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Clock genes regulate the feeding schedule-dependent diurnal rhythm changes in hexose transporter gene expressions through the binding of BMAL1 to the promoter/enhancer and transcribed regions

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Abstract

The expression of hexose transporter genes (SGLT1, GLUT5 and GLUT2) in mice with ad libitum feeding under light (7:00–19:00)–dark (19:00–7:00) cycle gradually increased from a basal level at 7:00 and reached a maximum at 19:00, coinciding with the start of dark phase feeding. The peaks of these gene expressions were shifted to 7:00 in mice that were subjected to a restricted feeding schedule from 9:00 to 17:00. The expression of BMAL1, a transcription factor driving the central feedback loop of the clock genes, was followed by the increase of hexose transporter gene expressions. The expressions of Per1-3, genes related to negative regulation of BMAL1, were the highest at or just after the time of maximal expression of the hexose transporter genes in both the group fed ad libitum and the restricted feeding group. Furthermore, chromatin immunoprecipitation assays revealed that the binding of BMAL1 to the promoter and/or transcribed regions of hexose transporters and Per 2 genes was associated with changes in their expressions. These results suggest that diurnal changes in expression of hexose transporter genes depend on the feeding schedule and are directly regulated by a feedback loop of clock genes.

Keywords: Hexose transporter; Diurnal rhythm; Clock genes; BMAL1; Feeding schedule; Small intestine

1. Introduction

Intestinal expression of genes related to hexose absorption in rodents exhibit diurnal periodicity with respect to feeding. Glucose uptake activity in rats fed ad libitum peaks late in the dark phase, or early in the light phase, and depends on the imposed feeding schedule rather than the light cycle [1–3]. As the food consumption pattern in rats fed ad libitum is established by the light cycle, the diurnal rhythmicity of glucose transport activity is "cued by feeding" rather than as the result of an inherent oscillatory behavior of the small intestine. Expressions of three hexose transporter genes: the sodium/glucose cotransporter (SGLT1) and glucose transporter type 5 (GLUT5), which transport hexose to enterocytes from the lumen, and glucose transporter 2 (GLUT2), which is a glucose/fructose/galactose transporter expressed in the basal membrane in enterocyte, peaks at the late light phase to early dark phase just prior to maximal glucose uptake. In addition, protein levels of SGLT1 and GLUT5, but less so for GLUT2, are associated with expression changes of these genes [4–6]. The expression of these genes is regulated by the imposed feeding schedule, because feeding rats only during the light phase shifts the peak of expression from late light-early dark phase to late dark-early light phase [3,7]. These results suggest that the diurnal rhythm of expression of intestinal hexose transporter genes is regulated by feeding rather than light, allowing adaptation to the greater amount of carbohydrate that needs to be digested and absorbed in the small intestine during the dark phase.

Recent studies have demonstrated that diurnal rhythms of gene expression were regulated by CLOCK and BMAL1 proteins, which are heterodimeric nuclear transcription factors. The heterodimer stimulates transcription of "negative regulators" [period 1, 2, and 3 (Per1–3) and cryptochrome 1 and 2 (Cry1–2)] by binding positive *cis*-elements, called E-boxes, located on their promoter/enhancer regions [8,9]. The protein products of these genes (Per1–3, Cry1–2) in turn oligomerize, enter the nucleus and suppress the activity of the CLOCK-BMAL1 heterodimer. Additionally, the transcription factor D site albumin promoter binding protein (DBP), is a positive-regulator for Per and Cry expressions, and E4BP4 is a negative-regulator of them. This negative feedback-loop is called the "core-feedback loop of clock genes" [10,11]. The nuclear receptor RAR-related orphan receptor (ROR) enhances BMAL1, CLOCK and E4BP4 gene expressions; on the other hand, Rev-erb is known to repress their expressions. This feedback mechanism for regulating BMAL1, CLOCK and E4BP4 genes expression is known as the "sub-loop of clock genes" [12,13]. Recent studies have shown that these feedback loops coordinately regulate diurnal rhythmical expression in the suprachiasmatic nucleus, which is known to regulate the central clock [14].

Recent studies also demonstrated that these clock genes are expressed rhythmically in peripheral organs in which they can presumably coordinate expression of a subset of tissue-specific genes,

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which might, in turn, impact directly on their physiological functions [9]. Although the central clock in the suprachiasmatic nucleus is light responsive and can be synchronized or reset by environmental cues, such as the light/dark cycle, peripheral clocks are unable to perceive light. These might be entrained by the central clock or independently by other physiological stimuli, such as feeding. Recent studies have demonstrated that feeding is one of the factors that regulate the diurnal rhythm of clock genes in the liver and colon [14]. However, it is not known whether expression of hexose transporters in the small intestine is regulated by these feedback loops of clock genes.

Based on the observation that the gastrointestinal tract is subjected to various 24-h rhythmic processes, we hypothesized that clock genes regulate expression of hexose transporter genes in the small intestine of the mouse. Our results suggest not only that diurnal changes in expression of hexose transporter genes depend on feeding schedule, but also that the expression of the hexose genes is directly regulated by the feedback loop of clock genes.

2. Material and methods

2.1. Animals

Seven-week-old male C57BL/6J mice (Japan SLC, Hamamatsu, Japan) were maintained under a light–dark cycle with 12 h of light and 12 h of darkness per day (light on 07:00, light off 19:00). Animals were divided into two groups. One group had free access to a standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan) for 2 weeks. The other group was subjected to a restricted feeding schedule; they had free access to the laboratory diet only for 8 h during the light period (9:00-17:00). The feeding restriction was performed by the removal of food pellets at 17:00 and re-feeding at 9:00 for 10 days. All animals had free access to drinking water. Samples from both groups were collected every 4 h throughout the whole diurnal cycle, starting at 11:00. The experimental procedure used in the present study met the guidelines of the Animal Usage Committee of the University of Shizuoka, Shizuoka, Japan.

2.2. Preparation of intestinal samples

The entire small intestine was removed and the jejunoileum was divided into two segments of equal length. The proximal segment of the jejunoileum was flushed twice with ice-cold 0.9% NaCl solution. One-cm segments (100 mg each) were excised from the middle region of the jejunal loop, and immediately used for RNA extraction. The remaining part of the jejunal loop was used for chromatin immunoprecipitation (ChIP) assays.

2.3. Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted by the acidified guanidine thiocyanate method, as described by Chomczynski and Sacchi [15]. The total RNA samples were stored at −80°C before use in quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Of the total RNA samples, 2.5 μg was converted into cDNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. To quantitatively estimate the mRNA levels and 18S rRNA (control), polymerase chain reaction (PCR) amplification was performed on a Light-Cycler instrument system (Roche Molecular Biochemicals, Tokyo, Japan). Real-time PCR reactions were carried out in a total volume of 20 μl containing 400 nM each of gene specific primers, cDNA and SYBR Premix Ex Tap (Takara, Shiga, Japan). The cycle threshold (CT) values of each gene and 18S rRNA detected by real-time RT-PCR were converted into signal intensities by the delta–delta method [16], which calculates the difference of one CT value as a twofold difference between each signal for each gene and the signal for a gene for normalization (18S rRNA). The formula used was $[2^{(\text{CT gene of interest} - \text{CT 18S rRNA})}]$. The sequences of the PCR primer pairs and the fragment sizes are shown in Supplemental Table 1.

2.4. Chromatin immunoprecipitation assay

The mucosa removed from the jejunum was incubated with fixation solution (1% formaldehyde, 4.5 mM Hepes pH 8.0, 9 mM NaCl, 0.09 mM EDTA, 0.04 mM EGTA) in phosphate-buffered saline (PBS) for 30 min at 37°C, before being terminated by the addition of glycine to a final concentration of 150 mM. After washing with [1×PBS (−) with 2% bovine serum and 0.05% NaN_3], the samples were sonicated in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)) in conditions to achieve a DNA size of 200–500 bp. One tenth of the volume of the sample was diluted in 10-fold dilution buffer (50 mM Tris–HCl pH 8.0, 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate, 0.5 mM PMSF) and stored at 4°C as the input fraction; the remaining solutions were divided into several tubes and incubated with 2 μg each of specific antibodies, i.e., anti-BMAL1, anti-RNA polymerase II (Pol II) or a control rabbit IgG. The protein–DNA–antibody complexes were immunoprecipitated by protein G Sepharose containing 100-μg/ml salmon sperm DNA and 1% bovine serum albumin in radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate) with 150 mM NaCl. Protein G Sepharose was washed once in RIPA buffer with 150 mM NaCl, seven times in RIPA buffer with 500 mM NaCl, twice in LiCl buffer (10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 1 mM EDTA pH 8.0, 0.5% NP-40, 0.5% sodium deoxycholate) and twice in TE pH 8.0 buffer. Protein–DNA–protein G sepharose complexes were obtained by immunoprecipitation, and the input fractions were subjected to dissociation of cross linkage in 200 μl ChIP direct elution buffer (10 mM Tris–HCl pH 8.0, 5 mM EDTA, 0.5% SDS, 300 mM NaCl) at 65°C for 16 h. After removal of RNA and protein by treatment with RNase A, proteinase K and phenol/chloroform extraction, the precipitated DNA was subjected to real-time PCR using primers corresponding to the indicated sites in the promoter/enhancer regions, transcription start region and transcribed region (Supplemental Table 2). The CT values of ChIP signals detected by real-time PCR were converted to the percentage of each ChIP signal for the input DNA, which were calculated by the delta–delta method [16], in that the difference of one CT-value between the CT-value for a ChIP signal and the input signal was calculated as a twofold difference. The formula used was $100\times[2^{(\text{CT IP sample} - \text{CT Input})}]$. All ChIP signals were normalized to the corresponding input signals.

2.5. Statistical analysis

Results were expressed as means±S.E.M. The significance of differences between groups was determined by Dunn's test based on Kruskal–Wallis's multiple range test, which involves nonparametric analysis. A level of $P<.05$ was considered to indicate statistical significance.

3. Results

3.1. Diurnal rhythm of gene expressions for hexose transporters in the jejunum of mice fed ad libitum

To explore whether intestinal genes for hexose transporters demonstrate diurnal rhythms in their gene expressions, we performed real-time RT-PCR using total RNA from the jejunum of the mice fed ad libitum. The expression of hexose transporter genes (SGLT1, GLUT5, and GLUT2) increased from basal levels at 7:00 to maximal levels at 19:00, decreasing thereafter until 3:00 (Fig. 1A).

3.2. Diurnal rhythm of expressions for clock genes in the jejunum of the mice fed ad libitum

Next, to explore whether clock genes are expressed and whether they exhibit diurnal rhythm in the small intestine of mice, we performed real-time RT-PCR for clock genes using total RNA from the jejunum of the mice fed ad libitum. First, we focused on the clock genes related to the core-loop of feedback mechanism. As shown in Fig. 1B, BMAL1 mRNA level peaked at 11:00; in contrast, BMAL2 mRNA level had a peak at 23:00. CLOCK mRNA levels did not change between 7:00 and 19:00, and its expression tended to increase at 23:00. Negative regulators for the BMAL-CLOCK heterodimer (such as Per1, Per2, Per3, Cry1 and Cry2) had a similar expression pattern, i.e. a peak at early/mid-dark phase (Per1, Per3 and Cry2 at 19:00, or Per2 and Cry1 at 23:00). The mRNA level of DBP, a positive transcriptional regulator for Per, was highest at 19:00 and declined until 3:00. mRNA expression of E4BP4, a negative regulator for Per, was higher during 23:00–11:00 than during 15:00–19:00. In the subloop of clock genes (Fig. 1C), RORα, RORβ, and RORγ, which are positive regulators for BMAL1, CLOCK and E4BP4, had a peak of expression at 23:00 [RORα (tendency), RORβ (tendency) and RORγ ($P<.05$)]. RORα and RORβ also tended to have a second peak at 11:00. The mRNA level of Rev-erbα, a negative regulator for BMAL1, CLOCK and E4BP4, was highest at 11:00, with expression gradually declining until 3:00. The gene expression of another subtype of Rev-erb, i.e., Rev-erbβ was higher during the period between 11:00 and 23:00 than between 3:00 and 7:00. CKIδ and CKIε, which are related to protein degradation for Per and Cry, tended to have higher mRNA levels during the period between 11:00 and 23:00 than between 3:00 and 7:00.

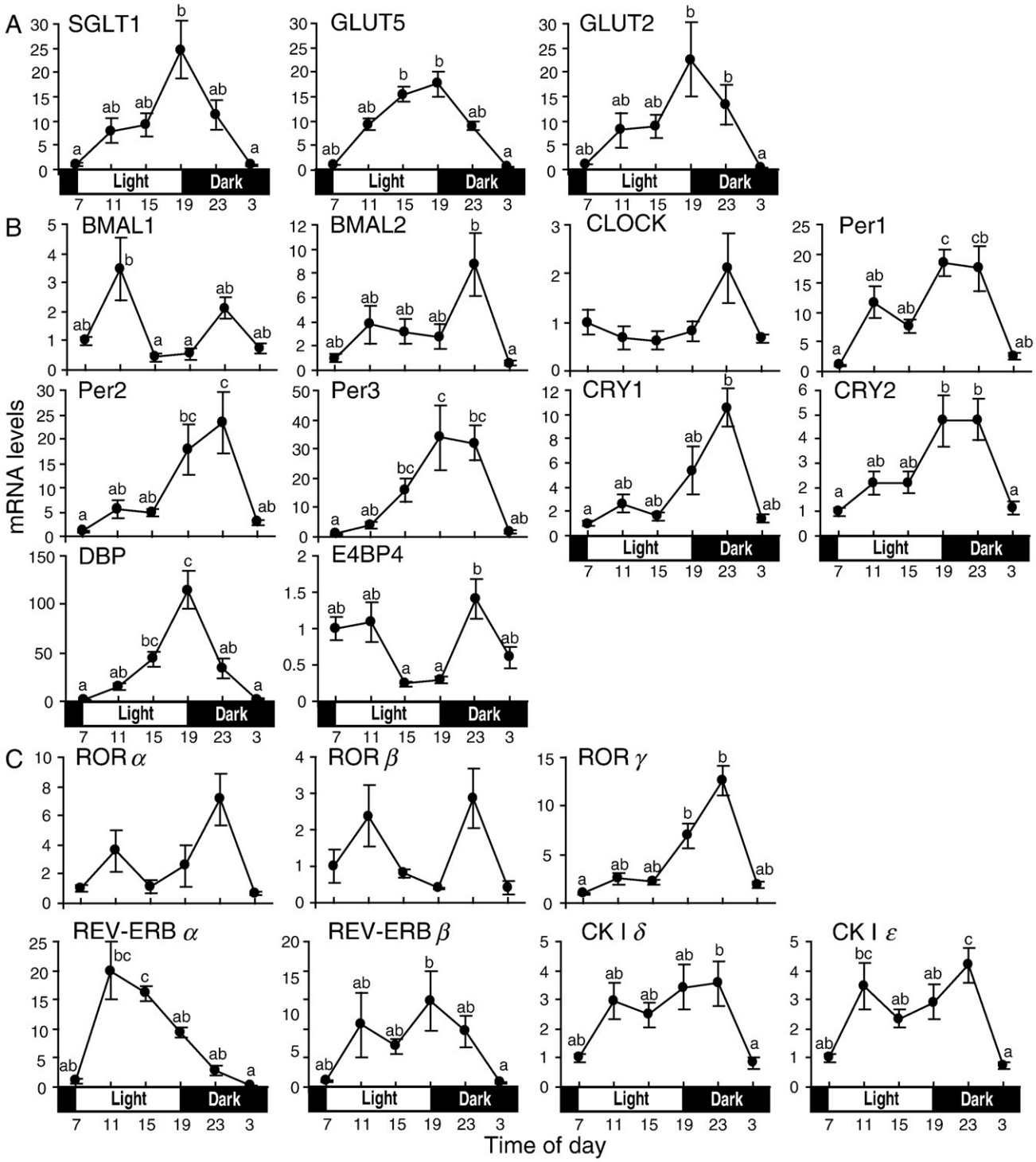

Fig. 1. Expression of jejunal genes in mice fed ad libitum. (A) Hexose transporters. (B) Core-loop feedback mechanism genes. (C) Sub-loop feedback mechanism genes. The open bar indicates the light period and the solid bar indicates the dark period. Total RNA was extracted from the jejunum and quantified by real-time RT-PCR. The mRNA levels were normalized to 18S rRNA abundance. Means±S.E.M. of seven to eight animals are shown. The mean of each mRNA at each time point was adjusted by the mean at time 7:00, which was regarded as 1. mRNA levels not sharing a common superscript (a,b,c) are significantly different ($P<.05$) from one another by Dunn's multiple range test.

3.3. Diurnal rhythm of gene expressions for hexose transporters in the jejunum of the mice undergoing feeding restriction

To examine which stimulus, feeding or light, alters jejunal gene expressions of hexose transporters in the diurnal rhythm, we performed real-time RT-PCR using total RNA from the jejunum of the mice with restricted food access [8 h during the light period (9:00–17:00)]. Hexose transporters (SGLT1, GLUT5 and GLUT2) showed a peak of mRNA levels at 7:00 with expression gradually decreasing until the nadir found between 15:00-23:00 [SGLT1 ($P<.05$), GLUT5 ($P<.05$) and GLUT2 (tendency)] (Fig. 2A).

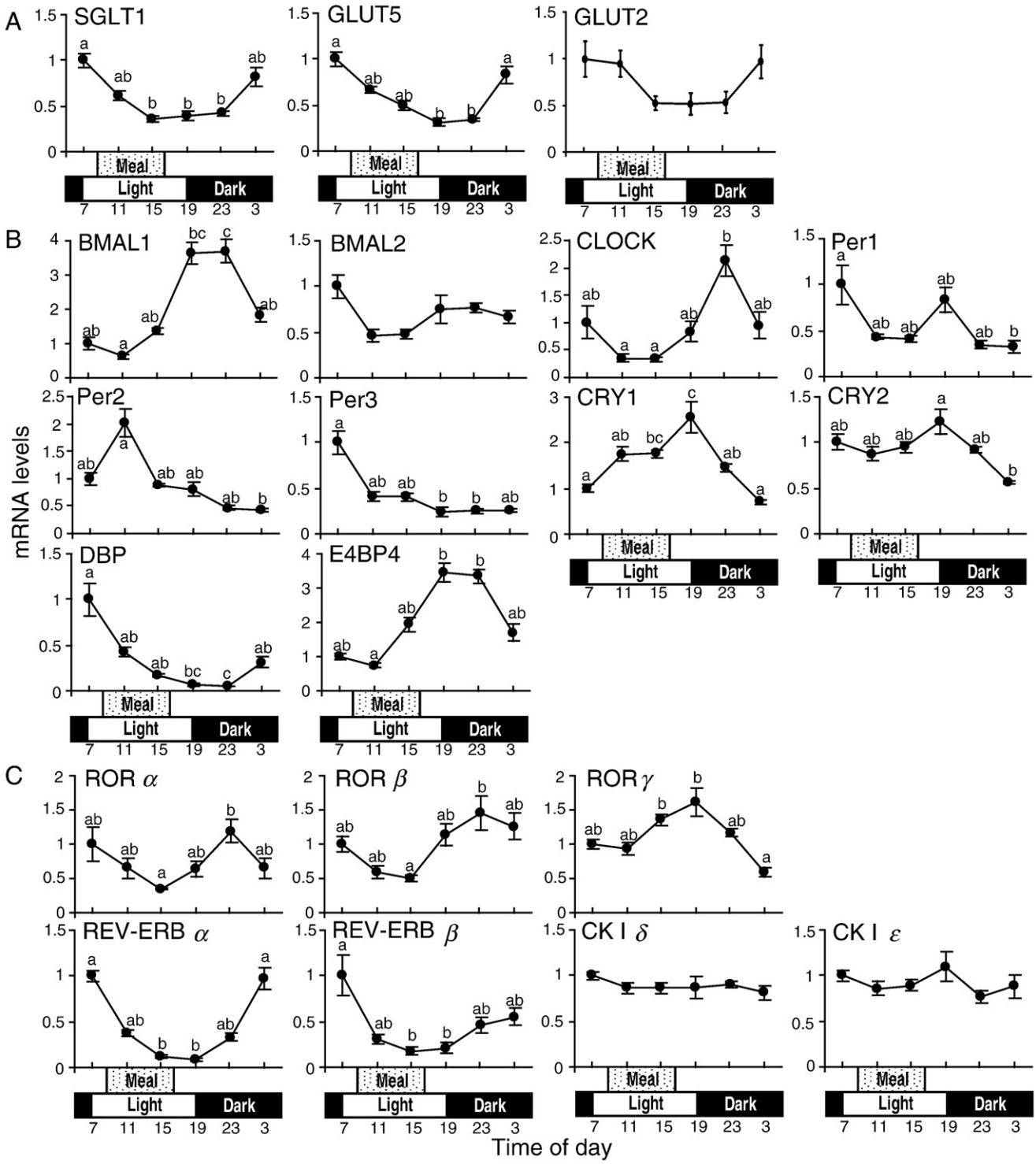

Fig. 2. Expression of jejunal genes in mice undergoing restricted feeding with access to food for only 8 hours in the light period (9:00–17:00). (A) Hexose transporters. (B) Core-loop of feedback mechanism genes. (C) Subloop of feedback mechanism genes. The restricted feeding schedule continued for 10 days. The open bar indicates the light period and the solid bar indicates the dark period. Total RNA was extracted from the jejunum and quantified by real-time RT-PCR. The mRNA levels were normalized to 18S rRNA abundance. Means±S.E.M. of seven to eight animals are shown. The mean of each mRNA at each time point was adjusted by the mean at time 7:00, which was regarded as 1. mRNA levels not sharing a common superscript (a,b,c) are significantly different ($P<.05$) from one another by Dunn's multiple range test.

3.4. Diurnal rhythm of expressions for clock genes in the jejunum of the mice undergoing feeding restriction

In the next study, we focused on the clock genes related to the core-loop of feedback mechanism in mice undergoing feeding restriction. The BMAL1 mRNA level rapidly increased from the lowest level at 11:00 to the highest level at 19:00–23:00. On the other hand, BMAL2 expression peaked at 7:00. CLOCK peaked at 23:00 and its expression decreased until 15:00. The expression of Per genes had peaks at 7:00 (Per1, Per 3) or 11:00 (Per 2) in early/mid light phase. Cry1 and Cry2

had a peak at 19:00. DBP mRNA level increased significantly from a basal level at 23:00 to the maximal level at 7:00. E4BP4 mRNA increased from a basal level at 11:00 until 19:00 (Fig. 2B).

For genes affecting the subloop of feedback mechanism of clock genes, the gene expression of subtypes of RORs had a peak at 19:00 (RORγ) or 23:00 (RORα, RORβ) in early/mid-dark phase. Rev-erbα and β were expressed at lower levels between 15:00–19:00; their expressions were elevated until 7:00. CKIδ and CKIε did not show clear peaks in gene expression at any time period during the day (Fig. 2C).

3.5. BMAL1 and RNA polymerase II binding to the hexose transporters and Per2 genes

To examine whether BMAL1 and RNA polymerase II (Pol II) bind to the promoter/enhancer and transcribed regions of the hexose transporter genes and whether such bindings are altered during diurnal periods, we performed ChIP assays using antibodies against BMAL1 and Pol II in mice fed ad libitum or undergoing restricted feeding. The ChIP signals for BMAL1 were detected at the −400-bp region and the region close to the transcription initiation site (+0) of

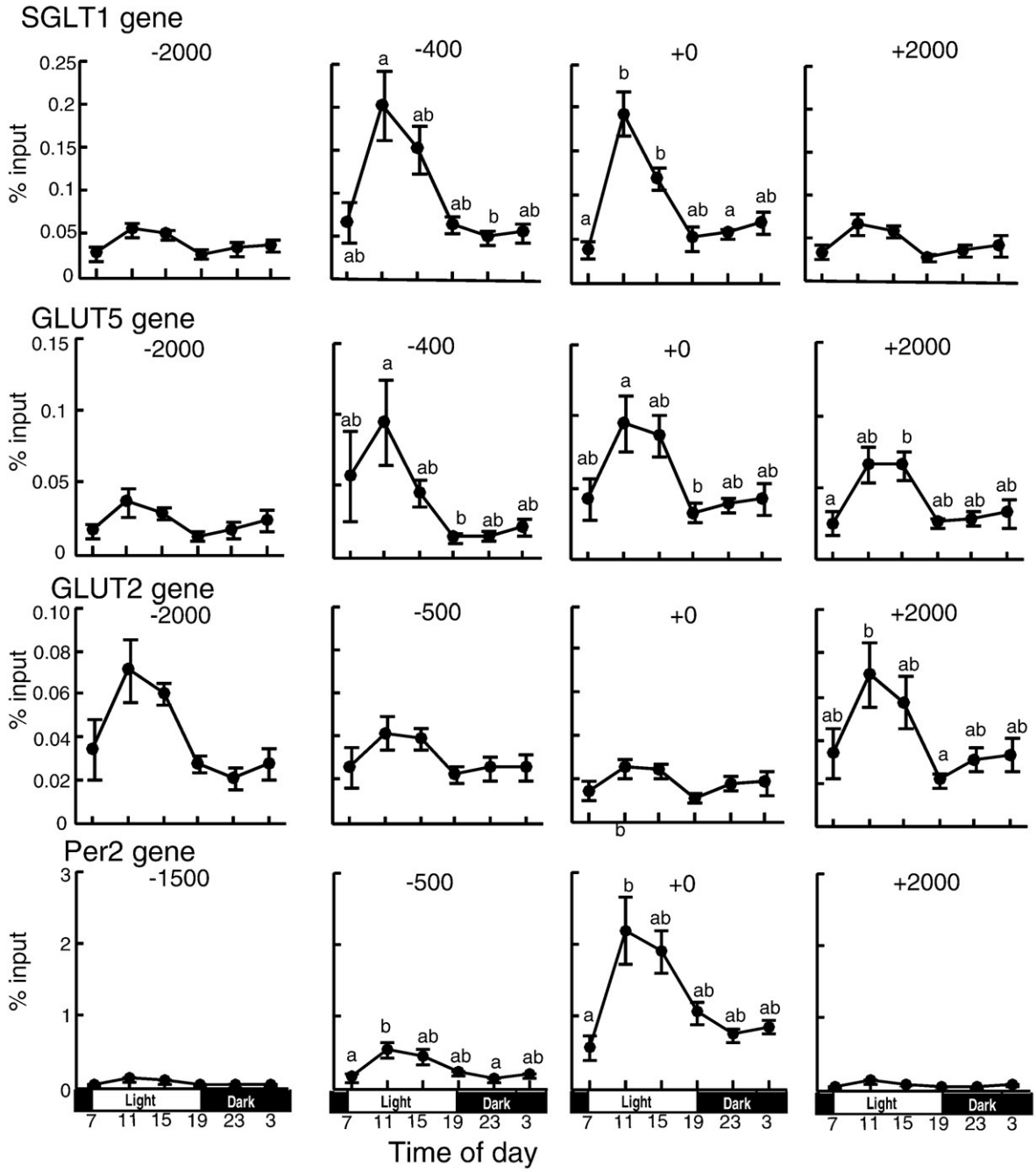

Fig. 3. The binding of BMAL1 to the promoter/enhancer and transcribed regions of hexose transporters and Per2 genes at various times of day in mice fed ad libitum. ChIP signals were detected by quantitative real-time RT-PCR and normalized to input signals. Genomic DNA, cross-linked to nuclear proteins in the mouse small intestine, was sonicated and precipitated by antibodies against BMAL1. The immunoprecipitated DNA fragments were amplified using the primers described in Supplemental Table 2 to detect the binding of BMAL1 to the indicated region on each gene, which is denoted relative to the transcription initiation site. Means±S.E.M. of seven to eight animals are shown. Signal levels not sharing a common superscript (a and b) on the same region of each gene are different from one another by Dunn's multiple range test.

the SGLT1 promoter, in the promoter/transcribed region from −400 bp to +2000 bp of the GLUT5 gene, in the regions of the promoter/enhancer (−2000 bp) and the transcribed region (+2000 bp) of GLUT2, and in the region close to the transcription initiation site (+0) of the Per2 gene (Figs. 3 and 5). Binding of Pol II was detected strongly around the transcription initiation sites in SGLT1, GLUT2 and Per2 genes, whereas on GLUT5, strong signals were detected around the transcribed region (+2000 bp) (Figs. 4 and 6). These ChIP signals were higher when using antibodies of BMAL1 and Pol II compared with normal IgG (Normal IgG signals were less than 0.03%). In addition, the ChIP signals for Pol II were higher in SGLT1 gene than in the GLUT5, GLU2 and Per2 genes. In the mice fed ad libitum, the ChIP signals for BMAL1 binding to the hexose transporters and Per 2 genes peaked at 11:00 and then declined to basal levels at 19:00 or 23:00 (Fig. 3), while in the mice undergoing restricted feeding, the ChIP signals peaked at 7:00 and then declined until 23:00 (Fig. 5). The ChIP signals for Pol II binding to the hexose transporter genes in the mice fed ad libitum had a peak at 11:00 and then decreased until 19:00 (Fig. 4), while those in the mice undergoing restricted feeding had a peak at 7:00 and then declined sharply until 11:00 (Fig. 6). The ChIP signals for Pol II binding to the Per2 gene in the mice fed ad libitum had a peak at 3:00 and then rapidly decreased until 7:00 (Fig. 4),

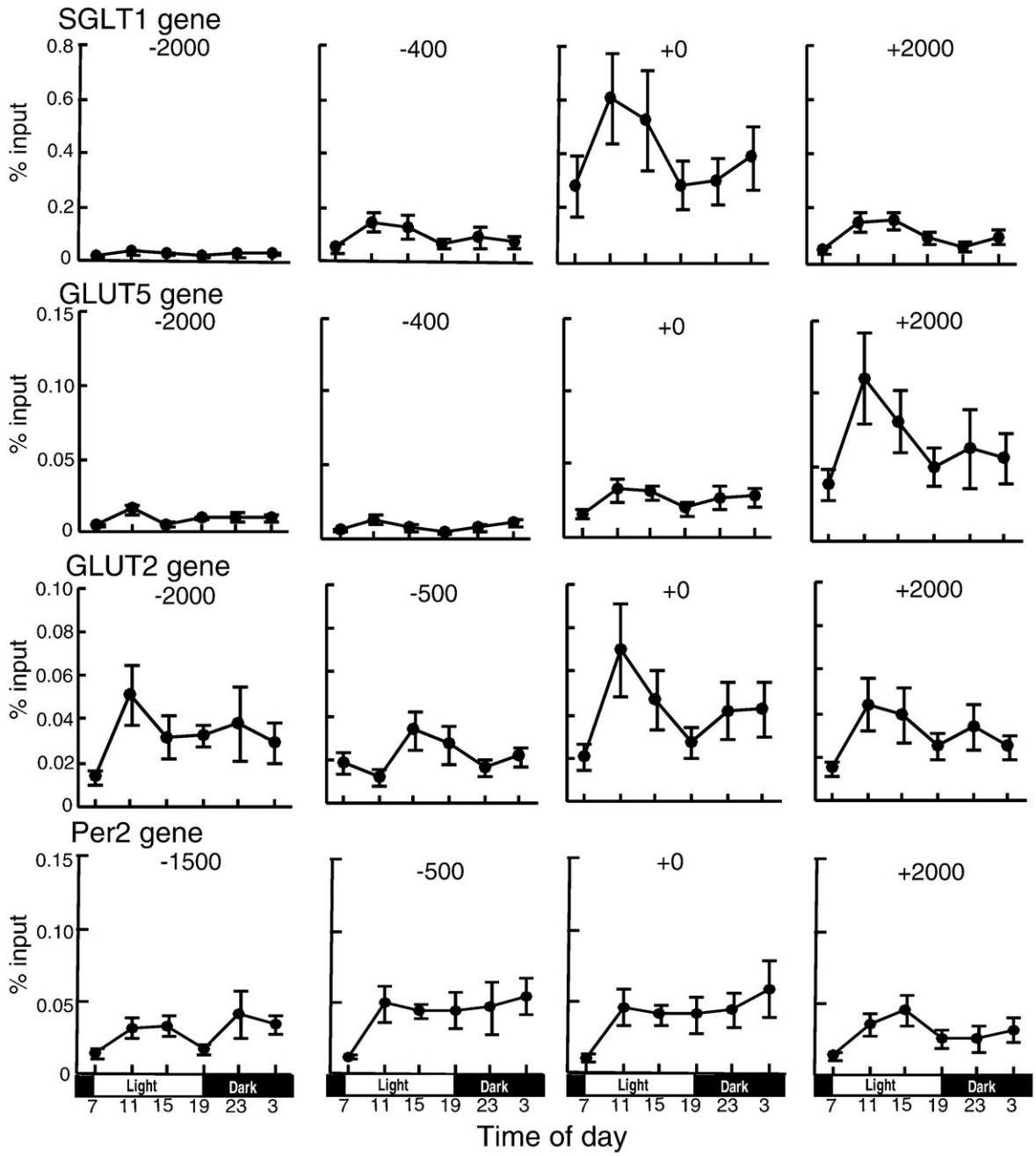

Fig. 4. The binding of Pol II to promoter/enhancer and transcribed regions of hexose transporters and Per2 genes at various times of day in mice fed ad libitum. ChIP signals were detected by quantitative real-time RT-PCR and normalized to input signals. Genomic DNA, cross-linked to nuclear proteins in the mouse small intestine, was sonicated and precipitated by antibodies against Pol II. The immunoprecipitated DNA fragments were amplified using the primers described in Supplemental Table 2 to detect the binding of Pol II to the indicated region on each gene, which is denoted relative to the transcription initiation site. Means±S.E.M. of seven to eight animals are shown.

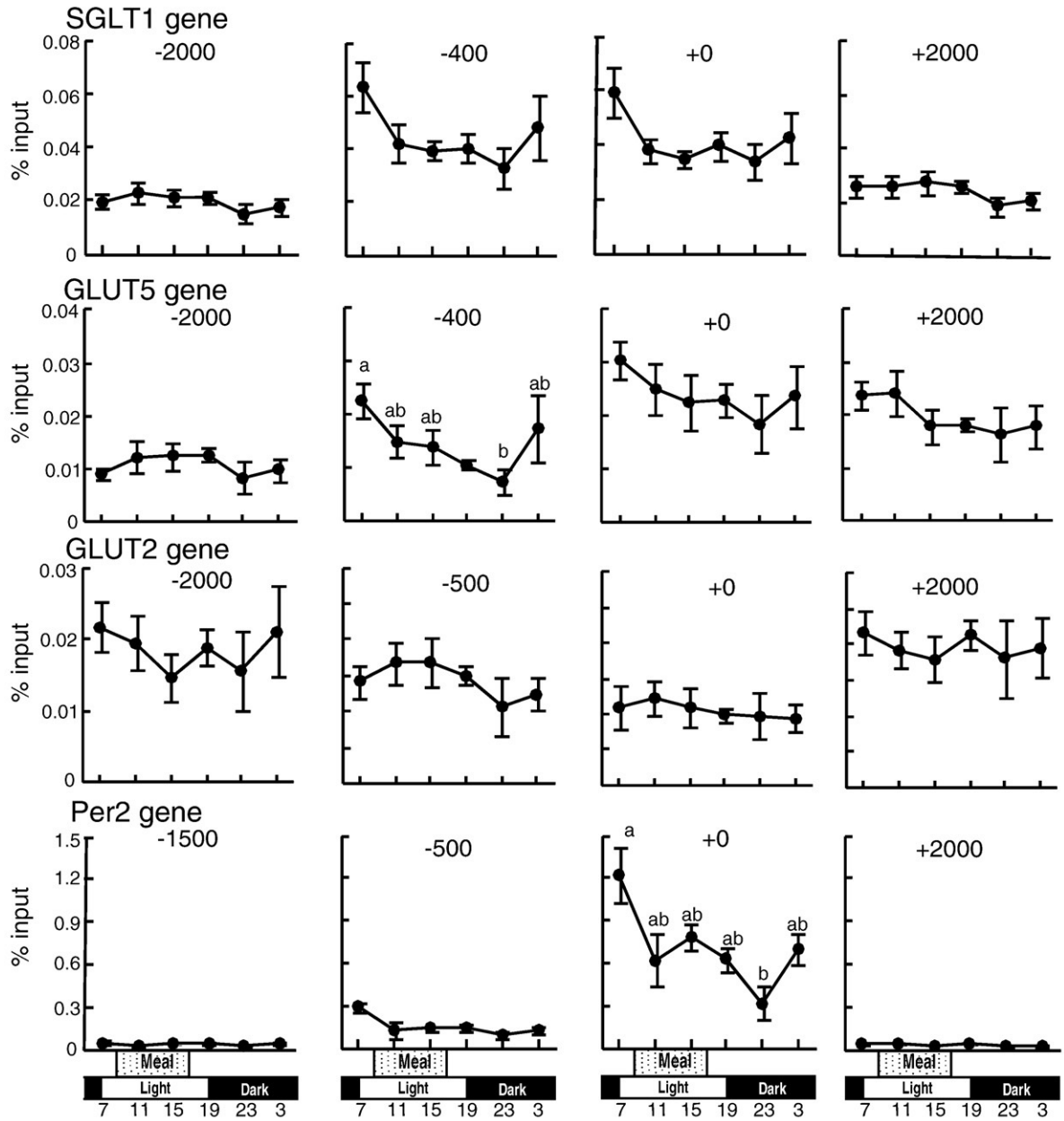

Fig. 5. The binding of BMAL1 to the promoter/enhancer and transcribed regions of hexose transporters and Per2 genes at various times of day in mice undergoing restricted feeding. ChIP signals were detected by quantitative real-time RT-PCR and normalized to input signals. Genomic DNA, cross-linked to nuclear proteins in the mouse small intestine, was sonicated and precipitated by antibodies against BMAL1. The immunoprecipitated DNA fragments were amplified using the primers described in Supplemental Table 2 to detect the binding of BMAL1 to the indicated region on each gene, which is denoted relative to the transcription initiation site. Means±S.E.M. of seven to eight animals are shown. Signal levels not sharing a common superscript (a and b) on the same region of each gene are different from one another by Dunn's multiple range test.

while those in the mice undergoing restricted feeding had a peak at 7:00 and then declined sharply until 23:00 (Fig. 6).

4. Discussion

Hexose transporters (SGLT1, GLUT5, GLUT2) show a diurnal rhythm in their expression in the small intestine of rodents [5,6]. In this study, we confirmed the results in mice (Fig. 1A). Additionally, we confirmed that the diurnal rhythm is regulated by the imposed feeding schedule (Fig. 2A). Recent studies suggest that the diurnal rhythm of gene expression is regulated by a feedback loop of transcriptional factors and their repressors, called “clock genes.” The feedback loop of clock genes regulates gene expression in peripheral tissues, as well as in the suprachiasmatic nucleus, which is known to be the location of the central clock [14,17,18]. The diurnal rhythm in peripheral tissues is believed to be regulated not only by the central clock, but also by other factors, such as feeding and secretion of hormones, although this is poorly understood. Recent studies have shown that feeding and hormones are factors for regulating the diurnal rhythm of some clock genes, as well as functional genes in tissues, such as the liver, stomach and colon [14,19–21]. However, there are no studies showing whether small intestinal gene expression is regulated by the feedback loop of clock genes.

As shown in Fig. 1B, BMAL1, an important transcription factor for driving the central feedback loop, is maximally expressed when the expression of many intestinal genes starts to increase (11:00) in mice

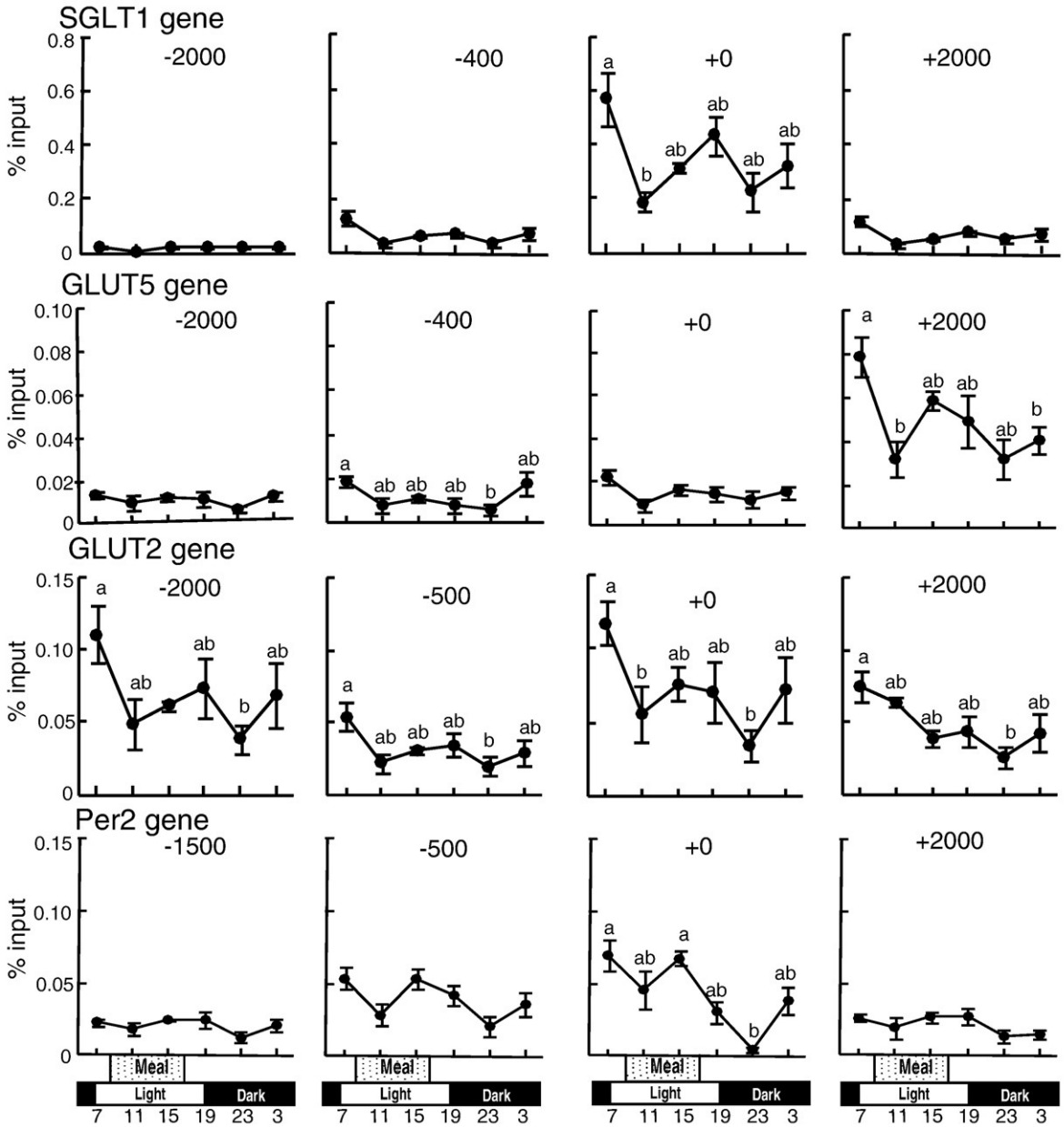

Fig. 6. The binding of Pol II to the promoter/enhancer and transcribed regions of hexose transporters and Per2 genes at various times of day in mice undergoing restricted feeding. ChIP signals were detected by quantitative real-time RT-PCR and normalized to input signals. Genomic DNA, cross-linked to nuclear proteins in the mouse small intestine, was sonicated and precipitated by antibodies against Pol II. The immunoprecipitated DNA fragments were amplified using the primers described in Supplemental Table 2 to detect the binding of Pol II to the indicated region on each gene, which is denoted relative to the transcription initiation site. Means±S.E.M. of seven to eight animals are shown. Signal levels not sharing a common superscript (a and b) on the same region of each gene are different from one another by Dunn's multiple range test.

fed ad libitum. Also, BMAL1 has a maximal peak at the period when the expression of repressors for the BMAL-CLOCK heterodimer (such as Per1, Per2, Per3, Cry1 and Cry2) start to increase, although another subtype of BMAL, BMAL2, has a peak of expression at the same time as these repressors. A previous study demonstrated that the protein level of Per2 in the small intestine was higher at the middle dark phase in an ad libitum feeding schedule [22]. Our results on the diurnal changes in expression of the Per2 gene in the ad libitum feeding schedule agree with the results of the previous study. Repressors such as Per1, Per2, Per3, Cry1 and Cry2 are known to be under the regulation of the BMAL-CLOCK heterodimer [8,9]. Thus, we speculate that BMAL1 is used to drive the central-loop of the small intestine of the mouse, whereas the function of BMAL2 is unclear. In addition, DBP, a positive regulator for Per and Cry, was highly expressed at the period just before the peak of expression of Per and Cry. Gene expression of E4BP4, a negative regulator for Per and Cry, was inversely associated with DBP gene expression in mice fed ad libitum (Fig. 1B). These results suggest that the central feedback loop of clock genes has a diurnal rhythm in the small intestine and is associated with changes in expression of intestinal genes for hexose transporters due to timing of feeding. RORα, RORβ and RORγ, which are activators for BMAL, CLOCK and E4BP4 genes expression, were inversely associated with gene expression of Rev-erbα, which is known as a repressor of BMAL, CLOCK and E4BP4 (Fig. 1C). Although these transcription factors (Rev-erb and ROR) show slightly different expression patterns in subtypes, these results indicate that the sub-loop of clock genes has a diurnal rhythm in the small intestine of the mouse.

Interestingly, the maximal expression of all genes in the core-loop that were tested, except for CLOCK, was shifted by feeding restriction. Particularly, BMAL1 was highly expressed at the period before food access (19:00–23:00), just prior to the increase in expression of the hexose transporter genes, as well as all Per subtypes. Additionally, the maximal expression of the DBP gene was shifted by feeding restriction from 19:00 to 7:00, at or just before maximal expression of Per subtypes. E4BP4 mRNA was inversely associated with DBP gene expression in the group undergoing feeding restriction (Fig. 2B). In the subloop of clock genes, a peak of Rev-erbα and Rev-erbβ gene expressions clearly shifted from the light phase in the group with free access to food, to the dark phase in the group undergoing feeding restriction (Fig. 2C). These results suggest that many genes in the diurnal rhythm of the core-loop and sub-loop of clock genes are regulated by the imposed feeding schedule rather than the light cycle. Since several genes, such as CLOCK, Cry1, Cry2, RORα, RORβ, RORγ, CKIδ and CKIε, did not show clear shifts of expression peaks under feeding restriction, these genes might be regulated by the light cycle rather than the imposed feeding schedule. Further studies are needed to investigate whether the expression of these diurnal rhythm-related genes changes in mice undergoing inversion between light and dark times or undergoing destruction of the suprachiasmatic nucleus.

To confirm whether these feedback loops of clock genes directly regulate diurnal rhythm of hexose transporter genes expression, we performed ChIP assays on hexose transporter genes using antibodies against BMAL1 and Pol II. ChIP assay using antibodies enables us to observe in vivo protein binding to DNA on a chromosome. Surprisingly, BMAL1 strongly bound to the −400 bp region of the SGLT1 promoter and around the transcription initiation site (+0), to the promoter/transcribed region from −400 bp to +2000 bp of the GLUT5 gene, to the regions of the promoter (−2000 bp) and transcribed region (+2000 bp) of GLUT2 gene during the period (11:00–15:00 in the group fed ad libitum and 3:00–7:00 in the group undergoing feeding restriction) when hexose transporter genes' expressions started to increase (Figs. 3 and 5). It also bound to the Per2 promoter region during the period. These bindings were also associated with Pol II binding on hexose transporter genes in both groups (Figs. 3 and 4). It should be noted that Pol II binding was observed strongly in the transcribed region of the GLUT5 gene, which is associated with an mRNA elongation reaction [23]. Thus, diurnal changes of GLUT5 gene expression would be strongly regulated at the level of mRNA elongation. In addition, it should be stated that Pol II binding to Per2 in the ad libitum feeding schedule group was high at 3:00, even though the mRNA level at that time was low. It is probable that other transcriptional/elongation complexes related to the Pol II activity on the Per2 gene were released at 3:00; however, this hypothesis needs to be examined by further study. It should be noted that diurnal changes of BMAL1 and Pol II on these genes were lower in the restricted feeding schedule group than in the ad libitum feeding schedule group. In addition, the magnitude of the response of mRNAs during the day was higher in the ad libitum-feeding schedule than in the restricted feeding-schedule; particularly for genes such as hexose transporters, BMAL2, Per1, Per2, Per3, CRY1, CRY2, RORα, RORβ and RORγ. These results suggest that light stimulation might also concerned with binding of BMAL1-Pol II on the hexose transporters and Per2 genes, and with diurnal rhythmical expression of hexose transporters and clock genes in the jejunum. Further study is required to determine whether light stimulation affects diurnal rhythmical expression of these genes and binding of BMAL1 on these genes in the jejunum. The promoter of the Per2 gene contains many E-box motifs, which are the binding sites for the BMAL1-CLOCK heterodimer [24]. Our results indicate that the BMAL1-CLOCK heterodimer directly binds to the promoter/enhancer and transcribed regions of the hexose transporter genes as well as to the Per 2 gene. Indeed, we found an E-box sequence from −158 to −152 bp (upstream region) of SGLT1, from +598 to +604 bp (second exon) of GLUT5 and from −1747 to −1742 bp and from +1874 bp to +1879 bp (upstream region and first intron, respectively) of GLUT2, as measured from the transcription initiation site in these genes. Around the sequences of these genes, we detected strong BMAL1-binding, although it is not clear whether it is functional. It should be investigated whether the BMAL1-CLOCK heterodimer binds these putative E-box elements on the hexose transporter genes in vitro and whether these bindings are disrupted by mutating the putative binding sites on these genes.

In conclusion, our results suggest that the diurnal changes in expression of the hexose transporter genes, which depend on the feeding schedule in the small intestine, are directly regulated by the core-feedback loop of clock genes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2010.02.012.

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