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# Mammalian Circadian Autoregulatory Loop: A *Timeless* Ortholog and *mPer1* Interact and Negatively Regulate CLOCK-BMAL1-Induced Transcription

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## Summary

**We report the cloning and mapping of mouse (*mTim*) and human (*hTIM*) orthologs of the *Drosophila timeless* (*dtim*) gene. The mammalian *Tim* genes are widely expressed in a variety of tissues; however, unlike *Drosophila*, *mTim* mRNA levels do not oscillate in the suprachiasmatic nucleus (SCN) or retina. Importantly, hTIM interacts with the *Drosophila* PERIOD (dPER) protein as well as the mouse PER1 and PER2 proteins in vitro. In *Drosophila* (S2) cells, hTIM and dPER interact and translocate into the nucleus. Finally, hTIM and mPER1 specifically inhibit CLOCK-BMAL1-induced transactivation of the *mPer1* promoter. Taken together, these results demonstrate that *mTim* and *hTIM* are mammalian orthologs of *timeless* and provide a framework for a basic circadian autoregulatory loop in mammals.**

## Introduction

Circadian rhythms are a fundamental property of living systems and impose a 24 hr temporal organization regulating the physiology and biochemistry of most organisms (Pittendrigh, 1993). In mammals, circadian rhythms are controlled by the suprachiasmatic nucleus (SCN) (Ralph et al., 1990). These rhythms have been shown to be under the control of cellular pacemakers (Welsh et al., 1995), which, in turn, are under genetic control (Vitaterna et al., 1994). Molecular components that comprise these pacemakers have been identified in a diverse set of organisms including the fruit fly, the mouse, and fungi (Takahashi, 1995; Dunlap, 1996; Young, 1998). However, until recently, it has remained unclear whether the circadian pacemakers of these various organisms share a common molecular mechanism (Dunlap, 1998).

Perhaps the circadian system that has been best characterized at the molecular genetic level is that of *Drosophila melanogaster* (Rosato et al., 1997a; Young, 1998). Two oscillator components, *period* (*per*) and *timeless* (*tim*), express rhythms in messenger RNA and protein abundance (Hardin et al., 1990; Edery et al., 1994; Sehgal et al., 1995; Myers et al., 1996). Mutations in these genes affect both overt circadian rhythms of eclosion and locomotor activity (Konopka and Benzer, 1971; Sehgal et al., 1994) as well as molecular oscillations of *per* and *tim* gene products (Hardin et al., 1990; Sehgal et al., 1995). The *per* and *tim* genes are involved in a negative autoregulatory feedback loop that underlies overt rhythm generation (Hardin et al., 1990; Zeng et al., 1994; Sehgal et al., 1995). The TIMELESS (dTIM) and PERIOD (dPER) proteins physically interact and regulate the expression of their own mRNAs following nuclear entry (Gekakis et al., 1995; Saez and Young, 1996).

Work over the past year has demonstrated a striking parallel between the mammalian and *Drosophila* circadian systems. The *Clock* gene regulates the period and persistence of circadian rhythms in mice (Vitaterna et al., 1994). The molecular identification of *Clock* first hinted at the conservation of the circadian system between flies and mammals as it encodes a novel basic helix–loop–helix (bHLH) PAS (PER-ARNT-SIM) transcription factor (Antoch et al., 1997; King et al., 1997). Subsequently, three mammalian *Per* homologs, *mPer1*, *mPer2*, and *mPer3*, have been identified. All three genes show oscillations in mRNA abundance in the SCN and retina (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Sun et al., 1997; Tei et al., 1997; Takumi et al., 1998a, 1998b; Zylka et al., 1998). Recent work has shown that CLOCK heterodimerizes with a bHLH-PAS partner known as BMAL1 or MOP3 (Gekakis et al., 1998; Hogenesch et al., 1998). The CLOCK-BMAL1 complex transactivates the *mPer1* promoter specifically via E box elements contained within the first 1.2 kb upstream of the gene (Gekakis et al., 1998). Concomitantly, the corresponding genes in *Drosophila* were discovered with the identification of *dClock*, a homolog of mouse *Clock*, and *cycle/dbmal*, a homolog of mammalian *BMAL1* (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998). Mutations in these two genes cause arrhythmicity in fly circadian behavior and abolish *per* and *tim* molecular oscillations (Allada et al., 1998; Rutila et al., 1998). dCLOCK, likely in partnership with dBMAL, activates the transcription of both *per* and *tim* through E box elements found in their respective promoters (Darlington et al., 1998). dPER and dTIM appear to feed back negatively by inhibiting the dCLOCK-induced activation on their own promoters (Darlington et al., 1998). Thus, the identification of dCLOCK-dBMAL defined a critical site for both positive and negative regulation of the circadian cycle in *Drosophila*.

Because a highly conserved picture of the *Drosophila* and mammalian circadian systems has emerged, we

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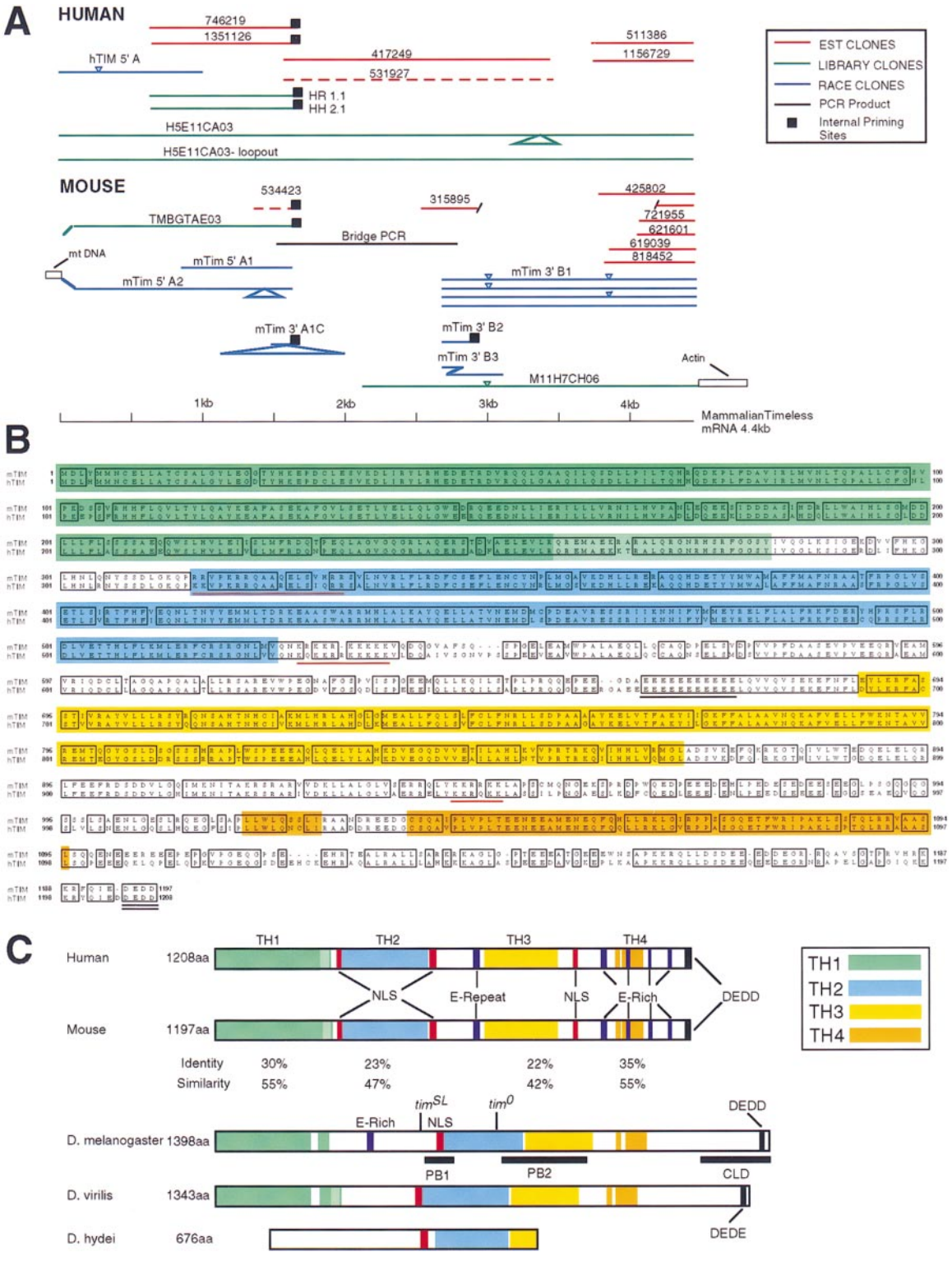


Figure 1. Cloning and Sequence Alignment of Mammalian *Tim*

(A) Clones obtained from the EST database (red), library screening (green), RACE (blue), and PCR (black) experiments are indicated for both *mTim* and *hTIM*. Introns are indicated by triangles below clones containing them. Insertions are delineated by small triangles above the clone. Internal priming sites (A-rich sequence) are identified by closed squares. Dashed lines indicate ESTs whose clones could not be recovered (likely misaddressed). The loopout clone derived by site-directed mutagenesis from H5E11CA03, which represents the full-length *hTIM* cDNA, is also indicated. Two clones were chimeric (mtDNA [mitochondrial DNA] and Actin [mouse actin cDNA fragment]). TMBGTAE03 and mtim5′A2 show splice variation at the 5′ end indicated by the slanted segments at their 5′ ends.

(B) ClustalW alignment of the hTIM and mTIM proteins. Highlighted are the *Tim* homology domains, designated TH1–TH4. The putative nuclear

sought to identify and characterize a mammalian ortholog of the *Drosophila timeless* gene. We initiated a database search for mammalian expressed sequence tags (ESTs) that correspond to such a homolog. We identified and subsequently cloned mouse and human genes, *mTim* and *hTIM*, that encode proteins that share extensive sequence homology with dTIM. Here, we report functional evidence that these mammalian *Tim* genes encode orthologs of the *Drosophila timeless* gene. Furthermore, we demonstrate that hTIM and mPER1 negatively regulate transcription of the *mPer1* promoter, closing the mammalian circadian loop.

## Results

### Molecular Cloning of Human and Mouse *Timeless* Orthologs

We initiated a search of the EST database to determine whether any mammalian *timeless* homologs could be identified. A search in September 1997 revealed one human EST, I.M.A.G.E. 746219 (Lennon et al., 1996), whose 5′ end sequence was recovered in a query with the full-length *Drosophila* TIM sequence (accession number AF032401) with a p value of 0.16 in a TBLASTN search of the EST database. While the score of the alignment was marginal, analysis of the 3′ end sequence of this clone indicated that the similarity to dTIM extended to both ends of the EST. The clone was obtained, sequenced, and used to search iteratively for additional ESTs sharing identity with 746219. This search revealed several EST sequences corresponding to human EST clones 417249 and 531927 and one mouse EST clone, 534423. The cDNA contig generated from the 746219 and 417249 clones revealed an uninterrupted open reading frame (ORF) of 2.5 kb. This ORF was incomplete, as no consensus start of translation or stop codons were present in this sequence.

The complete human cDNA sequence of *hTIM* was obtained by screening several libraries as well as using RACE PCR. We used 5′ RACE on human thymus cDNA to identify clones containing additional 5′ sequence. A 1 kb 5′ RACE product, htim5′A, was isolated, and we were able to identify the start of translation by comparison with the *Drosophila virilis* and *Drosophila melanogaster* TIM sequences (Myers et al., 1997; Rosato et al., 1997b; Ousley et al., 1998). To isolate a full-length clone, we screened an arrayed human placenta cDNA library using primers directed to the 5′ end and midportion of the *hTIM* coding sequence. A single clone, H5E11CA03, out of $5 \times 10^5$ clones screened was isolated and sequenced. This clone contained the complete ORF of *hTIM*; however, it also contained a retained intron at nucleotide position 3007. All clones isolated in these experiments are shown in Figure 1A. The complete *hTIM* cDNA sequence is 4414 bp in length. We cloned the mouse *Tim* cDNA in much the same fashion as *hTIM* and obtained a cDNA sequence 4438 bp in size (Figure 1A).

Translation of the *hTIM* ORF predicts a protein of 1208 amino acids, and translation of the *mTim* ORF predicts a protein of 1197 amino acids. The human and mouse *Tim* coding sequences are 82% identical at the nucleotide and 84% identical at the amino acid level (Figure 1B). hTIM and mTIM share four regions of sequence conservation with *D. melanogaster* and *D. virilis* TIM, and these are designated Tim homology (TH) 1–4 (Figure 1C). The degree of sequence similarity between the *Drosophila* and mammalian TIM proteins is comparable to or greater than that seen with dPER and each of the mPERs. The TH2 and TH3 domains in *Drosophila* span a stretch of amino acids implicated in dPER binding (PB2) (Saez and Young, 1996). Because TH2 and TH3 are separated in the mammalian TIM proteins, it appears likely that TH3 contains the functional dPER binding domain since it shares a larger overlap with PB2. Other functional domains identified in dTIM are also conserved in hTIM and mTIM. The PB1 domain contains the dTIM nuclear localization signal (NLS) sequence that is present in hTIM and mTIM; however, the rest of the domain is not conserved. Interestingly, the region containing the $tim^{SL}$ mutation (Rutila et al., 1996), which is just N-terminal to the PB1 domain, is not well conserved. The glutamate-rich sequence found in dTIM is also present in hTIM and mTIM as repeats of 13 and 11 glutamate residues at amino acid positions 665 and 662, respectively. The mammalian proteins also carry several other short stretches of glutamate-rich sequence that are not present in dTIM (Figures 1B and 1C). The significance of these sequences is not known. Finally, the cytoplasmic localization domain (CLD) in dTIM (amino acids 1228–1389) contains a tetrapeptide DEDD (in *D. virilis*, the sequence is DEDE) that is present at the extreme C termini of the hTIM and mTIM sequences. The C termini of the hTIM and mTIM proteins contain no other discernable sequence similarity to the dTIM CLD.

Besides *Drosophila* TIM, there are no closely related proteins to mammalian TIM in animals. The only other vertebrate example is a partial peptide purified and sequenced from bovine pituitaries (accession number AF041856), which likely represents the bovine ortholog of *tim*. mTIM and hTIM share some homology with a hypothetical yeast ORF of unknown function (accession number P53840), but the significance of this relationship is unclear. Finally, mTIM and hTIM show some weak sequence similarity with a *C. elegans* EST (accession number C43225).

### Splice Variants and Polymorphisms in Mammalian *Tim*

In the course of cloning *hTim* and *mTim*, we found several splice variants and polymorphisms in the two genes.

localization signal (NLS) sequences are underlined, as is the glutamate repeat. The DEDD sequence, which is conserved in the *D. melanogaster* cytoplasmic localization domain (CLD), is also indicated. Accession numbers for *mTim* and *hTIM* are AF098161 and AF098162, respectively. (C) Comparison of the human, mouse, *D. melanogaster*, *D. virilis*, and *D. hydei* TIM proteins. The percent identity and similarity between mouse TIM and *D. melanogaster* TIM in each of the four domains is indicated. Locations of functional sites of reference in the *D. melanogaster* TIM sequence are noted, including the $tim^{SL}$ and $tim^{0}$ mutation sites, the PER binding regions (PB1 and PB2), CLD, and NLS. Putative NLSs in the other proteins are also indicated.

In *mTim*, we found two different 5′ UTR sequences (nucleotides 1–67 of TMBGTAE03 are replaced by 55 nucleotides in *mTim*5′A2). Also, we found mouse clones that contained insertions of a CAG trinucleotide at two positions in the cDNA (at nucleotide positions 2988 and 3664), resulting in insertions of glutamine and alanine residues, respectively. These insertions are due to alternative splice acceptor usage at intron–exon boundaries. Genomic sequence shows that both insertions occur at defined intron–exon boundaries, and the sequence at both splice acceptor sites was CAGCAG. If the first AG is used, the splice would include the CAG trinucleotide. These two splicing events most likely occur independently, as clones of all four classes (± insertion at two positions) were identified (Figure 1A). A similar splice variant was found in the 5′ end of the human *TIM* gene. An AAG insertion was detected, resulting in a lysine insertion at amino acid 178.

Four single-nucleotide polymorphisms (SNPs) were found in *hTIM* by comparison of cDNA clone H5E11CA03 to sequence of the 746219 and 417249 ESTs. The first SNP, an A to G change, is a silent polymorphism occurring at nucleotide position 907. The second is a T to A at nucleotide 1505, which changes the coding sequence from a leucine to an isoleucine at amino acid 455. The third SNP is a G to A at nucleotide 1916, which alters the coding sequence from a valine to a methionine at amino acid 592. The fourth SNP that we detected was an A to G at nucleotide 2634, resulting in a glutamine to arginine alteration at amino acid 831. These SNPs may prove useful in examination of the genetic basis of circadian rhythm dysfunction in humans through association studies.

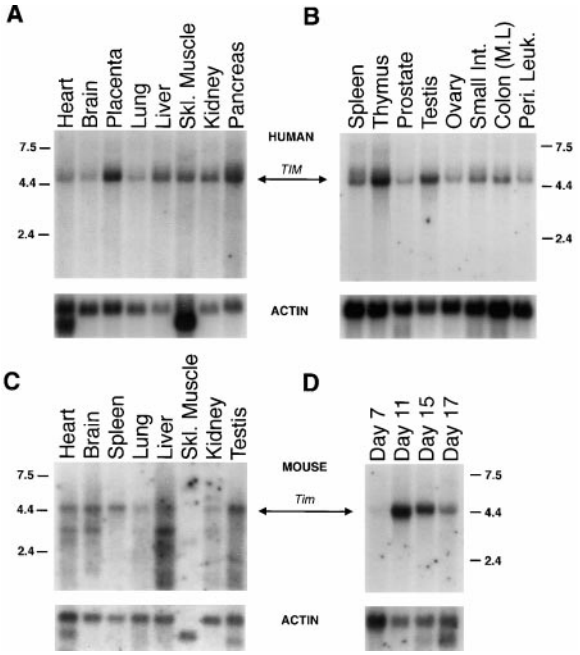


Figure 2. Tissue Distribution of *mTim* and *hTIM* mRNA Expression

(A) and (B) show human multiple tissue RNA blots. (C) and (D) show mouse multiple tissue and mouse embryonic tissue RNA blots. 2 μg of poly(A)$^+$ RNA of the indicated tissues was loaded on each lane. Primary transcript of 4.5 kb is evident in both mouse and human tissues. Blots were normalized with actin. M. L., mucosal lining.

### Mapping of *mTim* and *hTIM*

In order to map *mTim*, we PCR amplified genomic DNA, using primers designed within the 534423 EST sequence from various strains of mice to search for allelic variation in length. We found a length polymorphism between C3H/HeJ and C57BL/6J mice in the intronic sequence contained in the amplified PCR products. The C3H/HeJ allele is 11 bp shorter than the corresponding C57BL/6J allele. We used this polymorphic marker to map *mTim* in a backcross panel and found that *mTim* maps to the distal portion of mouse chromosome 10, ~1.7 cM distal to *D10Mit87* (1 recombinant out of 59 meioses, LOD score = 15.6). We named this marker *D10Nwu1-Tim*. We were unable to find an informative simple sequence length polymorphism (SSLP) marker located distal to *D10Nwu1-Tim* in this cross.

We mapped human *TIM* by radiation hybrid analysis on the Stanford G3 panel using a sequence tagged site (STS) to nucleotide positions 1253–1351. We found that WI−7760 is the closest linked STS marker to *hTIM*, with a LOD score of 11.05. This places *hTIM* on human chromosome 12 in a region of conserved synteny with mouse chromosome 10.

Unlike the mammalian *Per* gene family, which consists of at least three members (*mPer1*, *mPer2*, and *mPer3*), there are no obvious paralogs of the mammalian *Timeless* gene. Southern blot analysis in the mouse reveals only a single band when probed with an *mTim* cDNA probe. In addition, database searching with the mTIM and hTIM protein sequences did not reveal any closely related mammalian sequences. Thus, *mTim* appears to be a single-copy gene.

### mRNA Expression of *mTim* and *hTIM*

To examine the mRNA expression of *hTIM*, we performed Northern blot analysis on multiple tissue blots using EST clone 746219 as probe (Figures 2A and 2B). We found a single *hTIM* transcript of 4.5 kb in all human tissues analyzed. *hTIM* mRNA was widely expressed, with highest levels in the placenta, pancreas, thymus, and testis. In the mouse, a 4.5 kb *mTim* transcript was expressed in the heart, brain, spleen, liver, and testis with lower expression in the lung and kidney (Figure 2C). A minor 3 kb transcript was also seen in heart, brain, and liver. Mouse skeletal muscle contained two transcripts of 6 kb and 2.5 kb. During mouse development, *mTim* mRNA was highest at embryonic day 11 and then gradually decreased (Figure 2D).

Because *Drosophila timeless* exhibits circadian oscillations in both mRNA and protein and because the mammalian *Per* genes also have circadian rhythms in mRNA levels, we tested the hypothesis that *mTim* mRNA levels might cycle in either the SCN or retina. In situ hybridization studies demonstrated that *mTim* is expressed in the mouse SCN at low but detectable levels (Figure 3A). Analysis shows that the signal in the SCN from the antisense probe is twice the value of the sense control probe (antisense = 42.2 ± 6.7, sense = 18.5 ± 1.3, mean ± SEM) (compare Figure 3A with Figure 3B). To determine whether a circadian rhythm in *mTim* mRNA

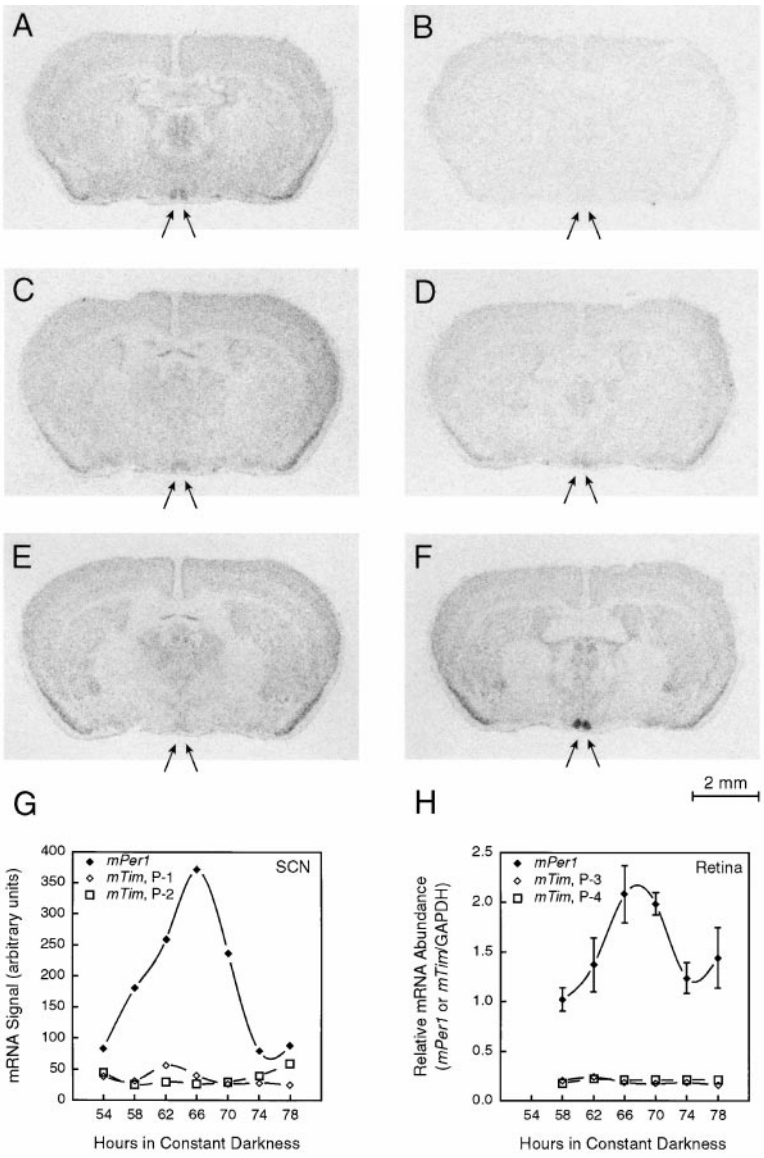


Figure 3. mRNA Expression of *mTim* and *mPer1* in SCN and Retina by In Situ Hybridization and Quantitative RT–PCR (TaqMan)

(A and B) Coronal sections of mouse brains showing representative antisense (A) and sense control (B) hybridization signals.

(C–F) Coronal sections of mouse brains at CT 18 (C and E) and CT 6 (D and F) showing hybridization with *mPer1* (E and F) and *mTim* (C and D). *mPer1* clearly demonstrates a circadian variation in abundance, whereas there is no apparent variation in *mTim* expression.

(G) Expression of *mPer1* and *mTim* mRNA levels in the mouse SCN. *mPer1* and *mTim* (two probes, P1 and P2) mRNA levels in the SCN were determined from adjacent sections of mouse brains obtained from three animals per time point indicated ($n = 2$ per probe).

(H) Expression of *mPer1* and *mTim* mRNA in the retina by quantitative RT–PCR. TaqMan RT–PCR assays were carried out on three independent RNA samples, each run in duplicate. Error bars indicated SEM. Error bars for the *mTim* quantitation are too small to be seen at this scale.

occurs, we examined SCN expression on the third circadian cycle in constant conditions (sampling every 4 hr starting at 54 hr into constant darkness [DD]). Experiments were performed using the two different *mTim* riboprobes, and we could find no evidence of *mTim* mRNA cycling with either probe (Figure 3G). As a positive control, *mPer1* mRNA levels in adjacent brain sections were measured, and as reported previously a high amplitude *mPer1* rhythm that peaked at 66 hr in constant darkness [approximately circadian time (CT) 6] was clearly seen (Figures 3E–3G). As indicated in Figure 3G, however, the *mTim* in situ hybridization signal was low relative to that seen with *mPer1* so that it is possible that an *mTim* mRNA cycle could not be reliably detected in these experiments. In the retina, in situ hybridization with *mTim* probes revealed expression in the outer nuclear, inner nuclear, and ganglion cell layers (data not shown). The pattern of *mTim* mRNA expression in the retina was identical to that seen with *mPer1*, *Clock*, and *BMAL1* (Gekakis et al., 1998). To determine whether the mouse retina exhibits circadian oscillations of *mTim* mRNA, we used TaqMan quantitative RT–PCR methods to measure both *mTim* and *mPer1* mRNA levels on the third circadian cycle in constant conditions (sampling every 4 hr starting at 58 hr into DD). As reported previously, *mPer1* mRNA levels in the retina were circadian with a peak between 66 and 70 hr in constant darkness (~CT 6–CT 10). Using two different *mTim* TaqMan probes, we found low but clearly detectable levels of *mTim* mRNA in the retina, which were greater than 4-fold above the assay's threshold for detection. Similar to that seen in the SCN, there was no significant circadian rhythm (Figure 3H). These two sets of experiments are consistent with at least five independent attempts to measure circadian rhythms of *mTim* mRNA in mice. We conclude that no circadian rhythms of *mTim* mRNA levels can be detected in the SCN and retina of mice.

### Biochemical Interaction of hTIM with *Drosophila* and Mammalian PER

In *Drosophila*, dPER and dTIM heterodimerize both in vivo and in vitro (Gekakis et al., 1995; Lee et al., 1996;

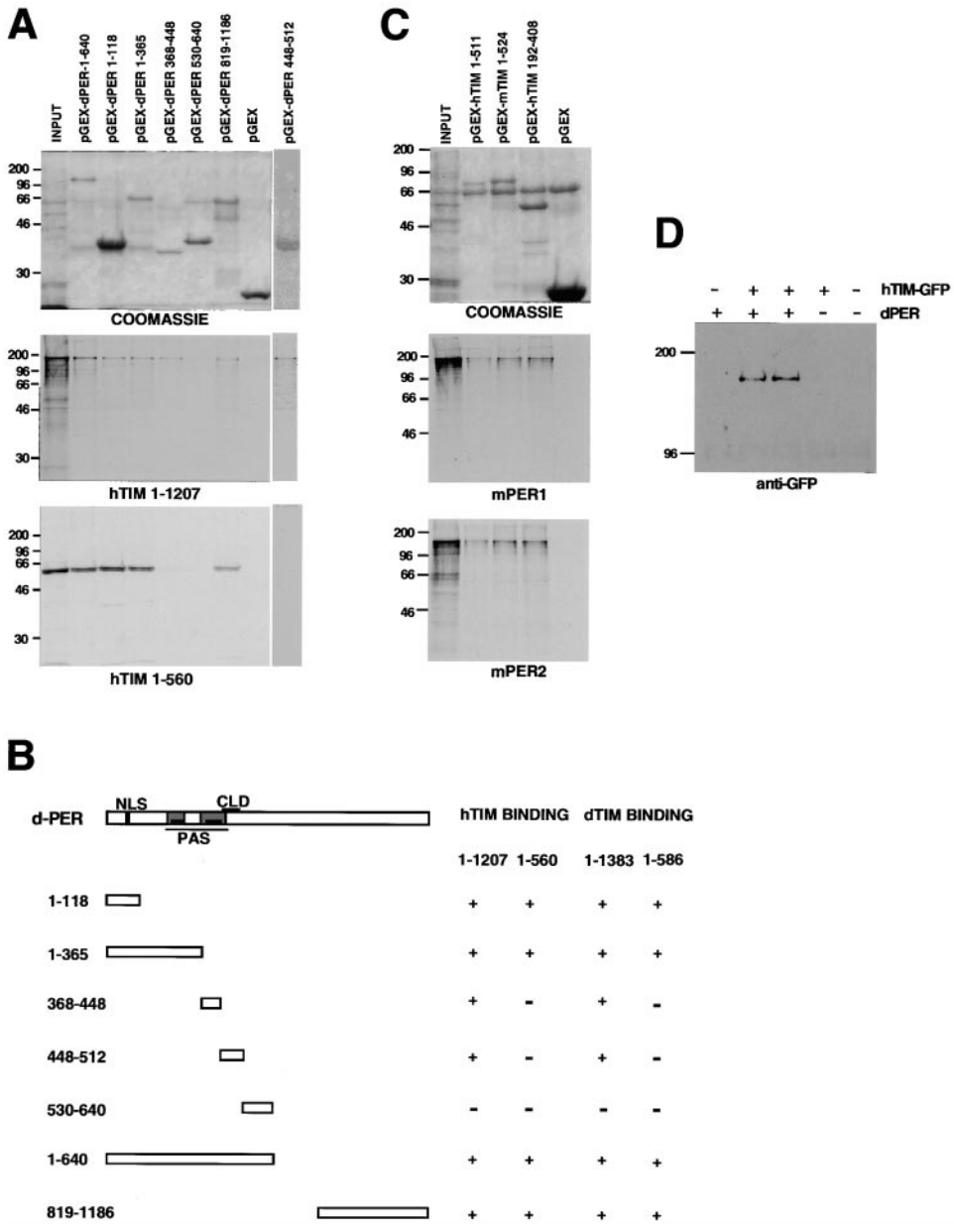


Figure 4. Physical Interaction of hTIM and mTIM with dPER, mPER1, and mPER2

(A) In vitro interactions of hTIM and dPER proteins. $^{35}$S-labeled *hTIM* (input) was incubated with GST alone or with GST-PER fusion proteins and analyzed for binding by SDS–PAGE and autoradiography as described (Saez and Young, 1996; see Experimental Procedures). The top panel shows a Coomassie-stained SDS–PAGE of the GST and GST-dPER fusion proteins used in the binding assay. Bottom panels show differential binding of in vitro translated hTIM 1–1207 and hTIM 1–560, respectively, to the indicated fusion proteins. The input lane shows the in vitro translated product before the binding reaction.

(B) Schematic representation and summary of the interaction of dPER with hTIM and dTIM. Positions of the dPER NLS, PAS, and CLD domains are indicated at top (Saez and Young, 1996). The dPER polypeptide fragments in the fusion proteins are indicated with respect to amino acid numbering of the full-length Canton-S *D. melanogaster* protein (Myers et al., 1997). The numbering of dTIM refers to amino acid sequence of *D. melanogaster* TIMELESS (Myers et al., 1997). Summary of dTIM binding drawn from Saez and Young (1996) and Saez and Young (unpublished data).

(C) hTIM and mTIM interact with the mouse PER1 and PER2 proteins in vitro. Full-length mouse PER1 and PER2 were in vitro translated, $^{35}$S-labeled, and incubated with GST-hTIM, GST-mTIM or GST alone as described. Top panel shows the Coomassie-stained SDS–PAGE gel of the GST fusions utilized in the binding assay. Bottom panels show autoradiographs of the in vitro translated mPER1 and mPER2 bound to the indicated GST-TIM fusion proteins. The input lanes show the indicated in vitro translation products before the binding reaction. Molecular sizes are in kilodaltons.

(D) Coimmunoprecipitation of PER and hTIM-GFP fusion protein in S2 *Drosophila* cells. Proteins from untransfected S2 cells, or from S2 cells transfected with *hs-per* and *hs-hTIM-gfp*, *hs-hTIM-gfp* alone, or *hs-per* alone were immunoprecipitated with anti-PER antibodies, separated by SDS–PAGE, and transferred to nitrocellulose, and coimmunoprecipitated hTIM-GFP was detected by Western analysis using anti-GFP antibodies (see Experimental Procedures). For *hs-hTIM-gfp*, GFP was fused to the C terminus of hTIM.

Saez and Young, 1996; Zeng et al., 1996). Thus, one functional test of the mammalian TIM proteins is their ability to interact with *Drosophila* and mammalian PER proteins. We first tested for direct association of hTIM and dPER polypeptide fragments in vitro (Figure 4A). Glutathione-S-transferase (GST) dPER fusion proteins or GST alone were expressed in bacteria, purified using glutathione-agarose beads, and incubated with in vitro translated, $^{35}$S-labeled hTIM fragments (hTIM 1–1207 and hTIM 1–560). SDS–PAGE analysis demonstrated that full-length hTIM binds to GST-dPER (1–640), GST-dPER (1–365), GST-dPER (1–118), GST-dPER (368–448), GST-dPER (448–512), and GST-dPER (819–1186) but not detectably with GST-dPER (530–640) or GST alone (Figure 4A). The hTIM 1–560 fails to bind GST-dPER (368–448) and GST-dPER (448–512) but recapitulates full-length hTIM interaction with the other dPER fragments. These experiments demonstrate that hTIM and dPER polypeptides can directly associate in vitro and, more importantly, that the pattern of dPER fragments that associate with hTIM is comparable to that seen with *Drosophila* TIM polypeptide fragments (Figure 4B). The sequence specificity of these in vitro physical associations suggests that elements of the dTIM sequence that promote interaction with dPER are conserved in hTIM.

To determine whether the mammalian *Per* homologs could interact with hTIM and mTIM, we also tested mPER1 and mPER2 for direct association in vitro (Figure 4C). GST-hTIM and GST-mTIM fusion proteins were expressed and purified as described above and incubated with in vitro translated, $^{35}$S-labeled mPER1 and mPER2 full-length proteins. SDS–PAGE analysis reveals that both hTIM and mTIM polypeptides are able to interact with mPER1 or mPER2 proteins. Thus, conditions previously shown to support physical interaction of dPER and dTIM in vitro also promote association of mammalian homologs of these proteins.

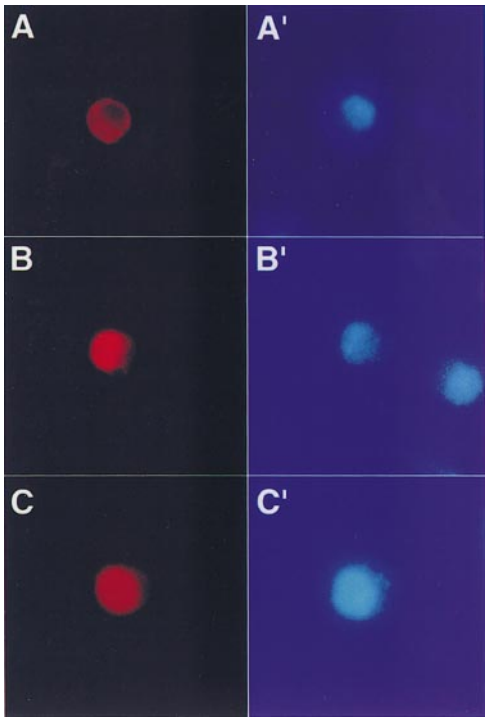


Figure 5. hTIM Promotes Nuclear Entry of dPER

S2 cells transfected with *hs-per* (A) or cotransfected with *hs-per* and *hs-hTIM*(B and C) were induced by heat shock, fixed 4 hr later, and immunostained with anti-PER antibodies. The antibody–antigen complex was detected with rhodamine-conjugated anti-rabbit IgG (red). Cells in (A) through (C) were also stained with Hoechst (blue) for detection of DNA in nuclei (A′–C′).

### Physical Association in *Drosophila* Cells and dPER Nuclear Localization by hTIM

To establish whether physical interactions between dPER and hTIM observed in vitro can also take place in *Drosophila* cells, we transfected *Drosophila* S2 cells with *hs-per* and *hs-hTIM-gfp*. As shown in Figure 4D, antibodies to PER coimmunoprecipitated hTIM-GFP from S2 cells that expressed both PER and hTIM-GFP but not from S2 cells that expressed only one of the two proteins.

Given evidence for physical interaction in S2 cells, we asked whether mammalian TIM could mimic a dTIM cellular function by promoting nuclear entry of dPER. An assay has previously been described in S2 cells that demonstrates that coexpression of dPER and dTIM are required for nuclear localization of either protein (Saez and Young, 1996). As shown earlier, expression of dPER alone in S2 cells results in cytoplasmic localization of the dPER protein (Figure 5A). When *hTIM* is coexpressed with dPER in S2 cells, dPER translocates to the nucleus (Figures 5B and 5C). Although *hTIM* expression is sufficient to promote nuclear localization of dPER, we have not examined whether hTIM is also translocated to the nucleus. hTIM and mTIM have several putative nuclear localization signals that suggest that they are indeed nuclear proteins. In spite of this caveat, the efficiency of hTIM in promoting nuclear entry of dPER is equivalent to that seen with *Drosophila* TIM. Thus, the functional similarity between dTIM and hTIM in this nuclear localization assay provides a second line of evidence that human *TIM* is an ortholog of *Drosophila tim*.

### Inhibition of CLOCK-BMAL1 Transactivation by mPER1 and hTIM

An additional important functional criterion for the correct identification of a putative mammalian ortholog of dTIM (or dPER) is the ability to engage in inhibition of *Per* gene transactivation by the CLOCK-BMAL1 heterodimer, a central feature of the negative feedback model of *Drosophila* and mammalian circadian clocks (Allada et al., 1998; Darlington et al., 1998; Gekakis et al., 1998; Rutila et al., 1998) that has received direct experimental support in the case of *Drosophila* PER and TIM (Darlington et al., 1998). To test this prediction with regard to hTIM and mPER1, we carried out luciferase reporter assays in cultured NIH-3T3 mouse fibroblast cells into which expression plasmids for full-length CLOCK, BMAL1, hTIM, and mPER1 had been transfected in various combinations.

Expression of CLOCK and BMAL1 together resulted in a robust activation of transcription from a 2.0 kb 5′ flanking fragment from the *mPer1* gene that included

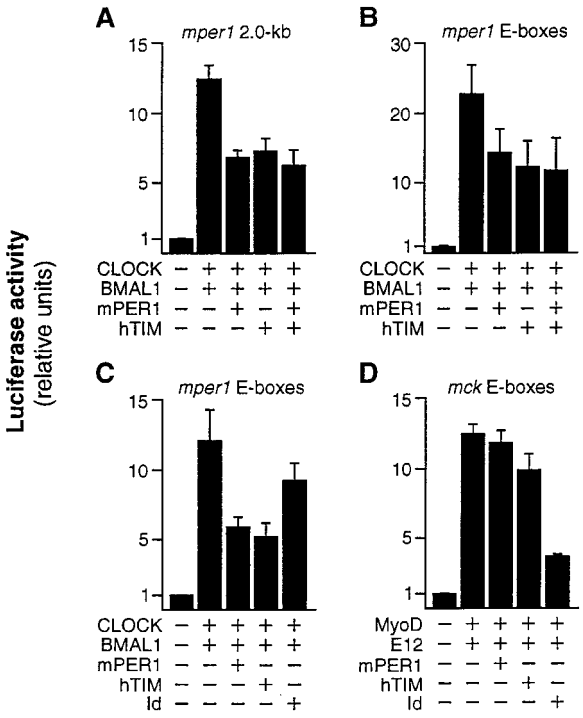


Figure 6. hTIM and mPER1 Inhibit *mPer1* Gene Transactivation by the CLOCK-BMAL1 Heterodimer

Transcriptional activation in mouse NIH-3T3 cells of a luciferase reporter gene from 5′ flanking sequences derived from the *mPer1* gene (A–C) or the muscle creatine kinase (*mck*) gene (D).

(A) Effect of hTIM, mPER1, or both on transactivation by the CLOCK-BMAL1 heterodimer from a 2.0 kb *mPer1* promoter fragment including three E boxes (CACGTG).

(B) Effect of hTIM, mPER1, or both on transactivation by the CLOCK-BMAL1 heterodimer from a 54 bp construct consisting of the three *mPer1* E boxes and their immediate flanking sequences linked together.

(C) Effect of hTIM, mPER1, or Id protein on transactivation by the CLOCK-BMAL1 heterodimer from the 54 bp *mPer1* construct.

(D) Effect of hTIM, mPER1, or Id protein on transactivation by the MyoD-E12 heterodimer from a 60 bp construct including four copies of the *mck* gene-specific E box (CAGGTG) and immediate flanking sequences. Plus or minus denotes an expression plasmid with or without, respectively, the indicated full-length cDNA insert. Shown are the mean and SEM of n = 6 independent experiments in each case. Some of the standard error bars are too small to be seen at this scale.

three E boxes (Figure 6A), as seen previously (Gekakis et al., 1998). Additional expression of either mPER1 or hTIM resulted in a significant inhibition of this transcriptional activation (45% and 42% inhibition, respectively; $p < 0.0005$ for each). Expression of mPER1 and hTIM together resulted in a level of inhibition only marginally greater than that seen with the expression of either one alone (52% inhibition). Expression of mPER1 or hTIM in the absence of CLOCK and BMAL1 expression resulted in no detectable inhibition of the basal transcriptional activity (data not shown). An essentially identical pattern of results was obtained using a 54 bp fragment in which the three *mPer1* E boxes and their immediate flanking sequences were linked together (Figure 6B).

We next examined the specificity of the inhibitory effects of hTIM and mPER1 on transactivation from E box sites. Once again, expression of either mPER1 or hTIM significantly inhibited transactivation by the CLOCK-BMAL1 heterodimer from *mPer1* E box sites (51% and 57% inhibition, respectively), but expression of Id, a known inhibitor of the MyoD-E12 heterodimer and related bHLH proteins (Benezra et al., 1990), caused only a small inhibition (24%) that was not statistically significant (Figure 6C). Conversely, expression of either mPER1 or hTIM produced little or no inhibition of transactivation by the MyoD-E12 heterodimer from a muscle creatine kinase (*mck*) gene E box site (4% and 19% inhibition, respectively), whereas expression of Id resulted in a large and highly significant inhibition (69% inhibition) as expected (Figure 6D).

These results indicate that both hTIM and mPER1 can inhibit CLOCK-BMAL1 transactivation from an *mPer1* gene regulatory element, as predicted if they represent mammalian circadian clock components. These experiments do not necessarily indicate that hTIM or mPER1 can act alone to inhibit CLOCK-BMAL1 transactivation; Northern analysis revealed that untransfected NIH-3T3 cells expressed both the 4.7 kb *mPer1* and the 4.5 kb *mTim* transcripts (data not shown). This contrasts with *Drosophila* S2 cells, for which transfection of *dper* and *dtim* is needed for strong regulation (Darlington et al., 1998) but neither *per* nor *tim* is endogenously produced (Saez and Young, 1996). Expression of these likely clock components in a mouse fibroblast cell line is not surprising, given that cultured rat fibroblasts have recently been shown to express *Per1* and *Per2* and to contain a functional circadian clock (Balsalobre et al., 1998). The most likely interpretation of these results is that exogenous hTIM or mPER1 interacted with endogenous mPER1 and mTIM, respectively, to inhibit CLOCK-BMAL1 transactivation. That coexpression of exogenous mPER1 and hTIM did not lead to significantly greater inhibition than either alone could be a limitation imposed by the transfection conditions as described above, or it could reflect a requirement for an endogenous factor other than mPER1 or mTIM that is limiting for the inhibition.

## Discussion

Sequence and functional analyses suggest that *hTIM* and *mTim* are mammalian orthologs of the *Drosophila* circadian gene *timeless*. *hTIM* and *mTim* show highest sequence similarity to the *Drosophila* TIM proteins and to no other known proteins. Comparison of the TIM proteins reveals four regions of similarity among the insect and mammalian proteins. These include regions of the *Drosophila* TIM protein involved in nuclear localization, protein–protein interaction with PER, and cytoplasmic localization. These structural similarities were tested for functional similarities, and indeed *hTIM* was found to associate physically with *Drosophila* and mouse PER proteins in vitro, to physically associate with and promote nuclear entry of *Drosophila* PER in S2 cells, and to inhibit CLOCK-BMAL1-driven transactivation of the *mPer1* promoter in NIH-3T3 mouse fibroblasts. Taken together, the results presented here indicate that *hTIM* and *mTim* are mammalian orthologs of the *Drosophila* circadian gene *timeless*.

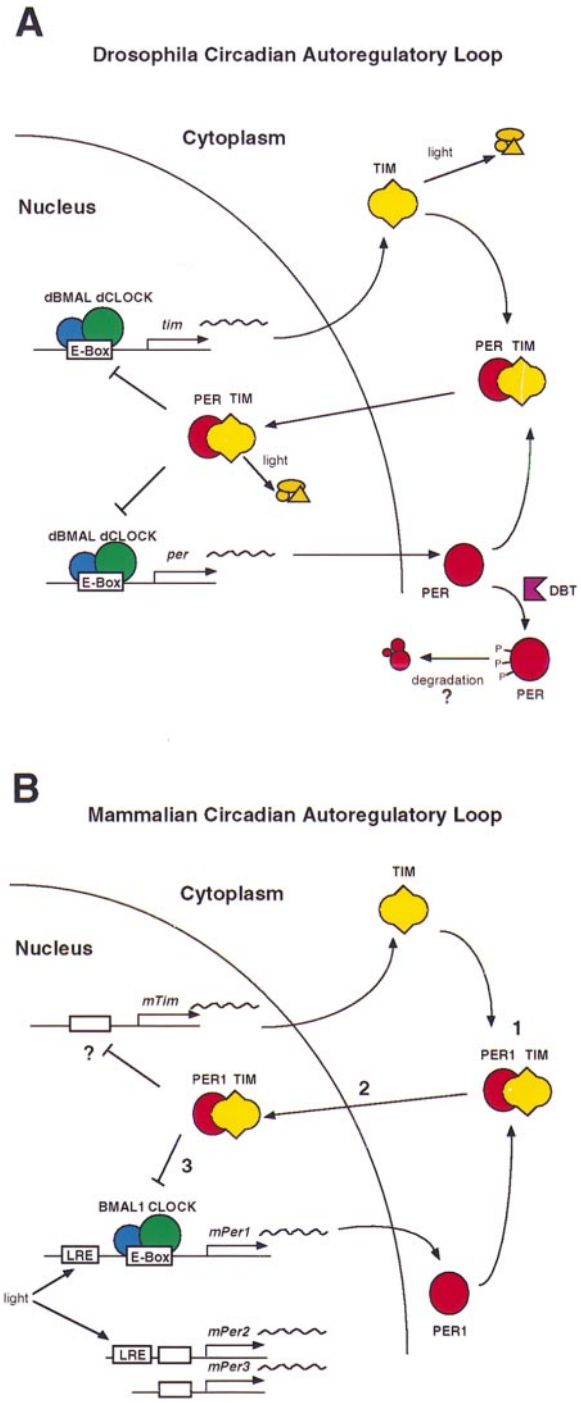


Figure 7. Summary of *Drosophila* and Mammalian Circadian Autoregulatory Loops

(A) The *Drosophila* circadian feedback loop. dCLOCK-dBMAL start the cycle by activating transcription of the *per* and *tim* genes. Once sufficient levels of PER and TIM are attained, heterodimerization can occur, thus allowing nuclear translocation. The cycle is closed with the inhibition of dCLOCK-dBMAL by nuclear PER-TIM complex. DOUBLE-TIME (DBT; Kloss et al., 1998; Price et al., 1998) is required for proper phosphorylation and turnover of PER and for cycling of the autoregulatory loop.

(B) The mammalian circadian autoregulatory loop showing activities of identified components suggested by this work: (1) direct association of PER1 and TIM, (2) ability of TIM to allow nuclear entry of PER1, and (3) inhibition by PER1 and TIM of CLOCK-BMAL1 induced activity of the *mPer1* promoter. LRE, light-responsive element.

The identification of a mammalian *Timeless* ortholog provides a framework for the description of a circadian autoregulatory loop in mammals involving *Clock*, *BMAL1*, the *mPer* paralogs, and *Tim*. Recent work has led to a similar model in *Drosophila* (Figure 7A), in which PER and TIM are coregulated in a circadian manner and interact to inhibit their own transcription mediated by dCLOCK-dBMAL transactivation via E box elements in the *per* and *tim* promoters (Darlington et al., 1998). While the same set of genes appears to be involved in both *Drosophila* and mammals, there are interesting similarities and differences apparent already.

At the level of the core circadian mechanism, the positive elements of the system, CLOCK and BMAL, are remarkably well conserved in *Drosophila* and mammals (Figures 7A and 7B). In both systems, the CLOCK-BMAL heterodimeric complex activates transcription of their respective *period* gene promoters via identical E box regulatory elements. In mammals, such an analysis has been demonstrated for the *mPer1* promoter but has not yet been reported for *mPer2* or *mPer3*.

An important variable, both among the various *Per* paralogs and among the various tissues in which they are expressed, is the difference in the phases of the *Per* mRNA rhythms in mammals. While the mRNA rhythms of *mPer1*, and to a lesser extent *mPer2* and *mPer3*, peak in the daytime in the SCN, the corresponding *Per* rhythms in the rest of the body peak much later, near dusk. Thus the phases of the *Per* mRNA rhythms in the SCN clearly differ from that seen in *Drosophila*; however, the rest of the body tissues in mammals express *Per* mRNA rhythms very similar to *Drosophila*. The expression of *mTim* mRNA in mice is apparently different from *Drosophila*, as it does not appear to oscillate. The lack of an *mTim* mRNA rhythm suggests that, unlike *Drosophila*, CLOCK-BMAL may not positively transactivate the *mTim* promoter—in this case, the lack of a rhythm would be due to the absence of CLOCK-BMAL as a target of negative feedback by PER-TIM. Alternatively, the lack of an *mTim* mRNA rhythm could be due to something as trivial as a long mRNA half-life—in this case, *mTim* transcription would still be circadian; however, the steady-state mRNA abundance of *mTim* would be constant. With the cloning of *mTim*, such issues can be addressed directly in the future.

If mammalian TIM protein levels are ultimately shown to be constitutive, is this inconsistent with a role for TIM as a "circadian clock gene" in mammals? In considering circadian oscillator models, we submit that it is not a requirement for both the PER and TIM proteins to oscillate as long as the oscillating proteins (in this case, the PERs) are rate limiting within their dynamic range. Given that TIM dimerization with the PERs is likely to be required for their nuclear entry, TIM expression would be necessary but would not have to be rhythmic. The existence of three different *Per* genes in mammals also could explain why mammalian TIM expression may be constitutive. Because the three *Per* genes have differently phased mRNA and presumably protein rhythms, and they are often expressed together in the same cell, constitutive expression of TIM could be an adaptation so that interactions with all three PER proteins would be possible. Indeed, it is difficult to see how circadian

cycling of a single *mTim* locus could be compatible with coexpression of multiple *mPer* genes. A second and perhaps more important reason to have only one TIM when there are multiple PERs would be to integrate this circadian pathway. By virtue of sharing a common dimerization partner, the three *Per* genes would be loosely coupled and would share regulation of the circadian autoregulatory feedback loop. Thus, one can already envisage a number of subtleties in the circadian transcriptional feedback loop oscillator system by comparison of *Drosophila* and mouse.

In addition to the components of circadian autoregulatory loop, the regulation of the circadian oscillator by environmental inputs also appears to differ between *Drosophila* and mammals. The most obvious difference is the way in which light acts in the mammalian SCN as compared to *Drosophila* (Figures 7A and 7B). In mice, recent work has shown that light exposure rapidly induces *mPer1* and *mPer2* mRNA levels in the SCN (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998a; Zylka et al., 1998). Interestingly, *mPer3* is not responsive to acute light stimulation (Takumi et al., 1998b; Zylka et al., 1998). In contrast, in *Drosophila* light does not directly affect PER but rather causes a rapid decrease in TIM protein levels (Hunter-Ensor et al., 1996; Lee et al., 1996; Myers et al., 1996; Zeng et al., 1996). As discussed previously (Dunlap, 1996; Young, 1998), these differences (increased transcription versus decreased protein level) make functional sense depending on whether the clock component (protein) peaks in the day or the night. A day-peaking protein is low at night; therefore, to reset its rhythm, light must increase the level or activity of the protein, which may be accomplished most efficiently by induction of transcription. By contrast, a night-peaking protein (such as *Drosophila* TIMELESS) is elevated at night; therefore, to reset its rhythm, light must decrease the level or activity of the protein, which may be best accomplished by regulation of the protein (via synthesis, degradation, or turnover). With the discovery of mammalian TIM, it will be of great interest to determine whether light regulates TIM levels in mice similar to that seen in *Drosophila*. Because the peaks of mammalian *Per* mRNA rhythms occur at dusk in most peripheral tissues, the mammalian PER proteins may in fact be reaching peak levels at night. If true, one would predict that light-induced decreases in TIM or PER protein levels would be expected to take place in these tissues. Thus, it is possible that a *Drosophila*-like model may apply more directly to peripheral tissues in mammals.

With clones of both mammalian *Per* and *Tim* now in hand, it will also be possible to examine posttranscriptional steps in the regulation of mammalian PER and TIM protein accumulation. These appear to be central to forming a circadian clock from a central autoregulatory loop in *Drosophila* (Edery et al., 1994; Dembinska et al., 1997; So and Rosbash, 1997; Kloss et al., 1998; Price et al., 1998). For example, in the absence of the *Drosophila* clock gene *double-time* (*dbt*), the central autoregulatory loop persists, but it fails to oscillate (Figure 7A). Given that entirely different but analogous sets of genes form circadian autoregulatory feedback loops of transcription in *Neurospora* and cyanobacteria (Dunlap, 1996; Ishiura et al., 1998), the extent to which higher order levels of regulation of the circadian mechanism are conserved will be fascinating to explore.

In conclusion, we propose that a circadian autoregulatory loop involving the positive elements, CLOCK and BMAL1, and the negative elements, PER and TIM, underlies the generation of circadian oscillations in mammalian cells. The recent demonstration of circadian rhythms in peripheral mammalian tissues and cell lines (Balsalobre et al., 1998; Zylka et al., 1998) underscores the significance of circadian rhythmicity in cells throughout the body. Now that a fundamental set of circadian components has been assembled in mammals, the task at hand is to understand how they function and interact to generate circadian rhythms in vivo.

## Experimental Procedures

### EST Clones

*D. melanogaster* TIM protein sequence (accession number AF032401) was used to search the EST database using the TBLASTN algorithm. I.M.A.G.E. clones of interest were ordered from Research Genetics and sequenced (Lennon et al., 1996). Full-insert EST sequences were used as queries to search the EST database using the BLASTN and TBLASTX algorithms in order to identify overlapping EST clones. This process was repeated with each identified clone until all EST clones corresponding to the *mTim* and *hTIM* genes were identified.

### cDNA Library Screening

#### *Genetrapper Library Screen*

We used the GibcoBRL Genetrapper Positive Selection System to screen a mouse brain plasmid library (Life Technologies). cDNA capture and second strand repair were carried out using the primer 5ttrap1: 5′-CTACAGCTCAGATCTGGGAAAGC-3′, an oligonucleotide designed from sequence of mtim5′A2. Screening was carried out as described in the protocol supplied by GibcoBRL.

#### *Phage Library Screens*

A Unizap XR human retinal cDNA library (Stratagene) was screened using 746219 insert as a probe by hybridization in 50% formamide, 10% Dextran sulphate, 1 M NaCl, 1% SDS, and 100 μg/ml sheared salmon sperm DNA and washing in 2× SSC 0.1% SDS for 30 min at room temperature, and then three times in 0.2× SSC 0.1% SDS for 45 min at 65°C. A λ-gt11 human hypothalamic cDNA library was also screened with a dual probe from the 746219 and 417249 inserts in the same manner.

#### *Arrayed cDNA Library Screens*

Master plates of the human placenta cDNA library and mouse embryonic cDNA library from Origene Technologies were screened by PCR. Mouse library screening was performed with primers mTim3GSP3 and mTim3GSP5 (sequence below). Primers for the human library screen were as follows: 417249.4, 5′-TGGAGCTGTTG TTCTGGAAG-3′, and 417249.5.1, 5′-ATATGACCCAGGACATCATC TGA-3′. Positive subplates (5E for the human library and 11H for the mouse library) were ordered and screened by PCR to identify positive subwells. Positive subwell stocks were plated out onto LB-Ampicillin (100 μg/ml) plates, and colonies were screened to identify *hTIM* and *mTim* cDNA clones.

### RACE PCR Cloning

RACE PCRs were performed using Clontech Marathon cDNAs (mouse muscle and brain, human thymus and hypothalamus) as template following the manufacturer's protocol with the following modifications. Conditions for PCR were as follows: 94°C for 30 s and then 5 cycles at 94°C for 5 s, at 72°C for 6 min, 5 cycles at 94°C for 5 s, at 70°C for 6 min, 25 cycles at 94°C for 5 s, and at 68°C for 6 min. Nested primers were used to increase specificity of amplified products for clones corresponding to *hTIM* and *mTim*. Four sets of mouse 5′ and 3′ RACE primers were used: Mtim5gsp5, 5′-TCCAACA TTTTGAGGAAGAGGTGGG-3′; Mtim5nsp5, 5′-GAAAGAGCGCCAG GAATAGTTCTCG-3′; Mtim5gsp3, 5′-CTGGCCACGGTGAACGAG

ATG-3′; Mtim5nsp3, 5′-ATGAGGCTGTTAGGGAGAGCAGTCG-3′; Mtim3gsp5, 5′-ATCCTTGTGGGCGAGGTATAGTTCC-3′; Mtim3nsp5, 5′-CTGAAGCTGGGCCTCTTCCTCAGG-3′; Mtim3gsp3, 5′-CCGTGA ACCAGAAAGCGTTTGTGG-3′; and Mtim3nsp3, 5′-GGAGCTGCTGT TCTGGAAGAACACC-3′. One primer set for human 5′ RACE PCR was used: Hstimgsp5, 5′-AGCTAAGCGTCCCTGCCCTACTCC-3′, and Hstimnsp5, 5′-GGGTTCTGGTCACGAAACATAAGGG-3′.

Appropriate bands were gel purified and subcloned into pGEM-T Easy for further analysis. Two 5′ mouse clones, mtim5′A1 and mtim5′A2, were isolated by RACE PCR initiated from the 5′ of 534423 sequence. Mtim5′A2 contains an intron near its 3′ end and also 70 bp of mitochondrial DNA at the very 5′ end. One 3′ mouse clone, mtim3′A1C, was isolated from RACE initiated from the 534423 sequence; however, it contained a ~1 kb intron and was internally primed.

We carried out 3′ RACE originating from 315895 sequence using gene-specific primers mTim3GSP3 and mTim3NSP3 and isolated six clones. Four clones appeared to be full length with two CAG splice variations alternatively present or absent (see text). A 1 kb gap in the mouse cDNA was closed by PCR with primers mTim3gsp5 and mTim5gsp3.

**DNA Sequencing and Sequence Analysis**

All sequencing was carried out on an ABI 377XL fluorescence-based automated sequencer. Sequencing reactions were carried out using the Big Dye Terminator kit at one-half to one-quarter chemistry depending on the template. Sequences were edited and assembled using Sequencher 3.0 (Genecodes, Ann Arbor, MI). Further protein and DNA sequence analysis was carried out using MacVector 6.0.1.

**Northern Blot Analysis**

Multiple tissue Northern blots were purchased from Clontech. A probe for the human MTN blots was generated by random priming the original I.M.A.G.E. clone 746219 insert using Pharmacia Ready-to-Go DNA labeling beads. A probe for the mouse embryonic and multiple tissue blots was generated from the mtim5′A1 clone. The blots were hybridized in Express Hybridization Solution (Clontech) and washed according to the manufacturer's protocols.

**Mapping**

PCR amplification from genomic DNA of various strains of mice revealed an intron at nucleotide position 1543 of the *mTim* cDNA. This intron was 107 bp long in C3H/HeJ mice as compared to 118 bp in C57BL/6J. This length polymorphism was used to map *mTim*. Primer sequences for *D10Nwu1-Tim* were 5′-ATGAGGCTGTTAGGG AGAGCAG-3′ and 5′-AACTTTCGAAAGAGCGCCAG-3′. We used 59 ([C3H/HeJ × C57BL/6J]F1 × C57BL/6J)$N_2$ mice as a mapping panel. 72 SSLP markers between the C57BL/6J and C3H/HeJ strains (Research Genetics) were tested for linkage to *D10Nwu1-Tim*. Once linkage was established to the distal arm of mouse chromosome 10, additional markers were scored to fine-map the *mTim* locus. The *hTIM* gene was mapped by STS screening of the Stanford G3 panel of 83 radiation hybrid cell lines (Research Genetics). Primers used for the *hTIM* STS were as follows: HstimRHfor, 5′-CAGCATGAT GAGACCTATTATATGTGG-3′, and HstimRHrev, 5′-ACTGAGGGTC TCAGAAACCAGG-3′.

**Site-Directed Mutagenesis**

In order to generate a full-length cDNA clone of *hTIM* for further functional studies, we used site-directed mutagenesis to delete the 216 bp intron from the H5E11CA03 clone. Mutagenesis was performed with the Quik Change Site-Directed Mutagenesis kit (Stratagene) using two oligonucleotides, SDEQuikTimS and SDEQuikTimAS (see below), designed with the 216 bp intron sequence deleted as per the manufacturer's directions, with the following modifications. Starting dsDNA template (200 ng) (H5E11CA03) was used with 375 ng of each primer and 3 μl of dNTP mix in a final 50 μl reaction volume using 18 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 23.5 min. Successfully mutated clones were identified by colony PCR, and the intron loopout was confirmed by sequencing: SDEQuik TimS, 5′-GTTGGCATCCTCCATCTTGCCAAATGGAGCGGAGTCC CTG-3′, and SDEQuikTimAS, 5′-CAGGGACTCCGCTCCATTTGGCA AGATGGAGGATGCCAAC-3′.

**Animals**

CD1 female mice were housed in 12 hr of light and 12 hr of dark (LD 12:12) for at least 1 week and then released into DD. Three mice were sacrificed in total darkness every 4 hr beginning at 54 hr in DD for one 24 hr cycle (seven time points). Optic nerves were severed under infrared light (15 W Kodak Safelight with number 11 filter). Brains were removed under dim red light (Kodak filter number 1A) and frozen on dry ice. In a separate experiment, (BALB/cJ × C57BL/6J)$F_5$ intercross female albino mice were housed in similar conditions, and three animals were sacrificed every 4 hr beginning at 58 hr in DD over six time points. Eyeballs were collected and frozen immediately in tubes on dry ice.

**In Situ Hybridization**

Coronal sections encompassing the SCN of 20 μm thickness were collected from each brain and thaw-mounted on gelatin-coated slides. Sections were fixed for 5 min in 4% paraformaldehyde in PBS and treated for 10 min in 0.1 M triethanolamine/acetic anhydride and then dehydrated through an ethanol series. Slides were hybridized overnight at 47°C in hybridization solution composed of 50% formamide, 300 mM NaCl, 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 1× Denhardt's, 10% dextran sulfate, 10 mM DTT and containing $5 \times 10^7$ cpm/ml of the relevant $^{33}$P-labeled probe. *mPer1* and two *mTim* probes (*mTim*-P-1 and *mTim*-P-2) were prepared using the Ambion MaxiScript in vitro transcription kit from templates containing nucleotides 468–821 of *mPer1* (accession number AF022992), 2392–2633 for *mTim*-P-1, and 764–1593 for *mTim*-P-2 (accession number AF098161).

**TaqMan Quantitative RT–PCR Assay**

A fluorescence-based real time quantitative RT–PCR method developed by Perkin Elmer ABI that is named "TaqMan" was used to measure mRNA levels in mouse retina. Total RNA from eyeballs was extracted using TRIZOL reagent (Life Technologies) according to the manufacturer's protocols. Total RNA (100 ng) from each sample was used in duplicate RT–PCR reactions consisting of 1× TaqMan EZ buffer; 3 mM manganese acetate; 300 μM each of dATP, dCTP, and dGTP; 600 μM dUTP, and appropriate primers and TaqMan fluorescent probe. Probes were labeled with 6-FAM on the 5′ end and with TAMRA on the 3′ end. For the *mPer1* and *mTim*-P-4 assays, *mPer1* primers and probe and GAPDH control primers and probe (Rodent GAPDH control Kit, ABI) were used in a single-tube assay. For the *mTim*-P-3 assay and GAPDH control, separate tubes were used. RT–PCR reactions were carried out in a Perkin Elmer ABI 7700 machine using the following thermal cycling parameters: 50°C for 2 min, 60°C for 30 min, and then 95°C for 5 min followed by 40 two-step cycles of 94°C for 20 s and 62°C for 1 min. *mPer1* and *mTim* relative mRNA abundance was calculated using the comparative Ct method as described in the ABI Prism 7700 Sequence Detection System, User Bulletin Number 2. Sequences of the various TaqMan probe sets are described below: *mPer1*-FOR, 5′-ACCTTGGCCA CACTGCAGTA-3′; *mPer1*-REV, 5′-CTCCAGACTCCACTGCTGG TAA-3′; *mPer1*-PROBE, 5′-ATTCCTGGTTAGCCTGAACCTGCTTG ACA-3′; *mTim*-P-3FOR, 5′-GCCCAGCTTCAGGAACTATACCT-3′; *mTim*-P-3REV, 5′-AGGTGCGCCAATATGGTTTC-3′; *mTim*-P-3PROBE, 5′-CACTACATCTTGACCTTCCACATCCTTGTGG-3′; *mTim*-P-4FOR, 5′-GGAGAAGGATGTCGTCTTTCACA-3′; *mTim*-P-4REV, 5′-CTGGGCTGCTTTCCCAGAT-3′; and *mTim*-P-4PROBE, 5′-AGG CCTTCACAATCTCCAGAACTACAGCTCA-3′.

**Biochemical Interaction**

hTIM, mPER1, and mPER2 polypeptide fragments labeled with [$^{35}$S]methionine were synthesized by coupled transcription–translation in vitro (TNT Lysate System, Promega). GST, GST-dPER, GST-hTIM, and GST-mTIM fusion proteins were produced in *E. coli* using the pGEX vector (Pharmacia) and purified using glutathione-agarose beads. $^{35}$S-labeled proteins were incubated with control (GST) or GST fusion beads for 30 min. The beads were washed with a buffer containing 0.5% NP-40 and 200 mM KCl. The proteins were denatured in Laemmli loading buffer and resolved by SDS–PAGE.

**Coimmunoprecipitation and Nuclear Localization Assays**

A Schneider 2 (S2) cell line was transiently transfected with *hs-hTIM* and *hs-dper* or *hs-dper* and *hs-hTIM-gfp* as described (Saez and

Young, 1996; Kloss et al., 1998). To induce the expression of the transfected genes, cell lines were incubated for 30 min in a 37°C water bath and allowed to recover at room temperature for 4 hr. For nuclear localization studies, heat shock–induced S2 cells were allowed to attach to a glass coverslip for 15 min and were fixed with 4% paraformaldehyde in PBS for 15 min. Fixed cells were washed with PBS and incubated with blocking solution containing 5% goat serum, 0.1% Triton in PBS. Cells were incubated overnight at room temperature with anti-dPER antibodies (1:5000) diluted in blocking solution, washed with PBS, and incubated with fluorescence-conjugated goat anti-rabbit IgG for 1 hr. Coverslips with stained cells were washed with PBS, incubated with 0.01% Hoechst (in PBS) for 10 min, washed in PBS, and mounted with Gel/mount (Biomeda). Coimmunoprecipitation studies were performed as described by Kloss et al. (1998).

**Transfection and Luciferase Reporter Gene Assays**

Transfection of NIH-3T3 cells with luciferase reporter and cDNA expression plasmids and assays of luciferase activity were performed essentially as described (Gekakis et al., 1998). Cells were transfected (LipofeCT Amine-Plus, GibcoBRL) in 6-well plates at 25%–50% confluence with 10 ng of the firefly luciferase reporter plasmid, 1 μg (total) of expression plasmids, and 0.5 ng of the internal control Renilla luciferase plasmid. Luciferase reporters were constructed in pGL3 promoter (Promega) with the following inserts: *mPer1*, a 2.0 kb promoter fragment (Gekakis et al., 1998) or a 54 bp fragment containing the three E boxes and immediate flanking sequences linked together in their native 5′-to-3′ order (Gekakis et al., 1998); and *mck*, a 60 bp fragment consisting of four iterations of the muscle creatine kinase right E box plus immediate flanking sequences (Skapek et al., 1996). Expression of full-length cDNAs was driven by the cytomegalovirus immediate-early promoter using the following expression plasmids: mouse *Clock* and hamster *BMAL1* inserts were in pcDNA3 (Invitrogen), *hTIM* insert was in pCMV6-XL3 (Origene), *mPer1* insert was in pCMV-SPORT2 (GibcoBRL), and *MyoD*, *E12*, and *Id* inserts were in pCS2 (Skapek et al., 1996). The total amount of each type of expression plasmid (250 ng each) was kept constant in any given experiment by including nonrecombinant expression plasmids in transfections, as necessary.

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**References**

Albrecht, U., Sun, Z.S., Eichele, G., and Lee, C.C. (1997). A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell *91*, 1055–1064.

Allada, R., White, N.E., So, W.V., Hall, J.C., and Rosbash, M. (1998). A mutant *Drosophila* homolog of mammalian *Clock* disrupts circadian rhythms and transcription of *period* and *timeless*. Cell *93*, 791–804.

Antoch, M.P., Song, E.J., Chang, A.M., Vitaterna, M.H., Zhao, Y., Wilsbacher, L.D., Sangoram, A.M., King, D.P., Pinto, L.H., and Takahashi, J.S. (1997). Functional identification of the mouse circadian *Clock* gene by transgenic BAC rescue. Cell *89*, 655–667.

Balsalobre, A., Damiola, F., and Schibler, U. (1998). A serum shock induces circadian gene expression in mammalian tissue culture cells. Cell *93*, 929–937.

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L., and Weintraub, H. (1990). The protein Id: a negative regulator of helix–loop–helix DNA binding proteins. Cell *61*, 49–59.

Darlington, T.K., Wager-Smith, K., Ceriani, M.F., Staknis, D., Gekakis, N., Steeves, T.D.L., Weitz, C.J., Takahashi, J.S., and Kay, S.A. (1998). Closing the circadian loop: CLOCK-induced transcription of its own inhibitors per and tim. Science *280*, 1599–1603.

Dembinska, M.E., Stanewsky, R., Hall, J.C., and Rosbash, M. (1997). Circadian cycling of a PERIOD-beta-galactosidase fusion protein in *Drosophila*: evidence for cyclical degradation. J. Biol. Rhythms *12*, 157–172.

Dunlap, J.C. (1996). Genetics and molecular analysis of circadian rhythms. Annu. Rev. Genet. *30*, 579–601.

Dunlap, J. (1998). Circadian rhythms. An end in the beginning. Science *280*, 1548–1549.

Edery, I., Zwiebel, L.J., Dembinska, M.E., and Rosbash, M. (1994). Temporal phosphorylation of the *Drosophila* period protein. Proc. Natl. Acad. Sci. USA *91*, 2260–2264.

Gekakis, N., Saez, L., Delahaye-Brown, A.M., Myers, M.P., Sehgal, A., Young, M.W., and Weitz, C.J. (1995). Isolation of timeless by PER protein interaction: defective interaction between timeless protein and long-period mutant $PER^{L}$. Science *270*, 811–815.

Gekakis, N., Staknis, D., Nguyen, H.B., Davis, F.C., Wilsbacher, L.D., King, D.P., Takahashi, J.S., and Weitz, C.J. (1998). Role of the CLOCK protein in the mammalian circadian mechanism. Science *280*, 1564–1569.

Hardin, P.E., Hall, J.C., and Rosbash, M. (1990). Feedback of the *Drosophila* period gene product on circadian cycling of its messenger RNA levels. Nature *343*, 536–540.

Hogenesch, J.B., Gu, Y.Z., Jain, S., and Bradfield, C.A. (1998). The basic-helix-loop-helix-PAS orphan MOP3 forms transcriptionally active complexes with circadian and hypoxia factors. Proc. Natl. Acad. Sci. USA *95*, 5474–5479.

Hunter-Ensor, M., Ousley, A., and Sehgal, A. (1996). Regulation of the Drosophila protein Timeless suggests a mechanism for resetting the circadian clock by light. Cell *84*, 677–685.

Ishiura, M., Kutsuna, S., Aoki, S., Iwasaki, H., Andersson, C., Tanabe, A., Golden, S., Johnson, C., and Kondo, T. (1998). Expression of a gene cluster kaiABC as a circadian feedback process in cyanobacteria. Science *281*, 1519–1523.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W., and Takahashi, J.S. (1997). Positional cloning of the mouse circadian Clock gene. Cell *89*, 641–653.

Kloss, B., Price, J.L., Saez, L., Blau, J., Rothenfluh, A., Wesley, C.S., and Young, M.W. (1998). The *Drosophila* clock gene *double-time* encodes a protein closely related to human casein kinase Iε. Cell *94*, 97–107.

Konopka, R.J., and Benzer, S. (1971). Clock mutants of *Drosophila* melanogaster. Proc. Natl. Acad. Sci. USA *68*, 2112–2116.

Lee, C., Parikh, V., Itsukaichi, T., Bae, K., and Edery, I. (1996). Resetting the *Drosophila* clock by photic regulation of PER and a PER-TIM complex. Science *271*, 1740–1744.

Lennon, G., Auffray, C., Polymeropoulos, M., and Soares, M.B. (1996). The I.M.A.G.E. consortium: an integrated molecular analysis of genomes and their expression. Genomics *33*, 151–152.

Myers, M.P., Wager-Smith, K., Rothenfluh-Hilfiker, A., and Young, M.W. (1996). Light-induced degradation of TIMELESS and entrainment of the *Drosophila* circadian clock. Science *271*, 1736–1740.

Myers, M.P., Rothenfluh, A., Chang, M., and Young, M.W. (1997). Comparison of chromosomal DNA composing timeless in *Drosophila melanogaster* and *D. virilis* suggests a new conserved structure for the TIMELESS protein. Nucleic Acids Res. *25*, 4710–4714.

Ousley, A., Zafarullah, K., Chen, Y., Emerson, M., Hickman, L., and Sehgal, A. (1998). Conserved regions of the timeless (tim) clock gene in *Drosophila* analyzed through phylogenetic and functional studies. Genetics *148*, 815–825.

Pittendrigh, C.S. (1993). Temporal organization: reflections of a Darwinian clock-watcher. Annu. Rev. Physiol. *55*, 17–54.

Price, J.L., Blau, J., Rothenfluh, A., Abodeely, M., Kloss, B., and Young, M.W. (1998). *double-time* is a novel *Drosophila* clock gene that regulates PERIOD protein accumulation. Cell *94*, 83–95.

Ralph, M.R., Foster, R.G., Davis, F.C., and Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. Science *247*, 975–978.

Rosato, E., Piccin, A., and Kyriacou, C.P. (1997a). Circadian rhythms: from behaviour to molecules. Bioessays *19*, 1075–1082.

Rosato, E., Trevisan, A., Sandrelli, F., Zordan, M., Kyriacou, C.P., and Costa, R. (1997b). Conceptual translation of timeless reveals alternative initiating methionines in *Drosophila*. Nucleic Acids Res. *25*, 455–458.

Rutila, J.E., Zeng, H., Le, M., Curtin, K.D., Hall, J.C., and Rosbash, M. (1996). The $tim^{SL}$ mutant of the Drosophila rhythm gene *timeless* manifests allele-specific interactions with *period* gene mutants. Neuron *17*, 921–929.

Rutila, J.E., Suri, V., Le, M., So, W.V., Rosbash, M., and Hall, J.C. (1998). CYCLE is a second bHLH-PAS clock protein essential for circadian rhythmicity and transcription of *Drosophila period* and *timeless*. Cell *93*, 805–814.

Saez, L., and Young, M.W. (1996). Regulation of nuclear entry of the Drosophila clock proteins Period and Timeless. Neuron *17*, 911–920.

Sehgal, A., Price, J.L., Man, B., and Young, M.W. (1994). Loss of circadian behavioral rhythms and per RNA oscillations in the *Drosophila* mutant timeless. Science *263*, 1603–1606.

Sehgal, A., Rothenfluh-Hilfiker, A., Hunter-Ensor, M., Chen, Y., Myers, M.P., and Young, M.W. (1995). Rhythmic expression of timeless: a basis for promoting circadian cycles in period gene autoregulation. Science *270*, 808–810.

Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F., Jr., and Reppert, S.M. (1997). Two *period* homologs: circadian expression and photic regulation in the suprachiasmatic nuclei. Neuron *19*, 1261–1269.

Shigeyoshi, Y., Taguchi, K., Yamamoto, S., Takekida, S., Yan, L., Tei, H., Moriya, T., Shibata, S., Loros, J.J., Dunlap, J.C., and Okamura, H. (1997). Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the *mPer1* transcript. Cell *91*, 1043–1053.

Skapek, S.X., Rhee, J., Kim, P.S., Novitch, B.G., and Lassar, A.B. (1996). Cyclin-mediated inhibition of muscle gene expression via a mechanism that is independent of pRB hyperphosphorylation. Mol. Cell. Biol. *16*, 7043–7053.

So, W.V., and Rosbash, M. (1997). Post-transcriptional regulation contributes to *Drosophila* clock gene mRNA cycling. EMBO J. *16*, 7146–7155.

Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G., and Lee, C.C. (1997). RIGUI, a putative mammalian ortholog of the Drosophila *period* gene. Cell *90*, 1003–1011.

Takahashi, J.S. (1995). Molecular neurobiology and genetics of circadian rhythms in mammals. Annu. Rev. Neurosci. *18*, 531–553.

Takumi, T., Matsubara, C., Shigeyoshi, Y., Taguchi, K., Yagita, K., Maebayashi, Y., Sakakida, Y., Okumura, K., Takashima, N., and Okamura, H. (1998a). A new mammalian period gene predominantly expressed in the suprachiasmatic nucleus. Genes Cells *3*, 167–176.

Takumi, T., Taguchi, K., Miyake, S., Sakakida, Y., Takashima, N., Matsubara, C., Maebayashi, Y., Okumura, K., Takekida, S., Yamamoto, S., et al. (1998b). A light-independent oscillatory gene mPer3 in mouse SCN and OVLT. EMBO J. *17*, 4753–4759.

Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M., and Sakaki, Y. (1997). Circadian oscillation of a mammalian homologue of the *Drosophila* period gene. Nature *389*, 512–516.

Vitaterna, M.H., King, D.P., Chang, A.M., Kornhauser, J.M., Lowrey, P.L., McDonald, J.D., Dove, W.F., Pinto, L.H., Turek, F.W., and Takahashi, J.S. (1994). Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science *264*, 719–725.

Welsh, D.K., Logothetis, D.E., Meister, M., and Reppert, S.M. (1995). Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron *14*, 697–706.

Young, M. (1998). The molecular control of circadian behavioral rhythms and their entrainment in *Drosophila*. Annu. Rev. Biochem. *67*, 135–152.

Zeng, H., Hardin, P.E., and Rosbash, M. (1994). Constitutive overexpression of the *Drosophila* PERIOD protein inhibits period mRNA cycling. EMBO J. *13*, 3590–3598.

Zeng, H., Qian, Z., Myers, M.P., and Rosbash, M. (1996). A light-entrainment mechanism for the *Drosophila* circadian clock. Nature *380*, 129–135.

Zylka, M.J., Shearman, L.P., Weaver, D.R., and Reppert, S.M. (1998). Three *period* homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron *20*, 1103–1110.

**GenBank Accession Numbers**

The accession numbers for the *mTim* and *hTIM* sequences reported in this paper are AF098161 and AF098162, respectively.