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RIGUI, a Putative Mammalian Ortholog of the Drosophila *period* Gene

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Summary

The molecular components of mammalian circadian clocks are elusive. We have isolated a human gene termed *RIGUI* that encodes a bHLH/PAS protein 44% homologous to Drosophila *period*. The highly conserved mouse homolog (*m-rigui*) is expressed in a circadian pattern in the suprachiasmatic nucleus (SCN), the master regulator of circadian clocks in mammals. Circadian expression in the SCN continues in constant darkness, and a shift in the light/dark cycle evokes a proportional shift of *m-rigui* expression in the SCN. *m-rigui* transcripts also appear in a periodic pattern in Purkinje neurons, pars tuberalis, and retina, but with a timing of oscillation different from that seen in the SCN. Sequence homology and circadian patterns of expression suggest that *RIGUI* is a mammalian ortholog of the Drosophila *period* gene, raising the possibility that a regulator of circadian clocks is conserved.

Introduction

In response to daily environmental cues, the physiology and behavior of all living organisms from bacteria to humans are controlled by circadian rhythms driven by endogenous oscillators (Dunlap, 1993; Takahashi, 1995). Alteration of the circadian rhythm in humans can lead to behavioral changes as typified by jet lag and sleep disorders including those associated with shift work (Arendt and Broadway, 1987; Vignau et al., 1993; Wehr, 1996). In addition, circadian rhythms have been linked to pathological effects such as an increased frequency of heart attacks during the morning period, and winter seasonal affective disorder (Kraft and Martin, 1995; Swaab et al., 1996; Teicher et al., 1997). Extensive physiological and behavioral studies have determined that the endogenous clock is characterized by a cycle approximately 24 hr in duration. When organisms are placed under invariant environmental conditions, this clock is self-sustaining, behaving as a pacemaker. The endogenous clock is further distinguished by its ability to be entrained (synchronized) by environmental cues such as light and temperature cycles (Pittendrigh, 1993; Takahashi, 1995). Primary culture of suprachiasmatic nucleus (SCN) neurons and SCN ablation and transplantation studies indicate that the circadian clock is cell autonomous and that in mammals it resides primarily in the SCN, which is located in the hypothalamus (Ralph et al., 1990; Welsh et al., 1995). A recent study has demonstrated the existence of independent circadian oscillators located in the retina (Tosini and Menaker, 1996). In constant darkness, the various circadian functions such as maintenance of body temperature, formation of urine, and secretion of cortisol become asynchronous (Aschoff, 1969). This suggests the existence of several independent clocks that each regulate specific rhythms. However, studies of the hamster *tau* mutant suggest that the molecular components that constitute the various clocks may be related (Tosini and Menaker, 1996).

The molecular components that constitute these oscillators in mammals are unknown. 2-deoxy [^{14}C]-glucose uptake experiments (Schwartz and Gainer, 1977) and studies using protein and RNA synthesis inhibitors suggest that circadian rhythms can be controlled by periodic expression of genes (Takahashi and Turek, 1987; Raju et al., 1991). It was recently found that a mutation in a single gene, *clock*, alters the period of the circadian clocks in mice (King et al., 1997). Whether *clock* is expressed in a periodic pattern is not known.

In Drosophila two genes, *period* (*per*) and *timeless* (*tim*), are essential components of the circadian clock (Reppert and Sauman, 1995). A heterodimer of Per and Tim proteins is thought to regulate the circadian process by creating a negative feedback loop controlling *per* and *tim* expression (Zeng et al., 1996). Two lines of evidence, the oscillatory nature of the *per* expression, and the phenotype of *per* mutants, portray the central role of the *per* gene in the circadian machinery of insects (Konopka and Benzer, 1971; Citri et al., 1987; Hardin et al., 1990; Hall, 1996). Immunohistochemical analysis of rat brain using a Drosophila Per antibody revealed staining in the SCN, suggesting the possibility of a conserved mammalian Per protein (Siwicki et al., 1992). However, in more than a decade since *per* was first isolated from Drosophila melanogaster (Bargiello et al., 1984; Citri et al., 1987), no mammalian *per* homolog has yet been reported.

This study describes the identification of a putative mammalian *per* ortholog designated as *RIGUI* (named after an ancient Chinese sundial). In the mouse this gene exhibits striking circadian changes of expression in the retina, the SCN, the hypophyseal pars tuberalis, and in the Purkinje cells of the cerebellum. As expected for a pacemaker gene, *RIGUI* oscillation is maintained under constant darkness. The cycle of oscillation can be entrained upon shifting the light/dark setting. Remarkably, *RIGUI* expression in the pars tuberalis, the retina, the Purkinje cells, and SCN is not in phase, indicating that expression in these tissues oscillates in an independent fashion.

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Taken together, our experiments suggest that *RIGUI* is a mammalian ortholog of the Drosophila *per* gene, and that a key regulator of circadian rhythms is thus conserved during evolution.

Results

Isolation and Characterization of *RIGUI* Transcripts

A partial cDNA for human *RIGUI* was obtained in an effort to identify human chromosome 17 specific transcripts (Lee et al., 1995). Out of 104 cDNAs identified this way, we selected five for further study. The deduced amino acid sequences of these five partial cDNAs indicated some degree of sequence homology to regulatory proteins (Sun et al., 1996). The sequence of one of the five cDNAs later revealed the presence of a basic helix-loop-helix (bHLH) motif and a Per-ARNT-Sim (PAS) domain (see below). This clone was referred to as *RIGUI* and maps to human chromosome 17p12 (Figure 1A). Using a 0.9 kb probe, derived from the initially isolated partial cDNA, 15 cDNA clones were isolated from a human heart muscle cDNA library. Ten clones ranged in length between 3 and 4.7 kb and, based on their DNA sequences, represent the *RIGUI 4.7* transcript (Figure 1B). Northern blots display a band at 4.7 kb possibly representing the 4.7 kb cDNA (Figure 1C) although a prominent larger species is also observed (see below). Three of 15 clones had high sequence homology to the 3′ region of *RIGUI 4.7*, but contained three segments inserted between the regions of homology (Figure 1B, *RIGUI 6.6*). Combining these partial cDNAs with the *RIGUI 4.7* sequence can account for the 6.6 kb band detected in the Northern blot (Figure 1C). The two remaining clones were 3.0 kb in length, and sequences from their 5′ and 3′ regions were identical to the corresponding 5′ and 3′ region of the *RIGUI 4.7* transcript; however, the clones were shorter due to an alternate splicing event. Although several *RIGUI* transcripts were isolated, the FISH studies (Figure 1A) and genomic-PCR experiments (data not shown) identify *RIGUI* as a single locus gene. The three transcripts shown in Figure 1B result from differential splicing.

RIGUI 4.7 can be translated into a protein sequence of 1301 amino acids (Figures 1B and 2; GenBank accession number: AF022991). The largest deduced open reading frame from *RIGUI 6.6* gave a peptide of 875 amino acids (Figure 1B). The initial 821 amino acids were identical to those of RIGUI 4.7, but more C-terminal residues diverged. The largest deduced reading frame of the *RIGUI 3.0* was 798 amino acids long (Figure 1B). RIGUI 4.7 and RIGUI 3.0 diverge at amino acid 758. Taken together, the *RIGUI* gene gives rise to at least three proteins that differ in their C-terminal regions.

BLAST and FASTA searches against peptide sequences revealed significant protein sequence similarity of RIGUI 4.7 ORF to D. melanogaster Per protein (Figure 2). A BLAST search yielded the following probability scores P(N) of homology: Per (various Drosophila species) between 5.1×10^{-26} to 5.2×10^{-20}, the next highest score was with the mammalian Aryl hydrocarbon receptor nuclear translocater (ARNT), which gave a P(N) of 5.9×10^{-14}, and the P(N) with mouse Single minded

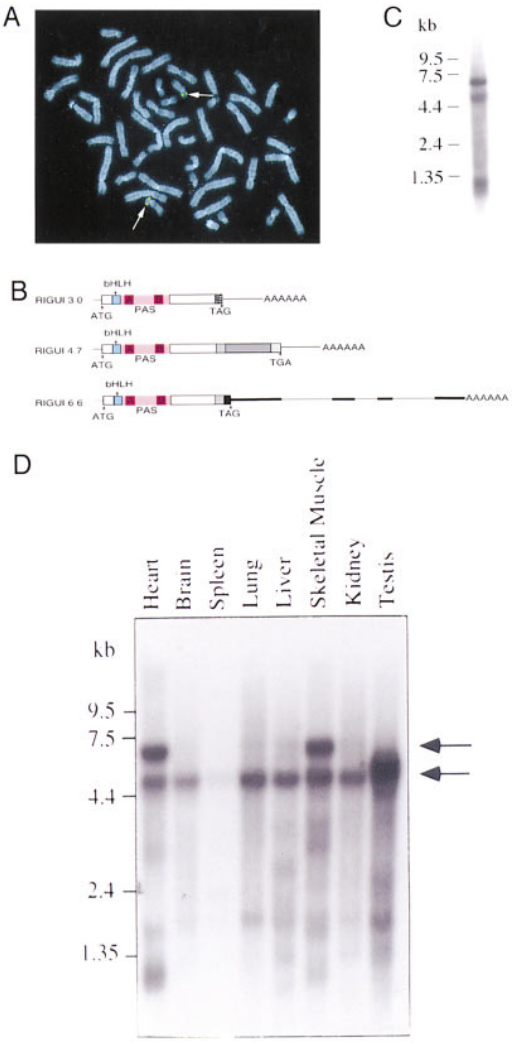

Figure 1. Characterization of the Human *RIGUI* Gene

(A) FISH mapping of a cosmid 39C2 (LA17NC01) containing the *RIGUI* gene to human metaphase chromosomes. The gene maps to the short arm of chromosome 17.

(B) Structure of three RIGUI isoforms deduced from extensive screening of a human heart cDNA library. The three isoforms are identical in regions indicated by the same hue/shading. The proteins diverge in their C-terminal portion. The blue box indicates a putative bHLH motif and the pink box represents the PAS domain with the two characteristic repeats A and B. Thick lines in RIGUI 6.6 3′ UTR represent sequences not present in the RIGUI 3.0 and RIGUI 4.7 variants.

(C) Northern blot of human heart muscle RNA probed with the *RIGUI* cDNA. Note the transcripts at 4.7 and 6.6 kb, possibly representing *RIGUI 4.7* and *RIGUI 6.6* cDNAs shown in (B).

(D) Northern analysis of adult mouse tissues. Transcript sizes (arrows) are similar to those seen in human.

(SIM1) protein was 0.52. The overall homology (identical amino acids, conservative and neutral substitutions) between RIGUI and Per of D. melanogaster is 44% and a slightly higher homology of 48% is found in the initial 500 amino acids that include the PAS domain. The PAS domain was initially observed in D. melanogaster Per, in human ARNT, and the Drosophila SIM protein. The PAS domain is approximately 260 amino acids in length

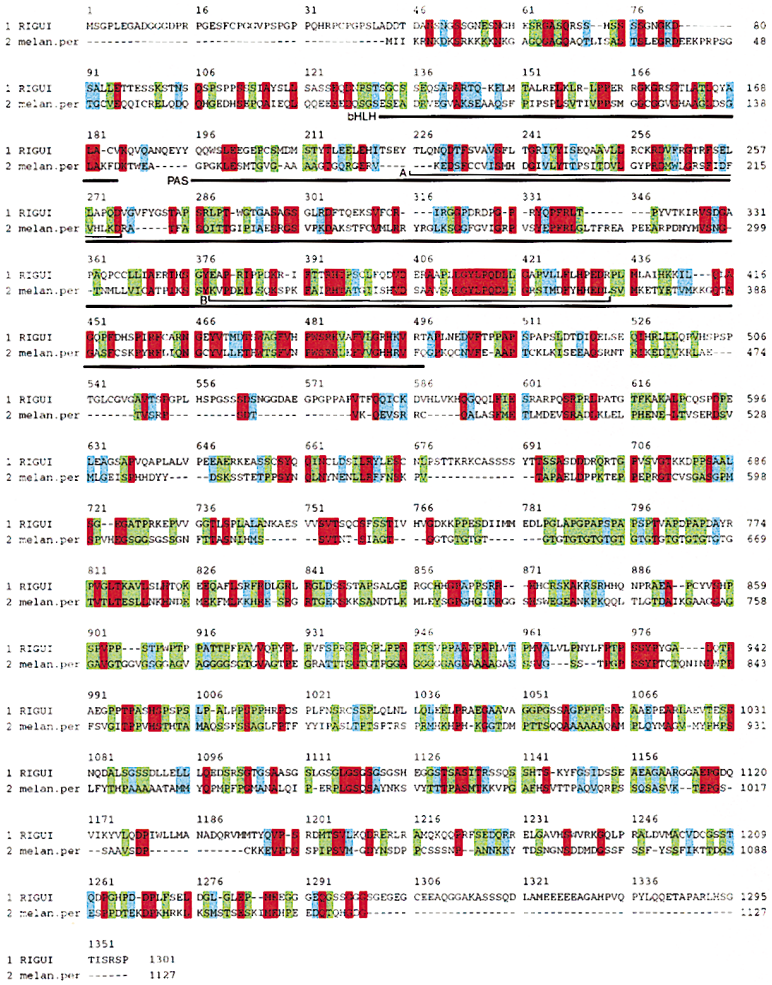

Figure 2. Comparison of the Predicted Protein Sequences of RIGUI and D. melanogaster Per

Alignment of the two protein sequences was carried out by the pattern-induced multisequence alignment program (PIMA) and revealed marked identity between them (Smith and Smith, 1992). The amino acids shaded in red are identical, those shaded in blue are conserved substitutions, and those shaded in green are neutral substitutions. The bHLH motif and the PAS domain are indicated by horizontal lines, and brackets A and B indicate the PAS A and B repeats.

and contains two direct repeats of 51 amino acids each (A and B; see Figures 1B and 2). Sequence homology in the Per A and B repeats is 39% and 61%, respectively. Importantly, many of the amino acids conserved between D. melanogaster Per and RIGUI reside outside of the PAS domain, emphasizing the similarity between the two proteins (Figure 2). In contrast, very little protein sequence identity was observed outside of the PAS domain of RIGUI or Per when compared to other PAS domain proteins including ARNT, SIM, AHR, NPAS1, NPAS2, and CLOCK.

Using the PHDsec program (EMBL) for secondary structure analysis, the N-terminal region of RIGUI was determined to contain a putative bHLH motif. Alignment of the bHLH region of RIGUI with that of other bHLH-PAS proteins revealed that several of the bHLH consensus amino acids are conserved (Figure 3). Analysis of the D. melanogaster Per protein with the same program did not reveal a bHLH motif. Taken together, RIGUI contains a bHLH-motif and a PAS domain thus emerging as a new member of a growing family of putative transcription factors that includes the recently identified gene products of *NPAS1*, *NPAS2* and *CLOCK* (King et al., 1997; Zhou et al., 1997). From our sequence analysis we proposed that RIGUI is a human ortholog of Drosophila Per.

Oscillation of *m-rigui* mRNA Expression in the Retina

As has been demonstrated for *per* and *tim* in Drosophila (Hardin et al., 1990; Sehgal et al., 1994) and the *frequency* gene in Neurospora (Dunlap, 1993), circadian oscillator genes are expressed in a periodic manner reflecting the 24 hr day/night cycle. To examine whether expression of *RIGUI* behaves in a similar way, we studied its expression in the mouse. A murine brain cDNA library was screened with the human *RIGUI 4.7* cDNA as probe, and a mouse homolog termed *m-rigui* was identified, encoding a protein of 1291 amino acids that has 92% amino acid identity with human RIGUI. The PAS and bHLH domains of the two proteins are 98% identical (GenBank accession number: AF022992). Northern analysis of several mouse tissues shows that *m-rigui* is expressed broadly (Figure 1D).

We next measured the level of *m-rigui* mRNA by RNase protection assays using RNA from the retinae of six adult male mice sacrified every 4 hr during a 12 hr light/12 hr dark cycle. The mammalian retina contains a circadian oscillator not dependent on that from the SCN (Tosini and Menaker, 1996). The level of *m-rigui* mRNA increased during the light phase from ZT4 to ZT12 (whereby zeitgeber time ZT0 is when lights were

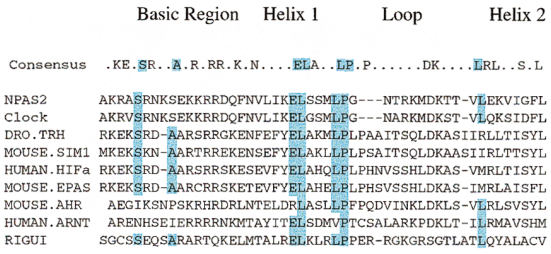

Figure 3. Alignment of the bHLH Motif of RIGUI and Other PAS Domain Gene Products

The amino acid sequence alignment of the basic-helix-loop-helix motif of RIGUI and 8 other products of the bHLH-PAS gene family was undertaken. The consensus line represents identical residues observed in >50% of the proteins listed. Amino acid residue conserved in RIGUI are in blue. The peptide sequences for the various bHLH-PAS were obtained from GenBank. Accession numbers for proteins listed are as follows: NPAS2 (U77969); clock (AF000998); Drosophila TRH (U42699); Mouse SIM1 (D79209); Human HIFα (U22431); Mouse EPAS1 (U81983); Mouse AHR (M94623); and Human ARNT (M69238).

turned on and ZT12 is when lights were turned off) and decreased during the dark phase between ZT16 to ZT24/ZT0 (Figure 4). In contrast, expression of *GAPDH*, an internal standard, remained constant during the same time span. When the level of *m-rigui* expression was normalized to that of *GAPDH*, the *m-rigui* RNA abundance was found to change 2.9-fold between the highest and the lowest levels in the daily cycle. We also determined *m-rigui* mRNA levels in the retina over a period of 3 days using reverse transcriptase PCR giving rhythms similar to those shown in Figure 4 (data not shown). These results indicate that the expression of the *m-rigui* transcript is circadian in nature and that this gene may be part of a molecular clock.

Oscillation of *m-rigui* Expression in the SCN, the pars tuberalis, and the Purkinje Neurons

In order to determine whether *m-rigui* was expressed in specific regions of the brain, we performed expression analysis by in situ hybridization. In situ hybridization was carried out using a 544 nt long riboprobe corresponding to the coding region of *m-rigui*. Adult male 129/SvEvBrd mice, kept in a 12 hr light/12 hr dark cycle, were sacrificed at 6 hr time intervals, and their brains were rapidly removed and fixed in ice-cold fixative. The four time points chosen were ZT6, ZT12, ZT18, and ZT24. To avoid the induction of immediate-early genes by light that could occur at ZT0 (Aronin et al., 1990; Kornhauser et al., 1992), we elected not to sacrifice animals at this time point but chose ZT24 instead.

An analysis of *m-rigui* expression on coronal and sagittal brain sections gave the following results (Figure 5). Expression in the SCN was high at ZT6 (Figure 5A), was much reduced at ZT12 (data not shown), was not detected at ZT18 (Figure 5B), and was very low at ZT24 (data not shown). In D. melanogaster the *per* amplitute of RNA rhythmicity is in the range of 8- to 10-fold (Hardin et al., 1990). Although quantitation by in situ hybridization cannot be reliably carried out, it can be estimated that *m-rigui* exhibits a similar rhythmicity amplitude in the SCN. Thus, as in the retina, there was a circadian

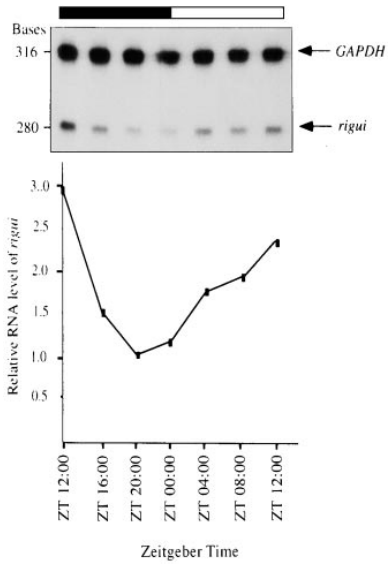

Figure 4. Circadian Regulation of *m-rigui* in the Mouse Retina of 129/SvEvBrd Male Mice

Retina RNA was isolated at different zeitgeber times (ZT) indicated on the abscissa. Amount of *m-rigui* expression was quantified by RNase protection assays and calibrated in reference to *GAPDH* mRNA levels using a Fuji BAS-100 phosphoimager. The graph illustrates the relative transcript level of *m-rigui* normalized to *GAPDH* as a function of ZT. The smallest value is adjusted as 1 and a peak level of 2.9 is observed at ZT12. The diurnal cycle is indicated by the light/dark bar.

pattern of *m-rigui* expression in the SCN. We also found such a temporal profile in the pars tuberalis, which surrounds the hypophyseal stalk of the pituitary gland (Figures 5C and 5D). However, in this case expression was highest at ZT24 (Figure 5C) and not detected at ZT12 (Figure 5D).

Another site of periodic *m-rigui* expression were the Purkinje neurons of the cerebellum (Figures 5E and 5F). A high level of expression in Purkinje neurons is seen at ZT12 (Figure 5F) and expression was minimal at ZT24 (Figure 5E). This expression profile is similar to that seen in the retina (Figure 4), but different from the situation in the SCN or the pars tuberalis.

When we examined expression of *m-rigui* in C57BL/6 male mice, the pattern of expression in the SCN was identical to that seen in 129/SvEvBrd males (data not shown). Remarkably, there was no expression of *m-rigui* in the pars tuberalis at any time point examined. Figures 5G and 5H illustrate the absence of *m-rigui* transcripts at ZT24 and ZT12. This result suggests strain-specific differences in the regulation of *m-rigui* expression in this tissue.

Other regions of the brain also expressed *m-rigui*, including the glomerular and mitral cell layers of the olfactory bulb (data not shown), the internal granular layer of the cerebellum (Figures 5E and 5F), the cornu ammonis and dentate gyrus of the hippocampus (Figure 5I), the cerebral and piriform (Figure 5J) cortices. No changes in *m-rigui* expression could be detected in these structures.

A recently identified mouse gene *clock* was also examined for changes in expression in the SCN and in

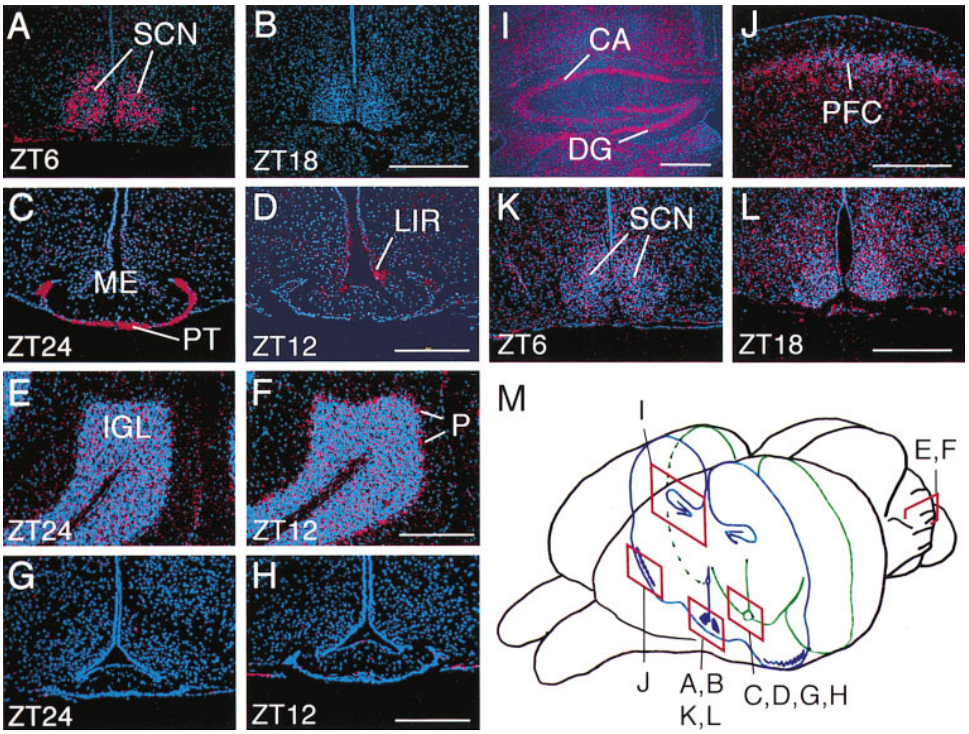

Figure 5. Circadian Regulation of *m-rigui* in the Mouse Brain

All micrographs were taken from brains of male 129/SvEvBrd mice, except for those shown in (G) and (H), which show tissue from C57BL/6 males. (A) High levels of expression of *m-rigui* in the suprachiasmatic nucleus (SCN) at zeitgeber time ZT6. (B) At ZT18, expression of *m-rigui* is no longer detected. (C) Expression of *m-rigui* in the pars tuberalis is high at ZT24. The pars tuberalis is a sheet of tissue surrounding the median eminence. (D) By ZT12, expression of *m-rigui* in the pars tuberalis is greatly reduced. Note weak expression in the lateral infundibular recess. (E and F) *m-rigui* is persistently expressed in the internal granular layer of the cerebellum. In contrast, Purkinje neurons express *m-rigui* strongly at ZT12. (G and H) Absence of expression of *m-rigui* in the pars tuberalis of C57BL/6 mice. (I) Expression of *m-rigui* in the hippocampus and piriform cortex (J) is constitutive. (K and L) Expression of the mouse *clock* gene in the SCN is constitutive. (M) Diagram illustrating plane and location of sections displayed in (A) to (L).

Abbreviations: CA, cornu ammonis; DG, dendate gyrus, IGL, internal granular layer; LIR, lateral infundibular recess; ME, median eminence, P, Purkinje neurons; PFC, piriform cortex; PT, pars tuberalis; SCN, suprachiasmatic nucleus. Scale bars correspond to 500 μm in all figures except (C), (D), (G), and (H), where the bars are equivalent to 300 μm.

other brain tissues. As previously reported (King et al., 1997), this gene is broadly expressed in the brain including the SCN. Two different probes, one corresponding to the 5′ coding region and the other corresponding to the 3′ UTR, failed to detect a change in expression in the SCN (Figures 5K and 5L).

Taken together, our data reveal striking diurnal changes in *m-rigui* expression. Intriguingly the times of maximal expression are not the same in SCN, retina, Purkinje neurons, and pars tuberalis. The asynchronous expression of *m-rigui* may thus reflect the fact that there are several independent circadian clocks in mammals.

Circadian Expression of *m-rigui* in the SCN Persists under Free-Running Conditions

To examine whether the absence of light affects *m-rigui* expression, we transferred 129/SvEvBrd males from a regular light/dark cycle to a dark/dark cycle. Then 72 hr later, animals were sacrificed every 6 hr and dissected under a 15 W safety red light lamp. *m-rigui* expression in the SCN and the pars tuberalis of these animals is depicted in Figure 6. At 5 P.M., corresponding to the subjective day Circadian Time CT12, expression in the SCN was visible but low (Figure 6A). Thereafter, transcript levels decreased to background levels (subjective day times CT18 and CT24; Figures 6B and 6C). At subjective CT6 (Figure 6D), however, expression was very high, comparable to ZT6 in Figure 5A. Expression in the pars tuberalis peaked at CT24 (Figure 6G), i.e., 6 hr ahead of that in the SCN (Figure 6D). We conclude that the oscillation of *m-rigui* expression is maintained under free-running conditions. This indicates that this gene is regulated by light-independent, endogenous mechanisms, a feature characteristic for a circadian clock gene.

Entrainment of *m-rigui* Expression by Light

Circadian pacemakers are eventually reset under the influence of a changing light source. To test whether *m-rigui* expression responds to such a change, C57BL/6 mice were transferred to a 12 hr light/12 hr dark cycle that had been advanced by 6 hr. Animals were analyzed at the day of transfer to the new cycle, and 3 and 8 days thereafter. In each case animals were sacrificed at four 6 hr time intervals. Expression analysis was focused on the SCN (Figure 7). A shift of maximal expression is clearly seen. At day 0, expression peaks at ZT6 (Figure 7D). At day 3 expression is about equal at ZT6 and ZT12 (Figures 7G and 7H). By day 8, the entrainment is complete and only ZT6 shows high *m-rigui* expression

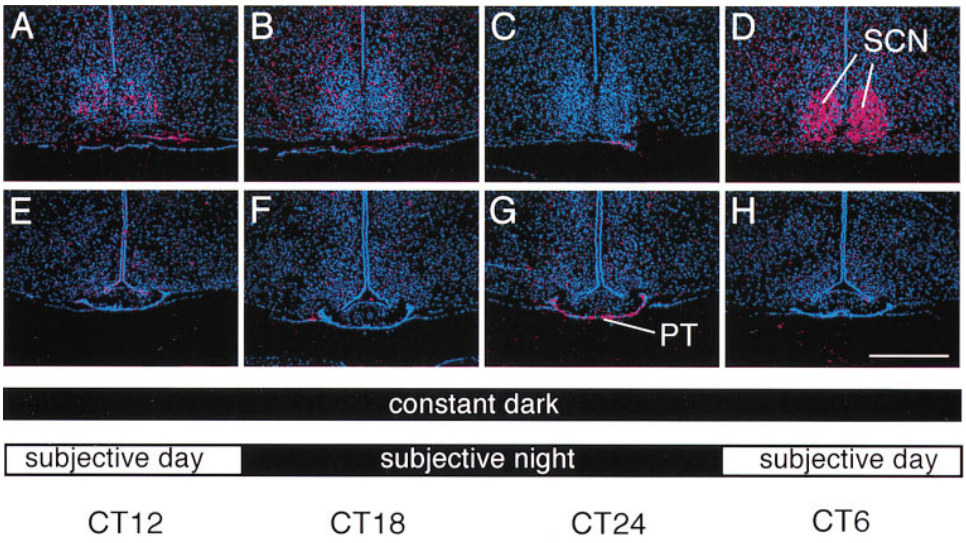

Figure 6. *m-rigui* in the SCN and pars tuberalis in Male 129/SvEvBrd Mice Kept in Constant Darkness

m-rigui in the SCN (A–D) and pars tuberalis (E–H). Animals were transferred from a 12 hr light/12 hr dark cycle to constant darkness. The bar at the bottom of the figure indicates the subjective time. Starting 72 hr after the transfer, animals were sacrificed every 6 hr at the times indicated in the figure. Strongest expression of *m-rigui* is seen at subjective circadian time CT6. For abbreviations see Figure 5. Scale bar corresponds to 500 μm.

(Figure 7K). From these data we conclude that, over a period of approximately one week, the regulation of the *m-rigui* gene is responsive to the light/dark cycles of the environment.

Discussion

Putative mammalian circadian regulator molecules should have the following characteristics. First, their expression should oscillate with a 24 hr rhythm. Second, they must be expressed in the suprachiasmatic nucleus (SCN), the master regulator of mammalian circadian rhythms. Third, circadian expression must persist in the absence of environmental cues such as light. Fourth, the intrinsic rhythm of expression should be reset by changes in the oscillation of environmental cues (entrainment). As judged from temporal mRNA expression studies, *m-rigui* fulfills these criteria. We find that, during a 24 hr period, the level of expression of this gene in the SCN goes through a distinct maximum and minimum. Maximum expression of *m-rigui* in the SCN occurs at ZT6, which suggests that this gene behaves like a "day-type" oscillator (Dunlap, 1996). Moreover, when animals are transferred to constant darkness, such oscillation persists in the SCN for at least four days. Whether the peak of expression is slightly shifted during that period of time cannot be resolved using expression data based on in situ hybridization. If animals are exposed to a light/dark cycle that is advanced by 6 hr, then a shift in the peak expression time in the SCN occurs gradually. Based on these data, we conclude that the expression of *m-rigui* can be entrained by a light cue. An additional piece of evidence suggesting that *m-rigui* and its human homolog are clock genes comes from the observation of marked amino acid sequence conservation between m-rigui and the D. melanogaster Per protein. Mutations in *per* cause abnormal circadian rhythms and its expression oscillates with a circadian rhythm (Konopka and Benzer, 1971; Hardin et al., 1990; Hall, 1996). Our sequence analyses identify RIGUI and its mouse homolog as a member of the bHLH-PAS domain family, which includes gene products such as ARNT, SIM, AHR, NPAS1, NPAS2, and the recently identified circadian regulator Clock. When compared with other members of this family, the best alignment within the PAS domain is found between RIGUI and Per. However, sequence identity is relatively low, explaining the difficulty of finding a vertebrate *per* homolog by cross-species hybridization approaches (Takahashi, 1995). Several studies using an anti-Per antibody have suggested the existence of a mammalian Per ortholog (Hall, 1990; Siwicki et al., 1992). It remains to be determined whether the antigen detected in these immunological analyses is encoded by RIGUI (note, a 14 amino acid long peptide used to generate the anti-Per antibody had only 29% sequence identity with the corresponding region of mouse Rigui protein). It is important to emphasize that there are many small clusters of amino acid sequence conservation outside the PAS domain that are conserved between RIGUI and Per. This supports our hypothesis that RIGUI is a mammalian ortholog of Per. Of note, RIGUI but not Per contains a bHLH motif. Whether this discrepancy disqualifies RIGUI from being a true Per ortholog remains to be determined by experiments in which these genes are functionally interchanged. Fluorescence in situ hybridization (FISH) and genomic PCR mapping indicate the presence of a single *RIGUI* locus. The existence of other *per*-like genes, not detected by these methods is a possibility. Nagase et al. (1997) have recently reported a protein sequence that is 40% identical to RIGUI. Taken together, our expression studies, in combination with the presence of Per-like motifs in RIGUI, raise the possibility that RIGUI is an essential

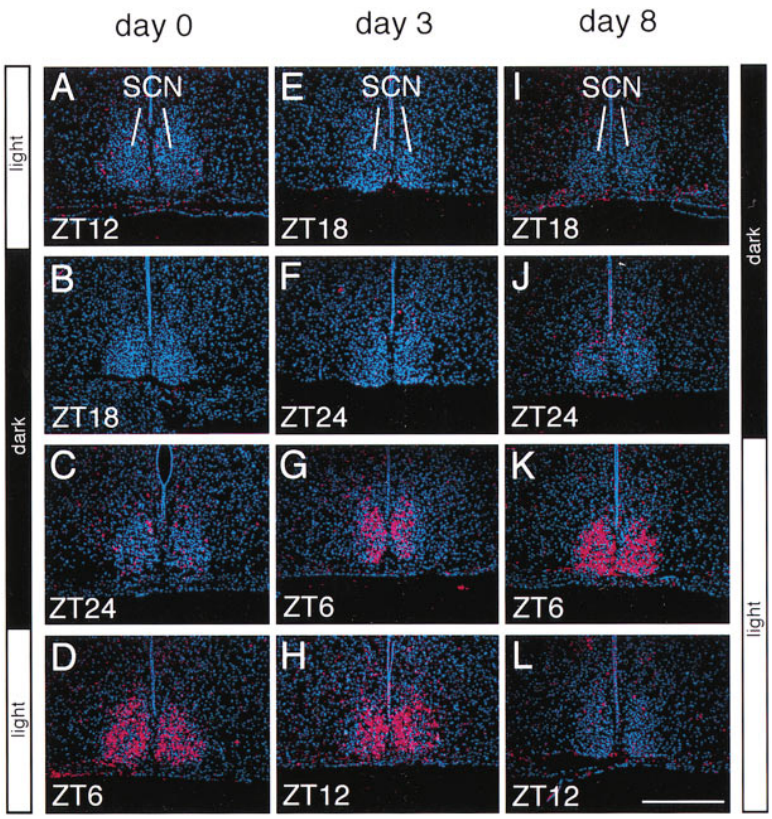

Figure 7. Entrainment in the SCN of *m-rigui* Expression by a Forward Shift of the 12 hr Light/12 hr Dark Cycle by 6 hr

Data are from C57BL/6 males. For abbreviations see Figure 5. Scale bar corresponds to 500 μm.

(A–D) SCN of a reference animal kept in the standard 12 hr light/12 hr dark cycle (symbolized by the vertical bar to the left). Note maximal expression at ZT6 (D).

(E–H) Animals sacrificed 3 days following the 6 hr shift of the 12 hr light/12 hr dark cycle (symbolized by the vertical bar to the right). High expression is now seen in (G) and (H) indicating an initiation of a phase shift in *m-rigui* expression.

(I–L) Animals sacrificed 8 days following the 6 hr shift of the 12 hr light/12 hr dark cycle. High expression is now seen only in (K) indicating a completion of the entrainment of *m-rigui* expression and acquisition of the new day/night cycle.

regulator of the mammalian clock. At the present time it is not entirely clear whether RIGUI is a component of the circadian clock or an output element of the circadian pathway.

In addition to the SCN, *m-rigui* is expressed in the internal granular layer and the Purkinje cells of the cerebellum, the hippocampus, the cerebral cortex, the olfactory bulb, the pars tuberalis, and the retina. We did not notice any periodic expression of *m-rigui* in the internal granular layer, the cerebral cortex and the hippocampus, but the other tissues show oscillatory *m-rigui* expression. Interestingly, the phases of these cycles are not synchronized to that of the SCN and differ amongst themselves. The nature of this phenomenon is not clear, but the data raise the possibility that there is tissue-independent regulation of *m-rigui* expression. It is tempting to speculate that this reflects the cell-autonomous expression of circadian clocks previously observed in cell and organ cultures and in transplantation studies (Ralph et al., 1990; Welsh et al., 1995; Tosini and Menaker, 1996). The existence of multiple oscillators suggests that *m-rigui* expression is probably not controlled by a single upstream regulator and raises the possibility of autoregulation, perhaps in conjunction with other proteins such as Clock.

What could be the significance of the oscillation of *m-rigui* expression in the pars tuberalis? This structure is a glandular epithelium surrounding the hypophyseal stalk of the pituitary gland and is in direct contact with the portal blood supply. The pars tuberalis also releases luteinizing hormone, which is negatively regulated by circulating melatonin (Nakazawa et al., 1991). The pars tuberalis has the highest concentration of melatonin receptors in the mammalian brain (de Reviers et al., 1989; Weaver and Reppert, 1990; Stankov et al., 1991; Fraschini and Stankov, 1993). These observations, in conjunction with the oscillating expression of *m-rigui*, suggest that the pars tuberalis is a target site for a melatonin feedback loop and confers a circadian rhythm to the body via hormonal pathways.

Interestingly, expression of *m-rigui* in the pars tuberalis was observed in 129/SvEvBrd mice but not in the C57BL/6 strain. C57BL/6 and the majority of the inbred mice strains (exceptions are C3H/H and CBA) are known to have a genetic defect for pineal melatonin biosynthesis. Thus C57BL/6 mice do not produce melatonin (Goto et al., 1989). The 129/SvEvBrd strain carries C3H/H alleles as a result of historical backcrosses with that strain (Simpson et al., 1997), and thus it is likely to generate melatonin. We propose that the strain-dependence of *m-rigui* expression in the pars tuberalis may reflect the difference in melatonin production. This would implicate melatonin as a regulator of *m-rigui* expression in this region of the brain.

The identification of *RIGUI* as a putative circadian clock gene provides a useful tool to explore the molecular mechanism of the mammalian circadian machinery. Using interaction screening approaches, it should be possible to find interacting proteins, perhaps in the form of a Drosophila Tim ortholog. Furthermore, promoter analyses of the *RIGUI* gene should uncover how light cues and possibly other environmental stimuli regulate the expression of this gene. Lastly, targeted disruption of the *m-rigui* gene, using stem cell technology, may

provide a valuable model system to study the various physiological and pathophysiological aspects of disrupting circadian rhythms.

Experimental Procedures

Mapping Studies by FISH and STS–PCR

Fluorescence in situ hybridization (FISH) mapping of a 39C2 cosmid on metaphase chromosomes was conducted essentially as previously described (Ijdo et al., 1992). STS-PCR mapping of *RIGUI* was carried out with the Corriel mapping panel #2 using the following primers 5′-CTCCCATCTGGGGAGGAGGT-3′ and 5′-GGACCATCTC CAGGAGTCCA-3′ with methods as previously described (Lee et al., 1995).

Screening of cDNA Libraries

The cDNA for *RIGUI* was isolated by reciprocal probing (Lee et al., 1995) of an arrayed human heart cDNA library with the LA17NC01 chromosome 17 cosmid library (Kallioniemi et al., 1994). This approach results in the simultaneous identification of genomic cosmid clones associated with a particular cDNA. The full-length cDNA for *RIGUI* and *m-rigui* was identified from a human heart and a mouse brain cDNA library (GIBCO–BRL), respectively. Radiolabeled probes generated from *RIGUI* cDNA fragments were used to screen the appropriate cDNA library using standard laboratory protocols (Sambrook et al., 1989). Both strands of the nucleotide sequence of the *RUGUI* and *m-rigui* cDNA were determined using DNA sequencing reaction kits and an ABI 373A instrument.

RNase Protection Assay

Six adult mice were sacrificed at 4 hr intervals. The eyes and brains were dissected and immediately homogenized in 4 M guanidinium isothiocyanate solution. Total RNA was isolated by CsCl ultracentrifugation as previously described (Chirgwin et al., 1979).

The *m-rigui* probe, a 280 bp fragment between the restriction site PstI at nucleotide position 722 and the SacI site at position 995, was subcloned into pBluescript II vector. This plasmid was linearized with EcoRI and a radiolabeled antisense probe was generated by T3 RNA polymerase with [^{32}P]CTP using a kit purchased from Promega. The 316 bp internal control *GAPDH* probe (Ambion) was generated using the same procedure except that the ratio of the cold to hot CTP was increased by 50-fold compared to the *m-rigui* probe. All the riboprobes were purified by gel electrophoresis and eluted in RNase free elution buffer (Ambion). For each time point, 20 μg of RNA was hybridized to 5×10^4 cpm of each riboprobes under the following conditions; 0.75 M NaCl, 0.075 M sodium citrate, and 0.05 Tris–HCl (pH 7.0) at 65°C for 16 hr. On completion of hybridization, the reaction mixtures were treated according the procedure of the RNase protection kit purchased from Boehringer-Mannheim.

Specimen Preparation and Histology

The 129SvEvBra and C57BL/6J mouse strains were provided by Dr. Allan Bradley. All animals were kept in separate cages under the condition of 12 hr light/12 hr dark cycle for at least two weeks prior to their use in the respective experiments. For free-running condition, the mice were maintained in a room with lights completely turned off. Both the 12 hr light/12 hr dark cycle and entrainment experiments were carried out as stated in the legends of the corresponding figures. Mice were sacrificed by cervical dislocation and the brain was removed, fixed in ice-cold 4% paraformaldehyde for 16 to 20 hr. Tissue was dehydrated and embedded in paraffin and sectioned at a thickness of 7 μm. Animals collected under dark conditions were dissected under a 15 W safety red light.

RNA In Situ Hybridization

In situ hybridization was carried out as described (Albrecht et al., 1997). Antisense and sense riboprobes were synthesized with T3 or T7 RNA polymerase in the presence of [α-^{35}S]UTP (1250 Ci/mmol; Du Pont NEN, Charlotte, NC). The *m-rigui* probe was made from a cDNA corresponding to nucleotides 620 to 1164 (GenBank accession number: AF022992). Two different *clock* probes were obtained from clones generated by PCR amplification of oligo-dT primed mouse brain cDNA. Probe 1 encompassed nucleotides 1352 to 2080 of the coding region, and probe 2 encompassed nucleotides 6331 to 7122 in the 3′ UTR (Genbank accession number: AF000998). Hybridization was done overnight at 55°C (*m-rigui* and both *clock* probes). Stringency washes were performed at 64°C (*m-rigui*), 65°C (coding region probe for *clock*), and 63.5°C (3′ UTR region probe for *clock*). Slides were dipped in NTB-2 emulsion and exposed for 6 to 10 days. Tissue was visualized by fluorescence of Hoechst dye-stained nuclei (blue color in figures). Silver grains (appear red in figures) were visualized by dark-field illumination. Images are videographs captured with Adobe Photoshop.

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