Intestinal Expression of Mouse Abcg2/Breast Cancer Resistance Protein (BCRP) Gene Is under Control of Circadian Clock-activating Transcription Factor-4 Pathway*

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Background: Intestinal expression of Abcg2/breast cancer resistance protein (BCRP) exhibits circadian oscillation, but the mechanism is unknown.

Results: ATF4, a molecular component of the circadian clock, induces circadian expression of Abcg2 in mouse small intestine.

Conclusion: The circadian clock-ATF4 pathway causes the oscillation of BCRP function and induces the circadian change in intestinal drug absorption.

Significance: ATF4 constitutes a novel molecular link connecting the circadian clock to xenobiotic detoxification.

ABCG2, encoding breast cancer resistance protein (BCRP), is a member of the ATP-binding cassette transporter family and is often associated with cancer chemotherapeutic resistance. BCRP is also expressed in a variety of normal cells and acts as a xenobiotic efflux transporter. Because intestinal BCRP limits systemic exposure to xenobiotics, alterations in the function and expression of this transporter could account for part of the variation in oral drug absorption. In this study, we show that ATF4, a molecular component of the circadian clock, induces circadian expression of the Abcg2 gene in mouse small intestine. Three types of leader exons (termed exons 1A, 1B, and 1C) are identified in the 5′-untranslated region of mouse Abcg2 transcripts. The exon 1B-containing Abcg2 transcript was the only isoform detected in mouse small intestine, and its mRNA levels oscillated in a circadian time-dependent manner. ATF4 bound time-dependently to the cAMP response element within the exon 1B promoter region of the Abcg2 gene, thereby causing the oscillation of BCRP protein abundance and its efflux pump function. The circadian clock-ATF4 pathway appears to enhance the function of BCRP during a specific time window and to modulate intestinal drug absorption. Our findings suggest a mechanism underlying circadian change in xenobiotic detoxification.

Daily variations in biological functions are thought to affect the efficacy and/or toxicity of drugs; a large number of drugs cannot be expected to have the same potency at different times of administration (1, 2). Dosing time-dependent differences in the therapeutic effects of drugs are, at least in part, due to circadian-related changes in drug disposition, e.g. absorption, distribution, metabolism, and elimination. The xenobiotic transporter breast cancer resistance protein (BCRP),4 encoded by the ABCG2 (ATP-binding cassette, subfamily G, member-2) gene, functions as an energy-dependent efflux pump by expelling cytotoxic substances. In mammals, BCRP is expressed in epithelial cells of several organs, including the liver, intestine, and kidney, and thus contributes to the biliary, intestinal, and renal elimination of many drugs (3). It is now well reported that the BCRP transporter acts as an intestinal barrier to limit oral drug absorption because BCRP recognizes a broad range of substances (4–6).

It has been reported that three types of ATP-binding cassette transporter, MDR1 (encoded by ABCB1), MRP2 (encoded by ABCCC2) and BCRP, are highly expressed in the apical membrane of the epithelium in the small intestine, and the expression of all three ATP-binding cassette transporters exhibits circadian oscillation in rodents (7). Among them, the circadian regulation mechanism of MDR1 and MRP2 has been evaluated regarding their pharmacokinetic significance, which might account for dosing time-dependent changes in the bioavailability and toxicity of various drugs. However, it remains to be clarified whether circadian oscillation of BCRP affects intestinal drug absorption.

In mammals, circadian rhythmicity in various biological processes is under the control of a molecular pacemaker that is composed of clock gene products (8–10). These gene products constitute an oscillatory mechanism that is based on self-sustained transcriptional/translational feedback loops. Clock is the first clock gene identified in mammals (11, 12). The Clock gene encodes the transcription factor, CLOCK, and dimerizes with BMAL1 to activate transcription of the Period (Per) and Cryptochrome (Cry) genes through E-box or E-box-like enhancer sequences. Once PER and CRY proteins have reached a critical

4 The abbreviations used are: BCRP, breast cancer resistance protein; CRE, cAMP response element; ZT, zeitgeber time; AUC, area under the plasma concentration-time curve.

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concentration, they attenuate CLOCK/BMAL1 transactivation, thereby generating circadian oscillation in their own transcription. In addition, the CLOCK/BMAL1, PER, and CRY proteins regulate the expression of ATF4 (activating transcription factor-4), which in turn sustains the transcriptional oscillation of the Bmal1, Per2, and Cry1 genes (13). This mechanism interconnects the positive and negative limbs of circadian clockwork circuitry and also regulates 24-h variation of output physiology through the periodic activation/repression of clock-controlled output genes (14). ATF4 also acts as an output component of the circadian clock and regulates the rhythmic expression of its target genes through the cAMP response element (CRE) (13). It has been shown that overexpression of ATF4 in malignant tumor cells induces the elevation of BCRP levels (15), but the potential contribution of ATF4 to circadian expression of Abcg2/BCRP has been little explored.

In this study, we found that ATF4 acts as a circadian regulator of intestinal expression of the Abcg2 gene in mice. Three types of leader exons (termed exons 1A, 1B, and 1C) are located in the 5′-untranslated region of mouse Abcg2 transcripts (16). Among them, the exon 1B-containing Abcg2 transcript was the only isoform detected in mouse small intestine. ATF4 time-dependently bound to the CRE within the exon 1B promoter region of the Abcg2 gene and caused the rhythmic expression of the exon 1B-containing Abcg2 transcript. We thus investigated how the time-dependent variation in the intestinal expression of Abcg2 affects the oral absorption of a typical substrate of BCRP.

EXPERIMENTAL PROCEDURES

Animals and Treatment—Clock mutant (Clock/Clock) mice of the ICR background and wild-type mice of the same strain were housed under a standardized light/dark cycle in a temperature-controlled room (24 ± 1 °C) at humidity of 60 ± 10%, with food and water ad libitum. They were adapted to the light/dark cycle for 2 weeks before the experiments. Under the light/dark cycle, zeitgeber time (ZT) 0 was designated as lights on, and ZT12 as lights off. Animals were treated in accordance with the guidelines stipulated by the Animal Care and Use Committee of Kyushu University.

Cells and Treatment—Immortalized small intestine epithelial cells (aMoS7), established from adult murine intestinal crypts (17), were kindly provided by Dr. M. Totsuka (Tokyo University). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma-Aldrich) supplemented with 5% Fetal bovine serum (FBS) (SAFC Bioscience, Kansas City, MO), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml insulin. To investigate the influence of clock gene products on the expression of endogenous Abcg2 mRNA, aMoS7 cells were transfected with expression plasmids encoding CLOCK, BMAL1, PER2, CRY1, hepatic leukemia factor, E4BP4, ATF4, retinoic orphan receptor-α, or REV-ERBα. At 24 h after transfection, mRNA levels of Abcg2 were determined by RT-PCR. To synchronize the circadian clocks in cultured aMoS7 cells, serum shock was performed as follows. Cells were grown to semiconfluence in DMEM supplemented with 5% FBS. On the day of serum shock, 50% FBS was added for 2 h, and the cells were incubated in DMEM supplemented with 1% FBS. Cells were harvested for RNA or protein extraction at the indicated times after serum treatment. To explore the role of ATF4 in the regulation of mouse Abcg2 mRNA expression, aMoS7 cells were transfected with retrovirus vector expressing shRNA targeting Atf4 and thereafter treated with 50% FBS to synchronize their circadian clocks.

Quantitative RT-PCR Analysis—Total RNA was extracted from mouse jejunum using RNeasy (Tokara Bio Inc., Shiga, Japan) at ZT2, ZT6, ZT10, ZT14, ZT18, and ZT22. cDNA samples were analyzed by real-time or semi-quantitative RT-PCR. Real-time PCR was performed using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo Co. Ltd.) and a 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The distinct forward primers used for detection of mouse Abcg2 isoforms were as follows: exon 1A, 5′-TTCTGTCTTCTCTGTCCTCTCCT-3′; and exon 1B, 5′-AGCTGTGCTCAGTGAGTG-3′; and exon 1C, 5′-GAAGAACCACACAAATAAGG-3′. The common reverse primer (5′-TGGAATGCGAGGTATTTAG-3′) was located in exon 4 of mouse Abcg2. The sequences of β-actin primers (internal control) were 5′-GAGGAGCCAGGTCTACATCTAATTTTTTTCAGCACTGTGT-3′.

Construction of Retrovirus Vectors Encoding shRNA Targeting Atf4—Specific silencing of endogenous Atf4 in aMoS7 cells was achieved using an shRNA-expressing retrovirus vector. Nucleotides 1114–1132 of the mouse Atf4 coding sequence (GenBank accession number NM_009716) were chosen as a target for shRNA. The Atf4 shRNA-encoding oligonucleotides were created as indicated below, each containing the 19-nucleotide target sequence of Atf4, followed by a short spacer and an antisense sequence of the target: 5′-GAGCATTCTTTAGTTTAGAGAGGTCTACATCTAATAAGG-3′. The Atf4 shRNA-encoding sequence was cloned into the BamHI and BglII sites of the pDON-AI2 vector (Takara Bio Inc.) and transfected into G3T-hi packaging cells. All infected cells were cultured in medium containing the appropriate antibiotics. The control shRNA-expressing retrovirus vector was made using the same procedure with the following oligonucleotide sequence: 5′-GAAGCAGTCCCTGAACTTGTTAGAGGAGGAGGTCTACATCTAATAAGG-3′.

Luciferase Reporter Assay—Cells were maintained in DMEM supplemented with 5% FBS at 37 °C in a humidified 5% CO2 atmosphere. The day before transfection, cells were seeded (1 × 10^5 cells/well) into 24-well plates. The next day, cells were transfected with 100 ng of reporter construct and 0.5 μg (total) of expression constructs using Lipofectamine LTX reagent (Invitrogen) according to the manufacturer’s instructions. To correct for the variations in transfection efficiency, 0.5 ng of pGL4.74[hRluc/TK] vector (Promega Biosciences LLC, San Luis Obispo, CA) was cotransfected in all experiments. The total amount of DNA/well was adjusted by adding the pcDNA3.1 vector (Invitrogen). At 48 h post-transfection, cells were harvested with 200 μl of passive lysis buffer, and 50 μl of the extracts was used for firefly luciferase and Renilla luciferase assays by luminometry. The ratio of firefly luciferase activity, expressed from reporter plasmids, to Renilla luciferase activity,
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expressed from pGLA.74[hRluc/TK], in each sample served as a measure of normalized luciferase activity.

Construction of Reporter and Expression Plasmids—For the transcriptional assay, a 5′-upstream fragment of the exon 1B promoter region of the mouse Abcg2 gene spanning bp −183 to +17 (number is distance from the putative transcription start site, +1; Ensembl transcript ID ENSMUST00000114294) was fused to the pGL3-Basic vector (Abcg2 E1B–Luc). The CRE in the exon 1B promoter region of the mouse Abcg2 gene was also mutated at bp −55 to −48 (TGACGTCA to TGGTACCA). The expression plasmids of clock and clock-related genes were prepared as follows. The coding regions of clock or clock-related genes were obtained by RT-PCR and used after their sequences were confirmed. All coding regions were ligated into the pcDNA3.1 vector.

Chromatin Immunoprecipitation (ChiP) Assay—To analyze the temporal binding of endogenous ATF4 to the exon 1B promoter region of the mouse Abcg2 gene in intestinal epithelial cells of mice, ChiP assay was performed as follows. Mouse small intestinal segments were cross-linked with 1% formaldehyde in PBS at 25 °C for 5 min. The reaction was stopped by adding glycine to a final concentration at 0.125 M. Each cross-linked sample was sonicated on ice and then incubated with antibodies against ATF4 (sc-200, Santa Cruz Biotechnology). DNA was isolated from immunoprecipitates and subjected to PCR using the following primer pairs: 5′-ATGGTGCCACAGCTGCCTGG-3′ and 5′-CACACTCAGACCTGCACAG-3′. The quantitative reliability of PCR was evaluated by kinetic analysis. As a negative control, ChiP was performed in the absence of antibody or in the presence of rabbit IgG. PCR products from these samples were not detectable by ethidium bromide staining.

Western Blotting—Cell membrane fractions or nuclear extractions from epithelial cells of mouse small intestine were prepared at six time points as described previously (18). Then, 20 µg of total protein lysates was resolved by 8 or 10% SDS-PAGE, transferred to a PVDF membrane, and probed with rat monoclonal antibodies against BCRP (BXP-53; sc-58224), ATF4, or actin (all from Santa Cruz Biotechnology) under reduced conditions. Specific antigen-antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibodies and SuperSignal chemiluminescent substrate (Pierce).

Determination of Sulfasalazine Concentration in Plasma—For oral administration of sulfasalazine, 2.0 g of compound was dissolved in 3 ml of 3 M NaOH and diluted with 17 ml of 0.5% methyl cellulose solution, and the pH was adjusted to 7.3. Mice were fasted for 2 h prior to drug administration and were orally administered 1 g/kg sulfasalazine at ZT2 or ZT14. Blood samples were drawn by cardiac puncture at 15, 30, 60, 120, and 180 min after sulfasalazine administration. Plasma was separated by centrifugation (1200 × g) and stored at −70 °C until HPLC analysis as described above.

Nonlinear Mixed Effect Model Analysis—The nonlinear mixed effect model (NONMEM®) is a computer program designed to analyze pharmacokinetics in study populations by pooling data (19). In this study, NONMEM was applied to the pharmacokinetic analysis of sulfasalazine concentrations in plasma. The population pharmacokinetic parameters were calculated using 60 plasma concentrations obtained from 60 mice following the one-compartment model with first-order absorption (the PREDPP program, subroutines ADVAN2 and TRANS2). Bayesian estimates of individual pharmacokinetic parameters were obtained by the NONMEM program’s post hoc method. The statistical moment parameters, such as the area under the plasma concentration-time curve (AUC), maximum concentration of sulfasalazine in plasma (Cmax), and time of maximum plasma concentration (Tmax), were calculated using the estimated individual pharmacokinetic parameters.

Statistical Analysis—The statistical significance of differences between groups was validated by the Bonferroni test for multiple comparisons and Student’s test for comparison between two groups. A 5% level of probability was considered to be significant.

RESULTS

Expression of Abcg2 Transcript Isoforms in Mouse Liver, Kidney, and Small Intestine—Comparison of the sequences of the expressed sequence tag clones with mouse genomic DNA sequences revealed that the distance of the leader exons (termed exons 1A, 1B, exon 1C) from the first coding exon (designated exon 2) in genomic DNA was 58.5 kb for exon 1A, 15.0 kb for exon 1B, and 5.1 kb for exon 1C (Fig. 1A). As reported previously (16), all three Abcg2 transcript isoforms were confirmed. All coding regions were ligated into the pcDNA3.1 vector.
were expressed in mouse liver and kidney, whereas the exon 1B-containing Abcg2 transcript was the only isoform detected in mouse small intestine (Fig. 1B). The fact indicates that three leader exons of the mouse Abcg2 gene are under the control of distinct transcriptional regulation.

Next, we investigated the temporal expression profiles of these three Abcg2 transcript isoforms in mouse liver, kidney, and small intestine. In all investigated tissues, the mRNA levels of the Abcg2 isoform containing exon 1B showed significant 24-h oscillation, with a peak occurring during the light phase (p < 0.05 for all) (Fig. 1, C–E). By contrast, the mRNA levels of both exon 1A and 1C isoforms in the liver and kidney failed to show a significant time-dependent variation (Fig. 1, C and D).

Clock/Clock mice have a point mutation causing the deletion of exon 19 of the Clock gene, thus synthesizing mutant CLOCK protein (CLOCKΔ19) deficient in transcriptional activity, and exhibit low amplitude rhythms in various gene expressions (11, 12). With respect to these effects of the Clock mutation on circadian gene expression, we used the Clock/Clock mice to determine whether the intestinal expression of Abcg2 mRNA is under the control of the circadian clock. Compared with wild-type mice, the oscillation in the intestinal expression of the...
Abcg2 exon 1B isoform was significantly attenuated in Clock/Clock mice (Fig. 1E). These results suggest that transcription of the exon 1B isoform of the Abcg2 gene is involved in the control of the circadian clock.

Role of ATF4 in Circadian Expression of Abcg2 Exon 1B Isoform in Small Intestinal Cells—Clock genes, consisting of core oscillation loops, generate circadian oscillations in output physiology through the periodic activation/repression of clock-related output genes (14, 20). To explore whether the products of clock genes and/or clock–related output genes affect the expression of the exon 1B isoform of Abcg2, we investigated the effects of the transfection of expression plasmids encoding CLOCK, BMAL1, PER2, CRY1, hepatic leukemia factor, E4BP4, ATF4, retinoic orphan receptor-α, or REV-ERBα on the mRNA levels of the exon 1B isoform of Abcg2 in aMoS7 cells. Considerable expression of exon 1B isoform mRNA was detected in aMoS7 cells (Fig. 2A). The mRNA levels of the Abcg2 exon 1B isoform were elevated significantly when cells were transfected with ATF4 expression plasmids (p < 0.05), whereas transfection with expression plasmids encoding other clock and clock–related genes had little effect on the endogenous levels of exon 1B isoform mRNA.

Previous studies have demonstrated that several compounds and high concentrations of serum are able to induce synchronous circadian gene expression in cultured cells (21). Brief exposure of aMoS7 cells to 50% FBS induced the rhythmic mRNA expression of the Abcg2 exon 1B isoform (Fig. 2B). A time-dependent change in the protein levels of ATF4 was also detected in the cells (Fig. 2C, lanes 1–3). The rhythmic phase of protein levels of ATF4 was nearly the same as that of mRNA levels of the Abcg2 exon 1B isoform. We thus used serum-shocked cells to investigate the role of ATF4 in the control of the rhythmic expression of intestinal Abcg2 mRNA. Infection of cells with retrovirus vectors expressing shRNA targeting ATF4 resulted in a decrease in the protein levels of ATF4 (Fig. 2C, lane 4–6). Although treatment of control shRNA-expressing retrovirus-infected cells with 50% FBS caused a significant time-dependent oscillation in the mRNA levels of the Abcg2 exon 1B isoform (p < 0.05) (Fig. 2D), no significant oscillations in the mRNA levels of the exon 1B isoform were observed in ATF4-down-regulated cells (Fig. 2D). These findings suggest that ATF4 participates in the circadian control of intestinal expression of Abcg2.

Transcriptional Regulation of Exon 1B Isoform of Abcg2 Gene by ATF4—ATF4 is responsible for the circadian expression of genes that are under the control of CRE-mediated transcription (13). A nucleotide sequence showing homology to the CRE was located at bp −55 to −48 upstream of the transcriptional start site of the exon 1B isoform of the mouse Abcg2 gene (Fig. 3A, upper panel). However, none of the other three clock-controlled DNA elements, E-box (CACGTG), PAR bZIP (proline- and acid-rich basic leucine zipper) protein response element (TATA/C/TGGTAA), and retinoic orphan receptor response element ((A/T)(A/T)NT(A/G)GGTCA), were identified within 3000 bp of the 5′-flanking region of the exon 1B isoform of the Abcg2 gene. Cotransfection of the Abcg2 E1B-Luc reporter with ATF4 resulted in a dose-dependent increase in promoter activity, but ATF4-induced transactivation was attenuated by the mutation of CRE (Fig. 3A, lower panel).

ATF4 protein was expressed in wild-type mouse small intestine and showed obvious circadian oscillation, with a peak occurring from the mid-light phase to the late light phase (Fig. 3B). The cyclic accumulation of ATF4 protein in the small intestines of wild-type mice was synchronized with that of mRNA expression of the exon 1B isoform of the Abcg2 gene. On the other hand, in Clock/Clock mice, ATF4 protein levels failed to show obvious time-dependent oscillation; protein levels were consistently reduced throughout the day.

The result of the ChIP assay also showed that, in the small intestines of wild-type mice, the amount of endogenous ATF4
binding to the exon 1B promoter region of the Abcg2 gene increased at the time of day corresponding to the peak of mRNA expression for the exon 1B isoform (Fig. 3C). In contrast, Clock/Clock mice failed to show time-dependent oscillations in ATF4 binding to the exon 1B promoter region of the Abcg2 gene (Fig. 3C). The amount of ATF4 binding decreased at both time points (ZT6 and ZT18). The correlation between the binding activity of this protein and the transcriptional regulation of the Abcg2 gene suggests that the CLOCK-regulated output pathway, probably through ATF4, contributes to the rhythmic expression of the exon 1B isoform of the Abcg2 gene.

Time-dependent Change in Intestinal Accumulation of Sulfasalazine—Because the mRNA levels of the exon 1B isoform of the Abcg2 gene oscillated time-dependently in wild-type mice small intestine, we investigated whether the function of BCRP also fluctuated in a circadian fashion. Obvious circadian variation in BCRP protein levels was observed in the small intestines of wild-type mice (Fig. 4A). The amount of ATF4 binding decreased at both time points (ZT6 and ZT18). The correlation between the binding activity of this protein and the transcriptional regulation of the Abcg2 gene suggests that the CLOCK-regulated output pathway, probably through ATF4, contributes to the rhythmic expression of the exon 1B isoform of the Abcg2 gene.

Dosing Time-dependent Change in Plasma Concentration of Sulfasalazine after Oral Administration—Because the efflux pump function of BCRP varied time-dependently, we explored whether the pharmacokinetics of sulfasalazine also varied depending on its dosing time. The plasma concentration peaked 15 min after oral administration of 1.0 g/kg sulfasalazine at ZT2 (Fig. 4C), whereas the peak time of plasma concentration of sulfasalazine was delayed significantly after oral administration of the drug at ZT14 (p < 0.01) (Table 1). The peak plasma concentration of sulfasalazine after administration at ZT2 was also significantly higher than that at ZT14 (p < 0.05) (Fig. 4C and Table 1). The final model derived from NONMEM analysis was as follows: CL/F (L/h) = 557 × 1.21DT, Vd/F (L) = 367 × 0.864DT, and ka (1/h) = 5.69 × 0.253DT, where CL is total body clearance, Vd is the apparent volume of distribution, and ka is the absorption rate constant. DT represents the dosing time: DT = 0 if injection of the drug was at ZT2, and DT = 1 if injection of the drug was at ZT14. Using the population parameters, individual pharmacokinetic parameters were calculated based on Bayesian estimates, and the AUC was derived from them. As shown in Table 1, AUC was significantly greater in mice administered sulfasalazine at ZT2 than at ZT14 (p < 0.05).

DISCUSSION

A recent study demonstrated that the intestinal expression of Abcg2 mRNA in Sprague-Dawley rats exhibits circadian oscillation (7), which might account for dosing time-dependent changes in the bioavailability of BCRP substrates; however, the mechanism remains to be clarified. In this study, we showed the underlying mechanism for circadian expression of Abcg2 mRNA in small intestines of mice. The oscillation of Abcg2 mRNA appears to affect the efflux pump function of BCRP. The regulation mechanism revealed a novel link between the circadian clock and the xenobiotic detoxification system.

Three types of leader exons are located in the 5’-untranslated region of mouse Abcg2 transcripts, although these isoforms encode the same BCRP protein (16). Among them, the exon
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Circadian genes is reduced in transcription (8, 9); therefore, the amplitude of the rhythm in many target genes together with BMAL1, but it fails to activate transcription of CLOCK isoform was significantly attenuated in ATF4-down-regulated aMoS7 cells. These in vitro data indicate that ATF4 directly regulates the circadian expression of Abcg2 mRNA in intestinal cells. In the small intestines of wild-type mice, the expression of ATF4 protein fluctuated corresponding to the mRNA rhythm of the Abcg2 exon 1B isoform. On the other hand, the protein levels of ATF4 did not exhibit obvious circadian oscillation in Clock/Clock mice, suggesting that the rhythmicity in the intestinal expression of ATF4 protein is also under the control of the function of CLOCK protein. Furthermore, time-dependent variation in ATF4 binding to the promoter region of the exon 1B isoform was not observed in Clock/Clock mice. Considering the ability of ATF4 to regulate the transcription of the exon 1B isoform of the Abcg2 gene, altered rhythms in the expression of ATF4 proteins could account for the blunted rhythm of the intestinal expression of Abcg2 mRNA in Clock/Clock mice. The transcriptional circuit underlying mammalian circadian clocks consists of at least four clock-controlled DNA elements: E-box, PAR bZIP protein response element, retinoic orphan receptor response element, and CRE (13, 23, 24). No putative sequences resembling known CREs and other clock gene response elements were detected in the upstream regions of the exon 1A and 1C isoforms of the Abcg2 gene. This may account for the arrhythmic expression of the exon 1A and 1C isoforms of the mouse Abcg2 gene.

Because BCRP acts as an intestinal barrier to limit oral drug absorption, alteration in the efflux pump function of this transporter may account for part of the variation in the drug pharmacokinetics (3–6). Sulfasalazine is well characterized as a substrate of BCRP in mice (22). After oral administration of sulfasalazine to Abcg2 knock-out mice, the bioavailability of the drug is ~10-fold higher than in wild-type mice. Circadian oscillation of the intestinal accumulation of sulfasalazine in wild-type mice was nearly antiphase to BCRP expression rhythm. Furthermore, the time-dependent variation in the intestinal accumulation of sulfasalazine was undetected in Clock/Clock mice. It has been reported that PAR bZIP proteins, hepatic leukemia factor, thyrotroph embryonic factor, and D-site-binding protein act as circadian output mediators regulating the expression of many enzymes and molecules involved in xenobiotic detoxification (14). Our present findings reveal that the efflux function of BCRP in mouse small intestine is under the control of the circadian clock-ATF4 pathway. The regulation mechanism appears to enhance BCRP function during a specific time window and to induce the circadian change in intestinal drug absorption. In fact, a significant dosing time-dependent difference was observed in sulfasalazine pharmacokinetics in wild-type mice. Maximum plasma concentration and the time to reach maximum plasma concentration varied according to the dosing time of sulfasalazine. The dosing time-dependent difference in the intestinal absorption process of sulfasa-

1B-containing Abcg2 transcript was the only isoform detected in mouse small intestine and expressed in a circadian fashion. CLOCLKA19 protein can still bind to regulatory elements on its target genes together with BMAL1, but it fails to activate transcription (8, 9); therefore, the amplitude of the rhythm in many circadian genes is reduced in Clock/Clock mice. Although the oscillation in the intestinal expression of the Abcg2 exon 1B isoform was significantly attenuated in Clock/Clock mice, cotransfection of aMoS7 cells with both CLOCK and BMAL1 had little effect on the mRNA levels of the exon 1B isoform of the Abcg2 gene. This finding suggests that CLOCK/BMAL1 heterodimers are unlikely to act directly on the intestinal Abcg2 gene to activate transcription.

The exon 1B isoform of the Abcg2 gene in cultured aMoS7 cells responded to the transfection of expression plasmids encoding ATF4. Furthermore, serum shock-induced oscillation in the mRNA expression of the exon 1B isoform was also significantly attenuated in ATF4-down-regulated aMoS7 cells.

**TABLE 1**

**Influence of dosing time on pharmacokinetic parameters of sulfasalazine after oral administration (1.0 g/kg)**

Values are shown as the means ± S.E. of 30 mice.

| Pharmacokinetic parameters | Dosing time | Statistical significance |
|----------------------------|-------------|-------------------------|
|                            | ZT2         | ZT14        | p value    |
| Tmax (min)                 | 17.5 ± 2.5  | 33.0 ± 6.7  | < 0.01     |
| Cmax (ng/ml)               | 79.1 ± 8.9  | 34.8 ± 3.1  | < 0.05     |
| AUC (ng/ml/h)              | 56.9 ± 8.6  | 44.8 ± 5.1  | < 0.05     |
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zine would be explained by the rhythmic change in the efflux pump function of BCRP. Although there is no previous report identifying the isoform of leader exons of the human ABCG2 gene, computer-aided analysis has indicated the existence of the first exon isoform in the ABCG2 gene in humans. The consensus sequence of the CRE is also located in the promoter region of one isoform of the human ABCG2 gene (GenBank accession number DB274954), suggesting that the expression of the ABCG2 gene in human tissues also fluctuates in a circadian fashion.

The individualization of pharmacotherapy has been achieved mainly by monitoring drug concentration. Consequently, dosage adjustment has been based on interindividual differences in drug pharmacokinetics; however, intra- as well as interindividual variability should be considered to aim at further improving rational pharmacotherapy because the pharmacokinetics of many drugs also vary depending on the rhythm of absorption, distribution, metabolism, and elimination (1, 2). Our results suggest a mechanism underlying the dosing time-dependent differences in drug pharmacokinetics and provide a molecular link between the circadian clock and xenobiotic detoxification.

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