View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by CiteSeerX

1521-0111/12/8105-739–747$25.00
MOLECULAR PHARMACOLOGY
Vol. 81, No. 5
76406/3766474
Copyright © 2012 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 81:739–747, 2012

Time-Dependent Interaction between Differentiated Embryo Chondrocyte-2 and CCAAT/Enhancer-Binding Protein α Underlies the Circadian Expression of *CYP2D6* in Serum-Shocked HepG2 Cells

Naoya Matsunaga, Miki Inoue, Naoki Kusunose, Keisuke Kakimoto, Kengo Hamamura, Yuichi Hanada, Ayumi Toi, Yuji Yoshiyama, Fuyuki Sato, Katsumi Fujimoto, Satoru Koyanagi, and Shigehiro Ohdo

Department of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan (N.M., M.I., N.K., K.K., K.H., Y.H., A.T., S.K., S.O.); Division of Community Pharmacy, Center for Clinical Pharmacy and Clinical Sciences, Kitasato University, School of Pharmacy, Tokyo, Japan (Y.Y.); Department of Pathology and Bioscience, Hirosaki University, Graduate School of Medicine, Hirosaki, Japan (F.S.); and Department of Dental and Medical Biochemistry, Hiroshima University, Graduate School of Biomedical Sciences, Hiroshima, Japan (K.F.)

Received October 17, 2011; accepted February 21, 2012

ABSTRACT

Differentiated embryo chondrocyte-2 (DEC2), also known as bHLHE41 or Sharp1, is a pleiotropic transcription repressor that controls the expression of genes involved in cellular differentiation, hypoxia responses, apoptosis, and circadian rhythm regulation. Although a previous study demonstrated that DEC2 participates in the circadian control of hepatic metabolism by regulating the expression of cytochrome P450, the molecular mechanism is not fully understood. We reported previously that brief exposure of HepG2 cells to 50% serum resulted in 24-h oscillation in the expression of CYP3A4 as well as circadian clock genes. In this study, we found that the expression of CYP2D6, a major drug-metabolizing enzyme in humans, also exhibited a significant oscillation in serum-shocked HepG2 cells. DEC2 interacted with CCAAT/enhancer-binding protein (C/EBPα), accompanied by formation of a complex with histone deacetylase-1, which suppressed the transcriptional activity of C/EBPα to induce the expression of CYP2D6. The oscillation in the protein levels of DEC2 in serum-shocked HepG2 cells was nearly antiphase to that in the mRNA levels of CYP2D6. Transfection of cells with small interfering RNA against DEC2 decreased the amplitude of CYP2D6 mRNA oscillation in serum-shocked cells. These results suggest that DEC2 periodically represses the promoter activity of CYP2D6, resulting in its circadian expression in serum-shocked cells. DEC2 seems to constitute a molecular link through which output components from the circadian clock are associated with the time-dependent expression of hepatic drug-metabolizing enzyme.

Introduction

Most living organisms exhibit behavioral and physiological rhythms with a period length of approximately 24 h. Some of these rhythms are controlled by a self-sustained oscillation mechanism called the circadian clock. Molecular studies of the circadian clock system have revealed that oscillation in the transcription of specific clock genes plays a central role in the generation of 24-h rhythms (Gekakis et al., 1998; Kume et al., 1999). In mammals, the core molecular mechanism of the oscillator consists of two transcriptional activators, CLOCK and BMAL1, and their transcriptional targets, PERIOD (PER) and CRYPTOCHROME (CRY). PER and CRY proteins act as negative regulators of CLOCK/BMAL1 activity, thus forming the major circadian autoregulatory feedback loop (Reppert and

This study was partially supported by the Japan Society for the Promotion of Science [Grant-in-Aid for Scientific Research (B) 21390047, Grant-in-Aid for Challenging Exploratory Research 21659041]; the Mandom International Research Grants on Alternative to Animal Experiments; and The Cosmetology Research Foundation.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

http://dx.doi.org/10.1124/mol.111.076406.

ABBREVIATIONS: DBP, D site-binding protein; E4BP4, E4 promoter-binding protein 4; P450, cytochrome P450; DEC2, differentiated embryo chondrocyte-2; bHLH, basic helix-loop-helix; HNF4α, hepatic nuclear factor-4α; C/EBPα, CCAAT enhancer binding protein-α; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; RT, reverse transcription; PCR, polymerase chain reaction; MAMC, 7-methoxy-4-(aminomethyl) coumarin; RORα, retinoic orphan receptor-α; siRNA, small interfering RNA; TSA, trichostatin A; HDAC1, histone deacetylase-1; HAMC, 7-hydroxy-4-aminomethylcoumarin.

Weaver, 2002). The expression of clock genes is also modulated by a second oscillation loop composed of two orphan nuclear receptors, REV-ERBα and retinoid-related orphan receptor-α (RORα), which drive the circadian oscillation in *Bmal1* transcription (Preitner et al., 2002; Akashi and Takumi, 2005).

The circadian oscillators in hepatic cells drive rhythmic physiology through these transcriptional factors, which in turn regulate the transcription of downstream genes (Gachon et al., 2006). D site-binding protein (DBP) and E4 promoter-binding protein 4 (E4BP4) are examples of such output mediators because they are transcriptionally regulated by core oscillator components (Ripperger et al., 2000; Ueda et al., 2005). Clock genes and clock-controlled output genes are expressed rhythmically not only in the suprachiasmatic nucleus, the center of mammalian circadian clock, but also in other brain regions and peripheral tissues (Sakamoto et al., 1998; Yamamoto et al., 2004). The master clock located in the suprachiasmatic nucleus follows a daily light/dark cycle and, in turn, synchronizes subsidiary oscillators in other brain regions and many peripheral tissues through neural and/or humoral signals (Balsalobre et al., 2000a; Terazono et al., 2003). These subsidiary oscillators coordinate a variety of biological processes, producing 24-h rhythms in physiology and behavior. Such rhythmic expression of clock genes is also observed in cultured cells after brief treatment with various compounds (high concentration serum, forskolin, phorbol-12-myristate-13-acetate, calcimycin, or dexamethasone) (Balsalobre et al., 1998, 2000a,b); therefore, the peripheral oscillator in cultured human cells could constitute an in vitro model for the molecular oscillator in human tissues.

The cytochrome P450 gene superfamily encodes a group of heme-containing monooxygenases, many of which metabolize compounds used as therapeutic drugs. CYP3A4 is the most abundant P450 expressed in the human liver and small intestine, contributing to the metabolism of approximately half of the drugs in clinical use today (Evans and Relling, 1999). The expression of CYP3A4 and its metabolizing activity exhibit significant circadian oscillation in serum-shocked HepG2 cells (Takiguchi et al., 2007). DBP and E4BP4 regulate the circadian expression of the *CYP3A4* gene, suggesting a molecular link between the circadian clock and xenobiotic metabolism. Differentiated embryo chondrocyte-2 (DEC2), also known as bHLHE41 or Sharp1, is a basic helix-loop-helix (bHLH) transcriptional repressor and acts as an output component of the circadian oscillator. DEC2 regulates the expression of genes involved in cellular differentiation, hypoxia responses, apoptosis, and circadian rhythms (Honma et al., 2002; Miyazaki et al., 2002). A previous study demonstrated that DEC2 also participates in the circadian control of hepatic metabolism by regulating the expression of P450s (Noshiro et al., 2004); however, the regulation mechanism remains to be fully understood.

Families CYP1, CYP2, and CYP3 encompass the most relevant xenobiotic-metabolizing P450s in humans (Evans and Relling, 1999). Despite the low content of the CYP2D6 isoform in human hepatic cells, a variety of drugs are metabolized by this enzyme (Daly et al., 1993; Wolf and Smith, 1999). However, circadian regulation of *Cyp2d* genes has been little explored, even in experimental animals. In this study, we found that brief exposure of HepG2 cells to 50% serum also induced a significant 24-h oscillation in the expression of CYP2D6. Although hepatic expression of CYP2D6 is dependent on both hepatic nuclear factor-4α (HNF4α) and CCAAT enhancer binding protein-α (C/EBPα), DEC2 repressed C/EBPα-induced transactivation of the *CYP2D6* gene. Therefore, we investigated the underlying mechanism of the circadian expression of CYP2D6 in serum-shocked cells by focusing on the transcriptional interaction between DEC2 and C/EBPα.

Materials and Methods

Cell Culture and Animals. HepG2 cells were supplied by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (SAFC Bioscience, Kansas City, MO) at 37°C in a humidified 5% CO_2 atmosphere. Male ICR mice were housed under a 12-h light/dark cycle (lights on at Zeitgeber time 0) with food and water ad libitum. They were cared for in accordance with the guidelines established by the Animal Care and Use Committee of Kyushu University. Primary cultures of hepatocytes were prepared by standard techniques, and cells were maintained in hepatocyte maintenance medium supplemented with 5% FBS, 0.1 μM insulin, 0.1 μM dexamethasone, 50 μg/ml gentamicin, and 50 ng/ml amphotericin B.

Experimental Design. To synchronize the circadian clocks in cultured HepG2 cells, serum shock was performed as follows. Cells were grown to semiconfluence in DMEM supplemented with 10% FBS and then incubated in serum-starved medium for 12 h. On the day of serum shock, 50% FBS or phosphate-buffered saline (control) was added for 2 h, and then cells were changed back to starvation medium. Cells were harvested for RNA extraction at 0, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, and 60 h after serum treatment. The mRNA levels of *CYP1A2*, *-2C9*, *-2C19*, and *-2D6* were measured by reverse transcription (RT)-polymerase chain reaction (PCR). To quantify the protein levels of CYP2D6 and its enzymatic activity, microsomes were prepared from HepG2 cells at 0, 6, 12, 18, 24, 30, 36, 42, 48, 54, and 60 h after serum treatment. The protein abundance of CYP2D6 was also determined by Western blotting. The enzymatic activity of CYP2D6 in the microsomal fraction was investigated using 7-methoxy-4-(aminomethyl) coumarin (MAMC). To investigate the influence of HNF4α and clock gene products on the expression of endogenous *CYP2D6*, HepG2 cells were transfected with expression plasmids encoding HNF4α, C/EBPα, CLOCK, BMAL1, PER2, CRY1, DEC2, DBP, E4BP4, RORα, or REV-ERBα. At 24 h after transfection, mRNA levels of *CYP2D6* were determined by RT-PCR. To clarify whether endogenous HNF4α and clock gene products affect the expression of CYP2D6 protein, HepG2 cells were transfected with small interfering RNA (siRNA) against HNF4α, C/EBPα, or DEC2. Twenty-four hours after transfection, the protein levels of HNF4α, C/EBPα, DEC2, or CYP2D6 were assessed by Western blotting. The transcriptional mechanism of *CYP2D6* by HNF4α, C/EBPα, and DEC2 was analyzed using luciferase reporter vectors containing the 5′-flanking region of the *CYP2D6* gene. The luciferase reporter assay was also performed in the presence or absence of trichostatin A (TSA), a histone deacetylase inhibitor. Interactions between DEC2 and C/EBPα were investigated by immunoprecipitation assay. The binding of endogenous C/EBPα, DEC2, or histone deacetylase-1 (HDAC1) on the CYP2D6 promoter in HepG2 cells was analyzed by chromatin immunoprecipitation. To explore the role of DEC2 in the circadian regulation of CYP2D6 expression, HepG2 cells were transfected with siRNA against DEC2 and thereafter treated with 50% FBS as described above. To explore whether the expression of the *Cyp2d* gene in the experimental animals exhibits circadian oscillation, the temporal profiles of Cyp2d9 and Cyp2d22 mRNA were assessed using serum-shocked primary culture of hepatocytes. We also investigated the temporal expression profiles of Cyp2d9 and Cyp2d22 mRNA in mouse liver kept under a 12-h light/dark cycle.

TABLE 1
Primer sequence used for quantitative RT-PCR analysis

Direction	Sequences (5′-3′)
Human *CYP1A2*	
Forward	5′-CATCCC CCACAGCACAACAA-3′
Reverse	5′-TCCCACTTGGCCAGGACTTC-3′
Human *CYP2C9*	
Forward	5′-AGCTTGGAAAACACTGCAGT-3′
Reverse	5′-CCTGCTGAGAAAGGCATGAAG-3′
Human *CYP2C19*	
Forward	5′-CCTGCTGAGAAAGGCATGAAG-3′
Reverse	5′-CCTGCTGAGAAAGGCATGAAG-3′
Human *CYP2D6*	
Forward	5′-CCCATATGACATCCCGTGACATC-3′
Reverse	5′-TTGGTGATGAGTGTCGTTCCCTTA-3′
Human *β-ACTIN*	
Forward	5′-GACAGGATGCAGAAGGAGATTACT-3′
Reverse	5′-TGATCCACATCTGCTGGAAGGT-3′
Mouse *Cyp2d9*	
Forward	5′-TGGCACAGATAGAGAAGGCCA-3′
Reverse	5′-TCACGCACCACCATGAGC-3′
Mouse *Cyp2d22*	
Forward	5′-GACACCCTTTCAGCCCTAACA-3′
Reverse	5′-GAAGCGTGGGTCATCGTACT-3′
Mouse *β-Actin*	
Forward	5′-CCAGGGTGTGATGGTGGGAA-3′
Reverse	5′-TTCACGGTTGGCCTTAGGGT-3′

Quantitative Reverse Transcription-PCR Analysis. Total RNA was extracted using RNAiso (Takara Bio Inc., Shiga, Japan). cDNA was prepared via reverse transcription of total RNA using a ReverTra Ace qPCR RT kit (Toyobo Co. Ltd., Osaka, Japan). Diluted cDNA samples were analyzed by real-time or semiquantitative RT-PCR. Real-time PCR was performed using THUNDERBIRD SYBR qPCR Mix (Toyobo) and the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The sequences of primer pairs are listed in Table 1.

Determination of CYP2D6 Activity. The enzymatic activity of CYP2D6 in HepG2 cells was determined by assessing the production rate of 7-hydroxy-4-aminomethylcoumarin (HAMC), the O-demethylated metabolite of MAMC (Onderwater et al., 1999). Microsomal fractions prepared from HepG2 cells were incubated in 0.1 M potassium phosphate buffer, pH 7.4, containing 10 μM NADPH and 0.4 mM EDTA. We added the inhibitory antibodies against CYP1A2 to the reaction mixture, because MAMC is also metabolized by CYP1A2 (Onderwater et al., 1999). In fact, antibodies against CYP1A2 decreased the production rate of HAMC in the HepG2 microsomal fraction by approximately 12%, whereas inhibitory antibodies to CYP2D6 reduced the activity by approximately 85%. After equilibration at 37°C, MAMC was added to the reaction mixture at a final concentration of 25 μM, and the real-time increase in fluorescence was recorded by spectrofluorometer with the excitation wavelength set at 405 nm and emission wavelength set at 480 nm. The production rate of HAMC was quantified from the resulting increase in fluorescence. Protein concentration of reaction mixtures was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The CYP2D6 activity was expressed as picomoles of HAMC during the 1-min incubation per milligram of protein.

Western Blotting. Samples (20 μg of protein) were separated on SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were reacted with antibodies against human CYP2D6 (Nihon Nosan, Kyoto, Japan), HNF4*α*, C/EBPα, DEC2, HDAC1, or ACTIN (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Specific antigen-antibody complexes were made visible using peroxidase-conjugated secondary antibodies and Chemi-Lumi One (Nacalai Tesque Inc., Kyoto, Japan).

siRNA. We designed siRNA for knockdown experiments using BLOCK-iT RNAi Designer (https://rnaidesigner.invitrogen.com/rnaiexpress/). The target sequences of *C/EBPα*, *HNF4α*, and *DEC2* genes are listed in Table 2. One day before transfection, HepG2 cells were seeded (5×10^5 cells/well) in six-well plates containing serum-free DMEM. siRNA against HNF4α, C/EBPα, DEC2, or scrambled control oligo (200 ng each) was transfected into the cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA).

TABLE 2
siRNA sequences for target gene

	siRNA Sequences (5′-3′)
Negative control	5′-UUCUCCGAACGUGUCACGU-3′
	5′-ACGUGACACGUUCGGAGAA-3′
Anti-*C/EBPα* siRNA	5′-GAUCGCACUAAGGAUGACGACACUAU-3′
	5′-AUAGUGUCGUCAUCCUUAGUGCGAUC-3′
Anti-*HNF4α* siRNA	5′-ACCCGGUACUCGUUGCUGUUCUUGU-3′
	5′-ACAAGAACAGCAACGAGUACCGGGU-3′
Anti-*DEC2* siRNA	5′-GCCUAACCAUUAGUACUUA-3′
	5′-UAAGUACUAAUGGUUAGGC-3′

Construction of Reporter Plasmids and Expression Vectors. To construct the luciferase reporter vectors of the human *CYP2D6* gene [*CYP2D6*-Luc], an approximately 1.4-kilobase pair fragment (−1399 to +89; +1 indicates the putative transcription start site) derived from the 5′-flanking region of the human *CYP2D6* gene (GenBank accession no. DQ211353) was amplified by PCR from genomic DNA of HepG2 cells. The PCR products were purified and ligated into the pGL3 basic vector (Promega, Madison, WI). The mutant construct of *CYP2D6*-Luc was prepared by changing the sequence from CATTGCACAATG to CAAAGCTTAATG (bases −1231 to −1220) and from AGGGCAAAGGCCA to AGGGTA-AGCTTCA (bases −55 to −43), respectively (Fig. 3A). Expression plasmids of HNF4α, C/EBPα, CLOCK, BMAL1, PER2, CRY1, DEC2, DBP, E4BP4, RORα, and REV-ERBα were prepared as follows: the coding regions of the transcriptional regulators were obtained by RT-PCR and used after their sequences had been confirmed. All coding regions were ligated into the pcDNA 3.1 vector (Invitrogen).

Transcriptional Assay. On the day before transfection, the cells were seeded (2×10^5 cells/well) into six-well plates containing DMEM supplemented with 10% FBS. Cells were transfected with 100-ng reporter constructs and 1-μg (total) expression vectors, using Lipofectamine-LTX reagent (Invitrogen) according to the manufacturer's instructions. To correct for variations in transfection efficiency, 0.5 ng of pRL-SV40 (Promega) was cotransfected in all experiments. The total amount of DNA per well was adjusted to 1.0 μg by addition of pcDNA3.1 vector (Invitrogen). The transfected cells were incubated in the presence or absence of TSA, a histone deacetylase inhibitor. At 48 h after transfection, cell extracts were prepared with 500 μl of passive lysis buffer (Promega), and 50-μl portions of the extracts were used for assays of firefly luciferase and *Renilla reniformis* luciferase by luminometry. The ratio of firefly luciferase activity (expressed from reporter plasmids) to *R. reniformis* luciferase activity (expressed from pRL-SV40) in each sample served as a measure of normalized luciferase activity.

Immunoprecipitation Assay. Nuclear fractions of HepG2 cells were prepared at the indicated time points after 50% serum treatment. The fractions were immunoprecipitated by anti-DEC2 or anti-C/EBPα antibodies on protein G-agarose beads. The specific bound proteins were released by resuspending beads in 20 μl of loading buffer, divided into equal amounts, and resolved by SDS-polyacrylamide gels. One gel was subjected to Western blotting with anti-DEC2, anti-C/EBPα, or anti-HDAC1 antibodies.

Chromatin Immunoprecipitation Assay. Cells were cross-linked with 1% formaldehyde in phosphate-buffered saline at 4°C for 10 min. Each cross-linked sample was sonicated on ice and then incubated with antibodies against C/EBPα, DEC2, or HDAC1. DNA was isolated from the immunoprecipitates and subjected to PCR using the following primer pairs: for the surrounding C/EBPα binding site in the CYP2D6 promoter (from base pairs −1312 to −1074), 5′-TGGTGAAACCCTATCTCTACTG-3′ and 5′-TCACTGCAGTCTC-GACATCA-3′; for CYP2D6 promoter that does not contain C/EBPα

binding sites (from base pairs −827 to −570), 5′-CCTGTTGCAAA-CAAGAAGCCATAG-3′ and 5′-GGACACGATTACACATGCAGAA-AAT-3′. As negative controls, chromatin immunoprecipitations were 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.

Statistical Analysis. The statistical significance of the differences among groups was analyzed by analysis of variance and Dunnett's test or the Bonferroni multiple comparison test. A 5% level of probability was considered significant.

Results

Rhythmic Expression of *CYP2D6* Gene in HepG2 Cells after Serum Treatment. We demonstrated previously that treatment of HepG2 cells with 50% FBS for 2 h induced the rhythmic expression of CYP3A4 and CYP2E1 (Takiguchi et al., 2007; Matsunaga et al., 2008). Treatment of cells with a high concentration of serum transiently induced the expression of CYP1A2 mRNA but did not affect the mRNA levels of CYP2C9 and CYP2C19 (Fig. 1A). On the other hand, serum-shocked HepG2 cells exhibited a significant oscillation in the expression of CYP2D6 ($P < 0.05$) (Fig. 1A). The rhythmic phase of mRNA levels for CYP2D6 was similar to that of DBP as reported previously (Takiguchi et al., 2007); however, the oscillation in the levels of CYP2D6 protein was delayed by approximately 8 h relative to its mRNA rhythm (Fig. 1B). The rhythmic pattern of CYP2D6 protein expression resembled the overall decreases and increases in the enzymatic activity of CYP2D6 (Fig. 1C). These in vitro results suggest that oscillation in the expression of the CYP2D6 gene is cell autonomous. The rhythmic change in mRNA levels of CYP2D6 seemed to cause the oscillation of its protein abundance and enzymatic activity.

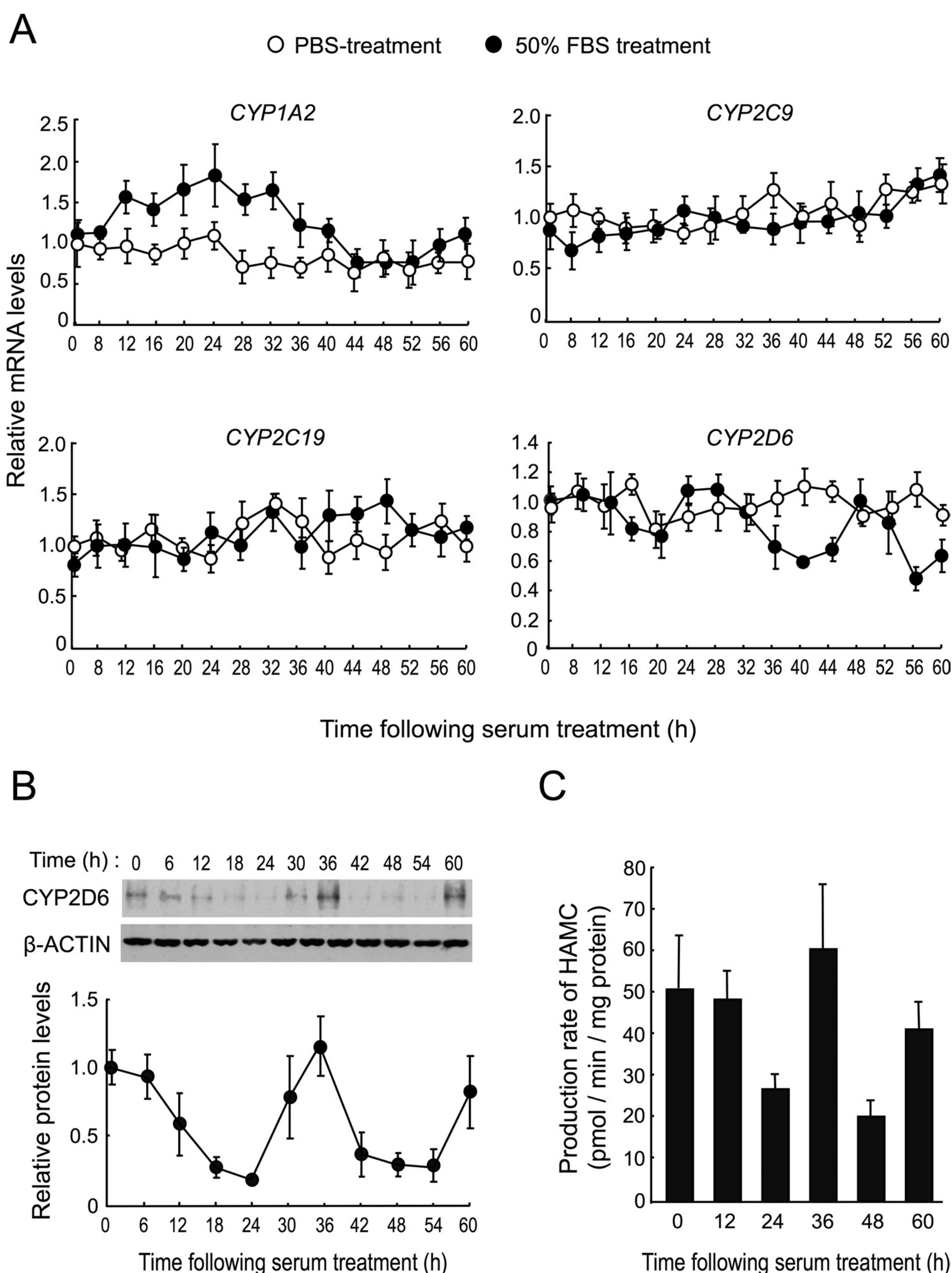

Fig. 1. Temporal expression profiles for *CYP* genes in HepG2 cells after serum treatment. A, quantification of temporal changes in CYP1A2, 2C9, 2C19, and 2D6 mRNA in HepG2 cells after treatment with 50% serum or phosphate-buffered saline. The basal level of mRNA (at time point 0) was set at 1.0. Each value is the mean with S.E.M. (n = 3–4). There is a significant time-dependent variation in the mRNA levels of CYP2D6 in serum-treated cells ($P < 0.05$, analysis of variance). B, temporal expression profile of CYP2D6 protein in HepG2 cells after treatment with 50% serum. Top, representative electrophoretic image of Western blotting of CYP2D6 protein in the serum-shocked HepG2 cells. Protein levels of CYP2D6 were normalized by those of ACTIN, and basal abundance (at time point 0) was set at 1.0. Each value is the mean with S.E.M. (n = 3–4). There is a significant time-dependent variation in the protein abundance of CYP2D6 ($P <$ 0.05, analysis of variance). C, temporal changes in enzymatic activity of CYP2D6 in HepG2 cells after serum treatment. The production rate of HAMC, the O-demethylated metabolite of MAMC, was assessed as an index of CYP2D6 enzymatic activity. Values are the mean ± S.E.M (n = 6). There is a significant time-dependent variation in the metabolizing activity of CYP2D6 ($P < 0.05$, analysis of variance).

Effects of Clock and Clock-Controlled Gene Products on the mRNA Levels of CYP2D6. Clock genes, consisting of core oscillation loops, generate 24-h variations in output physiology through the periodic activation/repression of clock-controlled output genes (Ripperger et al., 2000; Gachon et al., 2006). To explore whether the products of clock genes and/or clock-controlled output genes affect the expression of CYP2D6, we investigated the effects of the transfection of expression plasmids encoding CLOCK, BMAL1, PER2, CRY1, DEC2, DBP, E4BP4, RORα, or REV-ERBα on the mRNA level of CYP2D6 in HepG2 cells. Cells were also transfected with expression vectors coding HNF4α or C/EBPα as a positive control (Cairns et al., 1996; Jover et al., 1998). As shown in Fig. 2A, the mRNA level of CYP2D6 was elevated significantly when cells were transfected with HNF4α or C/EBPα expression plasmids ($P < 0.05$ for both), whereas transfection of cells with DEC2 significantly suppressed the endogenous expression of CYP2D6 mRNA ($P < 0.05$). Consistent with these findings, transfection of HepG2 cells with siRNA against HNF4α or C/EBPα resulted in an obvious reduction of CYP2D6 protein levels (Fig. 2B), whereas transfection with DEC2 siRNA increased CYP2D6 protein abundance. Among the products of clock genes and/or clock-controlled output genes, DEC2 seems to act as a repressor of CYP2D6. In this experiment, the protein levels of C/EBPα were decreased by transfection with the siRNA against HNF4α, but transfection of C/EBPα siRNA had little effect on the protein levels of HNF4α. These results also suggest that HNF4α may play a predominant role in hepatic C/EBPα expression.

Transcriptional Regulation of the *CYP2D6* Gene by HNF4α and DEC2. To investigate the transrepression mechanism of the *CYP2D6* gene by DEC2, we performed the luciferase reporter assay using native or mutated *CYP2D6* reporter constructs. Cotransfection of *CYP2D6*-Luc with HNF4α resulted in a 6-fold increase in promoter activity (Fig. 3B). A similar increase in promoter activity was also detected when *CYP2D6*-Luc was cotransfected with C/EBPα. These transactivation effects of HNF4α and C/EBPα were abolished when their binding sites were mutated (Fig. 3B). DEC2 repressed either HNF4α- or C/EBPα-mediated transactivation of *CYP2D6*. Although cotransfection with 1.0 μg of DEC2 plasmid partially inhibited HNFα-mediated transcription, transfection with the same amount of DEC2 plasmid suppressed the C/EBPα-induced promoter activity of *CYP2D6* at the basal level (Fig. 3C). These results suggest that DEC2 represses the transcription of CYP2D6 by mainly interacting with C/EBPα.

HDAC is often involved in transcriptional repression by bHLH transcription factors, and it has been reported that some actions of DEC2 are suppressed by HDAC inhibitors (Sun and Taneja, 2000; Garriga-Canut et al., 2001). We therefore examined the effects of the HDAC inhibitor TSA on DEC2-mediated repression. TSA dose dependently restored the DEC2-mediated repression of C/EBPα-induced CYP2D6 transactivation (Fig. 4A). The results of the immunoprecipitation assay revealed that DEC2 precipitated together with C/EBPα (Fig. 4B). Endogenously expressed HDAC1 proteins in HepG2 cells were also coimmunoprecipitated together with DEC2.

Although transfection of HepG2 cells with DEC2 expression vectors had little effect on the binding amounts of C/EBPα to its binding site in the *CYP2D6* promoter, the treatment-enhanced formation of the HDAC1-chromatin complex (Fig. 4C). The DEC2-enhanced HDAC1-chromatin formation seemed to be dependent on the C/EBPα protein, because no DNA bands were detected in HDAC1-immunoprecipitated chromatin by using primer pairs for amplifying the *CYP2D6* promoter region that does not contains C/EBPα binding site (Fig. 4C). These results suggest that DEC2 promotes HDAC1 recruitment on the *CYP2D6* promoter through the protein-protein interaction with C/EBPα. The correlation between the interaction of these proteins and the transcriptional regulation of CYP2D6 suggests that suppressive action of DEC2 on C/EBPα-mediated transactivation of CYP2D6 is attributable to the sustained recruitment of HDAC1.

Role of DEC2 in the Circadian Regulation of *CYP2D6* in Serum-Shocked HepG2 Cells. The levels of DEC2 mRNA also showed obvious 24-h oscillation in serum-shocked HepG2 cells (Fig. 5A). The rhythmic phase of DEC2 mRNA was similar to Per2 mRNA oscillation (Takiguchi et al., 2007). The oscillation in the expression of DEC2 protein was nearly antiphase to that in the mRNA levels of CYP2D6 (Fig. 5B), but the protein levels of C/EBPα and HDAC1 failed to show obvious circadian oscillation in serum-shocked cells. Immunoprecipitation experiments revealed that in serum-

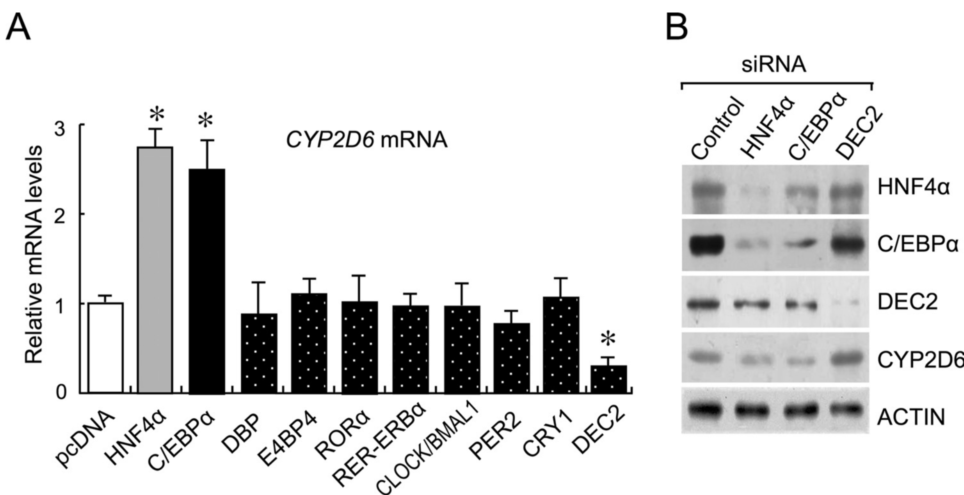

Fig. 2. Influence of clock gene products on the expression of CYP2D6 in HepG2 cells. A, cells were transfected with 1.0 μg of expression plasmids, encoding HNF4α, C/EBPα, DBP, E4BP4, RORα, REV-ERBα, CLOCK, BMAL1, PER2, CRY1, or DEC2. Total RNA was extracted from the cells 24 h after transfection and subjected to RT-PCR of CYP2D6 mRNA. For plots of intensity, the mean value of the control (pcDNA3) was set at 1.0. Each value is the mean with S.E.M. ($n = 3$). *, $P < 0.05$ compared with the control (pcDNA3) group using Dunnett's test. B, influence of the down-regulation of HNF4α, C/EBPα, or DEC2 on the expression of CYP2D6 protein in HepG2 cells. Cells were transfected with siRNA against HNF4α, C/EBPα, or DEC2 (20 nM each). Protein levels of C/EBPα HNF4α, DEC2, and CYP2D6 were assessed by Western blotting.

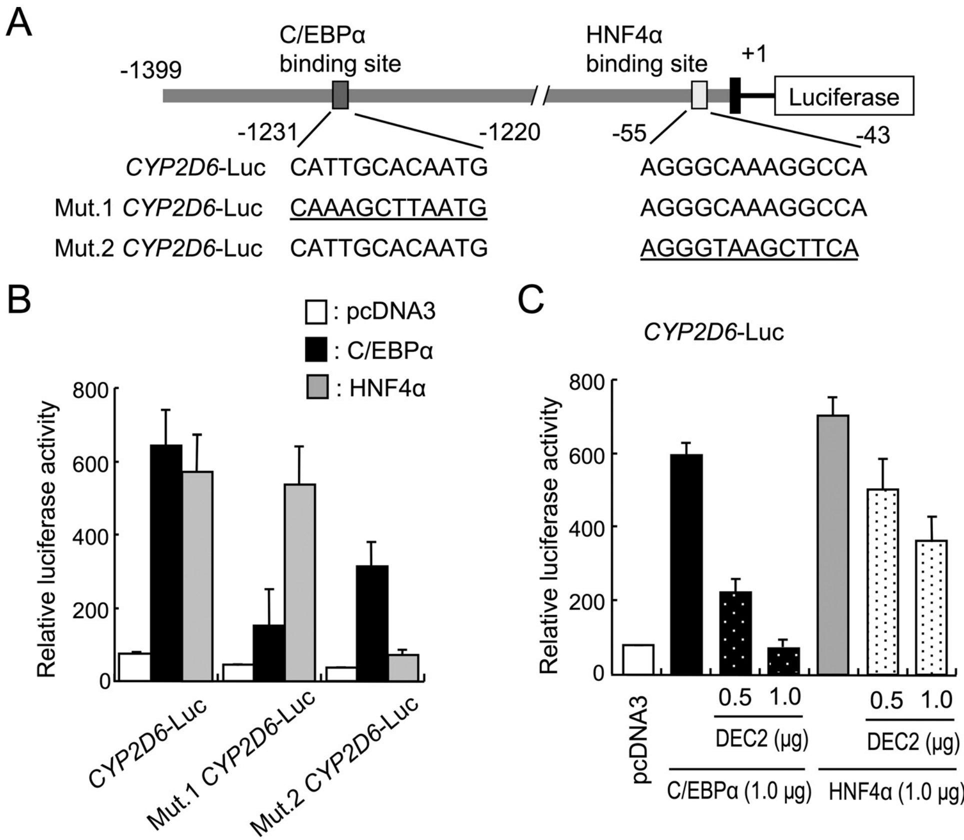

Fig. 3. Transcriptional regulation of CYP2D6 by C/EBPα and DEC2. A, schematic representation of human CYP2D6 promoter. Numbers below boxes are nucleotide residues in which C/EBPα and HNF4α binding sites are positioned relative to the transcription start site (+1). Underlined nucleotide residues indicate mutated sequences of C/EBPα- and HNF4α-binding sites. B, mutation of sequence of response elements abrogates C/EBPα- or HNF4α-induced *CYP2D6* promoter activity. C, repressive action of DEC2 on C/EBPα-induced *CYP2D6* promoter activity. Values are the mean ± S.E.M (n = 4).

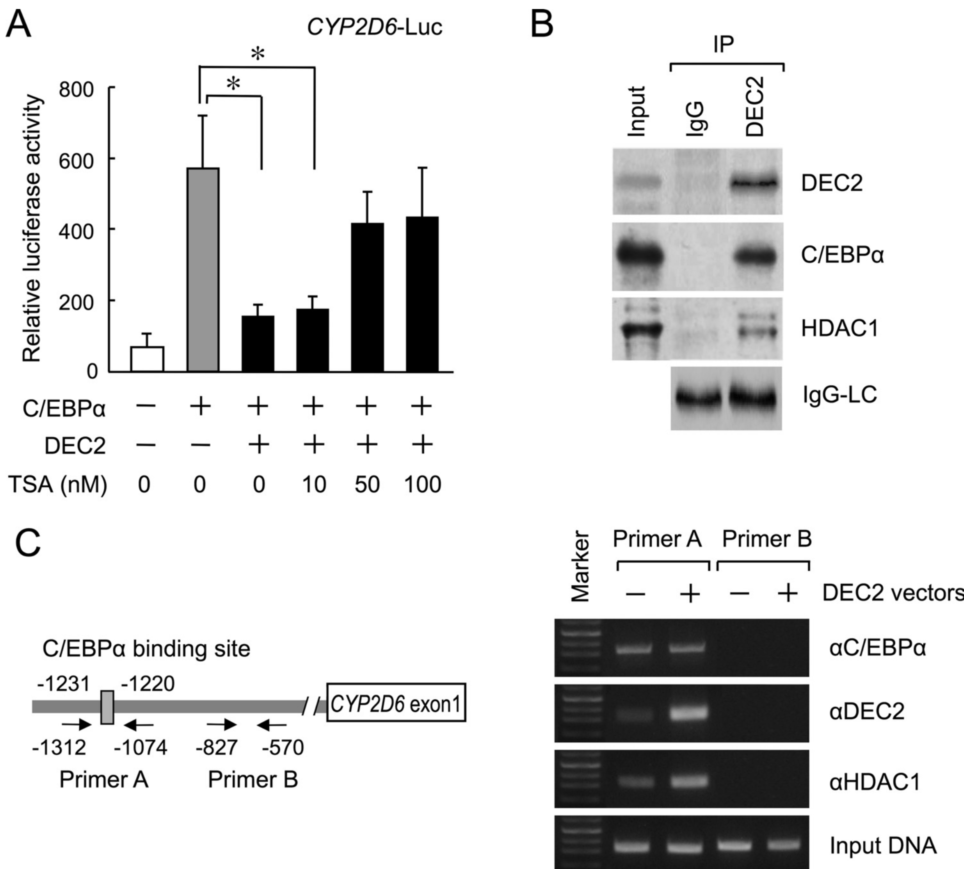

Fig. 4. DEC2 inhibits C/EBPα-mediated transactivation of CYP2D6 by HDAC1 recruitment. A, effects of the HDAC inhibitor TSA on DEC2-mediated transcriptional repression on C/EBPα and HNF4α. Values are the mean ± S.E.M (n = 4). *, $P < 0.05$ for comparison between two groups. B, DEC2 interacts with C/EBPα, accompanied by formation of a complex with HDAC1. Nuclear fractions of HepG2 cells were subjected to immunoprecipitation (IP) with anti-DEC2 or IgG antibodies and Western-blotted with biotinylated antibodies against DEC2, C/EBPα, or HDAC1. The light chain of IgG (IgG-LC) was the positive control. C, chromatin immunoprecipitation analysis of *CYP2D6* promoter in HepG2 cells. Left, schematic diagram of the CYP2D6 5′-flanking region. Solid line arrows represent amplification area by PCR. Numbers represent distances in bases from the putative transcriptional start site marked as +1. HepG2 cells were transfected with DEC2 expression vectors. Cross-linked chromatins collected from cells were immunoprecipitated with antibodies against C/EBPα, DEC2, or HDAC1. Right, representative electrophoretic image of PCR products are shown.

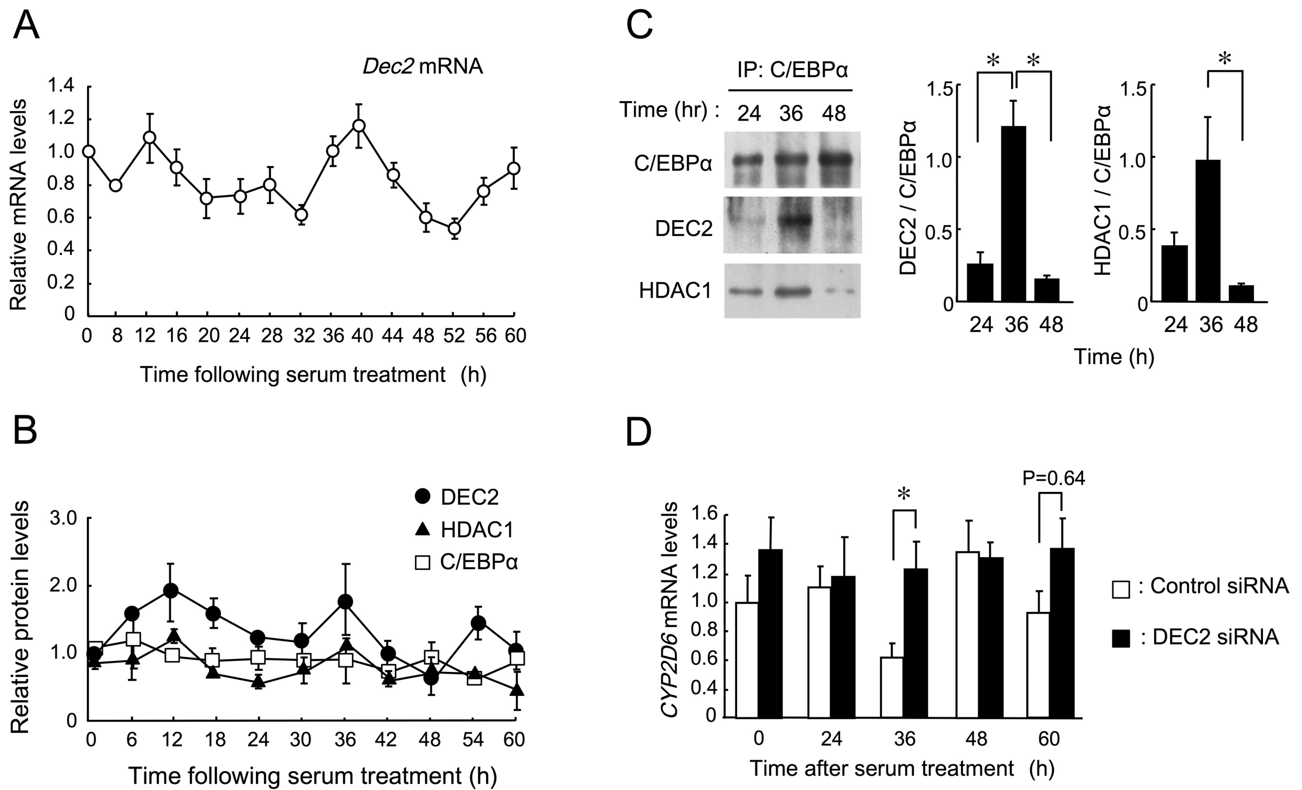

Fig. 5. DEC2 regulates the circadian expression of CYP2D6 in serum-shocked HepG2 cells. A, temporal expression profiles of DEC2 in HepG2 cells after serum treatment. B, temporal profiles of protein abundance of C/EBPα, DEC2, and HDAC1 in the nuclear fractions of HepG2 cells after serum treatment. Cells were exposed to 50% serum for 2 h, and thereafter nuclear fractions were prepared at the indicated times. C, time-dependent interaction between C/EBPα and DEC2 in serum-shocked HepG2 cells. Nuclear fractions were prepared at the indicated time points. Nuclear fractions were subjected to immunoprecipitation (IP) with anti-C/EBPα antibodies and Western-blotted with antibodies against DEC2, C/EBPα, or HDAC1. Band intensities of DEC2 and HDAC1 were normalized by C/EBPα and are shown as the mean ± S.E.M (n = 4–5). *, $P < 0.05$ for comparison between two groups. D, influence of down-regulation of DEC2 on the circadian oscillation of *CYP2D6* mRNA in serum-shocked HepG2 cells. Cells were transfected with control or Dec2 siRNA on 2 days before 50% serum treatment. For all panels, value are the mean ± S.E.M (n = 4–5). *, $P < 0.05$ for comparison between two groups.

shocked HepG2 cells, the amount of DEC2 associated with C/EBPα increased at the time corresponding to the trough of the CYP2D6 mRNA expression (Fig. 5C), whereas a decrease in the amount of DEC2-C/EBPα complex almost matched the peak of CYP2D6 expression. As shown in Fig. 2B, transfection of cells with siRNA against DEC2 caused an elevation of CYP2D6 protein levels. Furthermore, treatment of cells with DEC2 siRNA also prevented serum shock-induced oscillation in the expression of CYP2D6 mRNA (Fig. 5D). These results indicate that DEC2 protein interacts with C/EBPα in a time-dependent manner. Time-dependent interactions may underlie the circadian expression of CYP2D6 in serum-shocked HepG2 cells.

Rhythmic Expression of *Cyp2d9* Gene in Mouse Hepatocytes. In the final set of experiments, we explored whether the expression of *Cyp2d* gene in the experimental animals also exhibited circadian oscillation. Computer-aided analysis identified putative C/EBPα-binding sites in the promoter region of the mouse *Cyp2d9* gene, the murine homolog to human CYP2D6; however, putative binding sites were not found in the promoter region of the mouse *Cyp2d22* gene. The mRNA levels of Cyp2d9, but not of Cyp2d22, showed significant 24-h oscillation not only in the primary cultured mouse hepatocytes ($P < 0.05$) (Fig. 6A) but also in the liver of mice ($P < 0.05$) (Fig. 6B). These findings suggest that putative C/EBPα-binding sites in the promoter region of *Cyp2d9* gene are also functionally important for rhythmic expression of its mRNA.

Discussion

Members of the bHLH family of transcription factors have been shown to play critical roles in cellular differentiation, growth, apoptosis, hypoxia response, and circadian rhythm regulation (Honma et al., 2002; Miyazaki et al., 2002; Azmi et al., 2004; Takiguchi et al., 2007). DEC2 was originally found in rat brain (Rossner et al., 1997) and was subsequently identified in humans and mice (Rossner et al., 1997; Garriga-Canut et al., 2001). DEC2 is expressed in a variety of tissues (Lu et al., 1999; Fujimoto et al., 2001; Miyazaki et al., 2002); however, its role in the circadian regulation of hepatic metabolism has not been fully evaluated. In this study, we showed that DEC2 acts as a potent repressor of C/EBPα. The repressive action of DEC2 on C/EBPα seemed to be the underlying cause of circadian expression of CYP2D6 in serum-shocked HepG2 cells (Fig. 7). The expression levels of several types of P450s in HepG2 cells have been reported to be lower than those in primary human hepatocytes (Jover et al., 1998; Hara and Adachi, 2002; Westerink and Schoonen, 2007). However, considerable metabolic activity of CYP2D6 was detected in HepG2 cells, and the drug-metabolizing activity also varied in a circadian fashion.

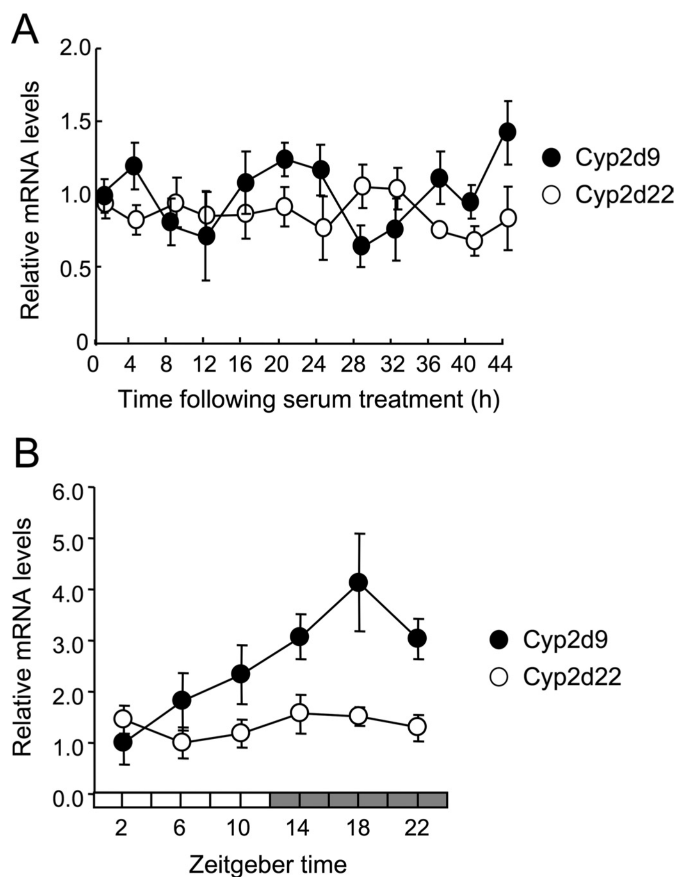

Fig. 6. Temporal expression profiles for the mRNA levels of Cyp2d9 and Cyp2d22 in serum-shocked mouse primary cultured hepatocytes (A) and in mouse liver (B). Values are shown as mean with S.E.M. ($n = 3–5$). For both panels, levels of Cyp2d9 mRNA significantly varied in a circadian time-dependent manner ($P < 0.05$; analysis of variance).

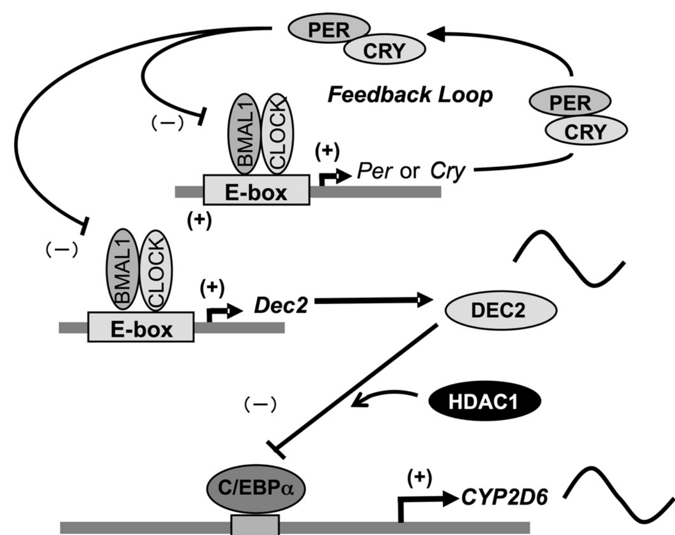

Fig. 7. Schematic representation of molecular mechanism regulating circadian expression of human *CYP2D6* gene in serum-shocked hepatic cells. Hepatic nuclear factors, HNF4α and C/EBPα, bind to their response elements in the *CYP2D6* promoter to activate its transcription. DEC2 periodically interacts with HNF4α and C/EBPα, thereby repressing their transactivation of the *CYP2D6* gene.

A recent report also demonstrated that DEC2 interacts with C/EBPα to suppress the expression of C/EBPα-target genes (Gulbagci et al., 2009). Protein-protein interaction is dependent on both the bHLH domain and carboxyl-terminal region of DEC2 protein. Furthermore, the suppressive actions of DEC2 on C/EBPα-mediated transactivation are suggested to be caused by sustained recruitment of HDAC1. The results of the immunoprecipitation assay revealed that DEC2 interacted with C/EBPα, accompanied by the association with HDAC1. The formation complex with HDAC1 seemed to underlie the suppressive actions of DEC2 on C/EBPα-mediated transactivation. DEC2 also functions as a corepressor of retinoid X-receptors (Cho et al., 2009). Retinoid X-receptors heterodimerized with pregnane X receptors or constitutive androstane receptors to regulate the expression of CYP3A4 (Pascussi et al., 2000a,b; Chen et al., 2010). Therefore, DEC2 may also contribute to the circadian regulation of *CYP3A4* gene in the serum-shocked HepG2 cells (Takiguchi et al., 2007).

CYP2D6, a member of the P450 superfamily, is responsible for the metabolism of approximately 25% of commonly prescribed drugs (Bertilsson and Dahl, 1996). The gene that encodes CYP2D6 has more than 90 variants (Ingelman-Sundberg, 2005; Beverage et al., 2007). Such polymorphism leads to a variety of enzymatic activities and different phenotypes. In fact, the activity of CYP2D6 ranges from complete deficiency to excessive activity, potentially causing medication toxicity or therapeutic failure even at the recommended drug dosage (Zanger et al., 2004; Ingelman-Sundberg, 2005; Beverage et al., 2007). CYP2D6 polymorphism is therefore regarded as the reason for interindividual differences in the pharmacokinetics and pharmacodynamics of drugs. In addition to interindividual variation, the present findings using an in vitro model of the hepatic circadian clock suggested that there was also intraindividual variation in CYP2D6 activity. In serum-shocked HepG2 cells, significant 24-h oscillation was detected not only in the mRNA levels of CYP3A4 but also in its metabolic activity (Takiguchi et al., 2007). Daily variation in CYP3A4 activity in humans has been suggested by the fact that the pharmacokinetics of several drugs, which are mainly eliminated by CYP3A4 metabolism, vary according to their dosing times (Smith et al., 1986; Min et al., 1997). Although the CYP2D6-mediated drug metabolism in human liver may also vary depending on its dosing time, it has not been clarified whether drug-metabolic activity of CYP2D6 in human liver exhibits circadian oscillation. Further studies are required to investigate this point. The mRNA levels of Cyp2d9, the murine homolog to human CYP2D6, exhibited significant circadian oscillation in mouse liver. The oscillation of Cyp2d9 mRNA levels was nearly antiphase to that of DEC2 expression (Noshiro et al., 2004). Because computer-aided analysis identified putative C/EBPα-binding sites in the promoter region of the mouse *Cyp2d9* gene, DEC2 may periodically repress the transcriptional activity of C/EBPα, thereby inducing the circadian expression of Cyp2d9 mRNA. Taken together, these findings suggest the possibility that the expression of CYP2D6 oscillates in human liver.

The individualization of pharmacotherapy has been achieved mainly by monitoring drug concentrations. Consequently, dosage adjustment is based on interindividual differences in drug pharmacokinetics; however, intraindividual as well as interindividual variability should be considered to aim for further improvement in rational pharmacotherapy, because the pharmacokinetics of many drugs also vary, depending on rhythmicity in absorption, distribution, metabo-

lism, and elimination (Ohdo et al., 2010). Although the contribution of 24-h variation in CYP2D6 expression to drug metabolism should be clarified, our results suggest a mechanism underlying the dosing time-dependent differences in the pharmacokinetics of drugs and provide a molecular link between the circadian clock and xenobiotic metabolism.

Acknowledgments

We are indebted to Dr. N. Watanabe (Daiichi Sankyo Co., Ltd., Tokyo, Japan) and M. Iwasaki (Daiichi Sankyo RD Novare Co., Ltd., Tokyo, Japan) for technical support.

Authorship Contributions

Participated in research design: Matsunaga, Koyanagi, and Ohdo.

Conducted experiments: Matsunaga, Inoue, Kusunose, Kakimoto, Hamamura, Hanada, Toi, and Koyanagi.

Contributed new reagents or analytic tools: Hanada, Toi, Sato, Fujimoto, and Koyanagi.

Performed data analysis: Matsunaga, Inoue, Yoshiyama, and Koyanagi.

Wrote or contributed to the writing of the manuscript: Matsunaga, Inoue, Koyanagi, and Ohdo.

References

Akashi M and Takumi T (2005) The orphan nuclear receptor RORα regulates circadian transcription of the mammalian core-clock Bmal1. *Nat Struct Mol Biol* **12:**441–448.

Azmi S, Ozog A, and Taneja R (2004) Sharp-1/DEC2 inhibits skeletal muscle differentiation through repression of myogenic transcription factors. *J Biol Chem* **279:** 52643–52652.

Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schütz G, and Schibler U (2000a) Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science* **289:**2344–2347.

Balsalobre A, Damiola F, and Schibler U (1998) A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* **93:**929–937.

Balsalobre A, Marcacci L, and Schibler U (2000b) Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr Biol* **10:**1291–1294.

Bertilsson L and Dahl ML (1996) Polymorphic drug oxidation: relevance to the treatment of psychiatric disorders. *CNS Drugs* **5:**200–223.

Beverage JN, Sissung TM, Sion AM, Danesi R, and Figg WD (2007) CYP2D6 polymorphisms and the impact on tamoxifen therapy. *J Pharm Sci* **96:**2224–2231.

Cairns W, Smith CA, McLaren AW, and Wolf CR (1996) Characterization of the human cytochrome P4502D6 promoter. A potential role for antagonistic interactions between members of the nuclear receptor family. *J Biol Chem* **271:**25269–25276.

Chen S, Wang K, and Wan YJ (2010) Retinoids activate RXR/CAR-mediated pathway and induce CYP3A. *Biochem Pharmacol* **79:**270–276.

Cho Y, Noshiro M, Choi M, Morita K, Kawamoto T, Fujimoto K, Kato Y, and Makishima M (2009) The basic helix-loop-helix proteins differentiated embryo chondrocyte (DEC) 1 and DEC2 function as corepressors of retinoid X receptors. *Mol Pharmacol* **76:**1360–1369.

Daly AK, Cholerton S, Gregory W, and Idle JR (1993) Metabolic polymorphisms. *Pharmacol Ther* **57:**129–160.

Evans WE and Relling MV (1999) Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* **286:**487–491.

Fujimoto K, Shen M, Noshiro M, Matsubara K, Shingu S, Honda K, Yoshida E, Suardita K, Matsuda Y, and Kato Y (2001) Molecular cloning and characterization of DEC2, a new member of basic helix-loop-helix proteins. *Biochem Biophys Res Commun* **280:**164–171.

Gachon F, Olela FF, Schaad O, Descombes P, and Schibler U (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. *Cell Metab* **4:**25–36.

Garriga-Canut M, Roopra A, and Buckley NJ (2001) The basic helix-loop-helix protein, sharp-1, represses transcription by a histone deacetylase-dependent and histone deacetylase-independent mechanism. *J Biol Chem* **276:**14821–14828.

Gekakis N, Staknis D, Nguyen HB, Davis FC, Wilsbacher LD, King DP, Takahashi JS, and Weitz CJ (1998) Role of the CLOCK protein in the mammalian circadian mechanism. *Science* **280:**1564–1569.

Gulbagci NT, Li L, Ling B, Gopinadhan S, Walsh M, Rossner M, Nave KA, and Taneja R (2009) SHARP1/DEC2 inhibits adipogenic differentiation by regulating the activity of C/EBP. *EMBO Rep* **10:**79–86.

Hara H and Adachi T (2002) Contribution of hepatocyte nuclear factor-4 to down-regulation of CYP2D6 gene expression by nitric oxide. *Mol Pharmacol* **61:**194–200.

Honma S, Kawamoto T, Takagi Y, Fujimoto K, Sato F, Noshiro M, Kato Y, and Honma K (2002) Dec1 and Dec2 are regulators of the mammalian molecular clock. *Nature* **419:**841–844.

Ingelman-Sundberg M (2005) Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics J* **5:**6–13.

Jover R, Bort R, Gómez-Lechón MJ, and Castell JV (1998) Re-expression of C/EBPα induces CYP2B6, CYP2C9 and CYP2D6 genes in HepG2 cells. *FEBS Lett* **431:** 227–230.

Kume K, Zylka MJ, Sriram S, Shearman LP, Weaver DR, Jin X, Maywood ES, Hastings MH, and Reppert SM (1999) mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell* **98:**193–205.

Lu J, Webb R, Richardson JA, and Olson EN (1999) MyoR: a muscle-restricted basic helix-loop-helix transcription factor that antagonizes the actions of MyoD. *Proc Natl Acad Sci USA* **96:**552–557.

Matsunaga N, Ikeda M, Takiguchi T, Koyanagi S, and Ohdo S (2008) The molecular mechanism regulating 24-hour rhythm of CYP2E1 expression in the mouse liver. *Hepatology* **48:**240–251.

Min DI, Chen HY, Lee MK, Ashton K, and Martin MF (1997) Time-dependent disposition of tacrolimus and its effect on endothelin-1 in liver allograft recipients. *Pharmacotherapy* **17:**457–463.

Miyazaki K, Kawamoto T, Tanimoto K, Nishiyama M, Honda H, and Kato Y (2002) Identification of functional hypoxia response elements in the promoter region of the DEC1 and DEC2 genes. *J Biol Chem* **277:**47014–47021.

Noshiro M, Kawamoto T, Furukawa M, Fujimoto K, Yoshida Y, Sasabe E, Tsutsumi S, Hamada T, Honma S, Honma K, et al. (2004) Rhythmic expression of DEC1 and DEC2 in peripheral tissues: DEC2 is a potent suppressor for hepatic cytochrome P450s opposing DBP. *Genes Cells* **9:**317–329.

Ohdo S, Koyanagi S, and Matsunaga N (2010) Chronopharmacological strategies: intra- and inter-individual variability of molecular clock. *Adv Drug Deliv Rev* **62:**885–897.

Onderwater RC, Venhorst J, Commandeur JN, and Vermeulen NP (1999) Design, synthesis, and characterization of 7-methoxy-4-(aminomethyl)coumarin as a novel and selective cytochrome P450 2D6 substrate suitable for high-throughput screening. *Chem Res Toxicol* **12:**555–559.

Pascussi JM, Drocourt L, Fabre JM, Maurel P, and Vilarem MJ (2000a) Dexamethasone induces pregnane X receptor and retinoid X receptor-α expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. *Mol Pharmacol* **58:**361–372.

Pascussi JM, Gerbal-Chaloin S, Fabre JM, Maurel P, and Vilarem MJ (2000b) Dexamethasone enhances constitutive androstane receptor expression in human hepatocytes: consequences on cytochrome P450 gene regulation. *Mol Pharmacol* **58:**1441–1450.

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, and Schibler U (2002) The orphan nuclear receptor REV-ERBα controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* **110:**251–260.

Reppert SM and Weaver DR (2002) Coordination of circadian timing in mammals. *Nature* **418:**935–941.

Ripperger JA, Shearman LP, Reppert SM, and Schibler U (2000) CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev* **14:**679–689.

Rossner MJ, Dörr J, Gass P, Schwab MH, and Nave KA (1997) SHARPs: mammalian enhancer-of-split- and hairy-related proteins coupled to neuronal stimulation. *Mol Cell Neurosci* **9:**460–475.

Sakamoto K, Nagase T, Fukui H, Horikawa K, Okada T, Tanaka H, Sato K, Miyake Y, Ohara O, Kako K, et al. (1998) Multitissue circadian expression of rat period homolog (rPer2) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. *J Biol Chem* **273:**27039–27042.

Smith RB, Kroboth PD, and Phillips JP (1986) Temporal variation in triazolam pharmacokinetics and pharmacodynamics after oral administration. *J Clin Pharmacol* **26:**120–124.

Sun H and Taneja R (2000) Stra13 expression is associated with growth arrest and represses transcription through histone deacetylase (HDAC)-dependent and HDAC-independent mechanisms. *Proc Natl Acad Sci USA* **97:**4058–4063.

Takiguchi T, Tomita M, Matsunaga N, Nakagawa H, Koyanagi S, and Ohdo S (2007) Molecular basis for rhythmic expression of CYP3A4 in serum-shocked HepG2 cells. *Pharmacogenet Genomics* **17:**1047–1056.

Terazono H, Mutoh T, Yamaguchi S, Kobayashi M, Akiyama M, Udo R, Ohdo S, Okamura H, and Shibata S (2003) Adrenergic regulation of clock gene expression in mouse liver. *Proc Natl Acad Sci USA* **100:**6795–6800.

Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y, Iino M, and Hashimoto S (2005) System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat Genet* **37:**187–192.

Westerink WM and Schoonen WG (2007) Cytochrome P450 enzyme levels in HepG2 cells and cryopreserved primary human hepatocytes and their induction in HepG2 cells. *Toxicol In Vitro* **21:**1581–1591.

Wolf CR and Smith G (1999) Pharmacogenetics. *Br Med Bull* **55:**366–386.

Yamamoto T, Nakahata Y, Soma H, Akashi M, Mamine T, and Takumi T (2004) Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. *BMC Mol Biol* **5:**18.

Zanger UM, Raimundo S, and Eichelbaum M (2004) Cytochrome P450 2D6: overview and update on pharmacology, genetics, biochemistry. *Naunyn Schmiedebergs Arch Pharmacol* **369:**23–37.

Address correspondence to: Dr. Shigehiro Ohdo, Department of Pharmaceutics, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: ohdo@phar.kyushu-u.ac.jp