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 263–324), including PAS-A (residues 263–175) 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, Antheraea pernyi, mouse and human. The tree was made using the UPGMA (Unweighted Pair Group Method with Arithmetic 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).
regions including the SCN, OVLT, VMH and Arc, and MeA (medial amygdaloid nucleus). Bar /H11005 of structures including the CiCx (cingulate cortex), DG (gyrus dentatus) signals in the OVLT, SCN, VMH and Arc, as well as cortical the mouse forebrain during the subjective day (CT4). Note the strong heterodimerization with Timeless (Huang et al., 1993), which forms the interaction domain for dPER’s 1.8 kbp (nucleotides 814–1955). The positions of RNA size markers are shown (left). The same filter subsequently was hybridized with a 1 mm. 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.
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

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
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 Leonardielli, 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; Slatinoff 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 mPer3-specific primers (F1 and F2 for 5'-RACE, R1 and R2 for 3'-RACE) were as follows: F1 (5'-TGC AGC AGG TCT ATG CCA GTG TA-3'), the sequence corresponding to nucleotides 1604–1626), F2 (5'-CTG CGA GTC CAT GGC CAT ACG ATC-3'), nucleotides 1659–1682), R1 (5'-GCA CTT CCT TTT CAA GGC CGG GA-3'), nucleotides 1883–1905) and R2 (5'-G TTC ATC TGC TGA TAG GAC-3'). The PCR protocol was as follows: 35 cycles 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 CT4, 8, 12, 16 and 20, and sacrificed 60 min after the initiation of the light exposure.

**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.

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**References**


**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 14C-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 were measured using a cryostat, and hybridized with the above-mentioned probes.

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Light-independent clock gene mPer3


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**Note added in proof**