Oscillation and Light Induction of *timeless* mRNA in the Mammalian Circadian Clock

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Circadian rhythms in *Drosophila melanogaster* depend on a molecular feedback loop generated by oscillating products of the *period* (*per*) and *timeless* (*tim*) genes. In mammals, three *per* homologs are cyclically expressed in the suprachiasmatic nucleus (SCN), site of the circadian clock, and two of these, *mPer1* and *mPer2*, are induced in response to light. Although this light response distinguishes the mammalian clock from its *Drosophila* counterpart, overall regulation, including homologous transcriptional activators, appears to be similar. Thus, the basic mechanisms used to generate circadian timing have been conserved. However, contrary to expectations, the recently isolated mammalian *tim* homolog was reported not to cycle. In this study, we examined mRNA levels of the same *tim* homolog using a different probe. We observed a significant (approximately threefold) diurnal variation in *mTim* expression within mouse SCN using two independent methods. Peak levels were evident at the day-to-night transition in light-entrained animals, and the oscillation persisted on the second day in constant conditions. Furthermore, light pulses known to induce phase delays caused significant elevation in *mTim* mRNA. In contrast, phase-advancing light pulses did not affect *mTim* levels. The *mTim* expression profile and the response to nocturnal light are similar to *mPer2* and are delayed compared with *mPer1*. We conclude that temporal ordering of *mTim* and *mPer2* parallels that of their fly homologs. We predict that mTIM may be the preferred functional partner for mPER2 and that expression of *mTim* and *mPer2* may, in fact, be driven by mPER1.

Key words: mtimeless (mTim)*; suprachiasmatic nucleus; light induction; circadian oscillation;* mPer*; mouse*

A consensus has emerged that even in the most complex of circadian oscillators, the mammalian suprachiasmatic nucleus (SCN), timekeeping is a property of single cells (Welsh et al., 1995; Herzog et al., 1998). Studies in *Drosophila* and *Neurospora* have demonstrated that the putative "intracellular" circadian clock is characterized by a molecular negative feedback loop (for review, see Sehgal et al., 1996; Dunlap, 1999). The molecular basis of SCN rhythmicity, like that of the *Drosophila* clock, is apparently regulated through the interactions of a limited number of genes and their protein products. The mammalian clock consists of at least six elements, three independent *period* genes (*mPers 1–3*) (Albrecht et al., 1997; Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997; Takumi et al., 1998a,b), *clock (mClk)* (Antoch et al., 1997; King et al., 1997), *bmal* (Gekakis et al., 1998; Hogenesch et al., 1998), and *timeless (mTim)* (Koike et al., 1998; Sangoram et al., 1998; Zylka et al., 1998b). Of these, only *mClk* is known to be essential for circadian rhythmicity (Antoch et al., 1997).

The molecular events that underlie mammalian circadian rhythmicity are largely unknown. However, the remarkable evolutionary conservation of potential clock elements renders the *Drosophila* model a useful predictor for the SCN. The *Drosophila* clock is based on a negative feedback transcription–translation oscillator using heterodimeric PAS domain-containing transcription factors that interact with paired negative elements. The core feedback loop requires four genes and their protein products, *dPer* (Konopka and Benzer, 1971), *dTim* (Sehgal et al., 1994, 1995; Voshall et al., 1994), *dClk* (Allada et al., 1998) and *Cycle* (*cyc,* the *Drosophila* homolog of *Bmal*) (Rutila et al., 1998). *dPer* and *dTim* transcription is activated by binding of a dCLK:CYC heterodimer to E boxes within *dPer* and *dTim* promoters (Darlington et al., 1998). Accumulation of dPER (Edery et al., 1994; Curtin et al., 1995) and dTIM (Hunter-Ensor et al., 1996; Myers et al., 1996) is followed by heterodimerization and nuclear entry, where they supplant the activity of dCLK:CYC, thereby inhibiting *dPer* and *dTim* transcription. Degradation of dPER, regulated

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in part by DOUBLE-TIME (Kloss et al., 1998; Price et al., 1998), and of dTIM releases transcriptional inhibition, completing the cycle.

Although molecular clock components are highly conserved between flies and mammals, subtle differences in timing and regulation are emerging. Each of three *mPers* oscillates independently. *mPer1* transcription, activated at least in part by interaction of CLK:BMAL (Gekakis et al., 1998; Hogenesch et al., 1998) with an E-box within *mPer1* promoter, begins before dawn. *mPer1* peaks at zeitgeber time 4 (ZT 4, 4 hr into the day of the light/dark cycle) and rapidly declines to basal levels before the end of the day (Sun et al., 1997; Tei et al., 1997). *mPer3* begins to accumulate at the beginning of day, and peak levels are maintained from ZT 4 through ZT 10, followed by decline to basal levels just after the onset of night (Takumi et al., 1998b; Zylka et al., 1998a). Accumulation of *mPer2* begins after *mPer1* and *mPer3* and peaks at the day-to-night transition (ZT 12) (Albrecht et al., 1997; Takumi et al., 1998a). Finally, light pulses at ZT 16 raise levels of *mPer1* and *mPer2*, whereas only *mPer1* is rapidly induced by light pulses at ZT 22 (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998a; Zylka et al., 1998a).

Recently, a mammalian homolog of *dTim* was identified (Koike et al., 1998; Sangoram et al., 1998; Zylka et al., 1998b). However, contrary to expectations, these researchers failed to detect a circadian oscillation of *mTim* mRNA. At the time of those publications, we also had successfully identified the same *mTim* homolog. We examined the circadian profile of this putative *mTim* under entrained lighting conditions, determined whether *mTim* expression changed in constant conditions, and investigated the effects of nocturnal light on acute expression of *mTim*.

Herein we report cycling of *mTim* mRNA in mice entrained to a light/dark (LD) cycle. After light entrainment, this oscillation persists in constant darkness (DD) with a dampened amplitude. Finally, light at ZT 16, but not at ZT 22, causes induction of the *mTim* transcript. These behaviors support a role for *mTim* in the circadian clock and suggest that mTIM and mPER2 may be functional partners.

MATERIALS AND METHODS

Northern blot. For all experiments, 6- to 8-week-old C57B6/J mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in a 12 hr LD cycle for at least 2 weeks before experimentation. Animals received food and water *ad libitum*. At zeitgeber time 12 (the time of lights off in the animal colony, ZT 12), animals were killed by cervical dislocation. Tissues were immediately removed and placed in Trizol reagent (Life Technologies, Gaithersburg, MD). Total RNA was immediately purified according to the manufacturer's protocol (Ambion, Austin, TX). Poly(A) RNA was then isolated using the Ambion Poly A Pure system. Poly(A) RNA (2–3 μg) was run on a 1% agarose/2.2 M formaldehyde gel and transferred to nitrocellulose. Membranes were prehybridized in solution (6× SSC, 2× Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA) and then hybridized in the same solution with the addition of a probe (described in Results) radiolabeled through PCR. Hybridization proceeded at 68°C for 16–24 hr. Membranes were washed in 1× SSC and 0.1% SDS at room temperature, followed by a wash in 0.2× SSC and 0.1% SDS at 68°C, and were exposed to x-ray film.

In situ *hybridization.* Mice were deeply anesthetized with 0.2 ml of sodium pentobarbital (30 mg/ml) and perfused intracardially with 5 ml of ice-cold 0.1 M PBS followed by 4% paraformaldehyde (60 ml). Brains were removed and post-fixed overnight at 4°C in 4% paraformaldehyde. Brains were transferred to 0.1 M PBS with 20% sucrose and maintained at 4°C until sectioning. Thirty micrometer sections were cut at −20°C on a cryostat. Slides were dried overnight at 40°C and then equilibrated in DEPC-PBS (0.1 M) for 10 min. Sections were permeabilized in DEPC-PBS with 0.1% Triton X-100, washed in DEPC-PBS, and treated with 2 μg/ml Proteinase K in Tris-EDTA buffer for 30 min at 37°C. Sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine before prehybridization (50% formamide in 4% SSC). Digoxygenin-labeled sense or antisense riboprobes (5 ng/μl) were applied in hybridization buffer (4× SSC, 40% formamide, 10% dextran sulfate, 1× Denhardt's solution, 10 mM DTT, 1 mg/ml yeast tRNA, and 1 mg/ml salmon sperm DNA) overnight at 42°C. Sections were washed in 2× SSC, followed by 1× SSC. Single-stranded RNA was digested with 20 mg/ml RNase A in NTE buffer (500 mM NaCl, 10 mM Tris, and 1 mM EDTA, pH 8.0) for 30 min. Sections were washed two times for 30 min each in 0.1% SSC. Alkaline phosphatase-labeled anti-digoxygenin antibody (1:100; Boehringer Mannheim, Indianapolis, IN) was applied for at least 2 hr at room temperature. Slides were washed in 100 mM Tris and 150 mM NaCl, pH 7.5. Alkaline phosphatase was visualized by incubating slides 4–8 hr in color solution (nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate, and 2.4 mg/ml levamisole). Coverslips were applied using an aqueous mounting medium. Analysis of *mTim*-positive cells was made throughout the rostrocaudal extent of each SCN by an individual blind to the experimental design and identity of the samples.

Ribonuclease protection assay. Mice were killed by cervical dislocation at ZT 0 and ZT 12 or circadian time 0 (CT 0, under DD, refers to the time of lights on in the previous lighting schedule) and CT 12 (lights off in the previous LD cycle). RNA was purified from SCN-containing ventral hypothalamus using the Trizol reagent (Life Technologies). Poly(A) RNA was isolated from total RNA using the Ambion Poly A pure system. Ribonuclease protection assay (RPA) was performed on either 1 μg of poly(A) RNA (LD samples) or 20 μg of total RNA (DD samples) with the RPAII kit (Ambion). RNA (1 μg/ml) was hybridized overnight with biotinylated antisense probes. Samples were digested with RNase T1 to remove unprotected RNA. Protected fragments were separated on a 5% polyacrylamide gel and transferred to a positively charged nylon membrane. Biotinylated probes were detected using the Bright Star Bio-detect kit (Ambion). Exposed films were quantitated by densitometry, and levels of *mTim* and *mPer2* were calculated relative to actin to control internally for variation in loading.

RESULTS

Characterization of a mouse *tim* homolog

We identified a *tim*-homologous expressed sequence tag (dbj/c88957) in the National Center for Biotechnical Information database and used primers based on this sequence to isolate *mTim* cDNAs from mouse brain (Marathon; Clontech). While this work was in progress, two other groups reported isolation of a *tim* homolog (Sangoram et al., 1998; Zylka et al., 1998b). Our 1743 nucleotide sequence is virtually identical to nucleotides 17–1760 of the sequence reported by Zylka et al. (1998b). Because the sequence of Sangoram et al. (1998) is the same as that of Zylka et al. (1998b), except for 67 nucleotides at the N terminus, we conclude that the same gene is being analyzed in all cases. The discrepancy at the N terminus is apparently produced by alternative splicing in the 5′ untranslated region (Sangoram et al., 1998).

Northern blots of multiple tissues collected at ZT 12 were probed with an RNA probe that corresponds to nucleotides 254–432 (Sangoram et al., 1998). We detected two major transcripts, 4.5 and ~2.3 kb in length, and a minor 1.0 kb transcript in the brain, heart, and testis (Fig. 1). These three species, as well as two others (~1.5 and 1.2 kb), were detected in the liver. Qualitatively, these data are similar to those published by Sangoram et al. (1998). On a commercial Northern blot, Sangoram et al. (1998) detected both a 4.5 kb band and a smaller band (although the size was 3.0 kb as opposed to 2.3 kb). Although they did not refer to it, there appeared to be considerable background hybridization to liver RNA, which could represent additional transcripts (Sangoram et al., 1998). We probed a similar Clontech blot with a 300 nucleotide probe (149–432) and observed a 4.5 kb band in all tissues (data not shown). Because of high background, the 1.0 kb transcript was not distinct in any tissue. The 2.3 kb band was detected in brain, lung, and spleen but was absent from heart, liver, and testis. Multiple bands were observed in skeletal muscle,

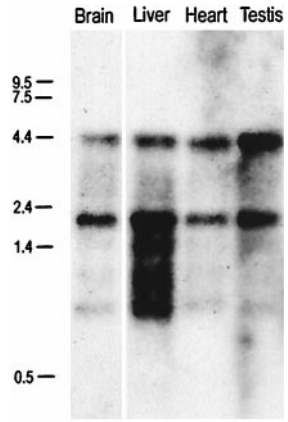

Figure 1. Northern analysis of poly(A) RNA (2–3 μg/lane) obtained at ZT 12. A PCR-generated, radiolabeled RNA probe corresponding to nucleotides 254–432 of the published *mTim* sequence (Sangoram et al., 1998) detected two major transcripts, 4.5 and ~2.3 kb in length, and a minor 1.0 kb transcript in the brain, heart, and testis. These three species as well as ~1.5 and 1.2 kb bands were detected in the liver.

including a 6.0 kb transcript (seen in heart as well) also reported by Sangoram et al. (1998). In contrast, Zylka et al. (1998b) reported only a 4.5 kb *mtim* transcript. It is also noteworthy that the tissue collection time for RNA samples on the commercial blot is unknown but likely was not ZT 12.

Cyclic expression of *mTim* RNA in the SCN

To examine the circadian pattern of expression of the mRNA encoded by the putative *mTim* gene under entrained lighting conditions, mice were killed at 4 hr intervals over a 24 hr period, and brains were prepared for *in situ* hybridization. Probes for *in situ* hybridization contained the same 180 bp sequence that was used for the Northern blot shown in Figure 1. No significant hybridization was observed at any time of day in the presence of the sense probe (data not shown). The *mTim* RNA signal was barely visible at ZT 0 and ZT 4; levels increased at ZT 8, peaked at the end of the day, ZT 12, and decreased steadily thereafter (Fig. 2*A,B*). The amplitude of this oscillation was approximately threefold (Fig. 2*B*). Analysis of *mTim* levels by RPA supports the *in situ* data. A 2.5-fold increase in the amount of the *mTim* protected fragment was observed at ZT 12 compared with ZT 0 (Fig. 2*C*). To assess relative levels of *mPer2*, a 300 bp probe corresponding to nucleotides 2242–2542 (Albrecht et al., 1997) was used. An increase similar to *mTim* was detected between ZT 0 and ZT 12 for *mPer2* transcript levels analyzed in the same samples (Fig. 2*C*).

To determine whether the observed *mTim* oscillation is clock-controlled, as opposed to being driven by light, we examined expression of *mTim* RNA in the SCN under constant conditions (DD). On the second day of DD, the *mTim* oscillation persisted, but with a considerably dampened amplitude (~1.5 fold; Fig. 2*D,E*). The dampening of the rhythm was largely attributable to an increase in trough levels (Fig. 2, compare *CT 0* in *E* with *ZT 0* in *B*) and not attributable to a reduction in peak expression levels (Fig. 2, compare *CT 12* in *E* with *ZT 12* in *B*). RPA of samples obtained at CT 0 and CT 12 confirmed the persistence of the *mTim* oscillation under constant conditions (Fig. 2*F*).

Induction of *mTim* RNA by light

Because the expression of *mPer1* and *mPer2* mRNAs is induced by nocturnal light at ZT 16, we sought to determine whether *mTim* mRNA displayed a similar response. Animals entrained to a 12 hr LD cycle were subjected to a light pulse (400 lux, 30 min) at either ZT 16 or ZT 22 and killed at successive intervals up to 4 hr after initiation of the light pulse. Light-entrained animals were chosen for these studies for two reasons. First, we were concerned that the dampening of the oscillation observed in DD, particularly the higher trough levels, might obscure the effects of light. Second, it alleviates the necessity of using a behavioral system to monitor circadian phase in free-running animals whose individual circadian periods vary. ZTs 16 and 22 were chosen because these times correspond to the times in C57B6/J mice when light causes a maximal phase delay, or phase advance, respectively (Daan and Pittendrigh, 1976). *mTim* mRNA was induced after the light pulse at ZT 16 (Fig. 3). At the end of the 30 min light pulse, *mTim* expression was not significantly increased, but at 60 min it was elevated ~2.5-fold compared with the unpulsed control (data not shown). Levels peak 90 min after the onset of the light pulse. *mTim* remained elevated 2 hr after the light pulse and returned to basal by 4 hr after initiation of light. In contrast, a light pulse at ZT 22 had no effect on *mTim* mRNA levels; expression of *mTim* after the ZT 22 light pulse was not different from unpulsed controls obtained at the same time intervals after ZT 22 (data not shown).

DISCUSSION

We have identified a homolog of the *Drosophila* clock gene *timeless* and provide compelling evidence that it may be an important component of the mammalian circadian clock. Using two independent measures, we demonstrate a significant diurnal pattern of expression for *mTim* mRNA in the SCN in animals entrained to an LD cycle. The oscillation persists under free-running conditions (DD) with a dampened amplitude. Furthermore, based on the responsiveness of *mTim* to light, our study implies that regulation of *mTim* may be similar to that previously demonstrated for *mPer2* and/or *mPer1* (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998a; Zylka et al., 1998a). Our results suggest that regulation of *mTim* is similar to *dTim* and that *mTim* could be a partner for *mPer2*.

Apparent discrepancies with the constitutive expression of *mTim* previously reported (Sangoram et al., 1998; Zylka et al., 1998b) may be explained by differences in experimental design and technique. We focused on *mTim* expression in the presence of LD cycles, whereas Zylka et al. (1998b) and Sangoram et al. (1998) assayed expression only on the first and third days of DD, respectively. Because we have demonstrated that the oscillation dampens significantly under constant conditions, it is not surprising that that cycling could have been missed.

Our staining is intense compared with the weak hybridization signals displayed in the previous publications, especially in the SCN. We used a nonradioactive digoxygenin-based system instead of ^{35}S for detection of riboprobes in *in situ* hybridization studies. This may increase sensitivity and/or the signal-to-noise ratio, thereby increasing the likelihood of detecting an oscillation. Furthermore, our smaller probe may better penetrate the tissue. Additionally, our probe was generated from a more 5′ region of the gene compared with those used previously. This becomes particularly important when comparing Northern blot data. In agreement with Sangoram et al. (1998), we identified multiple transcripts, whereas Zylka et al. (1998b) reported only a single 4.5 kb mRNA. Because the probe used by Zylka et al. (1998b) was considerably 3′ to our probe and also somewhat downstream of that used by Sangoram et al. (1998), it is conceivable that the

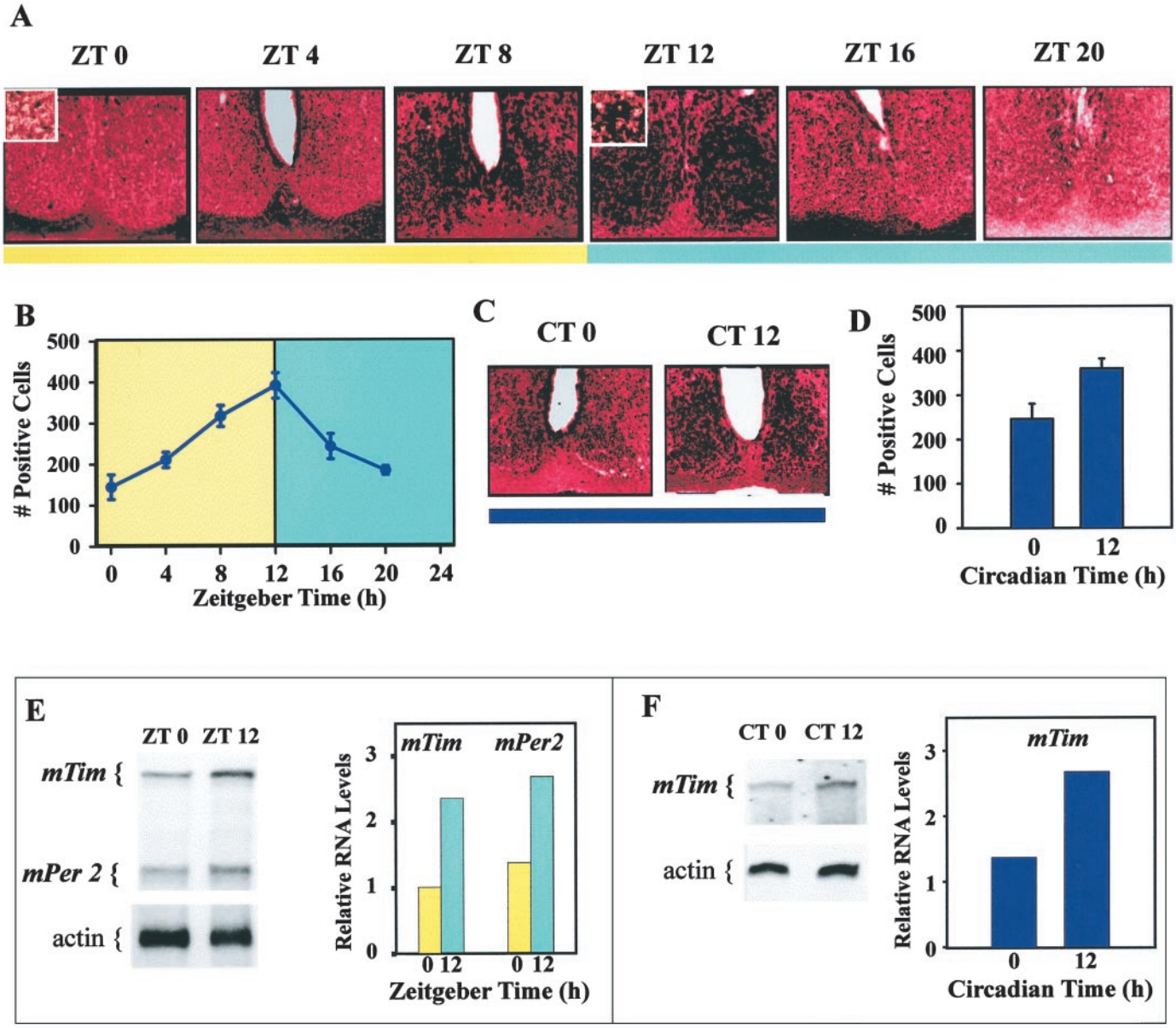

Figure 2. Oscillation of *mTim* mRNA in SCN under light-entrained and constant conditions. Expression of *mTim* mRNA was determined in the SCN of adult mouse brains using *in situ* hybridization and RPA. Coronal sections from mice maintained in 12 hr LD (*A*) or from entrained animals on the second day of DD (*C*) were probed with a riboprobe containing the same sequence of nucleotides used in Figure 1, and hybridization was detected using a digoxygenin nonradioactive system. Magnification, 200× unless indicated otherwise. Quantitation of LD and DD samples is shown in *B* and *D*, respectively. Data were analyzed blind to exclude bias. Cells positive for *mTim* staining were counted through the rostrocaudal extent of the SCN of every mouse and averaged, as described previously (Ding et al., 1997). For LD (*A, B*), the average ± SEM of five independent experiments is shown. The number of positive cells at *ZT 12* was significantly greater than that at *ZT 0, ZT 4, ZT 16*, and *ZT 20*. *ZT 8* was also greater than *ZT 0* and *ZT 20* (ANOVA, $p < 0.001$; Tukey test, $p < 0.05$). In DD (*C, D*), the number of positive cells was significantly greater at *CT 12* compared with *CT 0* ($p < 0.05$, Student's *t* test). Higher-magnification (400×) images are shown as *insets* in the *ZT 0* and *ZT 12* samples to reveal differences in the intensity of staining between these two times. Appearance of the optic chiasm was unpredictable and not correlated with cellular staining. Representative RPAs are shown in *E* and *F*. For the LD experiments, 1 μg of poly(A) RNA was analyzed. For DD, 20 μg of total RNA was used. In LD, the density of the protected fragment representing *mTim* was greater at *ZT 12* compared with *ZT 0* and greater at *CT 12* compared with *CT 0*. As a positive oscillating control in LD, *mPer2* levels were analyzed in the same samples. *mPer2* was higher at *ZT 12* compared with *ZT 0*.

smaller mRNAs represent alternatively spliced forms detected only by 5′ sequences of the gene. If these smaller isoforms make a major contribution to cycling, the inability to detect them could account for the absence of an oscillation on the first day of DD as reported by Zylka et al. (1998b; their 3′ *in situ* probe overlapped partially with their Northern probe). Thus, although on first glance our data appear to conflict with those published, we regard their data as complementary to ours.

We have demonstrated that the *mTim* oscillation does dampen in DD. It is noteworthy that dampening of *dTim*, as well as *dPer*, oscillations in DD also occurs in *Drosophila* (Hardin et al., 1990; Sehgal et al., 1995). Dampening could represent desynchronization of individual oscillators within the SCN. If so, it seems that desychronization would also cause dampening of the oscillations of the *mPers* under constant conditions. However, oscillations in *mPer1*, *mPer2*, and *mPer3* remain robust in constant conditions

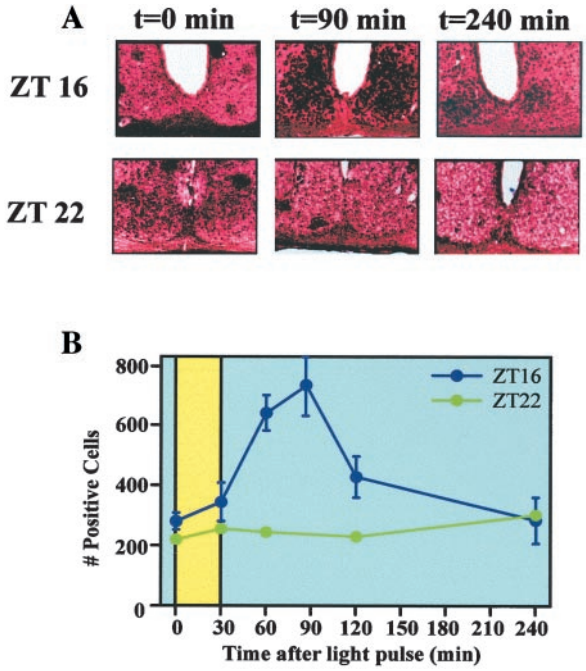

Figure 3. Response of *mTim* RNA to light. Mice were treated with a 30 min light pulse at the indicated time and collected 30, 60, 90, 120, and 240 min later. Sections were processed for *in situ* analysis as described in Figure 2. In *B*, data are means ± SEM for three independent experiments, except for the 90 min samples ($n = 2$). Sixty minutes after a light pulse at ZT 16, the number of positive cells increased significantly (ANOVA, $p < 0.001$; Tukey test, $p < 0.05$; $n = 3$). At 90 min *mTim* the number of positive cells appeared to be even greater. However, no increase in *mTim*-positive cells was observed after a light pulse at ZT 22 ($n = 1$ for each time point). *ZT 16* and *ZT 22* samples were processed simultaneously to reduce interassay variations in staining.

(Albrecht et al., 1997; Sun et al., 1997; Tei et al., 1997; Shearman et al., 1998; Takumi et al., 1998a,b; Zylka et al., 1998a). Because we measured only two points in DD, the actual peak and trough of the oscillation could have been missed, especially if the timing of the cycling shifts, as is characteristic of *mPer2* in entrained versus free-running conditions (Takumi et al., 1998a).

Our data suggest that mTIM is an important circadian clock component and that mPER2 is the most likely physiological partner for mTIM. The timing of *mTim* oscillation and its temporal gating with respect to light are remarkably similar to those of *mPer2* (Albrecht et al., 1997; Shearman et al., 1997; Shigeyoshi et al., 1997; Takumi et al., 1998a; Zylka et al., 1998a). The time course for the response of *mPer1* to light at ZT 16 is faster and more transient than that of *mPer2* and *mTim. mPer1* increases significantly 30 min after the light pulse, peaks at 60 min, and returns to basal levels by 180 min after light (Albrecht et al., 1997; Shigeyoshi et al., 1997; Zylka et al., 1998b). In contrast, *mPer2* (Takumi et al., 1998a; Zylka et al., 1998a) and *mTim* mRNAs are not elevated 30 min after light; peak induction occurs ~90 min after light, and their return to basal levels is not complete until 3–4 hr after light exposure. Furthermore, although rapid light responsiveness is also characteristic of *mPer1* at ZT 22, *mPer2* (Albrecht et al., 1997) and *mTim* do not change rapidly in response to light at ZT 22.

The delayed time course for elevation of *mPer2* and *mTim* after light suggests that these elements may not be required for the light-induced phase shift. Rather, their induction may be secondary to the phase shift; increased levels may occur only after the

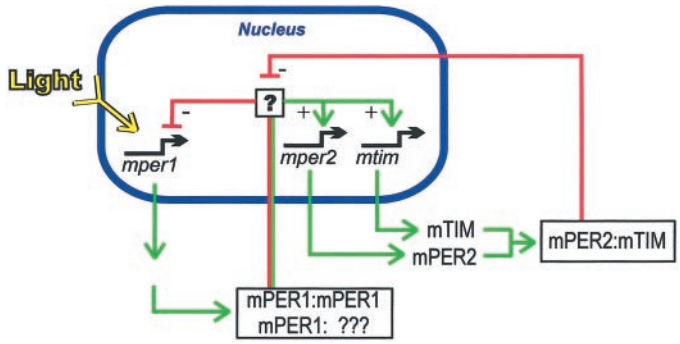

Figure 4. Model for regulation of the molecular elements of the mammalian clock that respond to light. We propose that mPER1 may, at least in part, drive the transcription of *mTim* and/or *mPer2*. This prediction is consistent with the endogenous profiles of these three clock elements, as well as the time courses of their light responses. *mPer3* is not included, because it does not respond to light (Takumi et al., 1998b; Zylka et al., 1998a).

clock has reset to a new time. At CT 16, light causes ~3 hr phase delay in wheel-running activity (Daan and Pittendrigh, 1976). Because the clock mechanism is likely reset within 1 hr of the light pulse (Best et al., 1999), we predict that the molecular state of the clock actually moves back in time, reorganizing in <1 hr, and resumes in a state approximating CT 13. This light-induced phase shift requires induction of *mPer1* (Akiyama et al., 1999). Thus, if light at CT 16 actually returns the clock to CT 13, clock cells must alter their function to realign with endogenous molecular events characteristic of CT13; *mPer2* and *mTim* mRNAs are elevated at CT 13. If rapid phase shifting also occurs after light-induced phase advances, then light at CT 22, which causes an ~1 hr phase advance, resets the clock to CT 23. Because light at CT 22 again causes rapid induction of *mPer1*, the mechanism for light-induced phase advances can also be predicted to require *mPer1*. At CT 23, the status of other clock components, such as subcellular localization and/or phosphorylation states of clock proteins, may not favor induction of *mPer2* and *mTim,* which would also account for their endogenously low levels in the late night–early day.

The putative molecular elements identified thus far are highly conserved between the circadian clocks of mammals and *Drosophila*. However, direct comparisons between species are currently restricted to analysis of mRNAs. Because each of the *mPers* displays a robust circadian oscillation, a central role for *mPer* in mammalian circadian timekeeping, similar to *Drosophila*, is generally accepted. The fact that that light induces both *mPer1* and *mPer2*, whereas light has no effect on levels of *dPer*, may simply reflect subtle species differences. In contrast to mammals, in which *mPer2* transcription is low at the time of the light-induced phase delay, in *Drosophila*, *dPer* continues to be expressed at maximal levels. Thus, further induction of the *dPer* transcript is likely unnecessary.

The role of *mTim* is controversial. Based on reported lack of cycling in DD, it has been proposed that *mTim* is either inconsequential (Zylka et al., 1998b) or acts merely as a mediator of *mPer* function (Dunlap, 1999). We favor an evolutionarily conserved mechanism with an essential role for *mTim*. We propose a model for molecular regulation of the mammalian clock based on those elements of the clock that show light responsiveness (Fig. 4). Transcription of *mPer1* is driven by CLOCK:BMAL (data not shown; Gekakis et al., 1998). The lag between initiation of transcription between *mPer1* and *mTim*/*mPer2* suggests that CLOCK:BMAL may not be sufficient to drive *mTim*/*mPer2* transcription.

Although little is currently known regarding the protein products of the putative mammalian clock genes, several experimentally testable predictions can be made. It is likely that translational events producing mPER1 begin morning to midday. mPER1 can be predicted to form homodimers or to heterodimerize with another PAS domain-containing protein (Zylka et al., 1998b) and enter the nucleus, where it could feed back to inhibit its own transcription as well as to promote the transcription of *mPer2* and/or *mTim*. Thus, mPER2 and mTIM would be produced at night, as in *Drosophila* (Edery et al., 1994; Curtin et al., 1995; Hunter-Ensor et al., 1996; Myers et al., 1996). The mPER2: mTIM heterodimers could then feed back and inhibit their own transcription. Although this does not exclude interactions of mPER1 and mTIM (Sangoram et al., 1998), the expression profiles of their mRNAs suggest otherwise.

mPer1 is an integral part of the signaling pathway by which nocturnal light resets the clock (Akiyama et al., 1999). Induction of *mPer1* in the early night may be directly responsible for the elevation of *mPer2* and *mTim*. Close temporal patterning of the mRNAs of *mTim* and *mPer2* based on circadian expression profiles and light responses predict that their protein products may be dimerization partners and, as such, co-regulate the molecular processes of the nocturnal domain. Therefore, despite apparent increased complexity of controls in the mammalian clock anticipated by the discovery of multiple forms of *mPer*, our data suggest that temporal ordering of some clock elements has been evolutionarily conserved, even between flies and mammals.

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