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Rhythmic Expression of *BMAL1* mRNA Is Altered in *Clock* Mutant Mice: Differential Regulation in the Suprachiasmatic Nucleus and Peripheral Tissues

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***BMAL1* is a putative clock gene which encodes a basic helix-loop-helix (bHLH)-PAS transcription factor. To examine whether the CLOCK protein is required for the circadian expression of *BMAL1* mRNA, *in situ* hybridization and Northern blot analysis were performed in the suprachiasmatic nucleus (SCN) and peripheral tissues of homozygous *Clock* mutant mice. In the SCN of *Clock* mutants, *BMAL1* mRNA did not oscillate significantly but apparently expressed with low levels, while in wild-type mice the mRNA was robustly oscillated in a circadian manner. The peak–trough amplitudes of *BMAL1* mRNA levels were 6.5-, 8.6-, and 6.7-fold in liver, heart, and kidney of wild-type mice, respectively. In *Clock* mutants, the amplitudes were extremely damped to 1.2-, 2.1-, and 1.4-fold, respectively. Furthermore, expressions of *BMAL1* mRNA in the peripheral of *Clock* mutant mice were close to the peak level in wild-type mice, whereas *mPer2* mRNA levels were severely blunted at trough values. Daily expression of albumin site D-binding protein (DBP), a clock controlled output gene (CCG), was also abolished at trough values by the *Clock* mutation in all tissues examined. These observations suggest that the circadian expression of *BMAL1* mRNA is affected by the CLOCK-induced transcriptional feedback loop in the SCN and peripheral tissues in a different way and that the regulation mechanism appeared to be different from those in *mPer2* and *DBP* expressions *in vivo*.**

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Many organisms display rhythms of physiology and behavior that are entrained to the 24-h cycle of light and darkness prevailing on Earth. Some of these internal biological rhythms are driven by endogenous oscillators called circadian clocks. Recently, the circadian oscillators are thought to be controlled by autoregulatory feedback loops in clock genes' expression in mammals as well as in *Drosophila* (1). *In vitro* studies imply the mechanisms about the transcriptional feedback loop at the level of protein-protein interactions (2–6), transcriptional activation/inhibition (2–5, 7), and translocation to the nuclear (2, 5). However, little is known about *in vivo* mechanisms of the circadian oscillators.

Clock is the first clock gene identified in vertebrate by *N*-ethyl-*N*-nitrosourea mutagenesis screening (8). When transferred from a light–dark cycle (LD) to a constant darkness (DD), homozygous *Clock* mutants exhibit abnormally long periodicity of behavior for initial 5 to 15 cycles, and consequently show a complete loss of circadian rhythmicity (8). The *Clock* gene encodes a basic helix-loop-helix (bHLH)-PAS transcription factor (9). *Clock* allele possesses a truncation and deletion of 51 amino acids in its transcriptional activation domain (9).

BMAL1 is a putative clock gene which also encodes a bHLH-PAS transcription factor (1). BMAL1 has been identified as a potential dimerization partner for mammalian CLOCK (4, 10). In *Drosophila*, *cycle* (*cyc*) is a homologue of mammalian *BMAL1*, and its homozygous mutants are completely arrhythmic (11). Homozygous *dClock* mutants (*Jrk*) are also arrhythmic, and express low levels of *period* (*per*) and *timeless* (*tim*) transcripts (12), which are essential elements of the circadian timing system in *Drosophila*. dCLOCK and dBMAL1 (CYC) form a heterodimer that drives *per* and *tim* transcriptions via the E-box (CACGTG motif) in their

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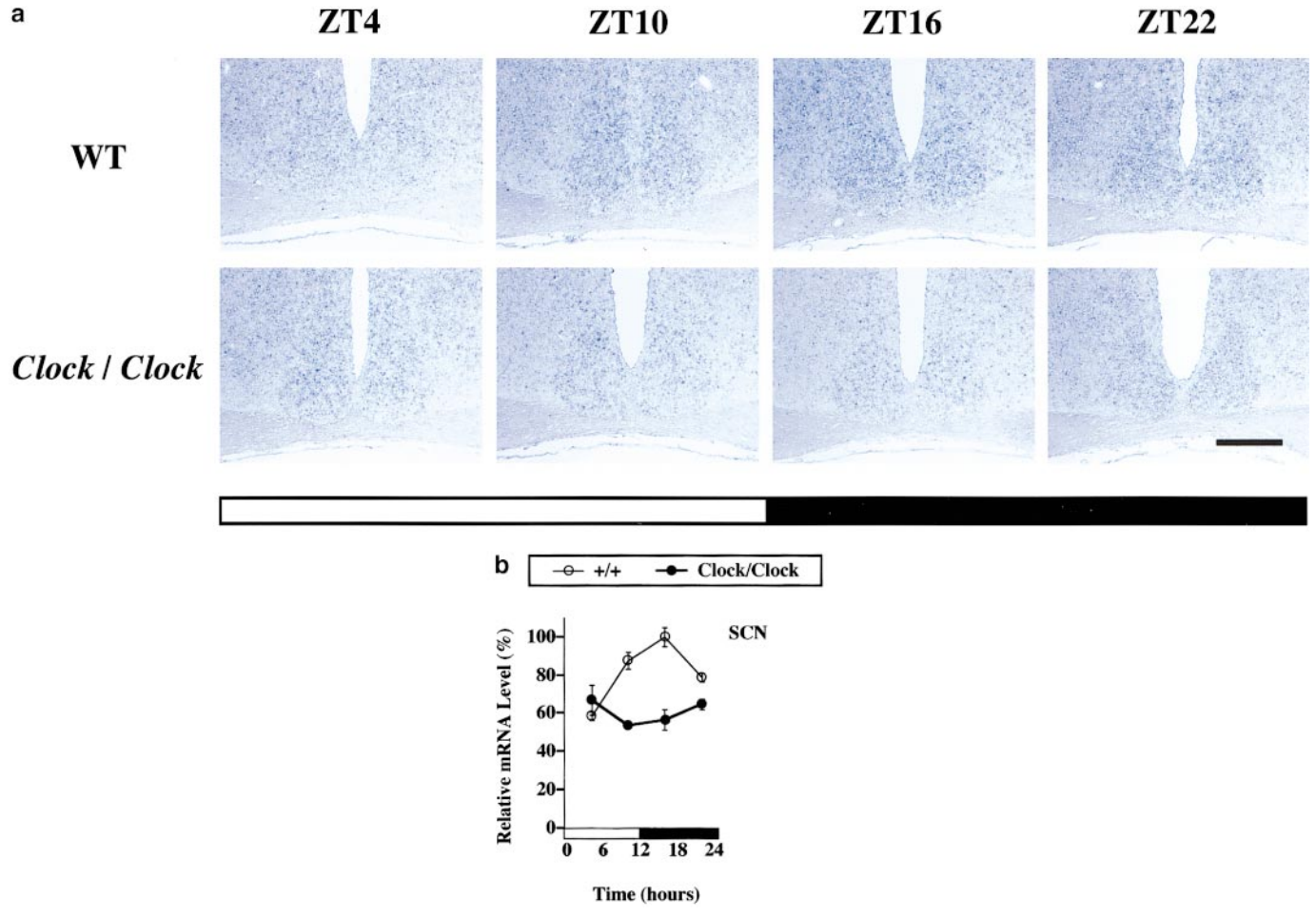


FIG. 1. Expression pattern of *BMAL1* mRNA in the SCN of wild-type and *Clock* mutant mice. Mice were housed in a 12-h light:12-h dark (LD) cycle (lights on at ZT 0). The open bar indicates the lights-on phase, and the dark bar indicates lights-off. (a) Representative *in situ* hybridization results on the SCN at four different time points. The blue signal shows the expression of *BMAL1* mRNA. Scale bar, 250 μm. (b) The graph depict a comparison of the expression patterns of *BMAL1* mRNA. Data are relative intensity of mRNA (%) to the value at ZT16 of wild-type animals. Values represent mean ± SEM. Significant daily variations were detected in the SCN of wild-type mice by two-factor factorial ANOVA ($p < 0.0001$). Scheffé's multiple comparison test revealed that the levels at ZT16 were significantly different ($p < 0.05$) from those at ZT4 in wild-type mice. Significant differences were found between the groups ($p < 0.0001$).

promoters, and in turn PER and TIM suppress their own transcription by inhibiting DNA binding activity of a dCLOCK–dBMAL1 heterodimer (13). Similar negative feedback mechanisms have been described for mammalian *per* homologues, since *mPer1, mPer2,* and *mPer3* mRNA levels are suppressed in the SCN of *Clock* mutant mice compared with those in wild-type animals (7). Gekakis *et al.* (4) showed that CLOCK–BMAL1 heterodimers activate transcription from E-box elements found in the *mPer1* flanking region, by using luciferase reporter gene assays in mouse NIH-3T3 cells. The three mPER proteins and mTIM are believed to be involved in the negative limb of the mammalian feedback loop, because they are able to inhibit CLOCK-BMAL1 driven transactivation in NIH3T3 cells (5, 7).

Previously, we found the circadian expression of *BMAL1* mRNA in the SCN and peripheral tissues of rats (14). Interestingly, the amplitudes of *BMAL1* and *rPer2* mRNA expressions were correlated between the different tissues (14). These findings suggest that the rhythmic expression of *BMAL1* mRNA plays an important role in circadian expression of mammalian *per* genes, and that there exist the circadian transcriptional feedback loops driven by CLOCK–BMAL1 heterodimer in peripheral tissues as well as in the SCN. However, the transcriptional regulation of *BMAL1* mRNA has been little known yet.

Albumin site D-binding protein (DBP) is a member of the proline- and acid-rich (PAR) domain subfamily of basic leucine zipper (bZIP) proteins and is involved in transcriptional regulation in the liver (15–18). The

mRNA is expressed according to a circadian rhythm in the SCN and peripheral tissues such as liver, kidney, spleen, lung, and testis (16), while the mechanism of the circadian expression of this gene is still unknown. Since *DBP* knockout mice are still rhythmic in behavior (19), *DBP* is thought to be a clock controlled output gene (CCG) (15–19).

In this study, to examine whether the CLOCK protein is required for the circadian expression of *BMAL1* mRNA, daily expression of *BMAL1* mRNA was investigated in the SCN and peripheral tissues of homozygous *Clock* mutant mice. Expression pattern of *mPer2* and *DBP* mRNAs were also examined and compared with that of *BMAL1* mRNA.

MATERIALS AND METHODS

Animals. *Clock* mutant mice were derived from animals supplied by J. S. Takahashi, Northwestern University (Evanston, IL). The animals provided had the *Clock* allele on a primarily BALB/c background. The breeding colony was expanded by further backcrossing to BALB/c mice (Clea Japan, Inc., Tokyo), and was subsequently maintained by interbreeding. Genotypes were determined using the PCR method provided by J. S. Takahashi (7). Homozygous wild-type and homozygous *Clock* mutant mice derived from this colony were used for this study. Animals were housed in a 12-h light–12-h dark cycle [LD 12:12; lights on at zeitgeber time (ZT) 0]. A white fluorescent lamp was used as a source of light during the day (150–200 lux at a level of the cages). Ten- to 13-week-old mice were used for this study.

In situ hybridization. Animals used for *in situ* analysis were anesthetized with pentobarbital and were perfused from the left ventricle with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), and fixed for 3 h at 4°C. Then the tissues were embedded in Tissue-Tek OCT compound (Miles). Eight-μm cryosections were cut. Digoxigenin-labeled RNA probes were generated from rat *BMAL1* cDNA fragment (bases: 27–683; GenBank Accession No. AB012600) for mouse *BMAL1*, and *rPer2* cDNA fragment (bases: 1–5437; GenBank Accession No. AB016532) for *mPer2*, using a DIG RNA labeling Kit (Boehringer-Mannheim). Hybridization and detection of the probes were carried out as described (20, 21). Densitometric analysis of hybridization intensity was accomplished using Q550IW (Leica). Effects of genotype were analyzed by two-factor factorial ANOVA, with Scheffé's post-hoc test for pairwise comparisons.

Northern blot analysis. Mice were decapitated, and tissues were dissected, quickly frozen, and kept in liquid nitrogen until used. In darkness, dissections were carried out under a dim red light. Total RNA was isolated from tissues by using Isogen (a guanidine–HCl/phenol procedure: Nippon Gene, Tokyo, Japan) and separated on a 1% agarose/0.7 M formaldehyde gel. RNA was transferred to a nylon membrane (GeneScreen Plus; DuPont, U.S.A.) by passive capillary transfer. Each lane contained 20 μg of total RNA from each tissue. A ^{32}P-labeled random primed probes were generated from rat *BMAL1* cDNA fragment (bases: 231–910; GenBank Accession No. AF015953) for mouse *BMAL1*, *mPer2* cDNA fragment (bases: 1123–1830; GenBank Accession No. AF036893), and rat *DBP* cDNA fragment (bases: 1138–1602, GenBank Accession No. J03179) for mouse *DBP*, then hybridization and detection were performed as described (22).

RESULTS

In wild-type mice, the strand-specific signal of *BMAL1* mRNA exhibited a robust cycling ($p < 0.0001$) with an approximately 2-fold peak-trough amplitude in the SCN by *in situ* hybridization (Fig. 1). The mRNA expression reached peak levels at ZT16 and trough levels at ZT4. In contrast to wild-type animals, the mRNA expression was nonrhythmic with low levels in homozygous *Clock* mutants (Fig. 1). In this study, diurnal lighting was used because of the marked difference in free-running circadian periods between wild-type animals (23.5 h) and homozygous *Clock* mutants (28 h) in DD (8).

Peripheral expression patterns of *BMAL1* mRNA in *Clock* mutant mice were compared with those in wild-type animals. In whole brain (except for the SCN), liver, heart, and kidney, Northern blot analysis showed robust daily expression of *BMAL1* mRNA in wild-type mice (Fig. 2), while the expression pattern was different from that in the SCN (Fig. 1). In peripheral tissues from wild-type mice, expression of *BMAL1* mRNA showed its peak around ZT22-ZT4 (Fig. 2), while in the SCN it reached the peak level at ZT16 (Fig. 1). In *Clock* mutants, the peak-trough amplitudes of *BMAL1* specific hybridization were extremely damped to 1.2-, 1.2-, 2.1-, and 1.4-fold in the brain, liver, heart, and kidney, respectively, whereas in wild-type animals, the amplitudes were 1.5-, 6.5-, 8.6-, and 6.7-fold, respectively (Fig. 2). Interestingly, in the brain, liver, and kidney from *Clock* mutants, the levels of *BMAL1* mRNA were continuously close to the peak level in wild-type mice (Fig. 2). It should be noted that the mRNA levels were obviously oscillate in heart from *Clock* mutant mice, while the amplitude was damped as described above (Fig. 2). These results suggest that daily expression of *BMAL1* mRNA is altered in peripheral tissues as well as in the SCN of *Clock* mutant mice.

To see whether the peripheral oscillations of *per* genes' expression are abolished as same as in the SCN (7), the expression patterns of *mPer2* mRNA were compared in tissues from wild-type and *Clock* mutant mice. In the SCN, robust expression rhythm of *mPer2* mRNA was damped in *Clock* mutants (Fig. 3). As we expected, in *Clock* mutants, expression rhythm of *mPer2* was extremely damped in all the tissues examined (Fig. 3). We also examined the expression patterns of *mPer1* and *mPer3* mRNAs in peripheral tissues of wild-type and *Clock* mutant mice (data not shown). As we predicted, both *mPer1* and *mPer3* mRNAs exhibited robust oscillations in all the tissues examined in wild-type animals, while in the mutants, they were extremely damped in the tissues.

We used the *DBP* mRNA oscillation as a marker for a component of the circadian output pathway. In all the tissues examined, daily expression of *DBP* mRNA which reached its peak level at light-dark transition was clearly observed in wild-type mice (Fig. 4). The peak-trough amplitude was small in brain (2.2-fold) and large in peripheral, 90.9-, 10.2-, and 24.4-fold in

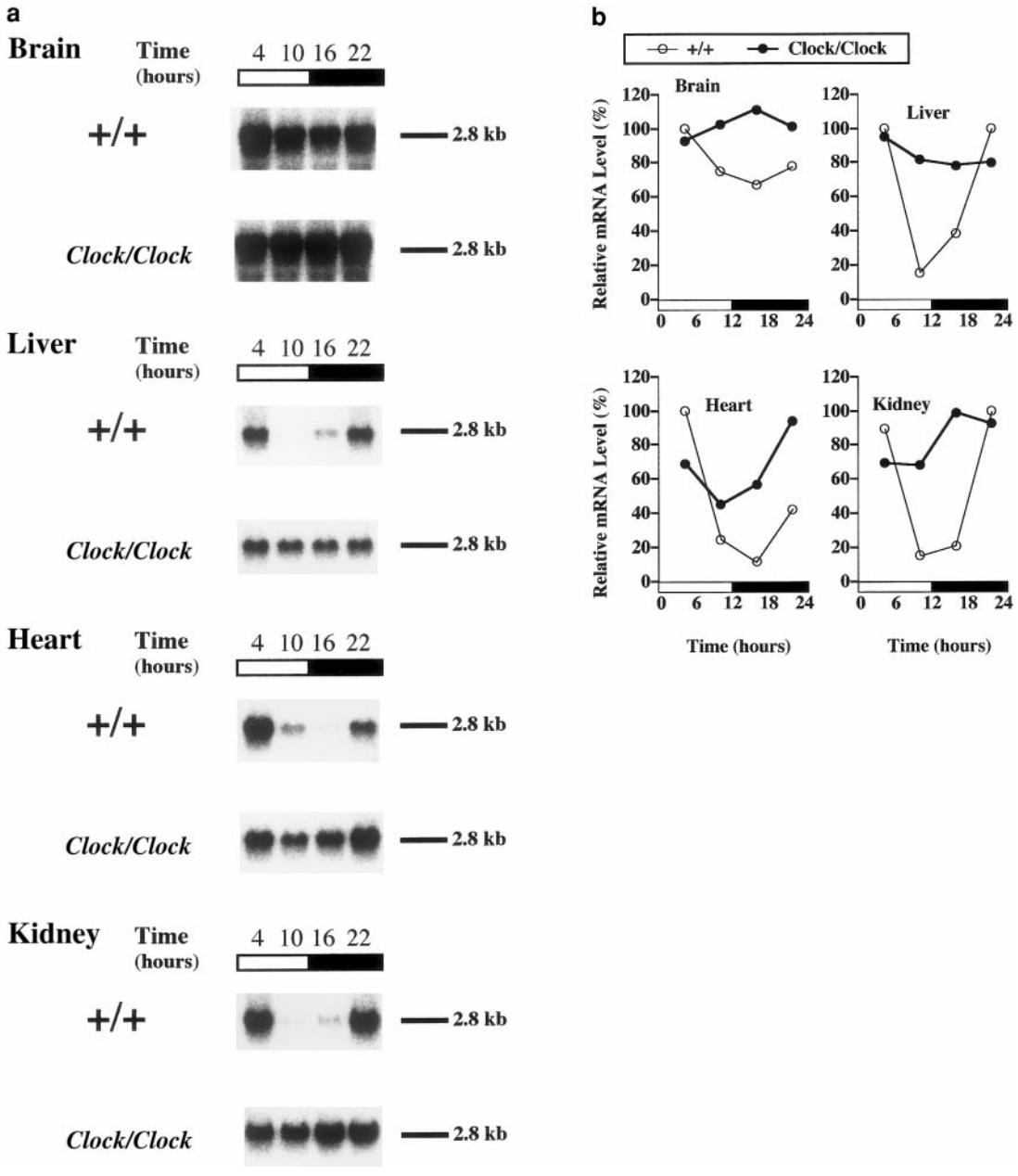
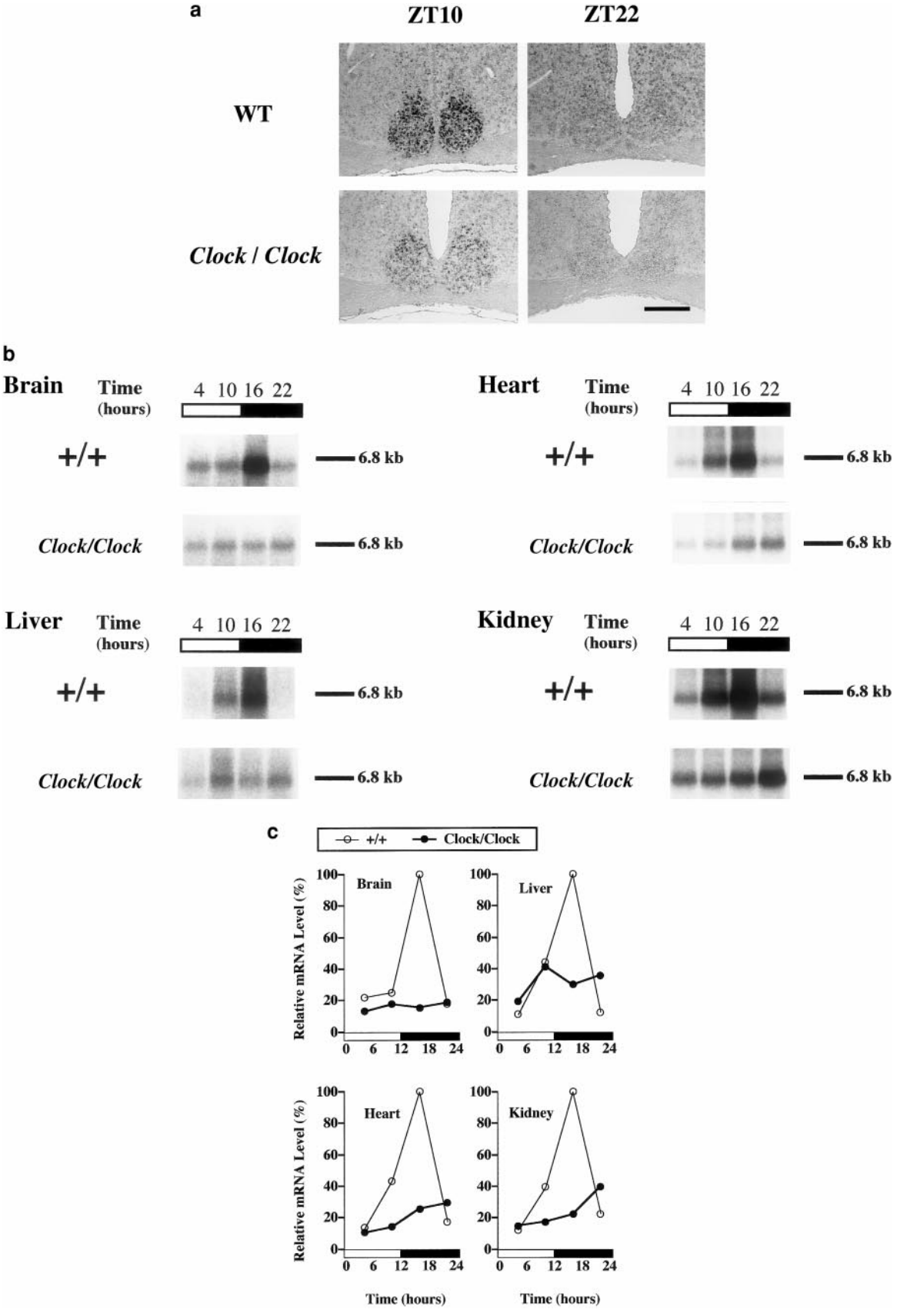


FIG. 2. Expression pattern of *BMAL1* mRNA in whole brain (except for the SCN) and peripheral tissues of wild-type and *Clock* mutant mice. The open bar indicates the lights-on phase, and the dark bar indicates lights-off. (a) Representative Northern blots of total RNA prepared from the tissues of each mice. (b) The graph depict a comparison of the expression pattern of *BMAL1* mRNA in each tissue of wild-type (open circles) and *Clock* mutant (closed circles) animals. The maximum value of wild-type mice was expressed as 100% in each tissue.

liver, heart, and kidney, respectively (Fig. 4). However, in *Clock* mutants, the rhythmic expression of *DBP* mRNA was completely abolished and blunted at the trough level in all the tissues examined (Fig. 4).

DISCUSSION

In this study, we showed that daily expression of *BMAL1* mRNA is altered in the SCN and peripheral tissues of *Clock* mutant mice. In peripheral tissues from wild-type mice, expression of *BMAL1* mRNA showed its peak around ZT22-ZT4, while in the SCN it reached the peak level at ZT16. These observations are consistent with the previous reports that the peripheral expression of *per* homologues had their peaks after 4–6 h with respect to the SCN in mice (1). While the expression mechanism of *BMAL1* mRNA is still unknown, it seems that *BMAL1* expression is differen-


a
ZT10
ZT22
WT
Clock / Clock
b
Brain
Time (hours)
4 10 16 22
+/+
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Clock/Clock
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Heart
Time (hours)
4 10 16 22
+/+
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4 10 16 22
+/+
6.8 kb
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6.8 kb
c
+/+
Clock/Clock
Brain
Liver
Heart
Kidney
Relative mRNA Level (%)
0
20
40
60
80
100
0 6 12 18 24
Time (hours)

tially regulated in each tissue, since in *Clock* mutant mice, the mRNA levels were constantly low in the SCN, constantly high in whole brain (except for the SCN), liver, and kidney, and rhythmic but extremely damped in heart.

In the SCN, CLOCK protein might be involved in the positive regulation of *BMAL1* mRNA expression, since the levels of *BMAL1* mRNA were constantly low in the *Clock* mutant mice. In *Drosophila*, *dClock* mRNA rhythm is abolished and the levels are trough values in *per* or *tim* mutants, suggesting that PER and TIM can act as transcriptional activators for *dClock* gene (23). But in the mammalian SCN, BMAL1 instead of CLOCK might be a pendulum of the central master clock, as the expression of *Clock* mRNA is not oscillate in mammals (1). If this is the case in mammals, the CLOCK-BMAL1 dependent transactivation of PER proteins might be involved in the transcriptional activation of *BMAL1* gene expression.

In peripheral, it appears that CLOCK is at least indirectly involved in the negative regulation of *BMAL1* gene expression, because the levels of the mRNA were continuously high in whole brain (except for the SCN), liver, and kidney of *Clock* mutant mice. These phenomena resemble the situation in *BMAL1* mutants of *Drosophila*, the levels of *dClock* mRNA are continuously close to the peak level in wild-type flies (24). Since CLOCK is thought to be a positive element driving the transcription of clock-related genes in mammals as well as in *Drosophila* (1), it is reasonably assumed that the negative regulation of *BMAL1* expression might be mediated by some transcriptional repressor whose expression is positively controlled by CLOCK.

It is worth noting that in heart of *Clock* mutant mice, *BMAL1* mRNA levels were apparently cycling, while the peak-trough amplitude was damped. Homozygous *Clock* mutant mice can entrain the locomotor activity with the environmental LD cycle (8). When transferred from LD to DD, the mutant animals exhibit the long periodicity of behavior for initial 5 to 15 cycles, and consequently show a complete loss of circadian rhythmicity (8). These observations imply the existence of CLOCK-independent oscillator(s) which can maintain the behavioral rhythm of the animal for a few days. In heart of *Clock* mutant mice, *BMAL1* mRNA expression might be partially regulated by a CLOCK-independent oscillator. Actually, it is reported that the peripheral circadian oscillator independent of a central master clock might exist in heart and kidney of zebrafish (25).

In mammals, three putative homologues (*mPer1*, *mPer2*, and *mPer3*) of *Drosophila* clock gene *per* have been identified (1). However, only *mPer2* has been proved as the mammalian clock gene, because when transferred from LD to DD, the homozygous *mPer2* mutants display a shorter circadian period in behavior, and then lose the rhythmicity (26). In this study, we showed that CLOCK participates in rhythmic expression of *mPer2* mRNA in peripheral tissues as well as in the SCN. In *Clock* mutant mice, rhythmic expressions of *mPer1* and *mPer3* mRNAs were also extremely damped even in peripheral tissues. These phenomena imply the existence of E-box dependent transcriptional activation in mammalian *per* genes in every tissues. However, both the oscillating phase and the response of light-induced transcriptional activation are obviously varied in mammalian *per* homologues (1, 27). While the expression of *per* genes might be differentially controlled in mammals, our results suggest that CLOCK molecule is required for the circadian regulation of three *per* genes.

In wild-type mice, the expression of *DBP* mRNA oscillated with a very large amplitude in liver, heart, and kidney, while the amplitude was very small in whole brain. Daily expression of *DBP* mRNA was severely abolished in brain and peripheral tissues of *Clock* mutant mice. Since DBP knockout mice show the rhythmic locomotor activity (19), considering with our data, DBP is a component of the circadian output pathway governed by a CLOCK-controlled oscillator system. Interestingly, *DBP* mRNA levels were still high in the brain of the mutant mice, although the levels were extremely low in peripheral tissues. It is reported that peak and minimum levels of *DBP* mRNA are reached about 4 h earlier in the SCN than in liver (19). Our results suggest that circadian expression of *DBP* mRNA is controlled by different mechanisms in the brain compared with those in peripheral tissues.

It appeared that clock-regulated rhythmic expression of transcription factors such as *BMAL1* and *DBP*, cause a variety of CCGs' transcriptional activation or suppression in various tissues in a time-dependent manner, and consequently the physiological and behavioral circadian rhythms are generated in the organism. Further elucidation of the molecular mechanisms

FIG. 3. Expression pattern of *mPer2* mRNA in the SCN, whole brain (except for the SCN), and peripheral tissues of wild-type and *Clock* mutant mice. (a) Representative *in situ* hybridization results at ZT10 (left) and ZT22 (right). The blue signal shows the expression of *mPer2* mRNA. Scale bar, 250 μm. (b) Representative Northern blots of total RNA prepared from the tissues of each mice. The open bar indicates the lights-on phase, and the dark bar indicates lights-off. (c) The graph depict a comparison of the expression pattern of *mPer2* mRNA in each tissue of wild-type (open circles) and *Clock* mutant (closed circles) animals. The maximum value of wild-type mice was expressed as 100% in each tissue.

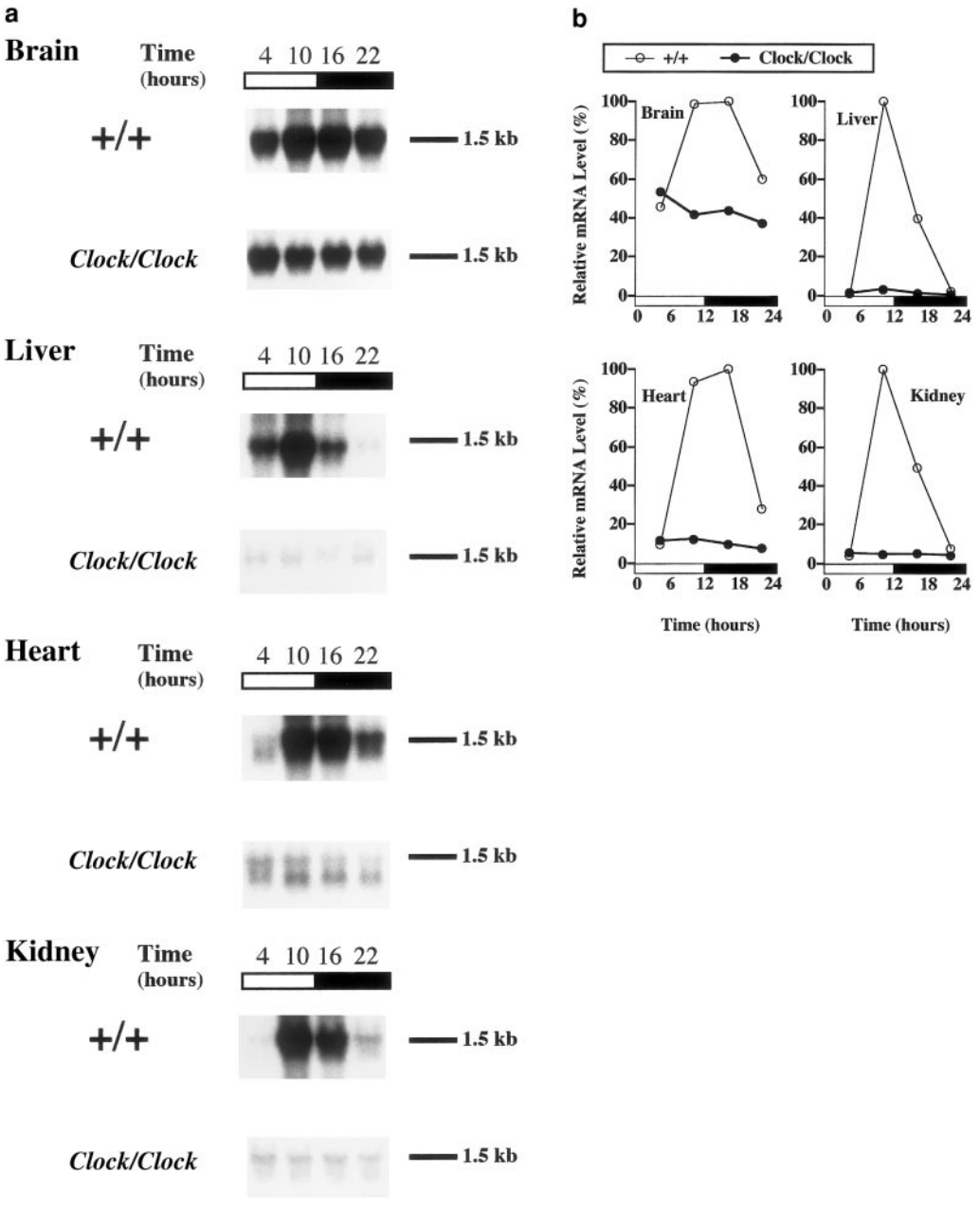

FIG. 4. Expression pattern of *DBP* mRNA in whole brain (except for the SCN), and peripheral tissues of wild-type and *Clock* mutant mice. The open bar indicates the lights-on phase, and the dark bar indicates lights-off. (a) Representative Northern blots of total RNA prepared from the tissues of each mice. (b) The graph depict a comparison of the expression pattern of *DBP* mRNA in each tissue of wild-type (open circles) and *Clock* mutant (closed circles) animals. The maximum value of wild-type mice was expressed as 100% in each tissue.

regulating the *BMAL1* and other transcription factors' expressions will provide an understanding of the mammalian circadian clock system.

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