Genome-wide Expression Analysis Reveals 100 Adrenal Gland-dependent Circadian Genes in the Mouse Liver

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Abstract

Recent progress in genome-wide expression analysis has identified hundreds of circadian genes not only in the suprachiasmatic nucleus (the mammalian master clock) but also in peripheral tissues, such as heart, liver and kidney of mammals. Glucocorticoid is thought to be a circadian time cue for mammalian peripheral clocks. To identify the genes of which the circadian expression is regulated by endogenous glucocorticoids, we performed DNA microarray analysis using hepatic RNA from adrenalectomized (ADX) and sham-operated mice. We identified 169 genes that fluctuated between day and night in the livers of the sham-operated mice. Among these, 100 lost circadian rhythmicity in ADX mice. These included the genes for key enzymes of liver metabolic functions, such as glucokinase, HMG-CoA reductase and glucose-6-phosphatase. The circadian expression of Lpin1, FKBP51 and S-adenosyl methionine decarboxylase was also abolished in the ADX mice. On the other hand, although the circadian expression of clock or clock-related genes, such as mPer2, DBP, E4BP4, mDec1, Usp2 and Wee1 remained almost totally intact in the liver of ADX mice, it was extremely damped in homozygous Clock mutant mice. The present findings suggested that one type of hepatic circadian genes in mice is transcriptionally regulated by core components of the circadian clock, such as CLOCK and BMAL1, and that the other depends on the adrenal gland.

Keywords: circadian rhythm; glucocorticoids; Clock; DNA microarray; liver

1. Introduction

Most organisms exhibit circadian rhythms in physiological processes, such as hormonal secretion and metabolic activities, as well as in behaviors, such as locomotor activity and feeding, which are regulated by an endogenous pacemaker. The central clock that controls most physiological and behavioral rhythms in mammals is located in the suprachiasmatic nucleus (SCN) of the brain. Recent studies at the molecular level on bacteria, fungi, plants and animals suggest that one or more negative feedback loops in gene expression drive circadian oscillators. Among the circadian clock genes involved in the feedback loops, the period (per) genes are thought to play critical roles in the generation of rhythm both in Drosophila and in mammals. Numerous studies have revealed the circadian expression of per genes not only in the SCN but also in various peripheral tissues, such as the heart, lungs, liver, kidneys and circulating blood cells in mammals, suggesting that an oscillatory system in peripheral tissues contains feedback loops similar to that in the SCN. Hundreds of tissue-specific circadian
clock-controlled genes that regulate an impressive diversity of biological processes have been identified by DNA microarray technology.\textsuperscript{6–11} Peripheral clocks seem to be governed by the central clock in the SCN, because the circadian expression of clock or clock-controlled output genes is abolished or considerably diminished in the periphery by surgical ablation of the SCN.\textsuperscript{5,12} However, the mechanism that underlies the circadian regulation of peripheral gene expression by the SCN remains obscure.

The circadian peak of blood glucocorticoids is controlled by the SCN via the paraventricular nucleus of the hypothalamus where neurons containing corticotropin-releasing hormone regulate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary.\textsuperscript{13} Glucocorticoid signaling is thought to be an important time cue for peripheral clocks from the SCN. The glucocorticoid analog dexamethasone (Dex) can induce the circadian expression of clock or clock-controlled genes in cultured fibroblasts.\textsuperscript{14} Temporal Dex injections \textit{in vivo} induce transient changes in the phase of clock gene expression in liver, kidney and heart in a phase-dependent manner.\textsuperscript{14} However, glucocorticoids cannot be the only signals that set the phase of peripheral clocks since the phase of oscillators is the same in glucocorticoid receptor (GR)-deficient hepatocytes and in the livers and kidneys of adrenalectomized (ADX) mice compared with intact wild-type mice.\textsuperscript{14,15}

CLOCK is a basic helix-loop-helix (bHLH)-PAS transcription factor that binds target DNA and modulates transcription after dimerization with BMAL1 (a bHLH-PAS transcription factor).\textsuperscript{3,4} The CLOCK/BMAL1 heterodimer drives the rhythmic transcription of the other clock genes \textit{period} (\textit{mPer}1, \textit{mPer}2 and \textit{mPer}3) and \textit{cryptochrome} (\textit{mCry}1 and \textit{mCry}2) through E-box (CACGTG) elements located in their promoters.\textsuperscript{3,4} Using microarray technology, we showed that a positive transcription regulator called E4BP4 is a component of the transcription/translation-based negative feedback loop of the circadian oscillator, CLOCK, is involved in the circadian transactivation of clock-controlled output genes in the mouse liver.\textsuperscript{11} The screened oscillatory genes perform various physiological functions, such as metabolism, transcription, translation, protein turnover, cell cycle, cell death, ion transport and signal transduction. The issue arising from these findings is how the oscillatory expression of only a few canonical clock genes regulates the oscillation of a vast number of clock-controlled genes.\textsuperscript{16} One possibility is that the CLOCK/BMAL1 heterodimer directly acts on circadian gene expression. This has in fact been demonstrated with respect to the rhythmic expression of several genes, such as \textit{vasopressin},\textsuperscript{17} \textit{albumin D-site binding protein (DBP)},\textsuperscript{18,19} \textit{plasminogen activator inhibitor-1 (PAI-1)},\textsuperscript{20} \textit{prokineticin 2},\textsuperscript{21} \textit{Wee1},\textsuperscript{11,22} and \textit{peroxisome proliferator–activated receptor \alpha (PPAR\alpha)}.\textsuperscript{23} Another possibility is that the CLOCK/BMAL1 complex acts indirectly through the transactivation of other transcription factors. Indeed, our previous study showed that many transcription factors seem to be transactivated by CLOCK protein.\textsuperscript{11} Yet another alternative is that clock molecules act indirectly through the circadian production/secretion of humoral signals that can affect the gene expression in peripheral tissues.

To examine the circadian regulation of gene expression in the liver, the present study analyzed genome-wide gene expression using hepatic RNA from ADX mice. We demonstrated using DNA microarrays and \textit{in vivo} injection analyses that 100 hepatic genes are transcriptionally regulated by endogenous glucocorticoids in a circadian manner. These included the genes for key enzymes involved in liver functions, such as \textit{glucokinase}, \textit{HMG-CoA reductase} and \textit{glucose-6-phosphatase}. The circadian expression of \textit{Lpin1}, \textit{FKBP51} and S-adenosyl methionine decarboxylase was also abolished in the ADX mice. Furthermore, we demonstrated that the circadian expression of several genes, such as \textit{mPer}2, \textit{DBP}, \textit{E4BP4}, \textit{mDec1}, \textit{Usp2} and \textit{Weel}, was almost totally intact in the liver of ADX mice, but extremely diminished in homozygous \textit{Clock} mutant mice. The present results suggested that at least two types of circadian controlled genes are located in the mouse liver: one is directly or indirectly dependent on endogenous glucocorticoids, whereas the other is governed by core components of the peripheral clock and is not affected by ADX.

2. Materials and Methods

2.1. Mice

Bilateral ADX was performed in male C57BL/6 mice aged 7–8 weeks using the dorsal approach under ketamine/xylocaine anesthesia (ketamine 91 mg/kg BW and xylocaine 3.6 mg/kg BW, i.m.). The ADX mice were given free access to standard chow (CE-2, Clea Japan Inc.) and to 0.9% NaCl. Sham-operated control mice were given standard chow and water \textit{ad libitum}. After the operation, apparently alert and healthy mice were housed in a 12:12 h light-dark cycle [LD12:12; lights on at zeitgeber time (ZT) 0] for at least 2 weeks before the day of the experiment. Liver samples were dissected, quickly frozen and stored in liquid nitrogen.

To examine the transient effect of glucocorticoid injection, 3 mg/kg BW of Dex was intraperitoneally injected into the ADX mice at ZT14. After 0.5, 1, 2, 3, 6, 12, 18 and 24 h, the mice were decapitated and the liver tissues were dissected.

Insulin-dependent diabetes was induced by a single intraperitoneal injection of the β-cell toxin streptozotocin (STZ) (200 mg/kg BW) into 7-week-old Jcl:ICR mice (Clea Japan, Inc., Tokyo) as described previously.\textsuperscript{24} 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 Cj:ICR mice.  

All animal experiments proceeded in accordance with the European Communities Council Directive of 24 November 1986 (86/EEC) and were approved by AIST, Japan.

2.2. Microarray analysis

Total RNA was purified from pools of three animal tissues collected at each time-point using ISOGEN (Nippon Gene Co., Ltd, Japan). Hybridization to Affymetrix GeneChip (MG-U74Av2) arrays proceeded as described.11

The difference (AD) value for each gene was provided by GeneChip software. To identify putative glucocorticoid-regulated circadian genes, we compared AD values between two time points (ZT2 and ZT14) in sham-operated and in ADX mice, because endogenous blood glucocorticoid levels peak at ZT11 to ZT14 in nocturnal rodents. Among 12 473 total probe sets, 47.0% (5861 probe sets) and 45.0% (5607 probe sets) of the genes were expressed at ZT2 and ZT14, respectively, in sham-operated mice. On the other hand, 46.5% (5806 probe sets) and 44.4% (5541 probe sets) of genes were expressed in ADX mice at ZT2 and ZT14, respectively. We applied three criteria to the selection of putative glucocorticoid-regulated circadian genes: (i) the AD value is marked as ‘present’ by the GeneChip software in at least one of two time points, (ii) the AD value exhibits a 2-fold or greater change in sham-operated mice because a 2-fold expression ratio is the approximate limit of sensitivity26 and (iii) the fold change is below 2-fold in ADX mice.

2.3. Northern blotting

Northern blotting proceeded as described previously.25 Random-primed 32P-labeled probes were generated from cDNA fragments of HMG-CoA reductase (bases: 1–694; GenBank accession no. M62766), glucokinase (bases: 330–962; GenBank accession no. BC011139), FKBPs (bases: 191–1069; GenBank accession no. U16959), S-adenosylmethionine decarboxylase (bases: 76–900; GenBank accession no. BC080791), Lpin1 (bases: 981–1884; GenBank accession no. AF412811), SAA4 (bases: 5061–5605; GenBank accession no. U65403), mPer2 (bases: 1123–1830; GenBank accession no. AF036893), mDec1 (bases: 722–1333; GenBank accession no. Y07836), Usp2 (bases: 89–384; GenBank accession no. AB041799), DBP (bases: 1138–1602; GenBank accession no. J03179), Wee1 (bases: 1089–2185; GenBank accession no. D30743), E4BP4 (bases: 61–770; GenBank accession no. U83148). Samples were normalized to the amount of GAPDH mRNA.

3. Results and Discussion

The liver is the central regulator of primary metabolism, and the circadian output reflects the physiological status of the organism and acts adaptively in nutrient assimilation and waste elimination.27,28 We identified 169 genes that fluctuated between day (ZT2) and night (ZT14) in the livers of sham-operated mice. Of these, the circadian expression of 100 genes was lost or diminished in ADX mice (Fig. 1). The key enzymes of metabolic and biosynthetic pathways, such as glycolysis, gluconeogenesis, cholesterol synthesis and polyamine biosynthesis, were among the putative glucocorticoid-dependent circadian genes. On the other hand, known components or targets of the circadian clock, such as mPer2, mDec1 (shown as Bhlhb2 in Fig. 2A), DBP, USP2 and Wee1, were among the 69 genes that remained rhythmic in the ADX mice (Fig. 2). The Gene Expression Omnibus (GEO) accession numbers for the expression data of this study are GSE2162 (GEO; http://www.ncbi.nlm.nih.gov/geo).

The key enzyme in the cholesterol biosynthetic pathway is 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase. Although daily variations in its liver activity have been recognized since 1969, circadian transcriptional regulation was not described until 2000. Notably, the underlying mechanism of circadian HMG-CoA reductase transactivation has remained unknown. The present study shows that the adrenal gland is involved in the circadian regulation of HMG-CoA reductase mRNA expression in the mouse liver (Fig. 1). Conversely, a single injection of Dex into ADX mice at the beginning of the dark phase transiently induced the expression of HMG-CoA reductase mRNA but not in a rhythmic manner (Fig. 3), suggesting that endogenous glucocorticoids directly regulate the expression of hepatic HMG-CoA reductase at the mRNA level. The effect of glucocorticoids on the activity of HMG-CoA reductase has been reported by many researchers over the past 30 years. However, the results are often contradictory. For example, ADX has no significant effect on its diurnal rhythm, greatly decreases the amplitude or abolishes the diurnal rhythm of HMG-CoA reductase. A single injection of glucocorticoids in ADX rats has no effect or restores the enzyme activity. These discrepancies appeared to result from the complexity of the regulation of HMG-CoA reductase activity, negative feedback regulation caused by cholesterol at the transcriptional level, stability and degradation of the mRNA and protein, and modulation of the enzyme activity by phosphorylation, thiois and substrate. The present results suggest that endogenous glucocorticoids play an important role in the circadian control of serum cholesterol levels by regulating the circadian expression of hepatic HMG-CoA reductase mRNA. The molecular mechanism of the transcriptional regulation of HMG-CoA reductase by glucocorticoids requires further elucidation.
| Sham | DEX | Probe | Accession | Symbol | Description |
|------|-----|-------|-----------|--------|-------------|
| Z12  |    |       |          |        |             |
| Z14  |    |       |          |        |             |
| Z12  |    |       |          |        |             |
| Z14  |    |       |          |        |             |

**Figure 1.** Continued.
The circadian fluctuation of HMG-CoA reductase activity might be regulated by changes in serum insulin concentrations, because hepatic HMG-CoA reductase mRNA expression levels are very low in the diabetic rat and are quickly restored by injected exogenous insulin. Serum insulin levels were severely decreased in the ADX mice (Supplementary Figure 1 is available at www.dnaresearch.oxfordjournals.org) as they are in ADX rats. We therefore examined the effect of decreased endogenous insulin levels on the circadian expression of HMG-CoA reductase mRNA using mice with STZ-induced diabetes (Fig. 4) in which plasma insulin levels remained consistently low throughout the day. The loss of insulin had surprisingly little effect on the circadian expression of hepatic HMG-CoA reductase mRNA in the diabetic mice, although the expression levels were slightly lower than those in control mice (Fig. 4). These observations support the notion that endogenous glucocorticoids are involved in the circadian expression of HMG-CoA reductase mRNA in vivo, whereas insulin is not.

Bile acids decrease whole body cholesterol levels and thus maintain cholesterol homeostasis in mammals. Here, we found that the mRNA expression of the bile acid biosynthetic enzymes, cholesterol 7α-hydroxylase (CYP7A1) and sterol 12α-hydroxylase (CYP8B1), fluctuated between day and night in the livers of sham-operated mice (Fig. 1) as reported previously. However, levels of the expression of both types of mRNA were continuously low in the ADX mice (Fig. 1). CYP7A1, a microsomal cytochrome P-450, is the rate-limiting enzyme of bile acid synthesis. Circadian expression of its mRNA is regulated by the basic leucine zipper (bZIP) transcription factor, DBP. On the other hand, the microsomal cytochrome P-450 enzyme, CYP8B1, is involved in the synthesis of cholic acid and it controls the ratio of cholic acid and chenodeoxycholic acid. Circadian expression of its mRNA is regulated by the suppressive effect of insulin. However, the present study found that the DBP expression levels were not affected by ADX as described below (Fig. 2). Serum insulin levels were continuously low in the ADX mice (Supplementary Figure 1 is available at www.dnaresearch.oxfordjournals.org). Thus, the ADX-induced down-regulation of CYP7A1 and CYP8B1 mRNA expression might due to the homeostatic regulation of bile acid synthesis, such as feedback inhibition of CYP7A1 and CYP8B1 expression by bile acids.

Glucokinase is a key enzyme of glycolysis and gluconeogenesis. Glucokinase mRNA levels are decreased when levels of plasma glucagon and insulin...
are high and low (e.g. fasting or diabetes), respectively.\textsuperscript{50,55} Transcription increases 20- to 30-fold within 30–60 min of an insulin injection into a diabetic rat, or after its addition to primary cultured hepatocytes.\textsuperscript{50} The loss of the circadian rhythmicity of glucokinase mRNA expression in the ADX mice might have resulted from an ADX-induced decrease in serum insulin levels throughout the day (Supplementary Figure 1 is available at www.dnaresearch.oxfordjournals.org). We therefore examined the effect of decreased endogenous insulin levels on the circadian expression of glucokinase mRNA using mice with STZ-induced diabetes (Fig. 4). The results demonstrated that glucokinase mRNA expression levels were remarkably reduced throughout the day in mice with STZ-induced diabetes (Fig. 4), although the circadian rhythm of serum corticosterone levels was maintained.\textsuperscript{24} Thus, the ADX-induced decrease in glucokinase mRNA expression in mice seems to be associated with a lack of insulin secretion.

The activity of glucose-6-phosphatase (G-6-Pase) (also a key enzyme of gluconeogenesis and glycolysis) shows a diurnal rhythm that increases during the day.\textsuperscript{56,57} However, its regulatory mechanism has remained unclear. We showed here that G-6-Pase mRNA levels were continuously low in the liver of ADX mice (Fig. 1). Reduced expression of the G-6-Pase mRNA in
the ADX mice suggested that gluconeogenesis is suppressed in ADX mice throughout the day.

The present study found that circadian expression of rate-limiting enzymes for glucose and cholesterol metabolism, such as glucokinase, G-6-Pase, HMG-CoA reductase, CYP7A1 and CYP8B1, was impaired by ADX. We cannot exclude the possibility that an ADX-induced decrease in food intake is responsible for the loss of circadian gene expression, because appetite parallels glucocorticoid levels both in humans and other animals. Nevertheless, the present results suggested that the circadian fluctuation of plasma glucocorticoids plays an important role in the time-keeping capability of an adaptive function that allows the prediction of mealtimes with consequently improved digestion and efficient energy usage.

The gene expression of FKBP51 (the 51 kDa FK506-binding protein) is induced by glucocorticoid in various cell lines and tissues. We previously showed the circadian expression of FKBP51 mRNA in the mouse liver. In the present study, ADX abolished its circadian expression (Fig. 1). Injecting ADX mice with Dex caused a transient induction of FKBP51 mRNA in the liver (Fig. 3). Thus, the circadian expression of FKBP51 might be directly regulated by endogenous glucocorticoid. FKBP51 down-regulates glucocorticoid signaling by binding to a dimer of heat-shock protein 90 (HSP90) that exists within a GR complex. Thus, FKBP51 might negatively regulate its own transcription by reducing the hormone sensitivity of GR. Therefore, the circadian expression of FKBP51 might affect glucocorticoid sensitivity at various times of day.

Polyamines are organic cations with multiple functions in cell proliferation and differentiation. Ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (SAMDC) are rate-limiting enzymes of polyamine biosynthesis. The circadian increase in ODC activity that is followed by that in mitotic activity in the liver has been understood since 1979. We showed here that SAMDC mRNA is expressed in the liver in a circadian manner and that such circadian expression was diminished in the ADX mice (Fig. 1). The circadian regulation of its mRNA seemed to be a direct effect of endogenous glucocorticoids, because Dex injection into the ADX mice transiently induced mRNA expression (Fig. 3). Attempts have been made to use specific inhibitors of SAMDC for cancer therapy since 1972, but polyamine-based drugs elicit toxic side effects. Further elucidation of the circadian regulatory mechanism of SAMDC expression might facilitate more effective chemotherapies by reducing side effects.

Lipin is the product of the Lpin1 gene that is mutated in mice with fatty liver dystrophy (fld) that exhibit several phenotypic abnormalities, including hyperlipidemia, defective adipocyte differentiation, impaired glucose tolerance and slow growth. Consistent with the lipodystrophic phenotype of fld mice, Lpin1 mRNA is prominently expressed in white and brown adipose tissues and in 3T3-L1 preadipocytes. In the ADX mice, the circadian mRNA expression of hepatic Lpin1 was abolished (Fig. 1) and transiently induced more than 10-fold by Dex injection (Fig. 3). This is the first report to describe the induction of Lpin1 mRNA by glucocorticoids. The circadian expression of the mRNA and the loss of rhythmicity caused by ADX were also evident in the adipose tissue as well as in the liver (data not shown), suggesting that glucocorticoids can affect adipocyte functions by...
regulating \textit{Lpin1} expression. Cushing’s syndrome is characterized by profound visceral adiposity and obesity as well as primary hypercortisolism.\textsuperscript{68} The mechanism for some physical features of this syndrome, such as dorsocervical-fat-pad enlargement that is called ‘buffalo hump’, remains unclear.\textsuperscript{69} Our results suggest that \textit{Lpin1} expression induced by hypercortisolism is related to the typical features of Cushing’s syndrome, such as central obesity and substantial fat deposition in the face, as well as supraclavicular and dorsocervical areas.

The serum amyloid A (SAA) family comprises differentially expressed apolipoproteins, acute-phase SAAs (A-SAAs) and constitutive SAAs (C-SAAs).\textsuperscript{70} Mouse \textit{SAA4} and the human \textit{SAA5} are the only known constitutively expressed \textit{SAA} genes.\textsuperscript{70} The present study, however, demonstrated the endogenous glucocorticoid-regulated circadian expression of \textit{SAA4} mRNA in the mouse liver (Fig. 1). Because the C-SAAs are associated with normal and acute-phase high-density lipoprotein (HDL),\textsuperscript{70} the circadian expression of \textit{SAA4} might affect the regulation of plasma HDL levels.

The functional presence of the SCN–adrenal connection is demonstrated by a light-induced fast decrease in plasma corticosterone that could not be attributed to a decrease in ACTH.\textsuperscript{71} Thus, the circadian expression of ADX-sensitive genes identified here might be affected by environmental lighting conditions via the glucocorticoid-mediated direct pathway.

We identified circadian oscillating genes of which the expression rhythms were abolished by ADX. Some of these genes were among the \textit{CLOCK}-regulated circadian output genes identified by microarray analyses using liver RNA isolated from homozygous \textit{Clock} mutant mice.\textsuperscript{11} For example, the circadian expression of \textit{glucokinase}, \textit{FKBP51}, \textit{Idb1} and \textit{Lpin1} is abolished in the liver of \textit{Clock} mutant mice\textsuperscript{11} and in that of ADX mice (present study). These circadian genes seemed to be indirectly affected by the \textit{Clock} mutation, because the circadian fluctuation of plasma corticosterone levels is obviously affected in homozygous \textit{Clock} mutant mice (Oishi, K., Ohkura, N., Shima, Y., Matsuda, J., Machida, K., Horie, S., and Ishida, N. manuscript submitted).

Among the 169 genes that fluctuated between day and night,\textsuperscript{69} were not affected by ADX (Fig. 2). These results were unpredicted, because the glucocorticoid hormones have been considered powerful time cues from the SCN to peripheral tissues.\textsuperscript{14} Figure 2 shows that the core clock component \textit{mPer2} and clock-controlled output genes,
such as mDec1, DBP, E4BP4 (shown as Nfil3 in Fig. 2A), Wee1 and USP2, were included, suggesting that endogenous glucocorticoids are not essential for the circadian expression of these genes. A comparison between the present and the previous11 data from microarray analyses suggested that among 69 genes rhythmically expressed in the liver of both sham-operated and ADX mice (Fig. 2), 13 were apparently transactivated by CLOCK protein. Actually, northern blot analyses revealed that the circadian expression of these genes was notably diminished (except for mDec1) in the livers of Clock mutant mice (Fig. 5), suggesting that the circadian expression of these genes is regulated by CLOCK protein and not affected by ADX.

The mRNAs of DEC1 and DEC2, which are negative regulators of bHLH transcription factors, are rhythmically expressed in the mouse SCN72,73 and peripheral tissues.74 They can repress CLOCK/BMAL1-induced transactivation of the mPer1 promoter and, therefore, might contribute to circadian clock regulation.75 The circadian expression of mDec1 mRNA is regulated by CLOCK in the SCN.76 However, CLOCK seems to be partly associated with its expression in the liver, because the rhythmic expression profile of mDec1 mRNA was slightly affected in the liver of homozygous Clock mutant mice (Fig. 5). In peripheral tissues, an autoregulatory negative feedback loop via the E-box elements located in the 5‘-flanking region of mDec1 gene75 might be maintained in the livers of Clock mutant mice.

The bZIP transcription factor, E4BP4, is expressed in a circadian manner in the SCN and peripheral tissues of mice.76 E4BP4 appears to act as a transcriptional repressor because it lacks a C-terminal PAR region.77 The phase of E4BP4 mRNA expression was almost opposite to that of DBP (Figs 2 and 5). The present study showed that the circadian expression of E4BP4 was not affected by ADX in the mouse liver (Fig. 2), although the mRNA expression is induced by glucocorticoids at least in vitro.78 Moreover, the expression levels of E4BP4 mRNA were continuously high in Clock mutant mice (Fig. 5), although they are continuously low in the liver of mCry1/mCry2 double knockout mice.76 These results and our present findings suggest that the circadian expression of E4BP4 mRNA is negatively regulated by some transcription factors, the expression of which is positively regulated by CLOCK protein.11 Antiphasic circadian expression of E4BP4 and DBP might augment the amplitude of downstream circadian genes via the common cis-element, because DBP activates and E4BP4 represses transcription.77

Glucocorticoids are not only important Zeitgebers for peripheral clock synchronization by the SCN14,15 but also strong homeostatic regulators of metabolic reactions against various environmental stresses. Circadian changes in metabolic reactions, such as gluconeogenesis/glycolysis and cholesterol biosynthesis, seem to be useful for normal sleep/wake cycles. However, organisms must adapt hepatic metabolic reactions to unusual environmental conditions, such as inflammation and starvation. Thus, the glucocorticoid-regulated gene expression of key enzymes for hepatic metabolism is apparently physiologically important for both circadian and homeostatic adaptation. On the other hand, the phase of endogenous clocks in peripheral tissues should be stable and resistant to daily stressors. In the present study, circadian expression of clock or clock-related genes, such as mPer2, DBP, mDec1 and E4BP4, remained almost intact in the liver of ADX mice. In addition, the steady-state phase of these genes was unaffected in the GR-deficient mouse liver.14 Terazono et al.79 reported that the stimulation of both

Figure 5. Temporal profiles of adrenalectomy-independent circadian expressing genes in livers of homozygous Clock mutant mice. Messenger RNA levels of genes were quantified from northern blots. Open and solid circles indicate mRNA levels in wild-type (WT) and homozygous Clock mutant mice, respectively. Maximal value of WT mice is expressed as 100%. Values are represented as means ± SE (n = 4).
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