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A light-independent oscillatory gene *mPer3* in mouse SCN and OVLT

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A new member of the mammalian *period* gene family, *mPer3*, was isolated and its expression pattern characterized in the mouse brain. Like *mPer1*, *mPer2* and *Drosophila period*, *mPer3* has a dimerization PAS domain and a cytoplasmic localization domain. *mPer3* transcripts showed a clear circadian rhythm in the suprachiasmatic nucleus (SCN). Expression of *mPer3* was not induced by exposure to light at any phase of the clock, distinguishing this gene from *mPer1* and *mPer2*. Cycling expression of *mPer3* was also found outside the SCN in the organum vasculosum lamina terminalis (OVLT), a potentially key region regulating rhythmic gonadotropin production and pyrogen-induced febrile phenomena. Thus, *mPer3* may contribute to pacemaker functions both inside and outside the SCN.

Keywords: circadian rhythm/mammalian clock gene/organum vasculosum lamina terminalis (OVLT)/*period*/suprachiasmatic nucleus (SCN)

Introduction

Most organisms produce a variety of behavioral or physiological rhythms with periods that are close to 24 h. The prevalence of such 'circadian' rhythms, and the recovery of mutations that alter them in *Cyanobacteria*, *Arabidopsis*, *Neurospora*, *Drosophila* and rodents (Dunlap, 1996; King and Takahashi, 1996; Rosbash *et al.*, 1996; Young *et al.*, 1996; Hastings, 1997), indicate an ancient, genetically determined mechanism.

The first and most completely characterized clock gene in the animal kingdom is the *Drosophila period* (*dPer*) gene. Recently, its structural homologs were isolated from mouse and human (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Sun *et al.*, 1997; Tei *et al.*, 1997; Takumi *et al.*, 1998). *mPer1*, the first identified mouse *period* gene, attracts intense attention because of its robust rhythmic expression in the suprachiasmatic nucleus (SCN), a mammalian center for circadian pacemaker function. *mPer1* expression is also strongly regulated by light in a fashion correlated with phase-shifting of overt behavioral rhythms. Subsequent discovery of *mPer2*, a second *period* gene homolog, indicates that mammalian *period* genes constitute a family. A remarkably high level of *mPer2* RNA accumulation was found in the SCN and, like *mPer1*, it shows a robust circadian rhythm in this tissue. However, the phases of cycling *mPer1* and *mPer2* expression differ (Albrecht *et al.*, 1997; Takumi *et al.*, 1998). These data suggested that the function of the mammalian *period* gene family in the SCN plays a central role in establishing circadian rhythmicity and entrainment as in *Drosophila* (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Shigeyoshi *et al.*, 1997; Sun *et al.*, 1997; Tei *et al.*, 1997; Takumi *et al.*, 1998).

Here we have characterized a third mammalian *period* gene, *mPer3*, whose pattern of expression and regulation by light differs substantially from that of *mPer1* and *mPer2*.

Results and discussion

The cloning of mPer3, a member of the mammalian period family

In an effort to identify new members of the mammalian *period* gene family, we performed a series of computer database searches. Basic Local Alignment Search Tool (BLAST) searches revealed significant sequence similarity of *mPer2* to human DNA sequence HS467L1. HS467L1 maps to human chromosome 1, whereas *hPer1* and *hPer2* map to chromosome 17p12–13.1 (Tei *et al.*, 1997) and chromosome 6 (Nagase *et al.*, 1997), respectively. In addition, a second clone (AA451523), which resembles a portion of HS467L1, was identified in the mouse expressed sequence tag (EST) database. Probes derived from the EST using rapid amplification of cDNA ends (RACE) allowed cloning of a full-length mouse cDNA. The cloned cDNA encodes 1115 amino acids (Figure 1A). The amino acid sequence encoded by *mPer3* exhibits 56 and 51% similarity overall to that of *mPer2* (Albrecht *et al.*, 1997; Shearman *et al.*, 1997; Takumi *et al.*, 1998) and *mPer1* (Sun *et al.*, 1997; Tei *et al.*, 1997), respectively.

Sequence alignments for mPER1, mPER2 and mPER3 are shown in Figure 1A. No typical basic helix–loop–helix (bHLH) motif or HLH was found in the N-terminal sequence of mPER3, although Sun *et al.* (1997) suggested the presence of a weak bHLH region in mPER1 and mPER2, and we suggested the presence of a possible HLH region without an adjoining basic region in mPER2 (Takumi *et al.*, 1998). The PAS domain (residues 126–324), including PAS-A (residues 126–175) and PAS-B (residues 263–312) repeats, which is regarded as a potential

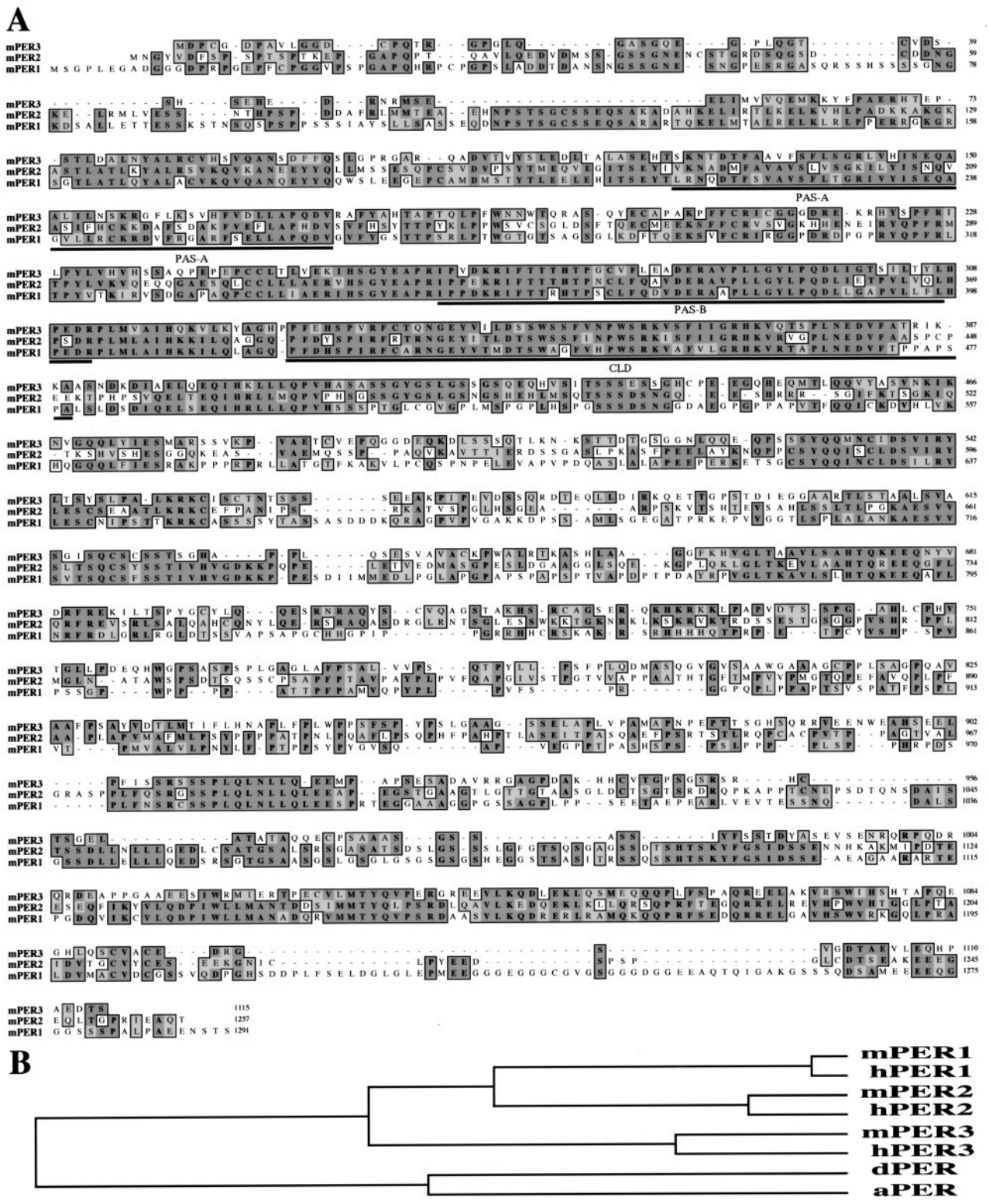

Fig. 1. Amino acid sequence comparison of the *Per* family. (**A**) Alignments of the complete amino acid sequences among the mouse *Per* family. The sequence of mPER3 in compared with mPER2 (Takumi *et al*., 1998) and mPER1 (Tei *et al.*, 1997). The amino acid sequences indicated with single-letter notations are aligned by inserting gaps (–) to achieve maximum homology. Amino acid identities and similarities are indicated by dark gray and light gray boxes, respectively. PAS-A, PAS-B and CLD are displayed by bold underlining. (**B**) An evolutionary tree of Period in *Drosophila*, *Antherea pernyi*, mouse and human. The tree was made using the UPGMA (Unweighted Pair Group Method with Arithmic Mean) Tree Window in Geneworks (IntelliGenetics, Inc., Mountain View, CA). Sequences used here are abbreviated as follows: mPER1, mouse PER1 (Tei *et al.*, 1997); hPER1, human PER1 (Tei *et al.*, 1997); mPER2, mouse PER2 (Takumi *et al*., 1998); hPER2, human PER2 (Takumi *et al*., 1998), mPER3, mouse PER3; hPER3, human PER3 (T.Takumi and H.Okamura, unpublished data); dPER, *Drosophila* PER (Citri *et al.*, 1987); and aPER, *A.pernyi* PER (Reppert *et al.*, 1994).

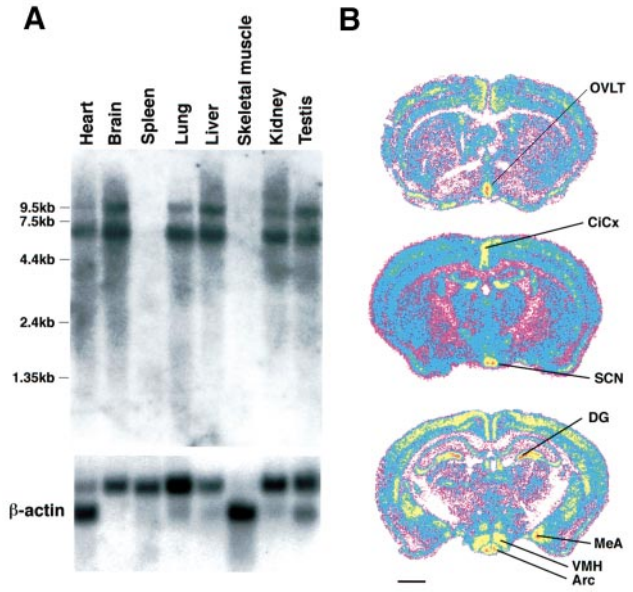

Fig. 2. Distribution of *mPer3* mRNA in mouse tissues. (**A**) Northern blot of RNA prepared from the indicated mouse tissues, probed with *mPer3* (nucleotides 814–1955). The positions of RNA size markers are shown (left). The same filter subsequently was hybridized with a 1.8 kbp β-actin probe. (**B**) *In situ* hybridization to *mPer3* transcripts in the mouse forebrain during the subjective day (CT4). Note the strong signals in the OVLT, SCN, VMH and Arc, as well as cortical structures including the CiCx (cingulate cortex), DG (gyrus dentatus) and MeA (medial amygdaloid nucleus). Bar = 1 mm.

PER dimerization domain (Huang *et al.*, 1993) and has been shown to form the interaction domain for dPER's heterodimerization with Timeless (Huang *et al.*, 1995), is highly conserved among all three mammalian *period* gene products. The cytoplasmic localization domain (CLD, residues 330–389; Saez and Young, 1996) and elements of the immediately downstream protein sequence were also well conserved. A single basic type of nuclear localization signal (NLS) was seen in residues 726–734 (RQKHKRKKL) of mPER3. No discernible NLS has been detected in mPER1, but a putative bipartite basic type NLS is found in mPER2 (Shearman *et al.*, 1997). In the C-terminal region of the predicted protein, several clusters of homologous sequence are also found (Figure 1A). These findings indicate that *mPer3* is a member of the mammalian *period* family.

We further compared protein similarity among the Period family including comparisons with *Drosophila* PER (Citri *et al.*, 1978; Jackson *et al.*, 1986; Baylies *et al.*, 1993) and *A.pernyi* PER (Reppert *et al.*, 1994). As illustrated in Figure 1B, PER1 and PER2 were most closely related to each other. PER3 was rather divergent in comparison with PER1 and PER2, and the divergence found between hPER3 and mPER3 was larger than that separating hPER1 and mPER1, and hPER2 and mPER2. This analysis shows that *period* forms a gene family encompassing vertebrate and invertebrate species.

mPer3 mRNA is expressed in the diencephalic regions including the SCN, OVLT, VMH and Arc

RNA blot analysis revealed that two size classes of *mPer3* mRNA, ~6.5 and 9.5 kb, are expressed in heart, brain, lung, liver, kidney and testis, but not in spleen and skeletal muscle (Figure 2A). The tissue distribution of *mPer3* mRNA was similar to that of *mPer2*, while *mPer1* transcripts were expressed more widely in various tissues (Sun *et al.*, 1997; Tei *et al.*, 1997). The distribution of *mPer3* mRNA in the mouse brain was examined by *in situ* hybridization (Figure 2B). The highest expression of *mPer3* in the brain was observed in the diencephalic regions including the SCN, the organum vasculosum lamina terminalis (OVLT), the ventromedial hypothalamic nucleus (VMH) and the arcuate nucleus (Arc), as well as telencephalic structures such as the gyrus dentatus and medial amygdaloid nucleus. Moderate signals were detected in the cingulate cortex, hippocampal pyramidal cells, cerebellar cortex and the nucleus tractus solitarius. In other brain regions, *mPer3* expression was low (e.g. cerebral cortex, caudate-putamen, thalamic nuclei, superior colliculus, inferior colliculus and dorsal horn of the spinal cord), or not detectable.

mPer3 transcripts show a clear circadian rhythmic expression in the SCN

To explore the time dependence and the daily rhythm in the expression of *mPer3* mRNA in the SCN, we examined the mouse SCN in 12 h light:12 h dark cycles (LD) and in constant darkness (DD) by a quantitative *in situ* hybridization method. These expression patterns were also compared with those of *mPer1* and *mPer2* under the same conditions (Figure 3A). In LD, the amount of *mPer3* mRNA began to increase in the morning, increasing steadily to the point of highest accumulation at ZT8 (ZT = Zeitgeber time used for assessing biological time in an LD cycle; ZT0 is lights-on and ZT12 is lights-off). *mPer3* mRNA levels subsequently decreased, forming a trough at ZT16–20 in the dark phase of the LD cycle. In DD (the second cycle of DD conditions), fluctuations in the level of *mPer3* mRNA were again observed, with highest accumulation at CT4 (CT = circadian time; CT0 is subjective dawn and CT12 is subjective dusk) and lowest accumulation at CT20 (Figure 3). The maximum *mPer3* mRNA level is 2- to 3-fold higher than the minimum under both LD and DD conditions. In this animal strain, the free-running locomotor activity period was 23.46 h (Shigeyoshi *et al.*, 1997).

These expression profiles of *mPer3* were compared with those of *mPer1* and *mPer2*. The pattern of *mPer2* expression differs in LD and in DD, during the subjective afternoon (Figure 3A). In a previous study, we also demonstrated that upon comparing the peak values of RNA accumulation, the *mPer2* transcript level was 37% higher in LD than in DD (Takumi *et al.*, 1998). In contrast, *mPer3* is expressed at a similar level in DD and LD. This was also observed for *mPer1* (Figure 3A).

In DD, *mPer1* mRNA oscillated with a phase that is advanced by ~4 h in relation to *mPer2*. The phase of accumulation of *mPer3* mRNA was similar to that of *mPer1*, but remained high for several hours. *mPer3* expression decreased with a phase similar to *mPer2*, and thus was delayed in relation to *mPer1*. The broader expression profile of *mPer3* corresponds well to previously documented circadian changes in electrical activity of the SCN (Inouye and Kawamura, 1979), which fits a sinusoidal curve unlike cycles of *mPer1* and *mPer2* expression. The different phases of expression of *mPer1*, *mPer2* and *mPer3* may reflect the function of a multioscillator system

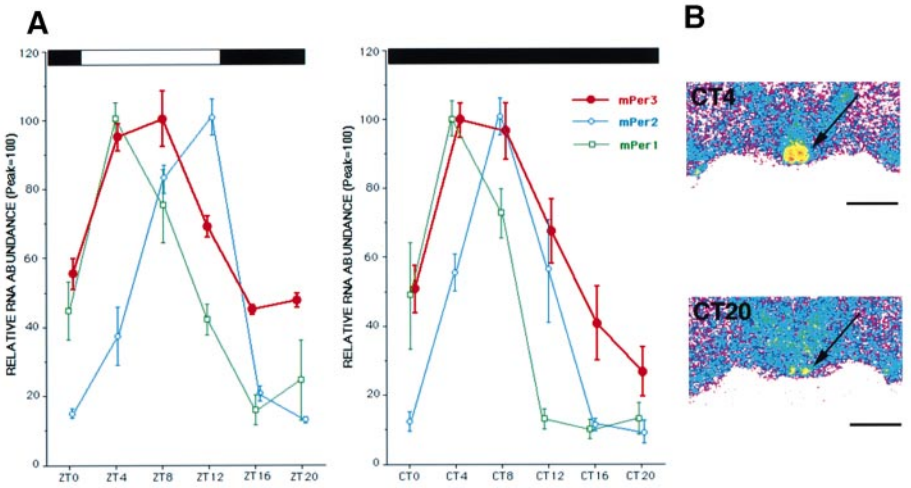

Fig. 3. Expression of *mPer3* mRNA in the SCN. (**A**) Rhythmic expression of *mPer3* (red) is plotted in comparison with *mPer2* (blue) and *mPer1* (green). The plot reflects signals detected by quantitative *in situ* hybridization in a light–dark cycle (LD, left) and constant darkness (DD, right). Each point is from five animals and is expressed as the mean ± SEM. Plots of RNA cycling set the mean peak value for *mPer1*, *mPer2* and *mPer3* to 100. Note that the value is the relative amount per molecule. (**B**) Representative *in situ* hybridization autoradiograms showing the SCN (arrows) at CT4 and CT20 in DD. Bar = 1 mm.

(Pittendrigh, 1960, 1993): from their expression patterns, *mPer1* behaves as a morning-phase oscillator, *mPer2* as an afternoon-phase oscillator, and *mPer3* as a broader, daytime oscillator. It is noteworthy that all three mammalian *period* genes produce mRNA cycles with a phase that differs from *dPer* mRNA, which accumulates during an early night phase (Hardin *et al.*, 1990).

mPer3 is the first light-independent gene within the mammalian period gene family

It is well established that single light pulses delivered in the early night induce phase delays in the locomotor activity rhythms of the mouse (Pittendrigh, 1960, 1993). Between CT16 and CT16.5 on the second day, one group of mice received a 30 min pulse of saturating light (incandescent light; 600 lux) at a time when light should result in a phase delay (Figure 4A) (Shigeyoshi *et al.*, 1997). Although expression of *mPer1* and *mPer2* was induced, as assayed 60 and 90 min after exposure to light, no induction of *mPer3* was found even when examined 270 min after the beginning of light exposure. Thus, in contrast to *mPer1* and *mPer2*, we conclude that the phase of *mPer3* gene expression is reset ultimately by a mechanism that differs from that resetting *mPer1* and *mPer2*.

To explore the possibility that *mPer3* might be induced by light delivered with an unanticipated phase, we examined the *mPer3* mRNA level in mice exposed to 30 min light pulses at various circadian times, and compared these with untreated controls (Figure 4B) at 60 min after the initiation of light exposure. However, we detected no difference between light-treated mice and untreated controls for any phase of light administration. For *mPer1*, RNA induction was observed in response to a light pulse during subjective night (CT12, CT16 and CT20), and light delivered at these times phase-shifts locomotor activity as previously shown (Shigeyoshi *et al.*, 1997). For *mPer2*, light-induced gene expression was found from the subjective afternoon to the first half of the subjective night (CT8, CT12 and CT16). Some of these inductions are not well

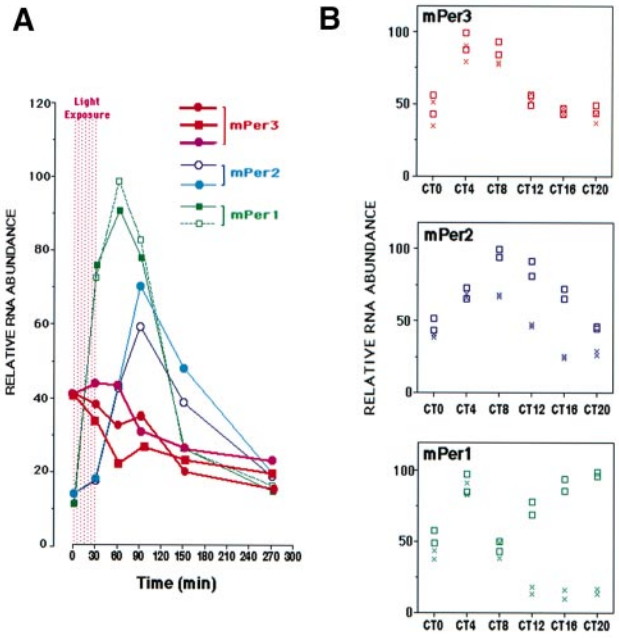

Fig. 4. A single brief exposure to light does not induce *mPer3*, but induces *mPer1* and *mPer2* in the SCN. (**A**) A 30 min light pulse (600 lux; incandescent light) delivered from CT 16.0 to CT 16.5 (pink dotted bar) induces *mPer1* and *mPer2* expression. The relative RNA abundance of *mPer1* was measured by quantitative *in situ* hybridization; each line represents one experimental series (three component experiments for *mPer*3, two for *mPer1* and *mPer2*). The value of the CT16 time point (0 min) plotted corresponds to the CT16 value of Figure 3 (i.e. 41.0 for *mPer3*, 11.2 for *mPer1*, 13.1 for *mPer2*). (**B**) The response of *period* gene expression to a brief exposure to light at various circadian time. Squares represent light-exposed mice, and crosses represent unexposed controls. Each point is from a single animal. Note that *mPer3* mRNA expression is not altered in response to pulses of light exposure, in contrast to *mPer1* (induced at CT12–20) and *mPer2* (induced at CT8–16). Plots of RNA set the peak value for *mPer1*, *mPer2*, and *mPer3* to 100.

correlated with times of light administration that produce behavioral phase-shifts. Since it is known that behavioral phase-shifts are most prominent around CT16 in this strain

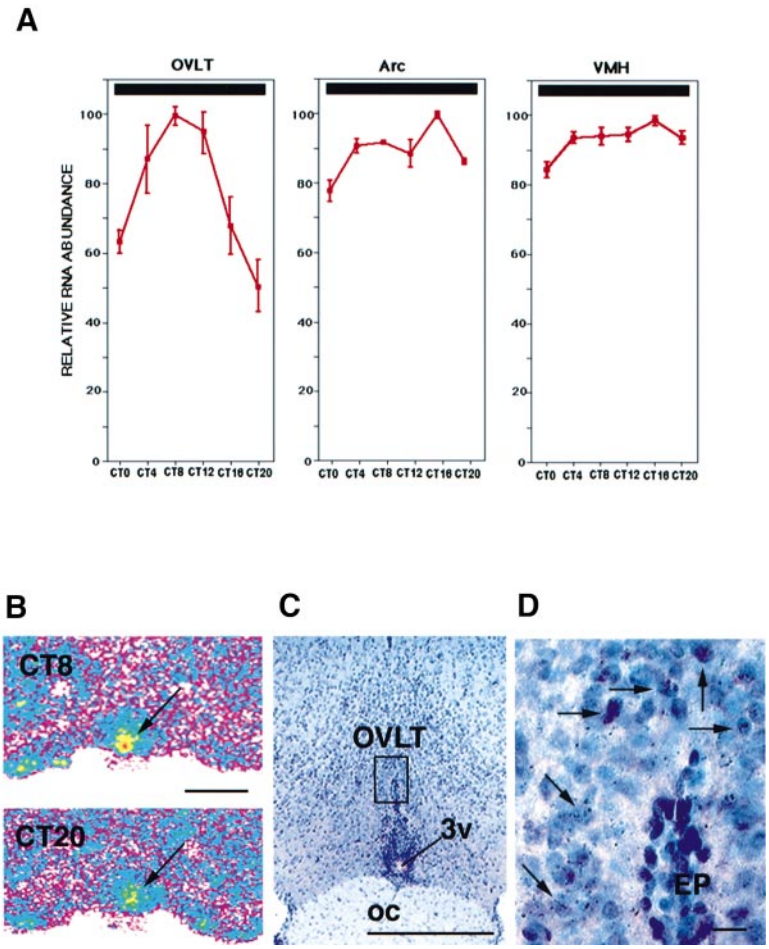

Fig. 5. *mPer3* in the extra-SCN area. (**A**) Circadian expression profiles of *mPer3* in the OVLT, Arc and VMH in DD. For each area, the relative RNA abundance was determined by quantitative *in situ* hybridization, with the peak value being adjusted to 100. (**B**) Representative *in situ* hybridization autoradiograms showing OVLT (arrows) at CT8 and CT20. (**C**) A low magnification photograph of the emulsion-coated section of *mPer3 in situ* hybridization counterstained with cresyl violet. The OVLT is located around the anteroventral pole of the third ventricle at the level of the anterior part of the optic chiasma. (**D**) A high magnification photograph of the boxed area of (C). Note that many cells (arrows) in the OVLT express *mPer3*-positive silver grains. oc, optic chiasma; EP, ependymal cells. Bar = 1 mm in (B), 500 μm in (C) and 20 μm in (D).

of mice (Shigeyoshi *et al.*, 1997), the *mPer2* gene response *per se* probably does not induce behavioral phase-shifts, but may contribute to a behavioral phase-shift probably influenced by *mPer1*.

The lack of a response to light at the level of *mPer3* gene expression also corresponds with the light-insensitivity of *period* gene expression in *Drosophila* (Hunter-Ensor *et al.*, 1996). Recently, Albrecht *et al.* (1997) reported that *mPer2* was not light-inducible. However, Shearman *et al.* (1997) and our own published results (Takumi *et al.*, 1998) indicate *mPer2*'s significant light inducibility. This apparent discrepancy might be derived from the different times of light exposure used in the former versus the latter studies, i.e. Albrecht *et al.* (1997) studied light responsiveness at CT22, while Shearman *et al.* (1997) examined light induction at CT14, and our studies involved light pulses centered on CT16. Consistent with this possibility, in the present study, we found that light induction of *mPer2* shows phase specificity, being highly inducible at CT8–CT16 and weakly responsive at CT20–CT24 (CT0). In contrast, *mPer3* was not induced at any phase of the clock by exposure to light. Thus, *mPer3* is the first truly light-independent gene within the mammalian *period* gene family. Since *mPer3* transcription is not altered rapidly by light, an alternative mechanism, such as that previously described in *Drosophila*, may set the phase of its oscillation in relation to LD cycles. The identification and characterization of mammalian *timeless* homologs could best address this issue.

mPer3 cycles in the OVLT in the anteroventral third ventricle

It may be important that *mPer3* is highly expressed in hypothalamic regions which are believed to provide hormonal and autonomic regulation in mammals. To begin to determine whether *mPer3* plays a role in organizing the daily rhythm of hormonal secretion and autonomic nervous activity in such brain regions, we performed time-dependent analyses of *mPer3* expression in the OVLT, Arc and VMH in DD (Figure 5). In all three areas, we found rhythmic *mPer3* expression but for Arc and VMH, the amplitude of these rhythms was low (18–22% peak–trough differences). The phase of these rhythms also differed from *mPer1–3* rhythms detected in the SCN, with peak RNA accumulation at CT16. Since a weak rhythm of electrical activity is produced with a similar phase in these extra-SCN brain regions, and these electrical rhythms are also out-of-phase with respect to the dominant SCN

electrical rhythm (Inouye and Kawamura, 1979), weak *mPer3* expression rhythms in Arc and VMH may be responses to molecular or electrical oscillations in the SCN.

In contrast to these two areas, the OVLT shows a clear circadian rhythm with maximum *mPer3* RNA accumulation at CT8 (trough at CT20). The peak–trough difference was ~2-fold. Thus *mPer3* cycles in the OVLT with a phase comparable with its expression in the SCN. It is known that the OVLT is involved in daily surge rhythms of luteinizing hormone (LH)/follicle-stimulating hormone (FSH), osmotic receptor function and pyrogen-induced febrile phenomena (Wenger and Leonardelli, 1980; Stitt, 1985; Bourque and Oliet, 1997; Vallieres and Rivest, 1997). The OVLT forms a portion of the anteroventral third ventricular (AV3V) system, and is tightly connected to the preoptic area, the chief regulatory center for body temperature (Blatteis and Banet, 1986; Berner and Heller, 1998). Although it is highly controversial (Eastman *et al.*, 1984; Satinoff and Prosser, 1988; Kittrell, 1991; Refinetti *et al.*, 1994), some SCN lesion studies have indicated that core body temperature rhythms persist when locomotor activity and drinking rhythms are completely abolished. Since *mPer3* cycles in the OVLT, future experiments of OVLT lesioning, sparing the SCN, or targeted disruption of *mPer3* might be interesting to address the role of OVLT in dictating rhythms of body temperature.

Materials and methods

cDNA cloning

RACE using a Marathon cDNA Amplification kit (Clontech, Palo Alto, CA) was performed as described previously (Takumi *et al.*, 1998). The sequences of the *mPer*3-specific primers (F1 and F2 for 3′-RACE, R1 and R2 for 5′-RACE) were as follows: F1 (5′-TGC AGC AGG TCT ATG CCA GTG TA-3′, the sequence corresponding to nucleotides 1604–1626), F2 (5′-CAT CGA GTC CAT GGC CAG ATC ATC-3′, nucleotides 1659–1682), R1 (5′-GCA CTT TCT TTT CAA GGC CGG GA-3′, nucleotides 1883–1905) and R2 (5′-G TTC ATC TGC TGA TAG GAC GAG CTG-3′, nucleotides 1818–1842). The PCR protocol was as follows: denaturation at 94°C for 1 min; followed by 94°C for 30 s; 68°C for 4 min for 25 cycles. The PCR products were electrophoresed on agarose gels and excised for subsequent subcloning and sequence determination. Both strands of the cDNA sequences were determined by automated DNA sequencers ABI PRISM310 and SHIMADZU DSQ1000 (Takumi *et al.*, 1997).

RNA blot hybridization

The membrane was purchased from Clontech and hybridized in Quick-Hyb hybridization solution (Stratagene, La Jolla, CA) at 68°C. The filter was washed in 0.1× SSC and 0.1% SDS at 60°C, and exposed to Fuji film at –70°C.

Animals

Male Balb-c mice (Japan Animal Care) 8–10 weeks old were used for all experiments. Mice were housed for at least 2 weeks of adaptation to the standard 12 h light:12 h dark (LD) cycle. In DD cycle experiments and light pulse experiments, we used the mice on the second day of the DD condition.

In situ hybridization

For the distribution study by *in situ* hybridization (Shigeyoshi *et al.*, 1997), we killed mice at ZT4 under deep ether anesthesia. The protocol of this research was accepted by the Committee for Animal Research at the Kobe University School of Medicine. Radiolabeled cRNA probes for *mPer3* (nucleotide positions 814–1955), *mPer2* (1–638) and *mPer*1 (538–1752) were made using [^{33}P]UTP (New England Nuclear) with a standard protocol for cRNA synthesis. Isotope-reacted *in situ* hybridization images were visualized by autoradiograms of BioMax film (Kodak) and by Ilford K5 nuclear track emulsion (Ilford).

Quantitative in situ hybridization

Serial coronal section (40 μm thick) of the mouse brain were made using a cryostat, and hybridized with the above-mentioned *period* probes by the quantitative *in situ* hybridization method, detailed previously (Shigeyoshi *et al.*, 1997). The radioactivity of the SCN of each section on the BioMax film (Kodak) was analyzed using a microcomputer interfaced to an image analyzing system (MCID, Imaging Research Inc., Canada) after conversion into the relative optical densities produced by the ^{14}C-autoradiographic microscales (Amersham, UK). Data were normalized with respect to the difference between signal intensities in equal areas of the SCN and the corpus callosum. The intensities of the optical density of the sections from the rostral- to the caudal-most areas of the SCN (10 sections per mouse brain) were then summed; the sum was considered a measure of the amount of *mPer3*, *mPer2* and *mPer1* mRNA in this region. Similarly to the SCN, we measured the radioactivity of the OVLT (five sections per mouse), Arc (10 sections) and VMH (10 sections). The values were expressed as means ± SEM (n = 5). We use 'relative RNA abundance', which refers to the peak value being adjusted to 100. For statistical analysis, one-way ANOVA followed by Sheffe's multiple comparisons was applied.

Methods for light treatments

Mice were exposed to an incandescent light stimulus (600 lux, 30 min) at CT16. *mPer3*, *mPer2* and *mPer1* induction experiments by light were performed in the second DD cycle. Animals were sacrificed 30, 60, 90, 150 and 270 min after the initiation of the light exposure. For gating experiences, animals were exposed to light at CT0, 4, 8, 12, 16 and 20, and sacrificed 60 min after the initiation of the light exposure.

DDBJ/EMBL/GenBank accession number

The nucleotide sequence data of *mPer3* will appear in the DDBJ/EMBL/GenBank databases with the accession number AB013605.

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