Neuron, Vol. 19, 1261–1269, December, 1997, Copyright ©1997 by Cell Press

Two *period* Homologs: Circadian Expression and Photic Regulation in the Suprachiasmatic Nuclei

Lauren P. Shearman,*‖ Mark J. Zylka,*†‖ David R. Weaver,* Lee F. Kolakowski, Jr.,‡ 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 Pharmacology
University of Texas Health Science Center at San Antonio
San Antonio, Texas 78284-7764

Summary

We have characterized a mammalian homolog of the Drosophila *period* gene and designated it *Per2*. The PER2 protein shows >40% amino acid identity to the protein of another mammalian *per* homolog (designated *Per1*) that was recently cloned and characterized. Both PER1 and PER2 proteins share several regions of homology with the Drosophila PER protein, including the protein dimerization PAS domain. Phylogenetic analysis supports the existence of a family of mammalian *per* genes. In the mouse, *Per1* and *Per2* RNA levels exhibit circadian rhythms in the SCN and eyes, sites of circadian clocks. Both *Per1* and *Per2* RNAs in the SCN are increased by light exposure during subjective night but not during subjective day. The results advance our knowledge of candidate clock elements in mammals.

Introduction

Biological clocks generate circadian rhythms in physiology and behavior (Pittendrigh, 1993). In mammals, the suprachiasmatic nuclei (SCN) of the anterior hypothalamus are the site of a master biological clock (reviewed in Reppert and Weaver, 1997). The SCN clock is entrained to the 24 hr day by the daily light–dark cycle, with light acting through both direct and indirect retina-to-SCN pathways (reviewed in Klein et al., 1991). The SCN is composed of an ensemble of multiple cell-autonomous circadian clocks (Welsh et al., 1995; Liu et al., 1997). Thus, the molecular machinery necessary for building a circadian clock is contained within single neurons. This finding has focused our attention on the molecular components and intracellular loops required for circadian oscillations.

Over the past year, significant progress has been made toward elucidating molecular components of the SCN clock. First, the mouse *Clock* gene was cloned. The *Clock* mutation, generated from a chemical mutagenesis screen, causes abnormally long circadian periods in behavior when monitored in constant darkness (DD), with homozygous mutants eventually becoming arrhythmic in DD (Vitaterna et al., 1994). A combination of positional cloning and functional rescue approaches revealed that *Clock* encodes a new member of the basic helix-loop-helix (bHLH)/PAS family of transcription factors (Antoch et al., 1997; King et al., 1997). Although *Clock* RNA levels are constitutively expressed (not rhythmic) in the SCN (Sun et al., 1997; Tei et al., 1997), *Clock* and its encoded protein still hold promise for being important components of an SCN clock mechanism (Reppert and Weaver, 1997).

A second discovery of potential importance for understanding molecular mechanisms in the SCN was the cloning of a putative mammalian ortholog of the Drosophila clock gene *period* (*per*). In the fruit fly Drosophila melanogaster, two clock genes, *per* and *timeless* (*tim*), are necessary elements of a circadian timing system that controls circadian rhythms in adult eclosion behavior and locomotor activity (reviewed in Hall, 1995; Reppert and Sauman, 1995). The RNA and protein products of both genes (PER and TIM) oscillate, and the cycling of each is dependent on PER–TIM dimerization and nuclear entry. Fly PER appears to function as a negative regulator of its own transcription, forming one arm of an autoregulatory feedback loop that constitutes a molecular gear of the circadian clock. In another insect, the silkmoth Antheraea pernyi, PER is also necessary for circadian function (Sauman et al., 1996), but the molecular details of PER interactions in circadian clock cells in silkmoth brain are different from those in Drosophila (Sauman and Reppert, 1996).

Cloning of the first mammalian *per* homolog (designated *Per1*) was accomplished independently by two groups (Sun et al., 1997; Tei et al., 1997). The *Per1* gene encodes a protein (PER1) with a PAS domain, which is highly homologous to the PAS domain of insect PER. PAS is an acronym for the first three proteins found to share this functionally important protein dimerization domain: Drosophila *P*ER, the human aryl hydrocarbon receptor nuclear translocater protein (*A*RNT), and the Drosophila single-minded protein (*S*IM) (reviewed in Hall, 1995). There are also several areas outside of the PAS domain that are conserved between PER1 and Drosophila PER (Tei et al., 1997). Importantly, mouse (m)*Per1* RNA levels exhibit a striking oscillation over the course of the day in the SCN and eyes (Sun et al., 1997; Tei et al., 1997). Thus, *Per1* is not only a structural homolog of Drosophila *per*, it may also be an ortholog.

We now evaluate a second *per* homolog (*Per2*) in mammals that is distinct from *Per1*. In a BLAST search against peptide sequences, human clone KIAA0347 surfaced with striking sequence homology to PER1 and Drosophila PER (Nagase et al., 1997). We have thus cloned the mouse (m)*Per2* cDNA and characterized its circadian oscillations, developmental expression, and light regulation. The results indicate that there is a family

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

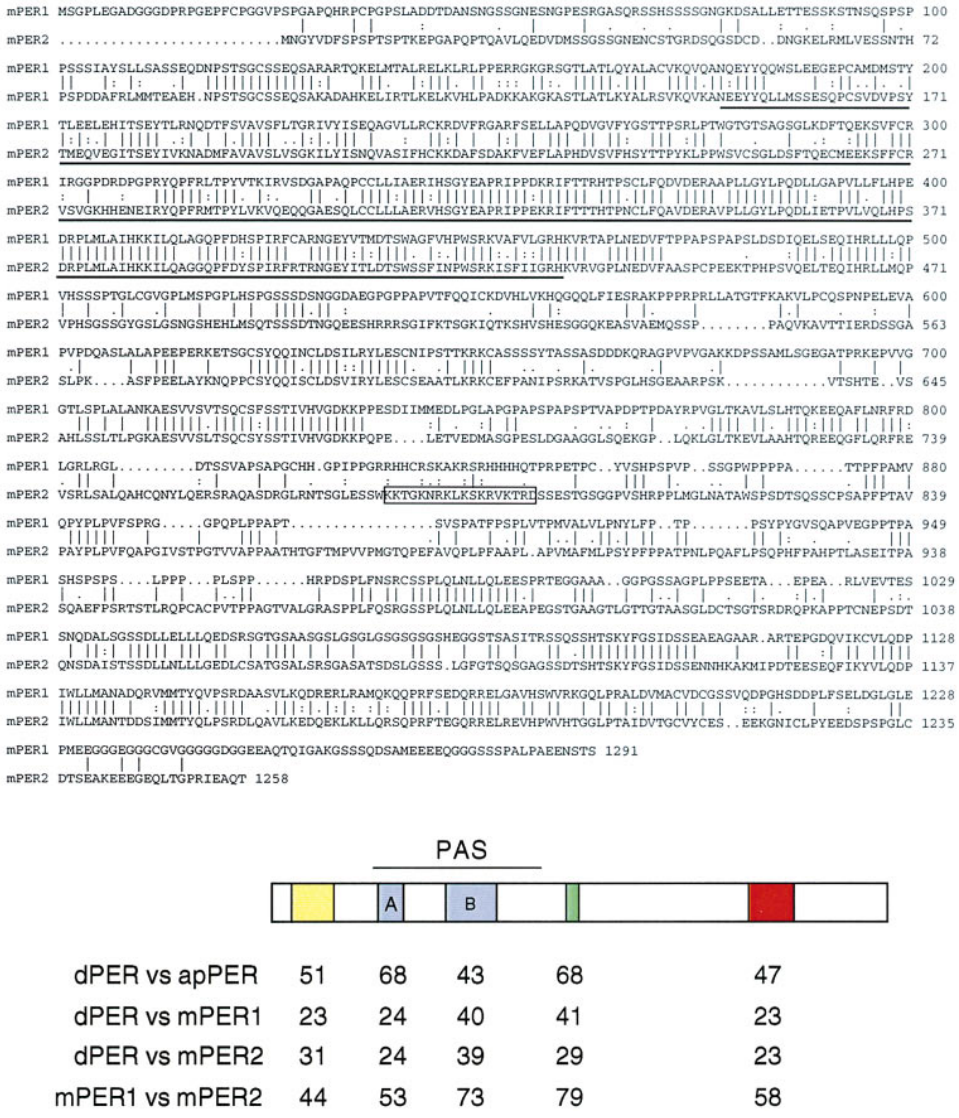

Figure 1. Comparison of PER Proteins

Upper: comparison of the deduced amino acid sequences of m*Per1* and m*Per2* cDNAs. The two sequences (mPER1 and mPER2) 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. The boxed region is a bipartite nuclear localization signal in mPER2. mPER1 sequence is from Sun et al. (1997), GenBank accession number AF022992.

Lower: homologous regions among Drosophila PER (dPER), A. pernyi PER (apPER), mPER1, and mPER2. A cartoon of mPER2 is shown with homologous regions highlighted by color: yellow, amino-terminal region; blue, A and B repeats of PAS; green, downstream from site of per^S mutation in Drosophila; red, region around the TG repeat in Drosophila. Highlighted regions are those previously delineated by Tei et al. (1997).

of mammalian *per* homologs and suggest that these genes may function as molecular components of a circadian clock mechanism.

Results

Structural Assessment of a Second Mammalian *per* Homolog

KIAA0347, human (h)*Per2*, was recently reported as one of 100 new cDNAs cloned from human brain as part of a cDNA sequencing project (Nagase et al., 1997). The deduced protein product of this gene contains a PAS domain with high homology to that of insect PER (Nagase et al., 1997). Because of this homology and the circadian features recently reported for the structurally related m*Per1* gene (Sun et al., 1997; Tei et al., 1997), we cloned m*Per2*. A mouse expressed sequence tag (EST) (accession number AA272850) showing >80% sequence identity to h*Per2* was obtained and sequenced. The resultant EST sequence was used to design specific oligodeoxynucleotide primers for 5′ and 3′ rapid amplification of cDNA ends (RACE) to clone the entire coding

region of the m*Per2* cDNA. The cDNA encodes a protein (PER2) of 1257 amino acids that is 77% identical to its human counterpart (data not shown).

BLAST analysis showed that PER1 and PER2 are most closely related to each other. GAP analysis between PER1 and PER2 showed 44% and 46% amino acid identity for the human or mouse proteins, respectively (Figure 1, upper). The human genes are distinct, as previous studies have shown that *Per1* maps to chromosome 17 (Sun et al., 1997; Tei et al., 1997), whereas *Per2* maps to chromosome 6 (Nagase et al., 1997). BLAST analysis also indicated that both PER1 and PER2 are highly related to Drosophila and silkmoth PER (Figure 1, lower). There is homology between the PERs of mammals and insects in the A and B repeat regions of the PAS domain, just downstream from the B repeat, and in two areas outside of PAS. The areas of homology outside of the PAS domain (previously identified for PER1 by Tei et al., 1997) include an amino-terminal region and a short region downstream from the site of the per^S mutation in Drosophila. One difference in conservation among the insect and mammalian proteins is a region of Drosophila PER that includes a TG repeat. Interestingly, there are no TG/SG repeat regions in A. pernyi PER (Reppert et al., 1994) or the human or mouse PER2 proteins, but short SG repeat regions are found in the PER1 proteins (Tei et al., 1997).

The two mammalian PER homologs were also analyzed for structural features with defined functional roles. By ProfileScan, human and mouse PER2 contain a PAS repeat and putative bipartite nuclear localization signals (three for the human sequence and one for the mouse sequence). Parallel analysis of the previously reported PER1 detected a PAS repeat but no nuclear localization signals. ProfileScan did not reveal the presence of a bHLH domain for either PER1 or PER2 proteins, while it did identify such a domain in previously characterized bHLH/PAS proteins (e.g., the Clock protein and MOP4/NPAS2). The PHDsec program (EMBL) for secondary structure also did not reveal a HLH motif in either human or mouse PER2; this same program did identify a putative HLH motif in PER1, as previously reported (Sun et al., 1997). However, it is important to point out that only three of the 23 amino acids immediately upstream of the putative HLH domain of PER1 are basic residues.

Phylogenetic Analysis Supports a Mammalian *Per* Gene Family

To provide further evidence that *Per1* and *Per2* are members of a family of mammalian *per* orthologs, we performed a phylogenetic analysis of the PAS domains of the 15 identified bHLH/PAS domain proteins, along with Drosophila PER, A. pernyi PER, PER1, and PER2. During the preparation of this manuscript, we became aware of a third *per* homolog (designated *Per3*; GenBank accession number Z98884, located on human chromosome 1). Thus, the PAS region of human PER3 (which has 30%, 52%, and 51% identity with the PAS regions of Drosophila PER, hPER1, and hPER2, respectively) was also included in the analysis.

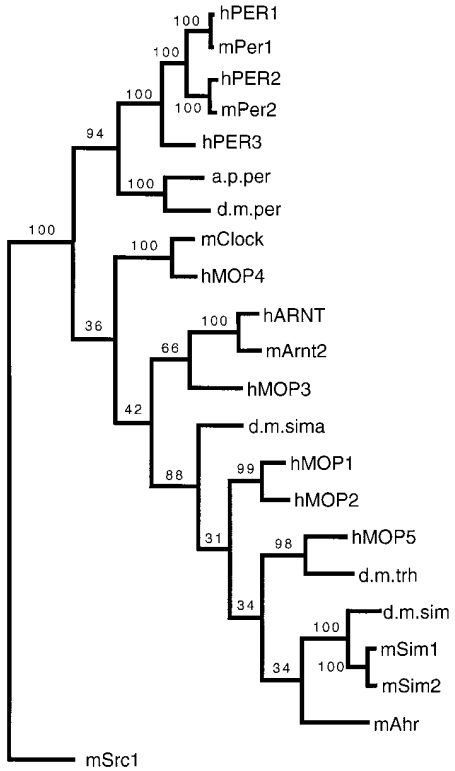

Figure 2. Phylogenetic Analysis of PAS-Containing Proteins

Maximum parsimony analysis of PAS domains of 15 proteins in the bHLH/PAS family of transcription factors and Drosophila PER, A. pernyi PER, human and mouse PER1, human and mouse PER2, and human PER3 shows that mammalian PER homologs are closely related to the invertebrate PER proteins. Sequences used in the analysis are abbreviated as follows: hPER1, human PER1 (AF022991); mPer1, mouse PER1 (AF022992); hPER2, human PER2 (AB002345); mPer2, mouse PER2 (AF035830); hPER3, human PER3 (Z98884); a.p.per, A. pernyi PER (U12769); d.m.per, Drosophila PER (M11969); mClock, mouse Clock (AF000998); hMOP4 (also hNPAS-2), human MOP4 (U51625); hARNT, human aryl hydrocarbon receptor nuclear translocator (U29165); mArnt2, mouse aryl hydrocarbon receptor nuclear translocator 2 (D63644); hMOP3 (also hBMAL; JAP3), human MOP3 (U51627); d.m.sima, Drosophila similar protein (U43090); hMOP1 (also HIF1alpha), human MOP1 (U29165); hMOP2 (also EPAS-1, mHLF), human MOP2 (U51626); hMOP5 (also hNPAS-1), human MOP5 (U51628); d.m.trh, Drosophila trachealess protein (U42699); d.m.sim, Drosophila single-minded protein (M19020); mSim1, mouse single-minded homolog 1 (U40575); mSim2, mouse single-minded homolog 2 (U40576); mAhr, mouse aryl hydrocarbon receptor (D38417); mSrc1, mouse steroid receptor coactivator-1 (U64828). Bootstrap values representing the frequency of branching in 1000 replicate searches are indicated on the horizontal branches. The horizontal length of branches indicates amino acid changes from the node. Vertical distances have no meaning. The alignment and data file utilized in the tree calculations are available on the Internet (http://www.neuron.org/supplemental/19/6/1261).

The PAS domains of all analyzed proteins were aligned, and maximum parsimony phylogenetic trees were computed. The data set converged on a single tree of length of 2051 changes (Figure 2). This tree showed that the seven PER proteins have significant bootstrap values and are thus highly related to each

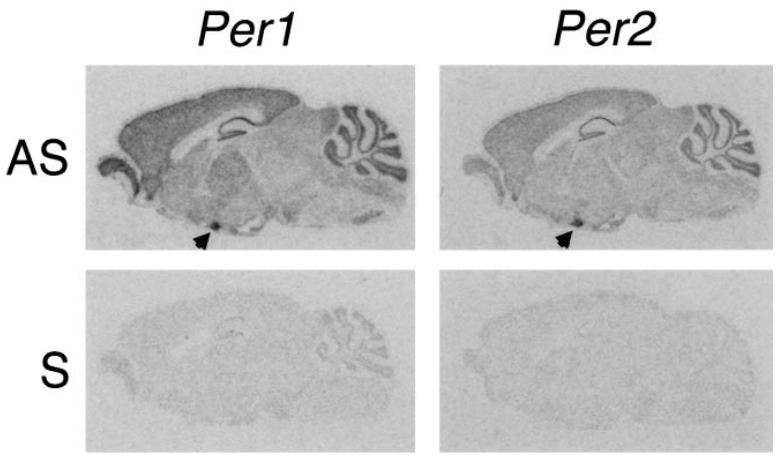

Figure 3. Patterns of m*Per1* and m*Per2* Gene Expression in Brain at CT 6

Parasagittal sections (15 μm) were examined by in situ hybridization for m*Per1* (left column) and m*Per2* (right column) RNA, using either antisense (AS, upper) or sense control (S, lower) cRNA probes. Arrows denote the SCN. Magnification, 3.3×.

other across vertebrate and invertebrate species. The remaining 15 PAS domains were less related to one another. The aryl hydrocarbon receptor (Ahr) and the steroid receptor coactivator-1 (Src1) PAS domains were more distantly related to the other members of this group.

Taken together, the structural and phylogenetic analyses strongly support the existence of a family of mammalian *per* homologs and suggest structural domains (e.g., the PAS region) that may have functional significance for circadian clock function in mammals. Our subsequent studies focused on various aspects of m*Per1* and m*Per2* gene expression (circadian oscillations, developmental expression, and light regulation) to further support their potential as clock genes.

m*Per2* RNA Levels Oscillate in the SCN and Eyes

If *Per2* is important for circadian clock function, then it should be expressed in the SCN, the master circadian clock in mammalian brain. We might also expect that its expression there would be rhythmic. We first examined the general brain distribution of the m*Per2* gene by in situ hybridization at circadian time (CT) 6 and compared it with that of m*Per1*. Both m*Per1* and m*Per2* RNAs were widely expressed in brain. Of all regions examined, the SCN contained the most intense hybridization signal with each probe (Figure 3). The expression of both m*Per1* and m*Per2* was detectable through the entire extent of the SCN. Moderate m*Per2* gene expression was present in piriform cortex, neocortex, hippocampus, and cerebellum. High levels of m*Per1* gene expression were evident in olfactory bulb, piriform cortex, neocortex, hippocampus, and cerebellum, as previously described (Sun et al., 1997). Expression of both m*Per1* and m*Per2* was evident at low but detectable levels throughout most other brain regions.

Next, we assessed the temporal profile of m*Per2* expression in the SCN by in situ hybridization and compared it with that found for m*Per1*. Hybridization signals were monitored at six time points over a 24 hr period on day 1 in DD. m*Per2* RNA levels exhibited a striking circadian rhythm in mouse SCN, with high levels during subjective day at CT 3, 6, 9 and at the first point in subjective night, CT 15 (Figure 4). As previously described (Sun et al., 1997; Tei et al., 1997), m*Per1* RNA levels also exhibited a prominent circadian oscillation in the SCN, with high RNA levels also during subjective day at CT 3, 6, and 9. Thus, the RNA rhythms for m*Per1* and m*Per2* in the SCN appear to be synchronous. Based on comparison with ^{14}C standards included in each film cassette, the abundance of specific hybridization in the SCN has a 4.0-fold peak–trough amplitude for m*Per1* RNA and a 7.6-fold amplitude for m*Per2*.

Recent evidence shows that circadian clocks also reside in rodent retinae (Tosini and Menaker, 1996). We thus assessed rhythmicity of expression of m*Per1* and m*Per2* in mouse eye. Eye RNA samples from the same animals that provided the SCN samples for the in situ hybridization experiments were analyzed by Northern analysis. Both m*Per1* and m*Per2* RNAs exhibited prominent circadian rhythms in whole eyes (Figure 5). Interestingly, the rhythms were also synchronous, but, in contrast to the SCN, the eye rhythms were characterized by high values at CT 9, 15, 18, and 21. In situ hybridization showed a specific hybridization signal for both m*Per1* and m*Per2* in retina (Figure 5, lower panel), suggesting that the eye rhythms for both genes are indeed generated from the retinae.

Per2 Transcripts Are Widely Expressed in Mice and Humans

The tissue distribution of *Per2* gene expression was examined to determine whether it is as widely expressed as that reported for *Per1* (Sun et al., 1997; Tei et al., 1997). Northern blot analysis of RNA from a variety of tissues showed that the *Per2* genes are expressed in all tissues examined (Figure 6). Hybridization with either a human or mouse *Per2* cDNA probe revealed a major hybridizing transcript of 7.0 kb in each species. In a few tissues (human heart and skeletal muscle), a smaller transcript of 1.8 kb was also detected with the probe (random prime-labeled). The Northern blots were then stripped and reprobed with human or mouse *Per1*-specific probes, which confirmed a major transcript of 4.6 kb with widespread distribution (Sun et al., 1997; Tei et al., 1997). Although the overall expression patterns were similar between *Per1* and *Per2* genes, *Per1* transcripts appeared to be more highly expressed than *Per2* in most human and mouse tissues.

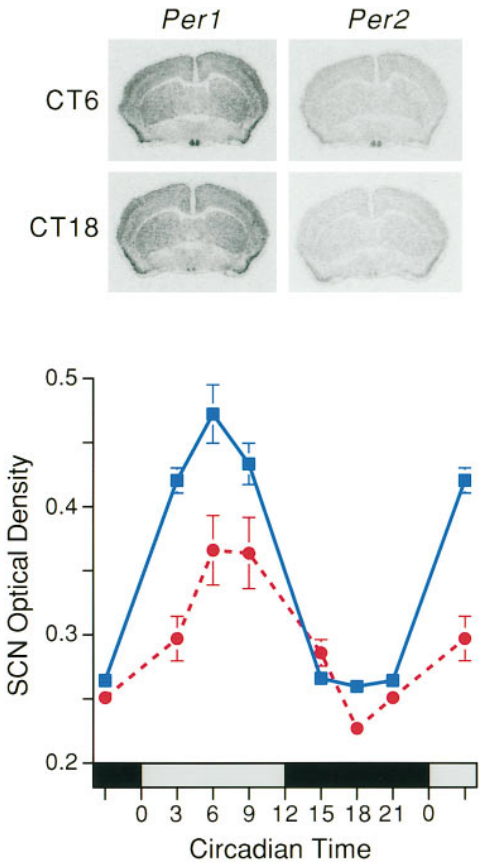

Figure 4. Circadian Expression of m*Per1* and m*Per2* in the SCN

Upper: expression of m*Per1* and m*Per2* in the SCN at CT 6 (upper) and CT 18 (lower). Coronal sections (15 μm) were examined by in situ hybridization using antisense cRNA probes. Magnification, 2.3×.

Lower: circadian patterns of m*Per1* (blue) and m*Per2* (red) expression in the SCN. Each value is the mean ± SEM of six to eight animals. The horizontal bar at the bottom of the panel represents the lighting cycle prior to placement in DD; stippled areas represent subjective day and the filled areas represent subjective night.

m*Per1* and m*Per2* Genes Are Expressed in the Developing SCN

In rodents, the SCN start functioning as a circadian clock during late fetal life, soon after the nuclei have completed neurogenesis (Reppert, 1994). Thus, if m*Per1* and m*Per2* genes are essential clock elements, they should be expressed in the SCN during late fetal and early neonatal life. In fact, in situ hybridization studies showed that both m*Per1* and m*Per2* are expressed in the developing mouse SCN on day 17 of gestation (3 days before birth; data not shown) and on the day of birth (Figure 7).

m*Per1* and m*Per2* RNA Levels in the SCN Are Regulated by Acute Light Exposure

If m*Per1* and m*Per2* are important for circadian clock function, they might respond to light applied at a time of the circadian cycle that normally produces phase shifts in the circadian clock. For example, in the fungus Neurospora crassa, RNA levels of the clock gene *frequency* (*frq*) are acutely regulated by light, providing a

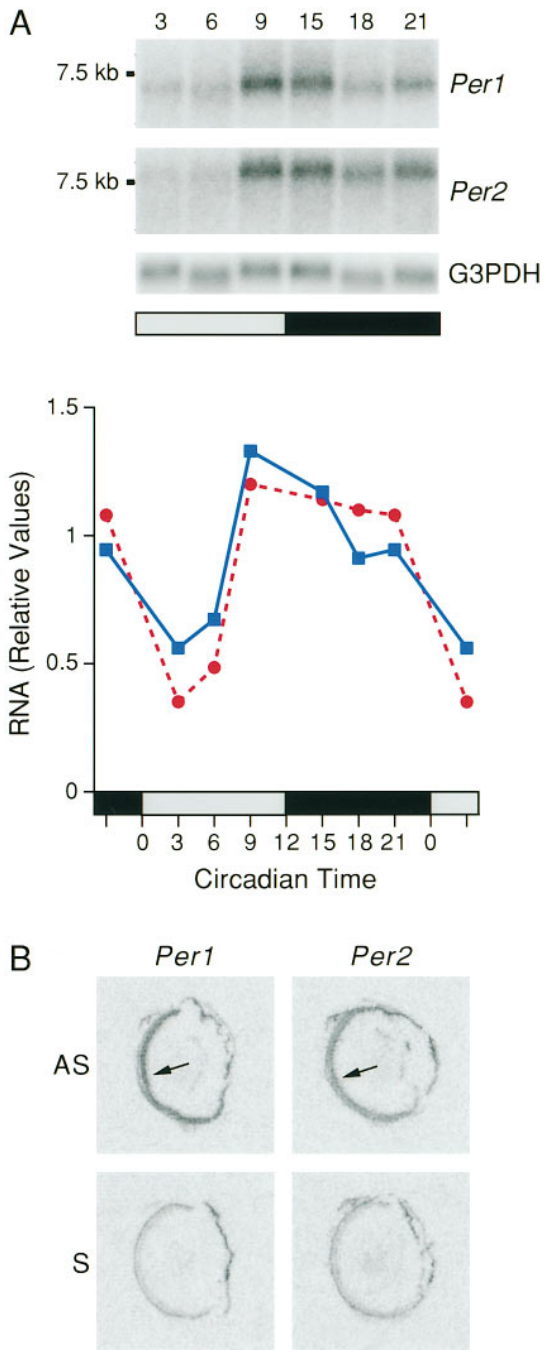

Figure 5. Circadian Expression of m*Per1* and m*Per2* in Eyes

(A) Northern blot analysis of m*Per1* and m*Per2* expression in eyes over a 24 hr period. The upper panel depicts hybridization densities for m*Per1*, m*Per2*, and G3PDH (control) at each circadian time. The lower panel shows quantitation of the data shown above for m*Per1* RNA (blue) and m*Per2* RNA (red). Relative RNA levels refers to ratios of m*Per1* or m*Per2*:G3PDH RNAs. The horizontal bar at the bottom of the panel represents the lighting cycle, with the shaded area representing subjective day.

(B) Expression of m*Per1* and m*Per2* in retina at CT 6. Sections (15 μm) were examined for m*Per1* (left) or m*Per2* (right) RNA by in situ hybridization using antisense (AS, upper) or sense control (S, lower) cRNA probes. Arrows denote retina signal. Magnification, 5.4×.

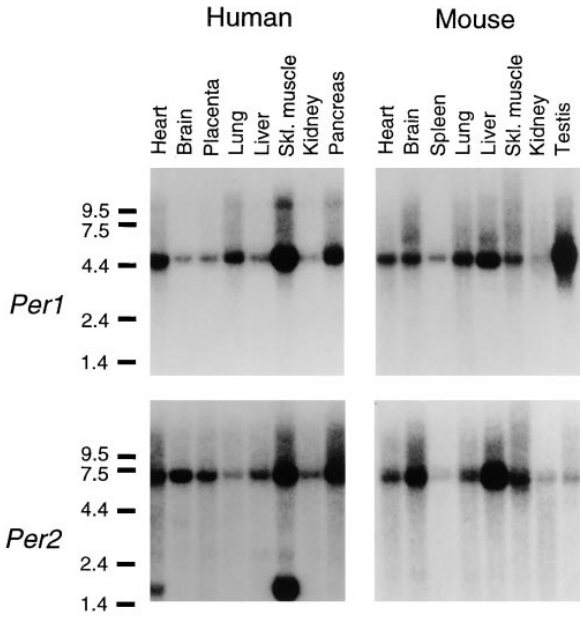

Figure 6. Northern Blot Analysis of *Per1* and *Per2* Gene Expression

Blots were purchased from Clontech. Each lane contained 2 μg of poly(A)$^+$ RNA. Human (left) and mouse (right) blots were probed first with species-specific *Per2* probes, then with *Per1* probes, and finally with actin (loading control). Exposure times were 2 hr for *Per1* blots and 12 hr for *Per2* blots.

molecular basis for light-mediated phase shifts in that organism (Crosthwaite et al., 1995). For this phase of study, we applied a 30 min light pulse at two biologically relevant circadian times: CT 3–3.5, a time of the circadian cycle when light does not induce phase shifts, and at CT 14–14.5, a time at which light would cause a phase delay in the behavioral activity rhythms regulated by the SCN (Schwartz and Zimmerman, 1990). RNA levels were

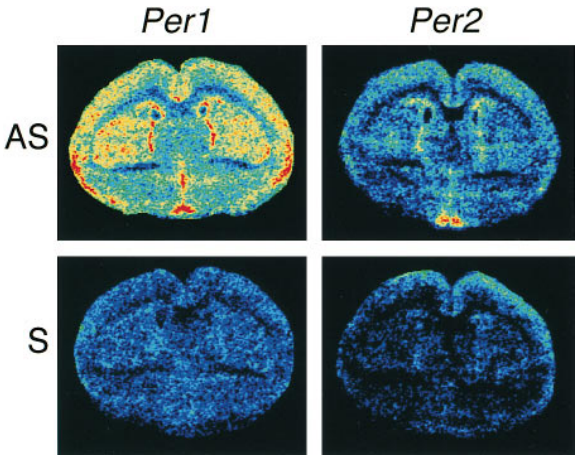

Figure 7. Pseudocolor Images Showing Expression of m*Per1* and m*Per2* in Developing SCN

Coronal sections (15 μm) through the neonatal SCN were examined by in situ hybridization for m*Per1* (left) and m*Per2* (right) RNAs, using either antisense (AS, upper) or sense control (S, lower) cRNA probes. Brains were sectioned in the skull; tissues outside the brain were digitally erased for clarity. Magnification, 6.1×.

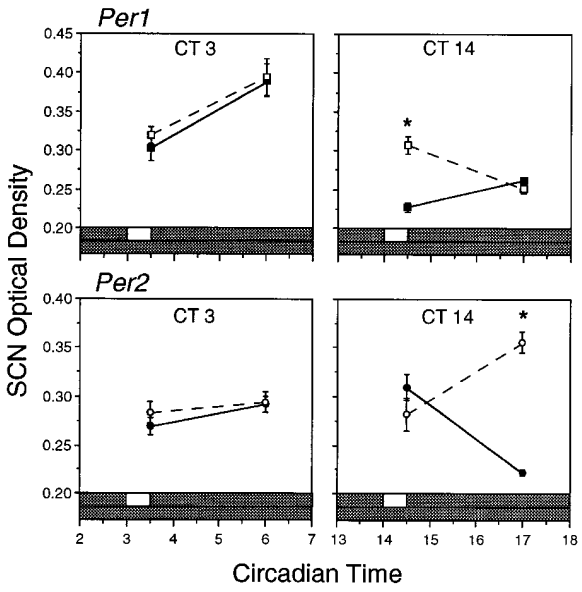

Figure 8. Light Regulation of m*Per1* and m*Per2* RNAs in the SCN

Animals were entrained to a 12 hr light:12 hr dark lighting cycle and then placed in DD. Light pulses (400 lux; 30 min duration) were applied during the first or second day in DD. Coronal sections (15 μm) through the SCN were examined for either m*Per1* RNA (upper row) or m*Per2* RNA (lower row) 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 (top bars; open symbols) and in time-matched controls maintained in DD (lower bars; closed symbols). Each value is the mean ± SEM of four to six animals. Asterisk, $p < 0.05$, Student's t test.

monitored by in situ hybridization of SCN sections from animals killed 30 min and 180 min after the beginning of light exposure.

Both m*Per1* and m*Per2* RNAs showed phase-dependent responses to light (Figure 8). Following light exposure from CT 3–3.5, neither transcript was detectably altered in its abundance at either the 30 or 180 min time points (relative to control animals not exposed to light). In contrast, both m*Per1* and m*Per2* hybridization intensities in the SCN were increased by light exposure at CT 14–14.5 (during subjective night). m*Per1* RNA levels increased rapidly following a light pulse during subjective night; RNA levels were significantly elevated after 30 min of light ($p < 0.05$, Student's t test) and had returned to basal levels 150 min later. m*Per2* RNA levels in the SCN increased more slowly in response to the light pulses; RNA levels were significantly elevated at 180 min ($p < 0.05$, Student's t test) but not at 30 min after light exposure. We also observed induction of m*Per1* and m*Per2* by light at night in two additional experiments. In one experiment, light exposure began at CT 15 and continued for 3 hr, while in the other, light exposure was limited to 30 min beginning at CT 19. In each experiment, m*Per1* RNA levels were significantly elevated at 30 min after initiation of the light pulse, while m*Per2* RNA levels were not elevated until the 3 hr time point (data not shown). Thus, for both *Per1* and *Per2*, a light pulse that would shift the phase of SCN-controlled behavioral rhythms altered the level of gene expression in the SCN.

Discussion

Our structural and functional evaluations of *Per2* indicate that this gene is a clock-relevant member of a mammalian *Per* gene family. Structural assessment shows that PER1 and PER2 are closely related to each other and share homology with several regions of Drosophila and A. pernyi PER (Figure 1). In addition, phylogenetic analysis clearly identifies a family of *per* homologs with at least three mammalian members. This finding suggests that there are conserved functions of PER proteins across a wide evolutionary distance. Our functional studies consisted of examining circadian oscillations, developmental expression, and photic regulation of m*Per1* and m*Per2* RNA levels. As elaborated below, results from our functional analysis show that m*Per1* and m*Per2* share several properties that suggest that they are important for clock function. Moreover, our light regulation data provide insights into a potential molecular mechanism of photic entrainment in the SCN.

Oscillating transcripts are a hallmark of the three most fully characterized circadian clock genes—*per*, *tim* (in Drosophila), and *frq* (in Neurospora). Thus, the prominent circadian rhythms of m*Per1* and m*Per2* RNA levels in the SCN support a role for both genes in the molecular working of the principal circadian clock in mammalian brain. Although there may be subtle oscillations in other brain regions (Sun et al., 1997), the m*Per1* and m*Per2* RNA rhythms in the SCN clearly stand out. In fact, at the peak of the oscillations, CT 6, m*Per1* and m*Per2* RNA levels in the SCN are the highest detected in all of mouse brain (Figure 3). The synchronous nature of m*Per1* and m*Per2* RNA oscillations in the SCN are reminiscent of the synchronous RNA (and protein) rhythms found for *per* and *tim* in Drosophila. Although protein data for m*Per1* and m*Per2* are not yet available and it is not yet known whether both *Per* genes are expressed in individual cells, the synchronous RNA oscillations (in the SCN and also in eyes; see below) suggest that the protein products of these genes may interact with each other through their PAS domains.

The functionally relevant PAS domain also provides a common thread between the protein products of mammalian *Per* genes and *Clock*. Since the Clock protein is a bHLH/PAS-containing protein that probably functions as a transcription factor (King et al., 1997), it is worth considering an array of potential interactions among PER1, PER2, and Clock proteins, through their PAS domains, that are fundamental to clock function. One interesting possibility is that rhythms in PER1 and PER2 form negative feedback loops that regulate the transcriptional activity of the Clock protein, which, in turn, drives expression of either or both *Per* genes. This sort of interaction would be consistent with the observations that the *Clock* RNA is not itself rhythmic (Sun et al., 1997; Tei et al., 1997), and yet the *Clock* gene product is required for normal circadian function (Antoch et al., 1997).

Prominent, synchronous rhythms of m*Per1* and m*Per2* RNAs in eye supply additional evidence that these genes are important for circadian function, since an autonomous clock also resides in rodent retina (Tosini and Menaker, 1996). The synchronous expression of m*Per1* and m*Per2* in eye further suggests that circadian clocks in mammalian SCN and retina use the same molecular components. Previous studies with *tau* mutant hamsters showed that the period alterations found in SCN-driven behavioral rhythms are also present in rhythms monitored from the retina in vitro (Tosini and Menaker, 1996) and in single SCN neurons in culture (Liu et al., 1997). This suggests that the gene that encodes the *tau* mutation is an important clock element shared by both the SCN and retina. A curious difference in the m*Per1* and m*Per2* RNA rhythms between the SCN and eyes is the phase of the rhythms relative to each other. The eye rhythms are clearly delayed by 3–6 hr relative to the SCN oscillations. Delineation of the temporal dynamics of mPER1 and mPER2 proteins in SCN and retinal clocks may help shed light on the biological significance of the phase difference.

We discovered that m*Per1* and m*Per2* RNA levels in the SCN are differentially regulated by light. The acute photic induction of m*Per1* RNA levels is reminiscent of the situation in Neurospora in which light pulses acutely induce *frq* RNA levels (Crosthwaite et al., 1995). The rapid induction of *frq* by light (RNA levels increase within 15 min of light exposure) correlates with the phase-shifting properties of light in the fungus. Dunlap and colleagues have suggested that *frq* induction is the initial clock-specific event involved in photic entrainment in that organism (Crosthwaite et al., 1995). Building on the *frq* model, the rapid photic induction of m*Per1* RNA levels during subjective night and the phase relationship of the m*Per1* RNA rhythm to circadian time are both consistent with m*Per1* RNA induction providing a clock-specific event for photic entrainment in the SCN.

The gate limiting m*Per1* RNA's photic induction to subjective night may simply reflect a limitation to further induction during daytime imposed by the height of the molecular oscillation and would fit with the peak of the m*Per1* RNA waveform occurring during subjective day (see RNA rhythm in Figure 4). This gating of the RNA induction correlates nicely with the well-known restriction of light-induced phase shifts in behavioral rhythms to subjective night. One could thus envision that an acute induction in m*Per1* RNA during early subjective night might extend its molecular oscillation and cause a phase delay, whereas acute RNA induction during late subjective night might prematurely elevate RNA levels, thereby advancing the rhythm. Further characterization is needed to clarify fully the functional importance of the m*Per1* RNA induction by light to a photic entrainment mechanism.

The delayed induction of m*Per2* RNA levels following a light pulse shows a clear dissociation between the regulation of *Per1* and *Per2* RNA levels. This delayed induction by light also suggests that the m*Per2* RNA rhythm is secondarily affected by light. Perhaps a cascade of events initiated by m*Per1* RNA induction or by immediate-early gene induction in the SCN (e.g., *c-fos*) (Takahashi, 1995) leads to altered m*Per2* transcription. The delayed photic induction of m*Per2* RNA levels may represent a transient distortion of its molecular oscillation as part of a clock resetting mechanism.

The elevations of both m*Per1* and m*Per2* RNAs in response to light pulses are not seen for Drosophila

per. Instead, light acutely decreases TIM levels (Hunter-Ensor et al., 1996; Myers et al., 1996; Zeng et al., 1996) with a resultant, secondary phase alteration in the *per* RNA rhythm by the next circadian cycle (Lee et al., 1996). Thus, if the *Per1* and *Per2* genes are shown to be central clock components in mammals, their mechanisms of regulation will be substantially different from those in Drosophila. There is precedent for species variation in the molecular details of PER regulation, as the dynamics of PER regulation in silkmoth brain are different from the dynamics of PER regulation in Drosophila (Sauman and Reppert, 1996).

The widespread expression of *Per1* and *Per2* genes was unexpected. Extensive studies over the past 25 years have revealed only one circadian clock structure in mammalian brain, the SCN (see Reppert and Weaver, 1997). More recently, circadian clocks have also been identified in mammalian retinae (Tosini and Menaker, 1996). Thus, the expectation was that mammalian clock elements would be limited in their distribution to these clock structures. However, in Drosophila, *per* is rhythmically expressed throughout the animal and in several brain regions, even though the clock that generates behavioral rhythms is felt to reside in a small cluster of "lateral neurons" (Ewer et al., 1992; Frisch et al., 1994). Moreover, recent studies in Drosophila show that light-sensitive, autonomous circadian clocks are present throughout the fly body (Plautz et al., 1997). Thus, it may be that numerous autonomous clocks also exist in mammals. Nevertheless, it is still possible that SCN- and retina-specific genes important for circadian function will be found in mammals.

Per1 and *Per2* define a growing family of putative mammalian *per* orthologs. With the recent identification of *Per3*, it will be important to determine the spatial and temporal expression patterns and regulatory features of this gene (as well as any other members yet to be identified) and determine how they compare with those described here for *Per1* and *Per2*. Future studies will also need to focus on the other criteria that will test the hypothesis that each mammalian *Per* gene is in fact an essential clock element (reviewed in Dunlap, 1993; Zatz, 1992). These include showing that (1) mutations of these genes alter basic clock properties (e.g., gene knockouts result in loss of rhythmicity); (2) acutely altering protein levels or activity resets the clock; (3) keeping protein levels constant stops the clock; and (4) the phase of the RNA and/or protein oscillations is reset by light within one cycle. The existence of a family of mammalian *Per* genes is an exciting discovery and should greatly enhance the molecular dissection of vertebrate circadian clocks.

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 Kit, Qiagen), subcloned into pCRII using a TA Cloning Kit (Invitrogen), and sequenced.

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

Phylogenetic Analysis

Members of the bHLH/PAS transcription factor gene family (n = 15) were aligned as proteins using Clustalw (Higgins et al., 1996). The aligned sequences within the PAS domain regions were further analyzed by maximum parsimony methods using the program PAUP (Swofford, 1991). A single tree was determined to be the shortest after 1,000 heuristic searches. The data set was subjected to bootstrap analysis using 1,000 repetitions of the heuristic algorithm. The results were analyzed using MacClade (Sinauer Press), and the tree was rooted using the mouse Src1 sequence. The alignment utilized in the phylogenetic analysis was processed using the ALSCRIPT program (Barton, 1993).

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.

To examine the distribution of *Per* gene expression in the neonatal brain, breeding pairs of C57BL/6 mice were maintained in 12L:12D, and the presence of newborn pups was assessed daily. Neonatal mice (postnatal day 0, less than 24 hr old) were killed by decapitation in the afternoon (6–9 hr after lights-on), and the heads were frozen and processed as described above.

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, 1996). Probe for m*Per1* (GenBank accession number AF022992) was derived from nucleotides 340–761. Probe for m*Per2* (GenBank accession number AF035830) was derived from nucleotides 9–489. 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, 1996). 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 optical density values for sections hybridized with the sense control probes (0.187 for *Per1* and 0.206 for *Per2* in this experiment). The amplitude is defined as the peak specific hybridization OD value divided by the trough specific hybridization OD value.

Northern Analysis

Total RNA was extracted from whole eyes using an Ultraspec RNA Isolation System (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 (SA = 2×10^6 cpm/

ml). The blots were hybridized with Express Hybridization Solution (Clontech) and washed following the manufacturer's protocol. Probes used were m*Per1* (nucleotides 468–821 of accession number AF022992); human *Per1* (nucleotides 430–1175 of accession number AF022991; m*Per2* (nucleotides 9–489 of accession number AF035830); and h*Per2* (nucleotides 28–496 of accession number AB002345). Blots were exposed at −80°C to Biomax film with two intensifying screens.

Acknowledgments

We thank Kathryn Picanso, Kurtis Gray, and Yvette Miller for technical assistance, Cheng Chi Lee for providing the sequence of the mouse-*rigui* cDNA prior to its availability in GenBank, and Steve Kay for information concerning Z98884. This work was supported by R37 HD14427. M. J. Z. was supported in part by NRSA MH11547.

Received November 24, 1997; revised December 2, 1997.

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GenBank Accession Number

The mPER2 sequence has been deposited in GenBank under the accession number AF035830.