Neuron, Vol. 20, 1103–1110, June, 1998, Copyright ©1998 by Cell Press

Three *period* Homologs in Mammals: Differential Light Responses in the Suprachiasmatic Circadian Clock and Oscillating Transcripts Outside of Brain

Mark J. Zylka,*†‡ Lauren P. Shearman,*‡
David R. Weaver,* 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

Summary

We have cloned and characterized the mouse cDNA of a third mammalian homolog of the *Drosophila period* gene and designated it *mPer3*. The mPER3 protein shows ~37% amino acid identity with mPER1 and mPER2 proteins. The three mammalian PER proteins share several regions of sequence homology, and each contains a protein dimerization PAS domain. *mPer3* RNA levels oscillate in the suprachiasmatic nuclei (SCN) and eyes. In the SCN, *mPer3* RNA levels are not acutely altered by light exposure at different times during subjective night. This contrasts with the acute induction by light of *mPer1* and *mPer2* RNA levels during early and late subjective night. *mPer3* is widely expressed in tissues outside of brain. In liver, skeletal muscle, and testis, *mPer* RNAs exhibit prominent, synchronous circadian oscillations. The results highlight the differential light responses among the three mammalian *Per* genes in the SCN and raise the possibility of circadian oscillators in mammals outside of brain and retina.

Introduction

The suprachiasmatic nuclei (SCN) of the anterior hypothalamus function as a circadian clock in mammalian brain, generating circadian rhythms in physiology and behavior (reviewed by Reppert and Weaver, 1997). The daily light–dark cycle acts through retina-to-SCN neural pathways to entrain (synchronize) the SCN clock and its output rhythms to the 24 hr day (reviewed by Klein et al., 1991). The SCN is a multioscillator clock with the entire oscillatory mechanism residing in single neurons (Welsh et al., 1995; Liu et al., 1997). Until very recently, knowledge of the molecular components and mechanisms underlying mammalian circadian clocks has been lacking. Substantial evidence in *Drosophila melanogaster* and *Neurospora crassa* shows that actual "clock genes" exist and that central clock mechanisms involve transcriptional/translational feedback loops (reviewed by Dunlap, 1996; Hall, 1996).

The cloning and characterization of two mammalian homologs of the *Drosophila* clock gene *period* (*per*) over the past year has provided tantalizing evidence that actual molecular components of the SCN clock have now been identified (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997). The protein products of these mammalian genes, designated *mPer1* and *mPer2* in the mouse, share several regions of homology with the *Drosophila* PER protein, including the signature PAS domain. Moreover, *mPer1* and *mPer2* RNA levels exhibit prominent circadian rhythms in the SCN (Albrecht et al., 1997; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997), and both *mPer1* and *mPer2* RNAs in the SCN are increased by light exposure during subjective night but not during subjective day (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). In fact, extensive correlative evidence suggests that *mPer1* RNA induction might be the initial clock-specific molecular event for photic entrainment in the SCN (Shigeyoshi et al., 1997).

In the present report, we have cloned and characterized a third member of the mammalian *Per* gene family in the mouse, designated *mPer3*. The results suggest that *mPer3* is a clock-relevant member of the mammalian *Per* gene family. The mPER3 protein contains a PAS region and its structure is most similar to mPER1 and mPER2. *mPer3* RNA levels exhibit a robust circadian variation in the SCN that is similar to RNA rhythms for *mPer1* and *mPer2*, but, unlike *mPer1* and *mPer2*, *mPer3* RNA levels are not affected by light pulses during subjective night. We also found that there are qualitative and quantitative differences in the response of *mPer2* RNA levels to light pulses during early and late subjective night. This differential response in *mPer2* gene expression may contribute to the differential behavioral responses to light exposure at these two circadian times. All three mammalian *Per* genes are rhythmically expressed in tissues outside of brain. These peripheral molecular oscillations raise the possibility that nonneural circadian oscillators exist in mammals.

Results

Cloning and Structural Assessment of *mPer3*

We previously identified a family of three mammalian PER proteins based on phylogenetic analysis of the PAS domains from several PAS-containing proteins (Shearman et al., 1997). PAS is an acronym for the first three proteins found to share this protein dimerization domain: *Drosophila* PER, the human aryl hydrocarbon receptor nuclear transporter (ARNT), and the *Drosophila* single-minded protein (SIM). Sequence of the third mammalian PER, designated hPER3, was found within a human chromosome 1 genomic contig (GenBank accession number Z98884). Using BLAST and FASTA algorithms, a mouse expressed sequence tag (EST; GenBank accession number AA451527) was found that was >80% identical at the amino acid level to sequence within the *hPer3* genomic contig. Likewise, sequence analysis indicated that AA451527 was similar to mPER1 and mPER2 within

‡These authors contributed equally to this work.
§To whom correspondence should be addressed.

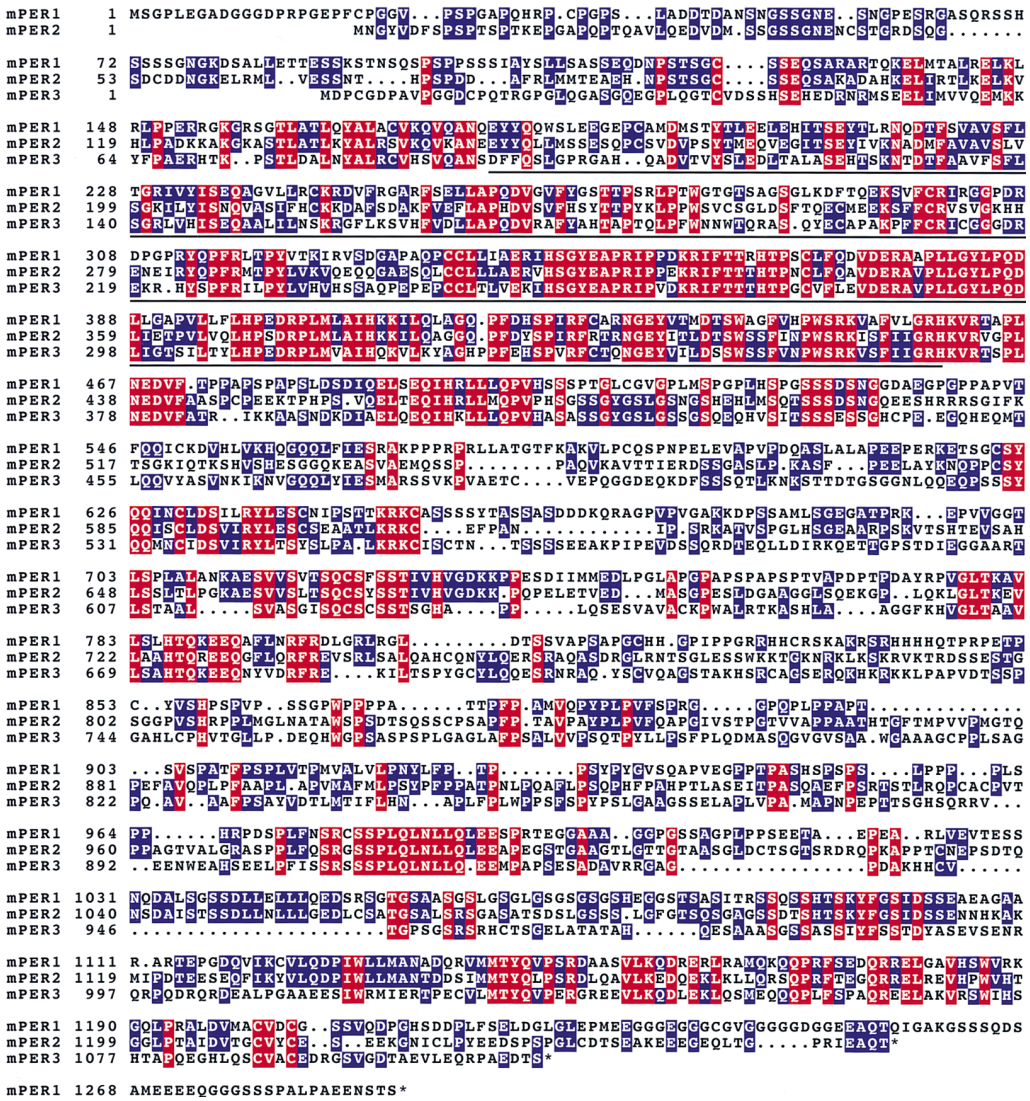

Figure 1. Comparison of Mammalian *Per* Proteins

The three sequences (mPER1, mPER2, and mPER3) were aligned using the GAP program. To maximize homologies, gaps (indicated by dots) have been introduced into the sequences. The underlined region denotes the PAS domain. Red indicates consensus among all three proteins; blue indicates consensus among two of the three proteins. mPER1 sequence is from Sun et al. (1997) (GenBank accession number AF022992). mPER2 sequence is from Shearman et al. (1997) (GenBank accession number AF035830). mPER3 sequence has been deposited in GenBank as accession number AF050182.

a short conserved region but was clearly not mPER1 or mPER2. Primers were then designed based on sequence from this EST to clone the cDNA by 5′ and 3′ rapid amplification of cDNA ends (RACE).

The cloned cDNA of *mPer3* contains an open reading frame (ORF) of 1113 amino acids. The first in-frame methionine contains a Kozak consensus sequence and is preceded by a stop codon. Sequence spanning the entire ORF could be detected within various exons of Z98884 with >80% amino acid overlap using TBLASTN. This suggested that Z98884 contains the entire *hPer3* gene and confirmed that the ORF of our cloned cDNA encodes the mouse homolog of *hPer3*.

BLAST analysis revealed that mPER3 is most similar to mPER1 and mPER2. Overall, mPER3 showed 36% and 37% amino acid identity to mPER1 and mPER2, respectively. This contrasts with the higher amino acid identity (46%) between mPER1 and mPER2. When the three proteins were aligned, several highly conserved regions were apparent (Figure 1). The largest conserved region is present within the PAS domain (underlined in Figure 1). There are several smaller regions of amino acid identity shared by the three proteins outside of the PAS region. Interestingly, mPER3 lacks several of the small conserved regions shared by mPER1 and mPER2. The absence of these conserved regions was confirmed by sequencing RT-PCR fragments spanning the *mPer3* cDNA and by searching for these motifs within Z98884.

ProfileScan revealed the presence of a PAS repeat in mPER3 but did not detect a basic helix–loop–helix

(bHLH) motif or nuclear localization signals. However, the PHDsec program (EMBL) detected an HLH motif. This motif was detected in mPER3 at the same location as the HLH motif previously defined in PER1 (Sun et al., 1997).

mPer3 Gene Expression Is Widespread in Brain

In situ hybridization was used to examine the distribution of *mPer3* gene expression in sagittal sections and in coronal sections of mouse brain at the level of the SCN. Hybridization with the antisense probe revealed highest levels of *mPer3* gene expression in the SCN, hippocampus, piriform cortex, and cerebellum (Figure 2 and data not shown). Lower levels of *mPer3* RNA were detected in neocortex. The sense (control) riboprobe had a reproducible, robust, anatomically specific pattern of hybridization that did not overlap with the antisense probe. Specifically, the sense probe labeled the supraoptic and paraventricular nuclei of the hypothalamus (data not shown). No variations in sense probe hybridization intensity were noted in the experiments detecting rhythmic expression of *mPer3* RNA.

mPer3 RNA Levels Exhibit a Circadian Oscillation in the SCN and Eyes

mPer3 RNA levels in the SCN were studied at six time points over a 24 hr period on the first day in constant darkness (DD). *mPer3* RNA levels were rhythmic ($p < 0.0001$, ANOVA), with highest levels during the subjective day at circadian time (CT) 6 and CT 9 (Figure 2). *mPer3* RNA levels at night (CT 15, 18, and 21) were significantly lower than at either of these daytime points, while the levels at CT 3 were intermediate (differing from CT 18 and CT 21 but not from CT 15; Scheffe's f test, $p < 0.05$). The peak–trough amplitude of the *mPer3* specific hybridization in SCN was 2.1-fold (see Experimental Procedures for amplitude calculation). The phase of the *mPer3* RNA rhythm was very similar to the phase of *mPer1* and *mPer2* rhythms in the SCN determined in previous experiments (Figure 2, bottom; *mPer1* and *mPer2* RNA patterns replotted from Shearman et al., 1997).

mPer3 also displayed a circadian rhythm in RNA abundance in eyes, synchronous with *mPer1* and *mPer2* eye rhythms (Shearman et al., 1997), with peak levels between CT 9 and CT 21 (data not shown). This represents a 3–6 hr difference in the *mPer* RNA rhythms between the eyes and the SCN. A specific hybridization signal for *mPer3* was found in retina using in situ hybridization (data not shown), as previously noted for *mPer1* and *mPer2* (Shearman et al., 1997). Thus, the RNA rhythms for all three *mPer* genes appear to originate from retina, the site of a known circadian clock (Tosini and Menaker, 1996).

Differential Light Regulation of *mPer1*, *mPer2*, and *mPer3* in the SCN

Previous studies have consistently shown that *mPer1* expression in the SCN is rapidly induced by exposure to light at night (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). With *mPer2*, however, the data are not consistent. We reported a significant

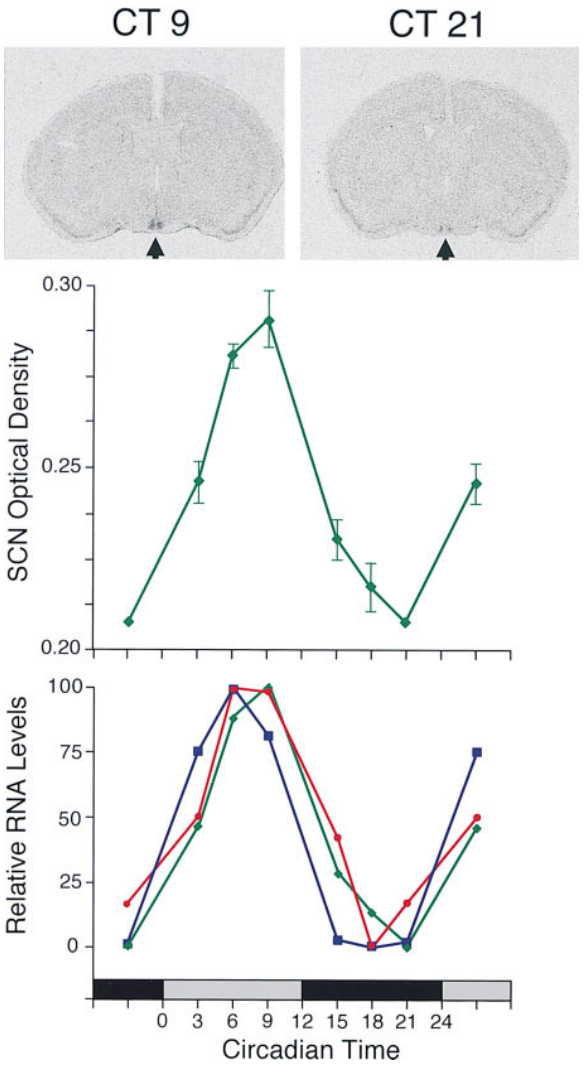

Figure 2. Circadian Expression of *mPer3* in the SCN

(Top) Expression of *mPer3* in the SCN at CT 9 (left) and CT 21 (right). Coronal sections (15 μm) were examined by in situ hybridization using antisense cRNA probes. Magnification, 4×.

(Center) Circadian pattern of *mPer3* expression in the SCN. Each value is the mean ± SEM of six to eight animals.

(Bottom) Comparison of the circadian patterns of *mPer1* (blue), *mPer2* (red), and *mPer3* (green) RNA levels in the SCN. To calculate relative RNA values while equalizing differences in rhythm amplitude and baseline, data are normalized so that the peak OD value equals 100% and the nadir value equals 0% for each probe. Original data for *mPer1* and *mPer2* are from Shearman et al. (1997). The horizontal bar at the bottom of the panel represents the lighting cycle prior to placement in DD; shaded areas represent subjective day and black areas represent subjective night. Data at CT 21 and CT 3 are double plotted.

increase in *mPer2* expression 3 hr after 30 min light exposures at CT 14 and at CT 19, whereas Albrecht et al. (1997) did not observe any effect of 15 min light exposure on *mPer2* at CT 22. This difference in response could be due to the difference in the circadian time of light exposure or in the time points analyzed. Our preliminary studies indicated that *mPer2* levels increased unexpectedly 30 min (but not 3 hr) after exposure to light at CT 0 (during the phase-advance portion

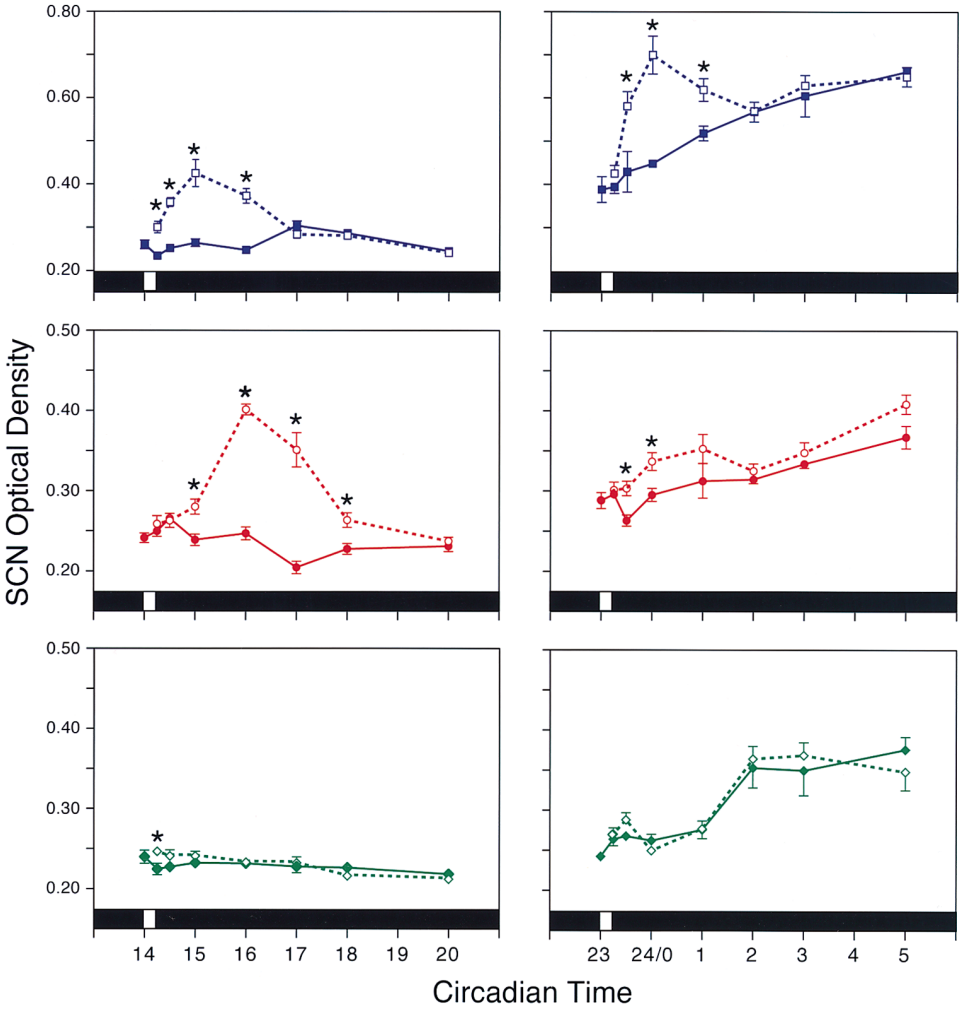

Figure 3. Light Regulation of *mPer1*, *mPer2*, and *mPer3* RNAs in the SCN

Animals were entrained to a 12 hr light:12 hr dark lighting cycle and then placed in DD. Light pulses (400 lux; 15 min duration) were applied at CT 14 (left) or CT 23 (right) during the first day in DD. Coronal sections (15 μm) through the SCN were examined for either *mPer1* RNA (blue; top), *mPer2* RNA (red; center), or *mPer3* RNA (green; bottom) by in situ hybridization using antisense cRNA probes. The horizontal bars below each panel indicate the lighting conditions for animals studied after exposure to light. Open symbols and dashed lines denote light-exposed animals; closed symbols denote time-matched controls maintained in DD. Each value is the mean ± SEM of four to six animals (*$p < 0.05$, Student's t test).

of the circadian cycle) and that *mPer3* levels were unaffected at either 30 min or 3 hr after light exposure at CT 3, CT 14, CT 19, and CT 0. These observations emphasized the need to examine a detailed time course of *mPer1*, *mPer2*, and *mPer3* gene expression in the SCN following light exposure. Circadian times 14 and 23 were chosen for study because light pulses given at these times produce phase delays and advances in behavior rhythms, respectively (Schwartz and Zimmerman, 1990).

mPer1 RNA levels increased rapidly and transiently after 15 min light exposure beginning at either CT 14 or CT 23 (Figure 3, top). *mPer1* RNA levels in the SCN were elevated within 15–30 min, peaked at 1 hr, and returned to control levels within 3 hr of exposure. *mPer2* RNA levels increased more slowly following light exposure at CT 14. *mPer2* RNA levels in the SCN were significantly elevated at 1 hr, peaked at 2 hr, and returned to control levels within 6 hr of exposure (Figure 3, center). In contrast, at CT 23, light exposure increased *mPer2* RNA levels rapidly (within 30 min) and briefly, with *mPer2* RNA levels returning to control levels within 2 hr of exposure. The amplitude of the response to light at CT 23 was also reduced compared to light exposure at CT 14 for *mPer2* but not for *mPer1*. Thus, there are both temporal and quantitative differences in *mPer2* regulation following light exposure at CT 14 and CT 23.

In marked contrast to the photic induction of *mPer1* and *mPer2* RNA levels during subjective night, *mPer3* RNA levels were unresponsive to light exposure at both CT 14 and CT 23 (Figure 3, bottom). A significant difference between light- and dark-exposed mice was observed at only one time, 15 min after light exposure at CT

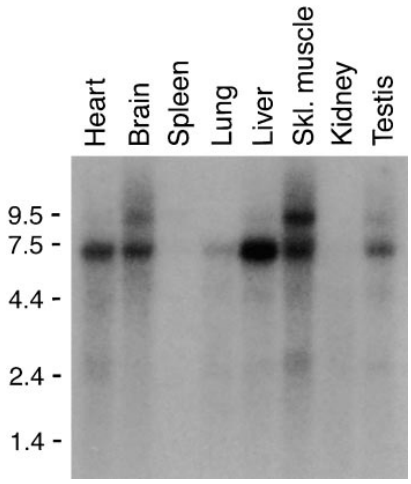

Figure 4. Northern Blot Analysis of *mPer3* Gene Expression in Multiple Tissues

Blot was purchased from Clontech. Each lane contained 2 μg of poly(A)$^+$ RNA. Exposure time was 40 hr.

14. This difference appeared due to low optical density values in the dark-exposed controls, which were also lower than the values from mice collected 15 min earlier.

mPer3 Transcripts Are Widely Expressed Outside of Brain and *mPer* Transcripts Exhibit Circadian Oscillations in Peripheral Tissues

We determined whether *mPer3* is as widely expressed in peripheral tissues as *mPer1* and *mPer2*. Northern blot analysis of RNA from several tissues showed that the *mPer3* gene is also widely expressed (Figure 4). A major transcript of 7.0 kb was detected in most tissues. Less abundant transcripts of 2.4 kb, 4.4 kb, and 9.0 kb were also detected. The levels of *mPer3* RNA detected in spleen and kidney were low in comparison with those in the other tissues examined.

If *mPer1*, *mPer2*, and *mPer3* are components of circadian clocks that reside in the SCN and retinae, why is the expression of these genes so widespread? Recent experiments using transgenic *Drosophila* expressing luciferase under the control of the *per* promoter identified light-sensitive, autonomous circadian clocks in many nonneural body parts that express *per* (Plautz et al., 1997). These observations hint that circadian clocks may exist in all or many of the mammalian tissues that express the *Per* genes. If this is indeed the case, then we would expect that expression of the three *Per* genes would be rhythmic in nonneural tissues in which they are expressed. We thus examined the daily profiles of *mPer1*, *mPer2*, and *mPer3* gene expression in three peripheral tissues collected from the animals used for the study depicted in Figure 2. The tissues examined included liver, skeletal muscle, and testis.

Circadian rhythms in RNA abundance were evident in all three tissues, with peak levels centered around CT 9–18 (Figure 5). Rhythms in *mPer3* were only clearly found for the 7.0 kb transcript in liver and testis. In skeletal muscle, there were synchronous *mPer3* rhythms for both the 7.0 kb and 9.0 kb transcripts (see autoradiograms in Figure 5). *mPer2* RNA levels were not quantified in the testis because of low levels of expression. All peripheral tissues, with the exception of liver, displayed broad peaks in *mPer* RNA abundance. Levels of all three *mPer* RNAs were sharply elevated at CT 15 in liver. In general, the phase of the circadian oscillations in *mPer* RNA levels in the three peripheral tissues was more similar to that in retina than in the SCN.

Discussion

Structural and functional analyses show that *mPer3* is a clock-relevant member of the mammalian *Per* gene family. Structurally, mPER3 contains a PAS domain and shows highest sequence identity/homology with mPER1 and mPER2. Outside of the PAS region, mPER3 shares several stretches of amino acid identity with mPER1 and mPER2. However, there appear to be more regions of amino acid identity shared between mPER1 and mPER2 than shared between mPER3 and either of the other two PER proteins. The functional significance of these structurally conserved non-PAS areas among the mammalian *Per* family members will be important to delineate.

Our functional analysis shows that *mPer3* RNA levels exhibit circadian oscillations in both the SCN and eyes, sites of known circadian clocks. Interestingly, in each tissue, *mPer3* RNA oscillations are similar to those for both *mPer1* and *mPer2*. The similar oscillations of these three *Per* genes in clock structures suggest the potential for protein–protein interactions through their PAS domains and participation in oscillating transcriptional feedback loops. The recently cloned *Clock* gene encodes a bHLH/PAS-containing protein that probably functions as an important transcription factor in the SCN clock mechanism (Vitaterna et al., 1994; King et al., 1997). In fact, *Clock* may be a positive element driving the transcription of any or all mammalian *Per* genes. In *Drosophila*, a strong case can be made for such CLOCK–*per* gene interactions. The *Drosophila per* promoter contains an E box element that is essential for high level *per* expression (Hao et al., 1997), the E box sequence is a bHLH DNA-binding motif (Murre et al., 1989), and a *Clock* homolog has been recently identified in the fly (dbEST AA698290).

Acute light regulation of the *mPer3* gene in the SCN is strikingly different from that of the *mPer1* and *mPer2* genes. *mPer3* RNA levels are unresponsive to light pulses applied throughout the circadian cycle. This contrasts strongly with the acute photic induction of both *mPer1* and *mPer2* RNA levels during subjective night (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997). This differential regulation among the three *mPer* genes suggests that each gene subserves a distinct function in the SCN and has distinct regulatory elements.

A provocative finding that surfaced from our current light pulse experiments is the different response of *mPer2* to light pulses applied during early and late subjective night. The behavioral effects of photic stimuli at

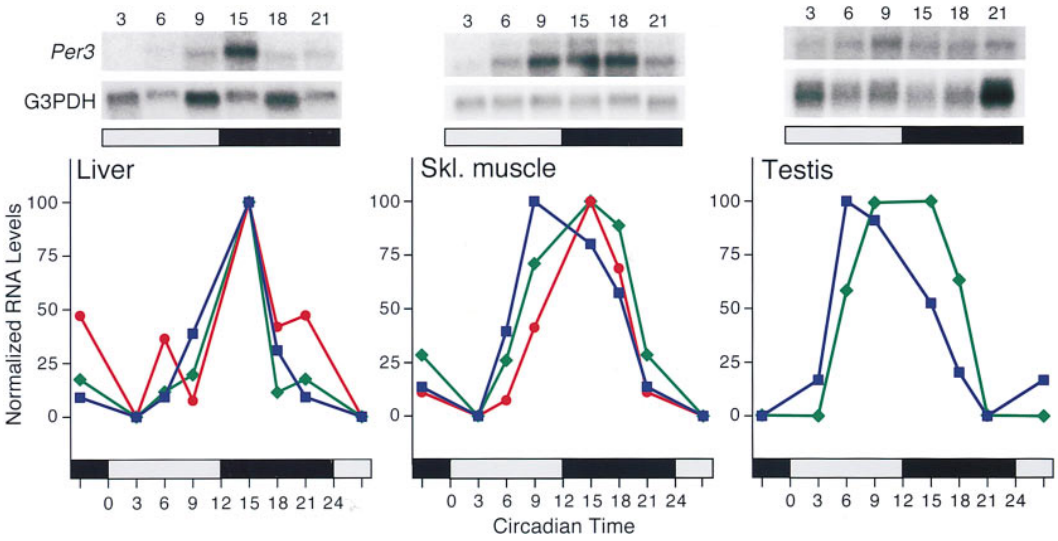

Figure 5. Circadian Oscillation in *mPer* Gene Expression in Nonneural Tissues

For each panel, the upper autoradiograms depict hybridization densities for *mPer3* (7.0 kb transcript) and G3PDH (control) at each circadian time. The graphs depict a comparison of the circadian patterns of *mPer1* (blue), *mPer2* (red), and *mPer3* (green) RNA levels in each tissue. To calculate relative RNA values while equalizing differences in RNA abundance and baseline, data were normalized so that the peak hybridization value equals 100% and the nadir value equals 0% for each probe. Data at CT 21 and CT 3 are double plotted. In skeletal muscle, the 9.0 kb *mPer3* transcript (weak upper hybridization signal in autoradiograms) exhibited a synchronous circadian oscillation with the major 7.0 kb transcript; the 2.4 kb transcript was also detectable but did not vary in abundance over time (data not shown). Since each blot was repeatedly probed and stripped with *mPer3*, *mPer2*, and *mPer1*, it was not possible to compare relative levels of RNA expression among the *Per* genes for each tissue. A replicate Northern blot of each tissue was probed with *mPer1* and showed similar rhythms to those depicted.

these two times are distinct in C57BL/6 mice, with light at CT 14 causing phase delays in behavioral rhythms and light at CT 23 causing phase advances (Schwartz and Zimmerman, 1990). The qualitative and quantitative differences in the photic induction of *mPer2* RNA levels to light applied at CT 14 and CT 23 suggests a molecular corollary between *mPer2* induction and the direction of the behavioral response to light (e.g., delay versus advance). This is quite distinct from the photic induction of *mPer1* RNA, which is very similar in magnitude and time course at both the phase delay and phase advance times of the circadian cycle (Shigeyoshi et al., 1997; Figure 3, top). Our data also suggest that at this point we cannot distinguish between the relative importance of *mPer1* or *mPer2* induction by light for ultimately phase shifting the oscillatory machinery of the SCN.

Although we previously thought that *mPer2* was a delayed response gene to light in the SCN (Shearman et al., 1997), our more detailed time course shows that *mPer2* is more rapidly induced by light (i.e., significant induction is observed 30–60 min after a light pulse). With this rapid time course of induction, it seems unlikely that either *mPer1* or *mPer2* induction would depend on the prior induction of protein products of immediate-early genes (e.g., *c-fos*, *fos-B*, *jun-B*, *zif268*, *nur77*, and *egr-3*), which are known to be acutely induced by light exposure throughout subjective night in the SCN (Takahashi, 1995; Morris et al., 1998). This does not, however, rule out complex interrelationships among these gene products, ultimately leading to phase shifts in behavior.

The photic induction of *mPer2* at CT 23 in our studies contradicts the lack of a photic response of *mPer2* RNA levels at CT 22 reported by Albrecht et al. (1997). Although the difference between the two studies could be due to the difference in the time of light exposure (CT 23 in our study versus CT 22 in their study) or strain differences, the most likely explanation is the lack of sensitivity for detecting a small induction in *mPer2* gene expression by light exposure at CT 22. Albrecht and colleagues (1997) did not quantitate their light responses, and the responses of the light-exposed group of animals were not compared to a nonlight pulse (control) group. Thus, the modest amplitude of the *mPer2* RNA induction found in our studies during the phase advance portion of the circadian cycle could have been easily overlooked.

One of the interesting aspects of our study was the finding of circadian oscillations in RNA levels for all three *Per* genes in nonneural tissues. This is reminiscent of the situation in *Drosophila*, in which *per* is widely expressed throughout the animal, and in most places where *per* is expressed its RNA abundance oscillates (the notable exception being the ovaries; Hardin, 1994). Furthermore, recent studies show that light-sensitive circadian clocks are present throughout the fly body, in all areas in which *per* oscillates (Plautz et al., 1997). Thus, the widespread expression of mammalian *Per* genes and the circadian oscillations of their RNA levels in the three peripheral tissues examined suggest that several peripheral clocks may also exist in mammals. For years, there has been circumstantial evidence suggesting the existence of such clocks in mammals, which has been largely ignored (Reeds et al., 1986; Wuarin and Schibler, 1990; Rienstein et al., 1998). However, circadian oscillations of putative clock elements (i.e., *Per*) in peripheral tissues make the possibility of peripheral circadian clocks more compelling. The ability to monitor *mPer* gene expression may

now allow definitive identification of circadian clocks in peripheral tissues.

Experimental Procedures

Cloning Studies

5′ and 3′ RACE were performed using the Marathon cDNA Amplification Kit (Clontech) with mouse brain cDNA as template. RACE products were eluted from agarose gels (Qiaex II Kit from Qiagen), subcloned into pCRII using a TA Cloning Kit (Invitrogen), and sequenced. RT-PCR using *mPer3* specific primers was used to generate two 2 kb fragments of the *mPer3* cDNA. These pieces were sequenced to confirm the sequence obtained by RACE.

Nucleotide sequences were determined from double-stranded template by the dideoxynucleotide chain termination method of Sanger, using Sequenase (U. S. Biochemical). Primers were synthetic oligodeoxynucleotides, either vector specific or derived from sequence information.

Animals and Tissue Collection

Male C57BL/6 mice, 6–10 weeks of age (Charles River Labs), were housed in ventilated environmental compartments within a temperature-controlled facility (23°C–24°C). The lighting cycle in each compartment consisted of 12 hr light:12 hr darkness (12L:12D), except as noted. The light was provided by cool white fluorescent bulbs. During periods referred to as darkness, dim red light was provided by special fluorescent fixtures (Litho Light #2, lower wavelength cutoff at 640 nm), which remained on at all times. Animals were killed by decapitation to avoid acute changes in gene expression. After decapitation (in light or darkness), brains were removed in the light, frozen in cooled 2-methylbutane, and stored at −80°C. Sections (15 μm thickness) through the SCN were cut in a Bright cryostat (Hacker Instruments) as a 1-in-8 series. Sections were collected on slides coated with Vectabond (Vector Labs), air dried, and stored at −80°C until use.

In Situ Hybridization

Antisense and sense cRNA probes were generated from each plasmid by in vitro transcription in the presence of [^{35}S]UTP (1200 Ci/mmol), as previously described (Weaver, 1993). Probe for *mPer1* (GenBank accession number AF022992) was derived from nt 340–761. Probe for *mPer2* (GenBank accession number AF035830) was derived from nt 9–489. Probe for *mPer3* (GenBank accession number AF050182) was derived from nt 1637–2223. Probe quality and size was confirmed by determining ^{35}S 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 (50–70 μl at 10^7 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 9–12 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. ^{14}C 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.

To calculate the amplitude of the RNA rhythms in SCN, the optical density (OD) of specific hybridization was first calculated by subtracting the SCN OD values for sections hybridized with the sense control probe (0.142 in this experiment). The amplitude is defined as the peak specific hybridization value divided by the trough specific hybridization value.

Northern Analysis

Total RNA was extracted from tissues using the Ultraspec RNA Isolation Reagent (Biotex 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 (specific activity = 2×10^6 cpm/ml). The blots were hybridized with Express Hybridization Solution (Clontech) and washed following the manufacturer's protocol. Probes used were *mPer1* (nt 468–821 of accession number AF022992), *mPer2* (nt 9–489 of accession number AF035830), and *mPer3* (nt 187–1139 of accession number AF050182). Blots were exposed at −80°C to BioMax film with two intensifying screens.

Individual blots were repeatedly probed and stripped with *mPer3*, *mPer2*, *mPer1*, and G3PDH, respectively. Autoradiograms were quantified by densitometry. Ratios between each *mPer* and G3PDH were calculated and normalized from 0–100 to facilitate comparison between genes.

Acknowledgments

We thank Kathryn Picanso, Kurtis Gray, and Yvette Miller for technical assistance. We also thank Steve Kay for first suggesting to us that genomic contig Z98884 might contain *hPer3*. This work was supported by National Institute of Child Health and Human Development grant R37 HD14427. M. J. Z. was supported in part by National Research Service Award MH11547.

Received March 18, 1998; revised April 17, 1998.

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GenBank Accession Number

The mPER3 sequence has been deposited in GenBank as accession number AF050182.

Note Added in Proof

Others have recently shown that *Per1* and *Per2* RNA levels exhibit robust circadian rhythms in cultured rat-1 fibroblasts treated with high concentrations of serum (A. Balsalobre, F. Damiola, and U. Schibler [1998]. Cell *93*, 929–937). This extends our in vivo demonstration of *mPer* RNA oscillations in peripheral tissues and further supports the existence of multiple circadian clocks in nonneural tissues of mammals.