitation on the Northern Analysis of Four CCGs, as Shown in Figure 7

Data are from two duplicate sets of animals sacrificed (three sets of animals for *mAlas1*). (a) *mCRBP1* probe; (b) *NG27* probe; (c) *mAlas1* probe; (d) *mAlas2* probe. The CCG signals are normalized with *Gapdh* levels. Error bar = SD. Note that due to the randomness in peak and trough expression, the expression pattern of the affected *mPer* mutants can vary widely from one experiment to another, resulting in large standard deviations that are not observed in wild-type animals.

mPer2, and *mCry1* (Shearman et al., 2000b; Zheng et al., 1999), indicating that mPER2 exerts a positive regulation on clock gene expression. Thus, the involvement of mammalian PERs in the clock appears to be mechanistically different from that of the *Drosophila* Per.

These observations raise the question as to how mPER1 regulates the mammalian circadian clock. Our studies of the peripheral clock suggest that mPER1 has a role in the clock at a posttranscriptional level. The loss of mPER1 results in an enhanced level of mPER2 in the mutant, suggesting that mPER1 normally represses mPER2 levels in vivo. As mPER1 and mPER2 have been shown to interact in vitro and in vivo (Field et al., 2000; Kume et al., 1999), this posttranscriptional regulation of mPER2 by mPER1 may be mediated through a direct protein-protein interaction. Whether posttranscriptional regulation by mPER1 mediates the circadian phenotype of the *mPer1* mutants remains to be determined.

One prominent difference between the *Drosophila* clock and the mammalian clock is the complexity of the output pathways that the clock controls. We searched for clock-controlled genes (CCGs) by comparing expression profiles at different times or in different genotypes using cDNA micro-array technology. Candidate genes were then analyzed by Northern analysis throughout a circadian cycle in wild-types and single and double *mPer* mutants. We identified at least 16 CCGs whose circadian expression depends on either or both mPER1 and mPER2. Although loss of circadian expression of CCGs in the *mPer2* mutants can be attributed partially to the loss of rhythms, this cannot apply to the *mPer1* mutants. The presence of CCGs whose circadian expression is abolished in *mPer1* but not *mPer2* mutants suggests that while mPER1 is not a major contributor of the RNA oscillation of the clock genes, it regulates some output pathways that are not shared by mPER2. Regardless of whether a particular CCG loses circadian expression in the *mPer1* or the *mPer2* mutants, the simultaneous loss of the circadian expression of many CCGs in the double mutants further supports a complete loss of clock function in these animals.

One of the CCGs we identified, *mCRBP1*, encodes a protein involved in vitamin A homeostasis. Recently, it has been shown that mice deficient in mCRBP1 are

essentially normal but, when reared on a vitamin A-deficient diet, develop abnormalities characteristic of postnatal hypovitaminosis (Ghyselinck et al., 1999). In the absence of vitamin A, *mCRBP1* mutant animals display a markedly altered electroretinogram, and the normal intimate contact between retinal pigment epithelium and outer segment is disrupted (Ghyselinck et al., 1999). Given our observation that expression of *mCRBP1* is under circadian control, it would be interesting to test whether mCRBP1 is involved in circadian photo response.

The observation that the genes encoding the rate-limiting enzymes for heme biosynthesis, *mAlas1* and *mAlas2*, are under circadian control is of particular interest. Circadian expression of *mAlas1* and *mAlas2* is completely disrupted in the double mutants, indicating that mPER1 and mPER2 regulates the availability of heme. The body level of cellular heme is tightly controlled, and this is thought to be achieved by a balance between heme synthesis and catabolism, the latter by heme-oxygenase. Two models have been proposed for the regulation of heme biosynthesis. The first model is based on a negative feedback of heme on *Alas1* message stability and on a posttranslational transport control (Hamilton et al., 1991; Kikuchi and Hayashi, 1981). A second model is based on a negative feedback of heme on the transcriptional control of *Alas1* expression (Kappas et al., 1968). Our observation here suggests a model based on a transcriptional control of *mAlas*1 expression by the circadian clock as a plausible mechanism for regulating levels of ALAS activity, and thereby heme levels, in mice. Our Northern analysis with the gene for heme-oxygenase 2 shows constant expression in all four genotypes (data not shown), suggesting that unlike its biosynthesis, the rate of heme catabolism by heme-oxygenase 2 occurs at a constant rate. The clock control of the availability of heme may have a wider implication for temporal control of the biochemical and physiological processes of an organism. It is well known that heme serves as a prosthetic moiety for many heme proteins that are involved in a vast array of biological functions. Among key proteins that contain heme are proteins involved in oxygen metabolism (myoglobin, hemoglobin, catalase, etc), electron transfer (cytochromes c and p450), and signaling (guanylyl cyclases, nitric oxide synthase) (Lucas et al., 2000; Ponka, 1999; Stuehr, 1997). It is thus possible that temporal control of heme biosynthesis could be a basis for a wider range of cascades in physiological processes. An interesting question raised here is whether the heme itself is important in regulating the clock. An independent link of heme to circadian regulation is that some PAS proteins are heme binding proteins. Recently, three bacteria PAS proteins, *Ax*PDEA1, Dos, and FixL, have been shown to be heme binding proteins (Chang et al., 2001; Delgado-Nixon et al., 2000; Gong et al., 1998). For at least one of these, FixL, the PAS domain, in addition to being required for protein to protein interactions, is also required for heme binding (Gong et al., 1998). PAS motifs from bacteria to mammals have highly conserved three-dimensional folds even though the amino acid sequence identity is low (Pellequer et al., 1998, 1999). Thus, it is possible that other PAS proteins, including those in mammals, are potential heme binding proteins. Indeed, the mouse NPAS2, a protein that is highly homologous to CLOCK, is a heme binding protein (S.L. McKnight, personal communication). Both NPAS2 and CLOCK can form heterodimer with BMAL1 protein to form an active transcription complex (Garcia et al., 2000). The regulation of heme level via mPER regulation of *mAlas1/2* expression may be part of an interface between the core clock mechanism and the cellular/subcellular environment. It can be envisaged that the availability of heme controls the function of heme binding PAS proteins like NPAS2, which in turn regulates their activity with their transcriptional partner protein BMAL1. The transcriptional activity of the BMAL1/NPAS2 or BMAL1/CLOCK would then regulate the expression of clock genes such as the *mPers*. The levels of mPER proteins in turn regulate heme biosynthesis via control of *mAlas1/2* expression. Such a model would provide a plausible interactive regulation between the biochemical/physiological conditions and the clock mechanism.

In summary, our data provide compelling evidence that *mPer1* and *mPer2* have distinct roles in the clock. mPER2 regulates the circadian cycle via a transcriptional control while mPER1 may operate via a posttranscriptional control. The loss of mPER1 and mPER2 results in a complete loss of circadian rhythms and is reflected at the molecular level by the loss of the rhythmic expression of both clock genes and CCGs. Our data demonstrate that some CCGs are differentially regulated by mPER1 and mPER2. Together with our studies that implicate roles for mPER1 and mPER2 in the input pathway of the clock (Albrecht et al., 2001), these studies indicate that mPER1 and mPER2 play multiple, nonredundant roles in circadian clock control. Finally, our studies imply that the regulation of cellular heme is under circadian control. The connection to heme biosynthesis would suggest both circadian control of physiological processes by regulating heme metabolism and a possible regulatory role of heme metabolism on the clock mechanism itself, possibly via PAS proteins that are heme binding.

Experimental Procedures

Constructions of the Targeting Vector

A genomic clone was isolated from a mouse 129S7 genomic library using a mouse *mPer1* cDNA probe. A replacement vector was constructed with *PGK-Hprt* as the positive selection marker and *HSVtk* as the negative selection marker. A 2.5 kb fragment was used as the 5′ homology region and a 5.3 kb fragment was used as the 3′ homology region. A 4.3 kb fragment between an XhoI site in exon 4 and a BglII site in exon 18 was deleted.

Characterization of the Circadian Phenotype

Unless stated otherwise, all animals were raised in LD 12:12 and used in respective experiments for the first time. Mice were housed in individual cages equipped with a running wheel in ventilated, light-tight chambers with controlled lighting. Wheel-running activity was monitored by an online PC computer equipped with the Chronobiology Kit (Stanford Software Systems, Santa Cruz, CA). The activity records are double plotted so that each day's activity is shown both to the right and below that of the previous day. To determine the period length, activity data for a 10 day interval upon release into DD were analyzed with a χ^2 periodogram (Sokolove and Bushell, 1978) using the Stanford Chronobiology Kit. Fourier periodogram analysis in the Chronobiology Kit was performed to assess the strength of any activity rhythm.

In Situ Hybridization

For DD data, light was turned off at ZT 12 and kept off throughout the rest of the experiment. Mice were sacrificed on the second day into DD by cervical dislocation under a 15 W safety red light. The *mPer1* probe corresponds to nucleotides 1 to 619 of GenBank accession number AF022992. The *mPer1* and *mPer2* probes are outside of the region deleted in the respective mutant. Quantification was performed by densitometric analysis of hybridization signals on X-ray films using NIH Image 1.6 software. For silver grain images, tissue was visualized by fluorescence of Hoechst dye-stained nuclei (blue color in figures), and silver grain signals artificially colored for clarity.

RT-PCR and DNA Sequence Analysis

For RT-PCR analysis, first strand cDNA was generated using SuperScript II reverse transcriptase (GIBCO-BRL) and oligo-dT priming from total kidney RNA. An aliquot of the first strand cDNA was then amplified by PCR with an m*Per1* exon 2 forward primer CCC TGT TTC GTC CTC CAC TGT ATG and an *Hprt* exon 6 reverse primer GCA TTG TTT TGC CAG TGT C. The RT-PCR product was then sequenced with an m*Per1* exon 3 forward primer ACA AAC TCA CAG AGC CCA TCC.

Western Blot Analysis

Rabbit antibodies to mPER proteins were raised from a His-tag fusion protein containing amino acids 974 to 1291 of mPER1 or amino acids 486 to 649 of mPER2. Animals from the respective CTs were sacrificed and protein samples were prepared from kidney tissues with standard protocols. For Western analysis, 150 μg of total protein (as determined by the Bradford assay, Bio-Rad) was used for each time point. Anti-sera against either mPER1 or mPER2 were diluted 1:5000 for these studies. Horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (Sigma) were used under the suggested working condition. Signals on film were detected by ECL kit (Amersham Life Technology).

Micro-Array and Northern Blot Analysis

Liver total RNA was prepared using the GTC/CsCl method (Chirgwin et al., 1979) from mature wild-type and *mPer* mutant animals that were maintained in 12:12 LD cycle since birth, and animals that had been maintained in DD for at least 24 hr. 1 μg mRNA was labeled by Thermoscript reverse transcriptase (GIBCO-BRL) using dNTP containing either Cy5-dCTP or Cy3-dCTP for signal detection. The cDNA probe generated was used to screen a mouse 6K micro-array assembled from a UniGene cDNA library. The conditions used are detailed in Cheung et al. (1999). We performed Northern analysis on total liver RNA using denaturing formaldehyde gels. For each sample, 15 μg of total RNA was used. For comparative purposes, the same blot was stripped and re-used for hybridization. The relative level of RNA in each lane was determined by hybridization with mouse *Gapdh* cDNA.

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