Regulatory mechanism governing the diurnal rhythm of intestinal H⁺/peptide cotransporter 1 (PEPT1)

Hirofumi Saito, Tomohiro Terada, Jin Shimakura, Toshiya Katsura, and Ken-ichi Inui

Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto, Japan

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Saito H, Terada T, Shimakura J, Katsura T, Inui K. Regulatory mechanism governing the diurnal rhythm of intestinal H⁺/peptide cotransporter 1 (PEPT1). Am J Physiol Gastrointest Liver Physiol 295: 395–402, 2008. First published June 26, 2008; doi:10.1152/ajpgi.90317.2008.—The intestinal H⁺/peptide cotransporter 1 (PEPT1) plays important roles as a nutrient and drug transporter. Previously, we reported that rat intestinal PEPT1 showed a diurnal rhythm and that this rhythm is closely related to the feeding schedule. Furthermore, we also demonstrated that transcription factors, Sp1, Cdx2, and peroxisome proliferator-activated receptor-α (PPAR-α) contribute to the basal, intestine-specific, and fasting-induced expression of PEPT1, respectively. In this study, to clarify the molecular mechanism governing the diurnal rhythm of PEPT1 expression, we compared expression profiles of these transcription factors under two kinds of feeding schedules. The intestinal Sp1 and Cdx2 did not show a circadian accumulation of mRNA or response to the daytime feeding regimen. Plasma free fatty acids, endogenous PPAR-α ligands, exhibited a robust circadian fluctuation in phase with that of PEPT1. However, subsequent experiments using PPAR-α-null mice revealed the absence of any association between the circadian rhythm of PEPT1 and PPAR-α. We then focused on the clock genes (Clock, Bmal1, Per1–2, and Cry1) and clock-controlled gene, albumin D site-binding protein (DBP). A robust and coordinated circadian expression of the clock genes was observed, and daytime feeding entirely inverted the phase except for Clock. The expression of DBP was in phase with that of PEPT1 in both groups. Electrophoretic mobility shift assays and reporter assays revealed that DBP has the ability to bind the DBP binding site located in the distal promoter region of the rat PEPT1 gene and induce the transcriptional activity. These findings indicate that DBP plays pivotal roles in the circadian oscillation of PEPT1.

SLC15A1; albumin D site-binding protein; clock gene; restricted feeding; small intestine

THE DEGRADATION OF INGESTED proteins results in a mixture of free amino acids and small peptides. Several studies have shown that these proteins are predominantly absorbed as di- and tripeptides rather than amino acids in the small intestine (14). H⁺-coupled peptide transporter (PEPT1) is located at the brush-border membrane of intestinal epithelial cells and mediates cellular uptake of these oligopeptides by using an inwardly directed H⁺ gradient across the brush-border membrane (5). Its broad recognition of substrates allows it to transport several pharmacologically active drugs, such as oral β-lactam antibiotics, the anticancer agent bestatin, and angiotensin-converting enzyme inhibitors (31). Moreover, targeting intestinal PEPT1 is clearly beneficial for improving the intestinal absorption of some drugs such as the antihypotensive drug midodrine (33). Thus PEPT1 plays important roles in the physiological, clinical, and pharmaceutical settings.

PEPT1 is regulated by various factors such as fasting and diurnal rhythm (1, 2, 10, 16–18), but the molecular mechanism behind its transcriptional regulation has not been well elucidated. Previously, we demonstrated that the transcription factor Sp1 contributes to the basal transcriptional regulation of PEPT1 (22). Additionally, we identified a transcription factor, caudal-related homebox protein 2 (Cdx2), involved in the intestine-specific expression of PEPT1 through interaction with Sp1 (24). As a physiological factor, starvation markedly increases the amount of mRNA and protein of PEPT1 in rats (10, 32), leading to altered pharmacokinetics of the substrates of PEPT1 (17). This fasting-induced upregulation of PEPT1 expression was mediated via a nuclear receptor, peroxisome proliferator-activated receptor-α (PPAR-α), with its endogenous ligands, free fatty acids (23). However, the molecular mechanism for transcriptional regulation of the diurnal rhythm of PEPT1 remains unclear.

Most organisms from eukaryotes to some prokaryotes show daily changes in physiology and behavior controlled by autonomous time-measuring oscillators called circadian clocks. In mammals, a dominant pacemaker resides in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The clock system consists of single-cell circadian oscillators that are composed of several clock genes (20). Genetic and molecular analyses have revealed that several clock genes form a transcription and translation-based negative feedback loop; the positive arm includes the transcriptional regulator Clock, Bmal1, whereas the negative arm includes three Period (Per1 and Per2) proteins and Cryptochrome (Cry1) protein acting as inhibitors of Clock/Bmal1 (8, 11, 20). Albumin D site-binding protein (DBP), the circadian expression of which is regulated by Clock/Bmal1-mediated activation and mPer- and mCry-mediated suppression through E-box motifs (CACGTG), is closely linked to the core circadian clockwork since it can additionally activate mPer1 gene expression (34). In mammals, these circadian clocks exist not only in the SCN but also in most peripheral tissues and cells, and the autonomous oscillations seem to be organized in a hierarchical manner. On this note, long-term peripheral circadian gene expression is attributed to a functional SCN pacemaker because robust cycling expression of transcripts could not be detected in SCN-lesioned mice (37). The central circadian clock in the SCN dominantly utilizes the solar light-dark (LD) cycle for adapting its circadian expression to the exact 24-h period of the day (20). On the other hand, peripheral clocks themselves are not responsive to...
LD cycles, rather, they are capable of responding to other nonphotic cues, for example, restricted feeding regimens (27). When nocturnal laboratory rodents are forced to eat only during the day, the phase of peripheral gene expression in many tissues is inverted completely in the course of about 1 wk, whereas SCN oscillators are refractory, suggesting that food is a dominant Zeitgeber for peripheral clocks (4).

A daily periodicity in intestinal transport activity and several digestive proteins has been well documented (7, 26). Previously, we demonstrated that the function and expression of intestinal PEPT1 had a diurnal rhythm with a peak near the onset of darkness in rats feeding ad libitum (17) and that this diurnal rhythm was inverted by daytime (0900 to 1500)-restricted feeding (18) though the mechanism underlying the circadian expression remains to be clarified. The aim of the present study is to clarify the molecular mechanism governing the diurnal rhythm of intestinal PEPT1 expression by comparing the daily fluctuations in intestinal PEPT1 mRNA expression with those of several transcription factors, Sp1, Cdx2, PPAR-α, and clock genes in rats on two kinds of feeding schedules.

MATERIALS AND METHODS

Materials. DBP recombinant protein was purchased from Abnova (Taipei City, Taiwan). The pCMV-DBP expression vector was purchased from OriGene Technologies (Rockville, MD), and restriction enzymes were from New England BioLabs (Beverly, MA). All other chemicals used were of the highest purity available.

Animals. Animal experiments were conducted in accordance with the Guidelines for Animal Experiments of Kyoto University. All protocols were previously approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. Male Wistar rats (7 wk old) were obtained from Japan SLC (Shizuoka, Japan). Male PPAR-α-null mice (B6.129S4-PPAR-αtm1Gonz N12) and wild-type mice (C57BL/6) (5–6 wk old) were purchased from Taconic (Germantown, NY). Rats and mice were kept under a 12-h LD schedule (0800–2000) and fed ad libitum at least 1 wk before the experiments. Rats were randomly divided into two groups; half were fed only during the daytime (0900–1500) (daytime feeding), and the other half were fed ad libitum (normal feeding) for 10 days. Water was freely available throughout all experiments. Animals were euthanized by cervical dislocation. The intestinal mucosa was then scraped and rapidly frozen in liquid nitrogen for the later preparation of total RNA.

Real-time PCR analysis. Real-time PCR analysis was performed according to our previous study (23). Briefly, isolated total RNA of intestinal mucosa was reverse transcribed, and the resultant reaction mixtures were used for real-time PCR. Real-time PCR was performed with an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). The forward and reverse primers for rat DBP were 5′-CCGAGGAACACTCCCCACAGACGTGT-3′, and 5′-TCTTTGCGTCTCTGACCTTCTT-3′, respectively. The sequence of the TaqMan probe for rat DBP was 5′-(FAM)TACTGGACCCGGAGGTATCAAGAACATGAA-(TAMRA)-3′. The primer probe sets for rat and mouse PEPT1 were designed previously (23). The primer probe sets used for rat PPAR-α, Sp1, Cdx2, Clock, Bmal1, Per1, Per2, and Cry1 were predeveloped TaqMan Assay Reagents (Applied Biosystems). The mRNA expression level of GAPDH was also measured as an internal control with GAPDH Control Reagent (Applied Biosystems). Two or three experiments with replicated methods were conducted, and representative results are shown.

Determination of the plasma free fatty acid level. Immediately before the isolation of the small intestine, whole blood was collected and placed on ice before being centrifuged for 10 min at 3,000 revolution/min. With the supernatant, the plasma concentration of free fatty acid was quantified by an enzymatic colorimetric assay (free fatty acid, Half-micro test; Roche Diagnostics, Penzberg, Germany).

Construction of reporter plasmids. For the reporter assay, the 2,268-bp flanking region upstream of the putative transcription start site of the rat PEPT1 gene was amplified by PCR from rat genomic DNA (Promega, Madison, WI). The PCR product was isolated by electrophoresis and subcloned into the firefly luciferase reporter vector, pGL3-Basic (Promega), at Xhol and HindIII sites. This reporter plasmid is hereafter referred to as −2,268/+19. The 5′-deleted regions (−1,556/+19, −821/+19, −392/+19, −128/+19, and −27/+19) were prepared by PCR from −2,268/+19 and subcloned into the same sites of pGL3-Basic as above. The 5′-flanking regions −8,992/−6,830 and −7,014/−4,844 of the rat PEPT1 gene were prepared in the same way as above and subcloned directly into −392/+19 at KpnI and Xhol sites or a Xhol site, respectively. The oligonucleotide sequences of primers used for the construction of reporter plasmids are summarized in Table 1. The nucleotide sequences of these constructs were confirmed with a multicapillary DNA sequencer RISA384 system (Shimadzu, Kyoto, Japan).

Cell culture, transfection, and reporter gene assay. Caco-2 cells were obtained from the American Type Culture Collection (ATCC CRL-1392) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Caco-2 cells were plated into 24-well plates (3 × 10^5 cells/well) and transfected the following day with 50 ng of the reporter constructs, a total of 500 ng of expression vector and 1 ng of the

Table 1. The oligonucleotide sequences of the primers

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′-3′)</th>
<th>Added Recognition Site For</th>
</tr>
</thead>
<tbody>
<tr>
<td>rPEPT1-2268F</td>
<td>AGCTCGAGAACACTCCCCACAGACGTGT</td>
<td>XhoI</td>
</tr>
<tr>
<td>rPEPT1+19R</td>
<td>AGAGGCTTACTGCGAACAGGAGTCTTACG</td>
<td>HindIII</td>
</tr>
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<td>XhoI</td>
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<td>XhoI</td>
</tr>
<tr>
<td>rPEPT1-8992F</td>
<td>AGCTGACATGCTGGATTATGGAAGTCTTCA</td>
<td>KpnI</td>
</tr>
<tr>
<td>rPEPT1-821F</td>
<td>AGCTGACATGCTGGATTATGGAAGTCTTCA</td>
<td>XhoI</td>
</tr>
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Recognition sites of restriction enzymes are underlined. rPEPT1, rat peptide cotransporter 1.
Renilla reniformis vector, pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen Japan, Tokyo, Japan) according to the manufacturer’s recommendations. The total amount of DNA per well was adjusted by adding the vector pCMV6-XL5 (OriGene). The medium was changed after 24 h. The firefly and Renilla activities were determined 48 h after the transfection with a dual luciferase assay kit (Promega) and a Mithras LB940 luminometer (Berthold, Bad Wild- baden, Germany). The firefly activity was normalized to the Renilla activity. Three or four experiments with replicated methods were conducted, and representative results are shown.

Electrophoretic mobility shift assays. The probes were prepared by annealing complementary sense and antisense oligonucleotides, followed by 3′-end labeling with digoxigenin (DIG) -11-ddUTP using terminal transferase (Roche Diagnostics) according to the manufacturer’s instructions. The binding mixture consisted of 8 mM HEPES (pH 7.9), 40 mM KCl, 0.4 mM MgCl2, 6.8% glycerol, 0.1 μg/μl of BSA, 0.8 mM DTT, 0.2 mM PMSF, 10 ng/μl of poly(dI-dC), and 50 ng of recombinant DBP protein. After preincubation on ice for 15 min, labeled probes (0.4 ng) were added and the binding mixture was incubated for a further 60 min at 30°C. The DNA-protein complex was then resolved on a 4% polyacrylamide gel in 0.5× Tris borate EDTA buffer. The DNA-protein complex was transferred to a positively charged nylon membrane (Hybond-N+; GE Healthcare UK, Buckinghamshire, UK) by electroblotting, followed by UV fixation of the oligonucleotide. The labeled oligonucleotide was detected using the DIG Luminescent Detection Kit (Roche Diagnostics).

Statistical analysis. Data are expressed as the means ± SE. The statistical significance of differences among groups was analyzed with the one-way ANOVA followed by Dunnett’s post hoc test. A 5% level of probability was considered significant.

RESULTS

Effect of restricted feeding on diurnal rhythm of intestinal PEPT1. At the end of entrainment periods (fed ad libitum, normal feeding, or fed only from 0900 to 1500, daytime feeding for 10 days), the phase of mRNA expression for intestinal PEPT1 was assessed using real-time PCR. As shown in Fig. 1, normal feeding rats showed a robust diurnal rhythm with a peak at 2000. Feeding restriction during daytime entirely shifted the peak of the rhythm from 2000 to 0800. These findings are consistent with our previous results of Northern blot analyses (18).

Daily variations in the expression of various transcription factors for PEPT1 and in the plasma level of free fatty acids. We previously identified three kinds of transcription factors, Sp1, Cdx2, and PPAR-α, as important regulators for transcription of the PEPT1 gene (22–24). To determine whether these transcription factors display circadian rhythm and response to daytime feeding, daily variations in their mRNA expression in rat small intestine were examined. As shown in Fig. 2, Sp1, which is involved in the basal transcriptional regulation of PEPT1, showed undetectable daily changes of mRNA expression, and daytime feeding had no effect on it. Similarly, mRNA expression of the intestine-specific transcription factor Cdx2 had no circadian rhythm and no responsiveness to daytime feeding. The amounts of intestinal PEPT1 mRNA and protein are markedly increased by fasting via the nuclear receptor PPAR-α with its endogenous ligands, free fatty acids. The accumulation of the mRNA of intestinal PPAR-α displayed slight diurnal rhythmicity with a peak at 2400 in the normal feeding condition. However, this rhythm was dampened by daytime feeding. Interestingly, daily variations in the plasma free fatty acid concentration were similar to those in the PEPT1 mRNA level in normal feeding rats. Furthermore, restricted feeding during daytime completely inversely the phase with a marked elevation at 0800, which corresponded to the peak of PEPT1 in daytime feeding.

PPAR-α deficiency could not impair PEPT1 diurnal rhythm in mouse small intestine. Previous study showed that fasting-induced PEPT1 expression was accompanied by a rise in serum free fatty acid levels, and this elevation contributes to the increase in PEPT1 mRNA expression by providing ligands for PPAR-α (23). Because the rats that fed only in the daytime (from 0900 to 1500) were in a temporary state of starvation at 0800, the coinciding of the highest increment in plasma free fatty acids with that in PEPT1 mRNA at 0800 suggested the possible involvement of PPAR-α in the diurnal rhythm of PEPT1. Supposing that PPAR-α plays a pivotal role in the regulation of the diurnal rhythm PEPT1, we then assessed the contribution of PPAR-α to the circadian regulation of PEPT1 using wild-type and PPAR-α-null mice. Mice were kept under the same conditions as normal feeding rats. In the mice, the plasma free fatty acid concentration fluctuated in a circadian manner and increased at 1600 with no change in the phase of rhythm between the genotypes (Fig. 3). In wild-type mice, intestinal PEPT1 mRNA expression was also rhythmic in a circadian manner with a peak at 1600. Although the PPAR-α-null mice showed lower basal levels of PEPT1 mRNA, the diurnal rhythm of PEPT1 displayed a similar phase angle to that of wild-type mice (Fig. 3). These results suggest that PPAR-α and plasma free fatty acids were not responsible for the diurnal rhythm of intestinal PEPT1 expression.

Circadian expression of clock genes in normal and restricted feeding schedules. We next focused on the clock genes as candidates for liable regulators of the diurnal rhythm of PEPT1. First, to consider the relevance between the phase of PEPT1 and that of several clock genes, the daily variation in the intestinal mRNA expression of clock genes and the effect of feeding time on them were determined (Fig. 4). A rhythmic expression was observed for all clock genes tested, Clock, Bmal1, Per1, Per2, and Cry1, and the clock-controlled gene DBP in the small intestine of normally fed rats. The mRNA expression of genes encoding positive regulators (Clock and
Bmal1) was in peak at the onset of daytime, 0800. In contrast, the rhythm of genes encoding negative regulators (Per1 and Per2) was in the opposite phase to that of Clock and Bmal1, namely, a zenith at around 2000 and a nadir at 0800. These results were in agreement with current molecular models of clock circuits depicted mostly in the SCN and in the liver or in other peripheral tissues (25, 35). In particular, the expression of the clock-controlled output gene DBP showed an overt circa-dian rhythm in parallel with that of PEPT1. In rats with restricted feeding, almost all the clock genes were expressed in a circadian manner, and the phases were shifted roughly 12 h apart from those in normal feeding rats. However, in the case of Clock, its circadian variation was disrupted by feeding restrictions during the daytime. Interestingly, the phase of DBP gene expression was shifted to the same extent by daytime feeding and consequently was approximately similar with the diurnal rhythm of PEPT1.

Luciferase assay. Because the mRNA expression of PEPT1 in both normal and daytime feeding rats was synchronized with that of DBP, we hypothesized that the diurnal rhythm of PEPT1 was regulated by DBP. To test this hypothesis, we investigated the effect of the overexpression of DBP on the rat PEPT1 (rPEPT1) promoter activity using a series of rPEPT1 5'-deletion luciferase reporter constructs. As a positive control regulated by DBP, a mouse Per1 (mPer1) reporter vector was also used (34). Cotransfection of the Per1 reporter vector with DBP resulted in a significant increase in luciferase activity. Among the rPEPT1 5'-deletion constructs, the 392/19 construct had the strongest promoter activity in the presence of DBP; however, this increase was not significantly different from that of pGL3-Basic (Fig. 6A). A database analysis revealed that there is no region that has high homology with the consensus DBP binding site, RTTAY-GTAAY (R; purine, Y; pyrimidine), within 3 kb of the 5' flanking region of the rPEPT1 gene. However, the same analysis covering the 10-kb promoter region of rPEPT1 unveiled three highly homologous putative DBP binding sites located in a more distal region 6 kb upstream of the transcription start site (Fig. 5). Therefore, the effect of DBP coexpression on the reporter constructs containing these regions was further investigated. These distal promoter regions were prepared as two segments, from −8,992 to −6,830 and from −7,014 to −4,844, and were subcloned, respectively, into the −392/+19 construct had the strongest promoter activity in the presence of DBP; however, this increase was not significantly different from that of pGL3-Basic (Fig. 6A). A database analysis revealed that there is no region that has high homology with the consensus DBP binding site, RTTAY-GTAAY (R; purine, Y; pyrimidine), within 3 kb of the 5' flanking region of the rPEPT1 gene. However, the same analysis covering the 10-kb promoter region of rPEPT1 unveiled three highly homologous putative DBP binding sites located in a more distal region 6 kb upstream of the transcription start site (Fig. 5). Therefore, the effect of DBP coexpression on the reporter constructs containing these regions was further investigated. These distal promoter regions were prepared as two segments, from −8,992 to −6,830 and from −7,014 to −4,844, and were subcloned, respectively, into the −392/+19 construct, which has enough basal promoter activity. Cotransfection of DBP produced a clear increase (approximately fourfold, \( P < 0.01 \)) in the transcriptional activity of the −7,014/−4,844-containing reporter construct but not in that of
the −8,992/−6,830 construct (Fig. 6B), suggesting the existence of a positive regulatory element between −6,830 and −4,844.

**Electrophoretic mobility shift assays.** Finally, to identify the region responsible for DBP-induced transcriptional activation, we performed EMSAs with a DIG-labeled probe. In the 5′ flanking region between −6,830 and −4,844, the sequence from −6,314 to −6,305 (GTTACGAAAC) showed the most homology to the optimal consensus sequence (9 of 10 bases are identical). As shown in Fig. 7B, an oligonucleotide probe containing −6,314/−6,305 (probe B in Fig. 7, A and B) was incubated with recombinant DBP protein, and a DNA–protein complex was detected. The failure of the probe with a mutation at −6,314/−6,305 (probe C) to form a DNA–protein complex with DBP confirmed the specificity of the binding of DBP to this region. A probe encompassing the optimal consensus sequence (probe D) was also used, and the corresponding complex was observed on incubation with DBP protein. In the 392-bp proximal promoter region, although the sequence from −202 to −193 shows the highest level of homology with the consensus sequence (7 of 10 bases are identical), similar binding of DBP was not observed (probe A). Taken together, these results suggested that DBP transactivates rPEPT1 expression via a DBP binding site from bp −6,314 to −6,305.

**DISCUSSION**

In normal rats, intestinal PEPT1 shows an overt diurnal rhythm in its mRNA and protein expression and also functionally influences the pharmacokinetics of its substrate, ceftibuten (16–18). In this study, we have undertaken the task of elucidating the mechanism regulating this diurnal rhythm by evaluating the association between PEPT1 and several transcription factors. Sp1 and Cdx2, which were characterized as PEPT1-regulating transcription factors, did not show a circadian rhythm in their mRNA levels in either feeding condition, but plasma free fatty acids, which act as endogenous PPAR-α ligands, exhibited a robust circadian fluctuation in phase with that of PEPT1. A recent study has indicated that PPAR-α is an important factor for the circadian regulation of lipogenic and cholesterogenic gene expression in the liver because daily variations in their expression were attenuated or abolished in mice in which the PPAR-α gene had been disrupted (9). Although intestinal PEPT1 is also known to have a circadian rhythm and be regulated by PPAR-α, the absence of any association between the circadian rhythm of PEPT1 and PPAR-α was confirmed by experiments in vivo using PPAR-α-null mice. Taken together, known PEPT1-regulating factors, Sp1, Cdx2, and...
PPAR-α, do not seem to participate in the circadian regulation of PEPT1 mRNA expression.

Most of the peripheral tissues harbor circadian oscillators referred to as circadian clocks (3, 15, 25, 36), and the circadian accumulation of some peripheral transcripts is under the control of the molecular clock. Microarray gene studies have revealed that ~5−10% of all genes exhibit circadian rhythms in peripheral organs such as the liver and heart (19, 28). Furthermore, it was reported that molecular circadian oscillators are also functional in the gastrointestinal tract (25), and the present results regarding the expression profile of clock genes support the function of the molecular clock in the small intestine. Hence, it is conceivable that the expression of some intestinal genes including the PEPT1 is under the control of clock genes as in other peripheral tissues.

Previous reports showed that the activity of some peripheral genes is directly regulated by the Clock/Bmal1 heterodimer through E-box enhancers. The circadian variation of Na+/H+ exchanger isoform 3 (NHE3) was proved to be directly regulated by Clock/Bmal1 via the E-box motif located in the NHE3 gene. The activity of the clock gene Per1 was also shown to be directly controlled by the NHE3 E-box motif, indicating that the expression of NHE3 is controlled by a clock gene.

![Fig. 6. Effect of DBP overexpression on the PEPT1 promoter activity.](image)

Fig. 6. Effect of DBP overexpression on the PEPT1 promoter activity. Caco-2 cells were transiently transfected with 50 ng of reporter constructs. A: series of 5'-deletion constructs. B: constructs with distal promoter region and 500 ng of the DBP expression vector. mPer1 reporter construct was used as a positive control. The total amount of transfected DNA was kept constant by adding empty vector. Firefly luciferase activity was normalized to Renilla luciferase activity. Data are reported as the relative fold increase compared with pGL3-Basic and represent the means ± SE of three replicates. **Significantly different from pGL3-Basic, P < 0.01.

![Fig. 7. Analysis of DBP binding to the rPEPT1 promoter.](image)

Fig. 7. Analysis of DBP binding to the rPEPT1 promoter. A: sequences of the probes used are shown. The numbering of the nucleotide residues indicates the distance from the transcription start site. The capitals indicate the sequence corresponding to the DBP binding site. B: EMSA was performed using the digoxigenin-labeled probe described in A. The formation of DNA-protein complexes were determined in the absence (-) or presence (+) of recombinant DBP protein.
promoter region, and the circadian rhythm in NHE3 expression was blunted in the Cry1/2 double knockout mice (21). We examined the sequence of the 5’ flanking region and first intron of the rPEPT1 gene to determine whether it contains an E-box. Although a perfect E-box (CACGTG) does not exist within 10 kb of the 5’ flanking region of rPEPT1 gene, a similar sequence to an E-box exists within the first intron of the gene. We performed reporter assays with reporter plasmids containing these sequences in Caco-2 cells, but coexpression of Clock and Bmal1 did not produce an increase in transcriptional activity of rPEPT1 reporter constructs although it vigorously transactivated the activity of the reporter construct encompassing the proximal promoter regions of arginine vasopressin used as a positive control (data not shown). These findings suggest that core-clock components do not directly participate in the establishment of the diurnal rhythm of rPEPT1.

Several studies have unveiled that circadian transcripts accumulate in many different phases (6, 19, 28). Thus it is unlikely that the core feedback loop solely mediates the circadian expression of several genes with diverse phases. The most probable way for the clock work to regulate downstream genes is to use circadian transcription factors that are regulated by the core feedback loop. DBP is a clear example of such a transcription factor in peripheral tissue because it is expressed rhythmically in many tissues. In the liver, DBP has been shown to regulate the rhythmic expression of a variety of genes encoding enzymes or regulatory proteins involved in metabolism such as a number of cytochrome P450 enzymes (e.g., Cyp2a4, Cyp2a5, and CYP3A4) involved in detoxification and elimination of dietary components (12, 30). The present study showed that PEPT1, involved in the absorption of food components, was regulated by the clock-controlled gene DBP, suggesting increased importance of the circadian clock and DBP in food processing and energy homeostasis. Almost all genes reported to be regulated by DBP possess their response element in the proximal promoter region (between −300 and −1) (12, 13, 30). However, the results of reporter assays and electrophoretic mobility shift assays revealed that the binding site for DBP within rPEPT1 was not located in the proximal promoter region (∼3 kb) but located in the distal promoter region, between −6,314 and −6,305.

In mouse liver, restricted feeding during the day completely inverts peripheral circadian gene expression, including of Clock, Bmal1, DBP, and also Cyp2a5 (4). In our experiments, the same food-induced phase shift of clock genes was observed in the small intestine. In the peripheral tissues, pulmonary clock genes are reported to be refractory to a restricted feeding schedule (27), suggesting clock genes in those organs essentially involved in food processing such as liver and small intestine are most sensitive to food loading. Nocturnal rodents kept under the usual 12-h LD cycle and offered ad libitum feeding consume ∼80% of their food during the active dark phase. Notably, rats feeding ad libitum showed a robust diurnal rhythm in intestinal PEPT1 mRNA expression with a peak at the onset of the dark phase, 2000, which was phase advanced relative to their all-out feeding. This kind of phase-advanced expression of PEPT1 mRNA was similarly observed in the daytime feeding rats, which were forced to eat during 0900–1500 and showed a diurnal PEPT1 mRNA expression with a peak at 0800. Furthermore, we previously demonstrated that the diurnal regulation of the PEPT1 mRNA level was maintained even in the 4-day fasted state, suggesting that the regulation of intestinal PEPT1 diurnal rhythmicity was not directly mediated by dietary components (17). In keeping with this conjecture, the present study showed that the clock-controlled gene DBP regulates this food-anticipatory diurnal expression of intestinal PEPT1, which seems to be a vital anticipatory mechanism to prepare PEPT1 for the expected luminal food exposure, because PEPT1 plays a pivotal role in the absorption of dietary protein.

In humans, it is not clear whether the expression of intestinal PEPT1 follows a diurnal rhythm or not. In a clinical study using a substrate of PEPT1, enalapril, the patients orally administered at 1000 more frequently complained of coughing, an adverse effect of enalapril, than the patients administered at 2200 (29). This was reported before the identification of PEPT1 diurnal rhythmicity; hence, PEPT1 is not mentioned as being responsible. However, this adverse effect might be ascribable to a diurnal rhythm of intestinal PEPT1 expression. Although the sequence around the DBP binding site of rPEPT1 identified here is not conserved in the human PEPT1 gene, another highly homologous DBP binding site is present in the promoter region of human PEPT1. To assess the diurnal rhythmicity of PEPT1 expression and contribution of DBP to PEPT1 expression in humans, further studies are needed.

In conclusion, we have demonstrated for the first time that a clock-controlled gene, DBP, regulates the circadian oscillation of PEPT1 expression under normal and restricted feeding conditions. These findings suggest that the circadian clock and DBP play pivotal roles in food processing and energy homeostasis. Other transcription factors that regulate PEPT1 gene expression (Sp1, Cdx2, and PPAR-α) were not responsible for the diurnal rhythm of PEPT1 expression.

GRANTS

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CIRCADIAN REGULATION OF PEPT1 GENE EXPRESSION


