CLOCK is a positive component of a transcription/translation-based negative feedback loop of the central circadian oscillator in the suprachiasmatic nucleus in mammals. To examine the CLOCK-regulated circadian transcription in peripheral tissues, we performed microarray analyses using liver RNA isolated from Clock mutant mice. We also compared expression profiles with those of Cryptochromes (Cry1 and Cry2) double knockout mice. We identified more than 100 genes that fluctuated from day to night and of which expression levels were decreased in Clock mutant mice. In Cry-deficient mice, the expression levels of most CLOCK-regulated genes were elevated to the upper range of normal oscillation. Most of the screened genes had a CLOCK/BMAL1 binding site (E box) in the 5’-flanking region. We found that CLOCK was absolutely concerned with the circadian transcription of one type of liver genes such as DBP, TEF, and Usp2 and partially with another (such as mPer1, mPer2, mDec1, Nocturnin, P450 oxidoreductase, and FKBP51) because the latter were damped but remained rhythmic in the mutant mice. Our results showed that CLOCK and CRY proteins are involved in the transcriptional regulation of many circadian output genes in the mouse liver. In addition to being a core component of the negative feedback loop that drives the circadian oscillator, CLOCK also appears to be involved in various physiological functions such as cell cycle, lipid metabolism, immune functions, and proteolysis in peripheral tissues.

Many organisms display rhythms of physiology and behavior which are entrained to the 24-h cycle of light and darkness which prevails on Earth. In mammals, the suprachiasmatic nucleus (SCN) is considered the master circadian pacemaker that controls most of the physical circadian rhythms including behavior (1, 2). Studies of clock genes have recently implied that oscillatory mechanisms function in peripheral organs and isolated cells and that they appear to be entrained to the SCN (1, 2). Although the peripheral oscillators seem to play an important role in regulating various physiological functions, little is known about the circadian oscillatory mechanism in peripheral tissues. Recent studies at the molecular level in bacteria, plants, and animals have provided a general model of the circadian pacemaker that is based on a self-sustained transcriptional/translational negative feedback loop (1, 2).

Clock was the first clock gene identified in vertebrates by forward mutagenesis using N-ethyl-N-nitrosourea in a behavioral screening (3). When transferred from a light-dark cycle to constant darkness, the periodicity of behavior exhibited by homozygous Clock mutants is abnormally long for the initial 5–15 cycles, and circadian rhythmicity is consequently lost (3). The Clock gene encodes a basic helix-loop-helix (bHLH)-PAS transcription factor (1, 2). Like other bHLH transcription fac-

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The Gene Expression Omnibus (GEO) accession numbers for the expression data of this work are GSM6792-7 (www.ncbi.nlm.nih.gov/geo).

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4 The abbreviations used are: SCN, suprachiasmatic nucleus; bHLH, basic helix-loop-helix; CT, circadian time(s); DBP, D site-binding protein; GADD, growth arrest and DNA damage-inducible; GC, glucocorticoid; HNF, hepatocyte nuclear factor; Id, inhibitors of differentiation/DNA-binding proteins; TEF, thyrotroph embryonic factor; WT, wild-type.
tors, CLOCK binds DNA and modulates transcription after dimerization with BMAL1 (a bHLH-PAS transcription factor) (1, 2). As the Clock allele is truncated and causes a deletion of 51 amino acids, the mutation presumably would not have a significant effect on the N-terminal bHLH and PAS domains, leaving CLOCK dimerization and DNA binding intact (1, 2). Actually, the mutant CLOCK protein can still form heterodimers with BMAL1 which bind to DNA, but the heterodimers are deficient in transactivation (1, 2). The CLOCK/BMAL1 heterodimer drives the rhythmic transcription of period (mPer1, mPer2, and mPer3) and cryptochrome (mCry1 and mCry2) genes through the E box (CACGTT) in Per and Cry promoters (1, 2). As the PER and CRY proteins are translated, they form multimeric complexes that are translocated to the nucleus. The CRY proteins are essential for the negative feedback loop that regulates the central clock (4, 5). The primary function of the CRY proteins in mammals is to inhibit CLOCK/BMAL1-mediated transactivation (1, 2). However, how the CRY proteins negatively regulate CLOCK/BMAL1-mediated transactivation remains obscure. A recent study has shown that H3 histone acetylation is a potential target of CRY inhibitory action, as CRY proteins increase the inhibition in CLOCK/BMAL1-mediated transactivation induced by histone acetyltransferase p300 (6).

Microarray analysis is a powerful tool with which to evaluate the expression of many genes in various experimental systems, and it can identify target genes for transcription factors. Hundreds of tissue-specific circadian clock-controlled genes, which regulate an impressive diversity of biological processes, have been identified by DNA microarray technology (7–13). CLOCK protein is involved in the transcriptional regulation of several circadian output genes as well as in the core circadian clock (7). We speculated that transcripts that are rhythmic in a circadian manner, down-regulated in homozygous Clock mutant mice, and elevated at high or intermediate levels in Cry-deficient mice, would potentially be direct targets of CLOCK protein. Thus, to examine the CLOCK-regulated genes that express in a circadian manner in mice, we performed oligonucleotide microarray analysis at circadian times (CT) 14 and CT2, when CLOCK/BMAL1 transcriptional activity is maximal and minimal, respectively (14), using RNA isolated from the livers of wild-type (WT), Clock mutant, and Cry-deficient mice. We also searched for an E box element in the 10-kb region 5′-upstream from these genes and their respective human homologs. Our results revealed that several circadian expressing output genes are probably regulated by CLOCK and CRY proteins via the E box element(s) in the mouse liver.

**MATERIALS AND METHODS**

**Mice**—Clock mutant mice were derived from animals supplied by J. S. Takahashi (Northwestern University, Evanston IL). The animals had the Clock allele originally on a BALB/c and C57BL/6J background. A breeding colony was established by further backcrossing with Jcl/ICR mice (14). Cry1 and Cry2 double knockout mice were generated as described previously (4, 5). C57BL/6J background mice were the WT control for the GeneChip analysis. Mice with WT Jcl/ICR and C57BL/6J backgrounds were Northern blot controls for Clock mutant and Cry-deficient mice, respectively.

All male mice of 8–10 weeks of age were maintained under a 12:12-h light-dark cycle for at least 2 weeks before the day of the experiment. After being placed in constant darkness (DD), the animals were sacrificed at CT2 and CT14 on the 2nd day of the DD. The livers were dissected, quickly frozen, and stored in liquid nitrogen.

**Samples and GeneChip Hybridization**—Total RNA was purified from a pool of two to three animal tissues collected at each time point using RNeasy (Qiagen). Poly(A)⁺ RNA was purified from the total RNA using an Oligotex®-dT30 mRNA Purification kit (Takara). Double stranded cDNA containing the T7 RNA polymerase promoter at the 5′-end was synthesized from 1 μg of poly(A)⁺ RNA using a SuperScript double stranded cDNA synthesis kit (Invitrogen).

Bioluminescent amplified RNA was then synthesized using a BioArray™ HighYield™ RNA transcription labeling kit (Enzo Life Sciences) and purified using an RNaseasy Mini kit (Qiagen). The labeled RNA was partially hydrolyzed and hybridized in duplicate (or more) to Affymetrix GeneChip (MG-UI74Av2) arrays. The arrays were hybridized, washed, and analyzed using standard Affymetrix reagents and protocols.

**Northern Blotting**—Northern blotting proceeded as described previously (14). Randi primed—labeled probes were generated from cDNA fragments of mPer1 (bases 2358–3114; GenBank AB002108), mPer2 (bases 1123–1830; GenBank AF036893), FKBPS1 (bases 191–1069; GenBank U16959), NADPH P450 oxidoreductase (bases 60–1013; GenBank B17571), mDec1 (bases 722–1333; GenBank Y07836), Nocturnin (bases 901–1730; GenBank AF199491), TEF (bases 1–1020; GenBank BC096891), Usp2 (bases 89–384; GenBank ABO14799) and DBP (bases 1138–1602; GenBank J08179). Samples were normalized to the amount of glyceraldehyde-3-phosphate dehydrogenase mRNA (data not shown).

**Quantitative Reverse Transcription-PCR and in Situ Hybridization**—Complementary DNA generated using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) was amplified by PCR using a LightCycler™ (Roche Applied Science). The primers were as follows: Usp2 forward, 5′-TGTATGCTGTTGCAATCA-3′ and reverse 5′-TATAGAACAAATAGGCGT-3′. To quantify and confirm the integrity of each RNA sample, glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. A standard curve was constructed with serial dilutions of cDNA obtained from hypothalamic RNA. Data were analyzed using LightCycler analysis software and are expressed as ratios to the highest value of the eight time points of the day.

In situ hybridization proceeded as described previously (14). In brief, mice were anesthetized with pentobarbital and perfused from the left ventricle with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The animals were dissected under a dim red light in the dark. The tissues were fixed for 2.5 h at 4°C, embedded in Tissue-Tek OCT compound (Miles), and cut into 8-μm cryosections. Digoxigenin-labeled RNA probes were generated from Usp2 (bases, 89–384; GenBank AB014799) using a DIG RNA labeling kit (Roche Applied Science). Transcripts were hybridized and detected as described (14).

**Screening of CLOCK-regulated Rhythmic Genes**—Average difference values for each gene were calculated using Affymetrix microarray analysis software, and average difference values of 1 or lower were set to 0, to avoid division by 0 or a negative number. Other studies have demonstrated that CLOCK-regulated gene expression levels in Clock (Clk1 Clk2) mutant mice are generally lower than those in the WT (14) and that an expression ratio of 2-fold is the approximate limit of sensitivity (15). Therefore, we applied three criteria to the selection of circadian-regulated genes: WT14 (the value of WT at CT14), WT14/WT02 > Clk02, and WT02 + WT14/2 > Clk02. Under these criteria, not only the genes that showed the damped expression in Clock mutants, but also the genes that show the changing in phase of circadian expression will be identified. Actually, known components of the CLOCK-regulated genes such as mPer1, mPer2, mDec1, and albumin D site-specific binding protein (DBP) were identified, suggesting that this screening strategy was appropriate for an examination of directly or indirectly CLOCK-regulated circadian gene expression in the mouse liver.

**Computational Analysis of E Box Motif**—The protein-coding sequences of each cycling gene were used for BLAST as queries for corresponding genomic contigs of mouse draft genome sequences (ftp://ensembl.org/pub/mouse-7.0/dna/golden_path). The consensus E box element (CACGTT) was searched against the 10-kb genome sequences upstream from the first methionine for the respective cycling genes (when the positions of coding regions were unavailable, we used the 10-kb genome sequences upstream from the mRNA start site instead).

**RESULTS AND DISCUSSION**

We identified 1216 genes that fluctuated day/night in the liver of WT mice. In these genes, 108 known genes (Fig. 1) and 27 unknown genes (Fig. 2) showed reduced expression levels in Clock mutant mice. Among them, known components of the circadian clock such as mPer1, mPer2, mDec1, and DBP were identified, suggesting that our current strategy was appropriate to examine CLOCK-regulated circadian gene expression in the mouse liver. Furthermore, BLAST search results of the screened expressed sequence tag clones revealed that our experiments were accurate because identical genes such as Nocturnin, long chain fatty acyl elongase (Lee), P450 oxidoreduc-
FIG. 1. Pseudo-color image of gene expression by WT, Clock mutant (Clk/Clock), and double knockout mice of Cry1 and Cry2 (WKO) under constant darkness. Relative expression changes to average difference of WT14 (the value of WT at CT14) are shown for each gene. Red and blue indicate high and low levels of expression relative to that of WT14, respectively. Black indicates unchanged expression compared with WT14. Genes are classified according to function. These latest annotations were retrieved from the NetAffx website. (45).
circadian expression of Hes3

range of normal oscillation in the peripheral tissues of Clock

found that progression in organisms ranging from flies to humans (19). We important for the control of differentiation and cell cycle pro-

act as dominant negative regulators of bHLH proteins and arepineal gland, whereas the related genes, Id-2 and Id-3, do not

expression of cell cycle regulatory genes. Id-1 exhibitscircuit rhythms of mRNA and protein expression in thepineal gland, whereas the related genes, Id-2 and Id-3, do not

We also showed that the circadian mRNA expression of Wee1 (this kinase inhibits mitotic cell division by inactivating the Mphase-promoting factor) was extremely reduced in the liver of Clock mutant mice, and the mRNA levels were continuouslyhigh in Cry-deficient mice (Fig. 1). A recent study has shown thatmPer2 protein is involved in tumor suppression by reg-

ulating DNA damage-responsive pathways (22). Growth con-

trol and the DNA damage response may be affected by Clock

mutation because the circadian expression of mPer2 mRNA isregulated by the CLOCK protein (14). In fact, some aged-Clock

mutant mice developed salivary gland hyperplasia like mPer2 mutant mice (data not shown). Considering the current data

and reported findings (22), mammalian cell cycle-regulating

mechanisms appear to be regulated by the circadian clock atthe molecular level.

The circadian expression of molecular chaperones such asHsp105 and mDj7 was depressed in Clock mutant mice (Fig. 1). Many heat-shock genes are expressed in a circadian manner both in the SCN and in the liver (7–9, 12). The decreased expression of these chaperones in Clock mutant mice suggests an altered response to external stress.

Ubiquitylation and proteasome-mediated protein degrada-

tion are involved in the core mechanism of the circadian oscil-

lator (23). The present study found that the circadian expres-

sion of ubiquitin-specific protease 2 (Usp2) and Rad23 mRNAswas regulated in a CLOCK-dependent manner in the mouse liver (Figs. 1 and 3). Rad23 contains a ubiquitin-like domain
and translocates ubiquitinated substrates to the proteasome (10). Usp2 has shown to be expressed in a circadian manner in the mouse liver but not in the SCN (7). Its expression is reduced significantly in the liver of Clock mutants (7). In this study, however, reverse transcription-PCR analysis and in situ hybridization showed that Usp2 mRNA is expressed in a circadian manner in the SCN (Fig. 4) as well as in the liver (Fig. 3) and that the expression levels were damped in Clock mutant mice. Most of the cyclic transcripts are tissue-specific, and only a few genes are rhythmically expressed in both the SCN and in the liver (7–9, 12, 13), suggesting that the Usp2 plays a key role in the core mechanism of the circadian oscillator.

The Nocturnin gene encodes a poly(A) tail-specific 3’-exonuclease that functions in the deadenylation process (24). The widespread expression and rhythmicity of the Nocturnin gene parallel the circadian expression of clock genes in various mammalian tissues including the SCN (11). Circadian changes in poly(A) tail length or mRNA stability appear to be important for maintaining proper control of clock-regulated mRNA levels (25). We showed here that the circadian expression of Nocturnin mRNA is damped in the liver of Clock mutant mice and that the mRNA levels are continuously high in Cry double mutant mice (Fig. 3). These data suggest that Nocturnin plays an important role in clock function or as a circadian clock effector in mammals.

The closely related PAR basic leucine zipper proteins, DBP and thyrotroph embryonic factor (TEF), are key elements in the liver specific transcriptional regulation of genes. Both proteins recognize the same DNA sequences in vitro (26). However, although TEF stimulates transcription from the albumin promoter more potently than DBP, only DBP can efficiently activate transcription from the cholesterol 7α-hydroxylase (CYP7) promoter (26). We found that the circadian expression of both DBP and TEF genes was depressed in Clock mutant mice (Fig. 4).

**Fig. 3.** Temporal patterns of CLOCK-regulated circadian expressing genes in the mouse liver. Messenger RNA levels of genes were quantified from Northern blots. Solid circles and bars represent levels in WT mice. Open circles and bars represent levels in Clock mutant and double knockout (Cry1 and Cry2) mice, respectively. Maximal value of WT mice is expressed as 100%. Values are represented as the means ± S.E. (n = 3 and n = 4 in Clock mutant and Cry-deficient mice, respectively).

**Fig. 4.** Usp2 mRNA expression in the SCN of WT and Clock mutant (Clk/Clk) mice. A, quantitative reverse transcription-PCR of Usp2 mRNA in the SCN of WT and Clk/Clk mice. B, representative in situ hybridization of Usp2 mRNA in the SCN of WT mice at CT14.
3), suggesting that the expression of these PAR basic leucine zipper transcription factors is positively regulated by CLOCK via E box elements in vivo. Plasma albumin levels were decreased significantly at all time points in Clock mutant mice compared with those in WT mice, suggesting that these PAR basic leucine zipper transcription factors function in albumin synthesis in vivo. Because DBP is a major transcription factor controlling circadian expression of the steroid 15α-hydroxylase (Cyp2a4) and coumarin 7-β-hydroxylase (Cyp2a5) genes in the mouse liver (27), steroid hormone-associated physiology may be affected in Clock mutant mice.

The mRNA expression of hepatocyte nuclear factor 3 (HNF-3), a forkhead winged helix family transcription factor, was reduced by the Clock mutation and up-regulated in Cry-deficient mice (Fig. 1). CRG-1, a transcription factor that is similar to the N-terminal domain of HNF-3, is regulated by the circadian clock genes timeless (tim) and per in Drosophila (28). HNF-3 binds to cis-regulatory elements in hundreds of genes encoding gluconeogenic and glycolytic enzymes, including glucokinase (29). Glucokinase is a key and rate-limiting enzyme of glucose metabolism. Glucokinase mRNA expression is also rhythmic and damped by the Clock mutation as shown in Fig. 1. Thus, transcription of the glucokinase gene seems to be regulated both directly (E box-dependent transactivation) and indirectly (HNF-3-dependent transactivation) by CLOCK in the mouse liver. Adenoviral vector-mediated overexpression of glucokinase in diabetic mice normalizes blood glucose levels (30), suggesting that glucokinase is an important regulator of glucose storage and disposal in the liver. Therefore, the reduced expression of HNF-3 and glucokinase mRNAs may affect glucose metabolism in Clock mutant mice.

Fatty acids are synthesized de novo via several steps. After malonyl-CoA is produced from acetyl-CoA mediated by acetyl-CoA carboxylase, malonyl-CoA is altered to fatty acids in a complex of fatty acid synthetase. The mRNA expression levels of fatty acid synthetase and another two lipogenic enzymes, Lee and acyl-CoA synthase 4, were decreased in the liver of Clock mutant mice. Fatty acid synthetase can elongate fatty acids in two-carbon increments to produce palmitic acid. The synthesis of long chain fatty acids (with more than 16 carbon atoms in the backbone), are produced by other mechanisms. The enzyme Lee catalyzes the initial long chain fatty acid elongation and is therefore key and rate-limiting, so it plays an important role in the de novo synthesis of long chain fatty acids (31). Acyl-CoA synthase 4, located in peroxisomes and in the mitochondria-associated membrane, catalyzes the activation of long chain fatty acids to fatty acyl-CoA. Although a pharmacological approach predicted that acyl-CoA synthase 4 would be linked to triacylglycerol synthesis (32), a recent localization study suggests that acyl-CoA synthase 4 is involved in peroxisomal oxidation (33). Our results showed that transcription of the three key enzymes that catalyze fatty acids and fatty acyl-CoA de novo synthesis was affected by the Clock mutation. Actually, we found that lipid parameters in the plasma of Clock mutant mice were altered. Moreover, the expression levels of Lipin1, which is a candidate lipodystrophy gene (34), were also depressed in Clock mutant mice (Fig. 1). Lipin might be involved in the regulation of lipids and sugars because mutations of this gene cause several phenotypic abnormalities, including those of lipid and glucose metabolism. CYP51 (lanosterol 14a-demethylase) mRNA levels were also reduced in Clock mutant mice, suggesting that Clock mutations affect cholesterol biosynthesis, because CYP51 is a cytochrome P450 enzyme involved in the heptosqualene phases of cholesterol biosynthesis (35). The above evidence indicates that CLOCK participates in the regulation of lipid metabolism.

The amplitude of P450 oxidoreductase mRNA expression was reduced in Clock mutant mice, although the expression rhythm still remained in the mutant mice (Fig. 3). NPA52 (a transcription factor highly homologous to CLOCK) might compensate for the functional deficit of CLOCK in the periphery of Clock mutant mice (36). Rutter et al. (36) showed that DNA binding of the NPA52/BMAL1 transcription factor is stimulated by a reduction in the amount of NADPH and is inhibited by oxidized nicotinamide adenine NADP+. This might explain how the cellular metabolism and energy status impinge upon the timing of the circadian rhythm. The expression levels of P450 oxidoreductase mRNA are significantly increased and phase shifted by restricted feeding, which imposes feeding time during the light period upon both nocturnal WT and Clock mutant mice. Thus, P450 oxidoreductase might play an important role in the redox regulation-associated with food-entrainable oscillators (37) in the peripheral tissues of mice.

The expression levels of DBP were extremely decreased at all time points examined, whereas the expression levels of most of the CLOCK-regulated circadian genes were depressed but still rhythmic in Clock mutant mice (Fig. 3). Thus, CLOCK is absolutely concerned (all/nothing) with the circadian transcriptional regulation of some genes (such as DBP, TEF, and Usp5) and partially with that of others (such as mPer1, mPer2, mDec1, and Nocturnin). In the SCN, the expression levels of CLOCK-regulated clock genes such as mPer1 and mPer2 are reduced extensively (1, 2, 14), suggesting that CLOCK is indispensable in the SCN but not in the periphery for the rhythmic expression of some genes. CLOCK in the periphery seemed to play an important role in the transcriptional regulation of clock-regulated output genes such as DBP and TEF.

A daytime restricted feeding changes the phase of circadian gene expression in peripheral tissues but does not affect that of cyclic gene expression in the SCN (38). We previously found a CLOCK-independent oscillating mechanism in the peripheral tissue of mice under restricted feeding conditions (14). Clock mutant mice on a Ch-ICR background (as used in this study) exhibit abnormally longer behavioral periodicity (about 27.5 h, compared with about 23.8 h in WT mice) under constant darkness (14). Thus, a CLOCK-independent oscillating mechanism appears to be induced by the food-entrainable oscillators in Clock mutant mice because of rhythmic feeding. The rhythmic feeding behavior seemed to induce the expression rhythm of several genes such as mPer1 and mPer2 in the liver of Clock mutant mice, although the amplitude was smaller than that of WT mice. The acrophase of circadian expressing genes such as mPer1, mPer2, P450 oxidoreductase, and mDec1 was delayed for 6, 3, 3–6, and 12 h, respectively, in Clock mutant mice compared with WT mice (Fig. 3). These findings might be related to the lengthened behavioral periodicity caused by the extended firing rhythm periodicity of the SCN neurons from Clock mutant mice (19).

The present study showed that the circadian expression of FKB51 (51-kDa FKB51) was depressed in Clock mutant mice (Fig. 3). FKB51 associates with heat shock protein 90 and appears in functionally mature steroid receptor complexes, which leads to steroid hyposensitivity (39). On the other hand, low FKB51 gene expression is easily induced by glucocorticoid (GC), which also fluctuates in a circadian manner, suggesting an autoregulatory mechanism between steroids and FKB51. The induction of FKB51 mRNA by GCs might be a suitable marker with which to assess individual GC sensitivity, in vivo GC potency, and in vivo GC bioavailability (40). Thus, the transcriptional regulation of FKB51 might provide new ther-

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2 K. Oishi, manuscipt in preparation.
CLOCK-regulated Circadian Output Genes

Table I

| Symbol | Position | Symbol | Position |
|--------|----------|--------|----------|
| Per1   | −6246   | −6062  | −2968   | −2222  | −1658 |
| Per2   | −6748   |        |         |         |       |
| Bhlhb2 | −1245   | −646   |         |         |       |
| Dmp    | None     |        |         |         |       |
| Amt2   | −9226   |        |         |         |       |
| E4f1   | −8256   | −1244  |        |         |       |
| Kif13  | None     |        |         |         |       |
| Ccnr41 | −5229   |        |         |         |       |
| Nrk2c1 | −8423   | −8220  | −2928  |        |       |
| Tef    | −8920   | −8375  | −523   | −412   | −190  |
| Gdnf   | −4428   | −2412  |        |         |       |
| Hes3   | −3831   | −2050  | −51    |        |       |
| Hes8   | −3865   |        |         |         |       |
| Idb1   | −9285   | −8546  |        |         |       |
| Lig3   | −4338   |        |         |         |       |
| Pa2g4  | −5350   | −4352  | −1511  |        |       |
| Se11b  | −6468   | −2810  |        |         |       |
| Cpr2-pending | −2610 | | | | |
| Ccnt1  | −7446   |        |         |         |       |
| Ddit3  | −933    | −11    |        |         |       |
| Gadd45a| None    |        |         |         |       |
| Kfizzoa| 1940    |        |         |         |       |
| Men1   | None    |        |         |         |       |
| Mina   | −1975   |        |         |         |       |
| Gadd45b| −9078   | −820   | −185   |        |       |
| Rap2b  | −6032   |        |         |         |       |
| Weel1  | 4437    | −948   | −304   | −104   |       |
| Fac4l  | −3675   |        |         |         |       |
| Alox12e| −3495   |        |         |         |       |
| Abcb1  | −4069   | −3912  |        |         |       |
| Car1   | None    |        |         |         |       |
| Chr1   | −9697   | −7920  | −4705  | −3599  |       |
| Cyp51  | −2787   |        |         |         |       |
| Cyp26a1| −3027   |        |         |         |       |
| Eef2k  | −556    | −62    |        |         |       |
| Fasn   | −4856   |        |         |         |       |
| Foxa3  | −5884   | −1352  | −1036  | −460   |       |
| Gck    | −724    |        |         |         |       |
| H2-Ke6 | −4069   | −3014  | −1311  | −498   |       |
| Lpin1  | −8924   | −5672  |        |         |       |
| Lce-pending | −9309 | −9252  | −8959  | −6685  | −1416 |
| Por    | −8480   | −7837  | −4876  | −1702  | −1511 |
| Piga   | −4499   | −2553  |        |         |       |
| Srr    | −8972   | −8869  | −8145  | −2114  |       |
| Tnnd4f7| 9579    | −3096  | −108   |        |       |
| Dnajb9 | −7420   | −7172  | −6415  |        |       |
| Hsf2   | −207    |        |         |         |       |
| Hsp105 | −2913   | −258   |        |         |       |
| Tor1b  | −5420   | −1614  |        |         |       |
| Chst3  | 6800    | −6666  | −2055  |        |       |
| Lgals7 | None    |        |         |         |       |
| Has2   | −9970   | −9216  |        |         |       |
| Lgals8 | −2345   | −894   |        |         |       |

apotic modalities with which to treat allergic diseases such as steroid-resistant asthma. The regulation of FKBP51 expression by the CLOCK/BMAL1 heterodimer via the E box element is a novel mechanism. Considering the usefulness of chronotherapy in treating allergic diseases, this circadian change of FKBP51 might be a new target for the therapeutic effects of steroids.

The present study identified more than 100 putative CLOCK-regulated circadian expressing genes in the mouse liver (Fig. 1). Panda et al.’s (7) reported that only 9 genes are regulated by CLOCK in a circadian manner in the mouse liver. They compared gene expression between WT and homozygous Clock mutant mice at one time point, zeitgeber time 8, then screened genes with at least a 2-fold change in expression levels (7). In their screening, however, CLOCK-regulated genes whose amplitude of circadian transcription is small in WT mice might be omitted. In the present study, however, to identify the CLOCK-regulated circadian output genes that fluctuated with small amplitude in WT mice, we screened genes whose expression levels were smaller in Clock mutant mice than the average value of normal fluctuation (see “Materials and Methods”). Compared with Panda’s study (7), we identified 4 (mPer2, Usp2, mDec1, and Ak4) of 9 putative CLOCK-regulated circadian expressing genes (Fig. 1). Furthermore, we identified more than 100 putative genes that are, directly or indirectly, transactivated by CLOCK. The difference between the present and Panda’s (7) studies seems to be largely dependent on the screening procedures as mentioned above.

Most of these identified genes had the potential CLOCK/ BMAL1 binding site, called E box, in the genomic sequence located 5’ of the start of transcription in mice (Table I). Although it is difficult to estimate which E box (or E box-like) element functions in the circadian transactivation by CLOCK, the present results imply that a relatively large number of circadian expressing genes are regulated directly by CLOCK via the E box. We also found that mouse and human 5’-upstream sequences around the E box elements were highly conserved for mDec1, TEF, and Usp2 (Fig. 5). Comparative genomics has long been used to study promoters of specific genes in

| Symbol | Position |
|--------|----------|
| Fasn   | −4856    |
| Foxa3  | −5884    |
| Gck    | −724     |
| H2-Ke6 | −4069    |
| Lpin1  | −8924    |
| Lce-pending | −9309 |
| Por    | −8480    |
| Piga   | −4499    |
| Srr    | −8972    |
| Tnnd4f7| 9579     |
| Dnajb9 | −7420    |
| Hsf2   | −207     |
| Hsp105 | −2913    |
| Tor1b  | −5420    |
| Chst3  | 6800     |
| Lgals7 | None     |
| Has2   | −9970    |
| Lgals8 | −2345    |
many organisms. The recent genome alignments of four (41) and six (42) Saccharomyces species revealed that typically short (6–15 bp) conserved sequence motifs reside upstream from genes with similar functional annotations or similar expression profiles or those bound by the same transcription factor. The highly conserved sequences especially around the E box element of these genes between mice and humans (Fig. 5) suggest that this element is important for transcriptional regulation by CLOCK. In this study, however, the expression of circadian oscillating genes such as mPer1 and mPer2 was still rhythmic in Clock mutant mice, although the amplitude was reduced by the Clock mutation (Fig. 3). Thus, CLOCK-regulated genes appear to consist of two types. The involvement of CLOCK is absolute in the circadian transcriptional regulation of one type of genes (such as DBP, TEF, and Usp2) and partial for the other. The circadian clock genes mPer1 and mPer2 have shown to be transactivated not only via the E box elements but also via the cAMP-responsive elements that reside upstream from these genes (43). Thus, factors other than E box elements might also play an important role in the expression of circadian output genes (13).

In addition to being a core component of the negative feedback loop that drives the circadian oscillator, CLOCK also appears to be involved in various physiological functions such as the cell cycle, lipid metabolism, immune response, and proteolysis in peripheral tissues. A single nucleotide polymorphism located in the 3′-flanking region of the human Clock gene might be responsible for the human preference for morn-

**Fig. 5.** Comparison of mouse and human sequences around E box element 5′-upstream from mDec1 (A), TEF (B), and Usp2 (C). Conserved E box (CACGTG) elements are shaded in red.
CLOCK-regulated Circadian Output Genes

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159–171

172–182

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193–205

206–213

214–225

226–237

238–245

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