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Transcriptional regulation of carboxylesterase gene by glucocorticoid receptors and epigenetic marks

千葉科学大学

大学院薬科学研究科

薬科学専攻

堀　武志

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Transcriptional regulation of carboxylesterase gene by glucocorticoid receptors and epigenetic marks

Takeshi Hori

Chiba Institute of Science

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Contents

Abbreviations

5-aza-dC	5-Aza-2'-deoxycytidine
ADME	Absorption, distribution, metabolism, excretion
AID	Activation-induced deaminase
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide
BBB	Blood-brain barrier
BER	Base excision repair
cDNA	Complementary DNA
CES	Carboxylesterase
CHX	Cycloheximide
cMOAT	Canalicular multispecific organic anion transporter
CpG	Cytosine guanine dinucleotide
CPT-11	Irinotecan
C_t	Threshold cycle
CYP	Cytochrome P450
ΔC_t	Delta C_t
$\Delta\Delta C_t$	Delta delta C_t
DEX	Dexamethasone
DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
FBS	Fetal bovine serum
Fkbp5	FK506 binding protein 5
GR	Glucocorticoid receptor
HAB	Human and Animal Bridging
HDAC	Histone deacetylase
HFL	Human fetal liver
HPLC	High performance liquid chromatography
HXEL	Histidine-Xxx-Glutamic acid-Leucine
MeCP2	Methyl-CpG binding protein 2
MPHS	Methylprednisolone 21-hemisuccinate
MQ	Milli-Q
mRNA	Messenger RNA

Mrp4	Multidrug resistance-associated protein 4
ND	Not detected
NS	Not statistically significant
Oat3	Organic anion transporter 3
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PNPA	*p*-Nitrophenyl acetate
PXR	Pregnane X receptor
QUMA	Quantification tool for methylation analysis
rCES2	Rat carboxylesterase 2
rRNA	Ribosomal RNA
RU-486	Mifepristone
SD	Standard deviation
SD rat	Sprague-Dawley rat
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SN-38	7-Ehtyl-10-hydroxycamptothecin
Sp1	Specificity protein 1
TA	Triamcinolone acetonide
TAT	Tyrosine aminotransferase
TET	Ten-eleven-translocation
TSA	Trichostatin A
TSS	Transcription start site
UDG	Uracil-DNA glycosylase
UDP	Uridine 5'-diphosphate
UGT	UDP-glucuronosyltransferase

General introduction

Therapeutic and toxic actions of drugs are associated with concentrations of the drugs in the body. The concentrations of drugs are related to the extent and rate of absorption, distribution, metabolism, and excretion (ADME) of drugs. Metabolism is a key event in alteration of drug concentrations. Therefore, drug-metabolizing enzymes that play pivotal roles in drug metabolism have been taken into account in drug development.

In recent years, development of new drugs is often performed *in silico* on the basis of intermolecular interaction between drugs and target molecules. However, this often contributes to some traits such as an unduly low absorption ratio that make drugs unusable in clinical practice (Imai, 2009). Modification such as esterification of drugs can often improve such traits, and modified drugs are called prodrugs. Prodrugs acquire their pharmaceutical activities after metabolism. One of the key families in prodrug metabolism is the family of carboxylesterases (CESs: EC 3.1.1.1).

CESs are encoded by a multigene family (Satoh et al., 2002) and are members of an α,β-hydrolase-fold family (Bencharit et al., 2003). CESs fall into the B-esterase group because their activities can be inhibited by organophosphorus compounds (Aldridge, 1993; Satoh and Hosokawa, 2006). CESs catalyze hydrolytic reactions in a variety of xenobiotic and endobiotic substrates because of their ability to hydrolyze a broad spectrum of ester, amide, thioester, and carbamate compounds (Sanghani et al., 2004). According to the homology of amino acid sequences, we previously classified CESs into five major groups, CES1-5 (Satoh and Hosokawa, 2006), and found that many of the identified CESs belong to the CES1 or CES2 family. The two families have different substrate specificities. CES1 mainly hydrolyzes a substrate that can be converted to a small alcohol moiety and a large acyl moiety. This is in contrast to CES2, which mainly hydrolyzes a substrate that can be converted to a large alcohol moiety and a small acyl moiety.

CESs are prominently involved in many pharmaceutical agents. Many esterified drugs such as cocaine and meperidine are metabolized by CESs into inactive products (Potter and Wadkins, 2006). On the other hand, a number of ester-containing prodrugs, whose pharmacological activities are generally masked, are hydrolyzed by CESs and then exert their activities. Examples of prodrugs are the anticancer drug CPT-11 (irinotecan), which is metabolized by CES1 and CES2 to the active metabolite SN-38 (Humerickhouse et al., 2000), and the anti-influenza drug oseltamivir, which is metabolized by CES1 to Ro 64-0802 (Shi et al., 2006). CESs are related to transporters and conjugation enzymes and are involved in not only drug metabolism but also drug disposition. For instance, CESs convert temocapril, an angiotensin-converting enzyme inhibitor, to the active metabolite temocaprilate, which is transported by canalicular multispecific organic anion trans-

porter (cMOAT, ABCC2) (Ishizuka et al., 1997). Furthermore, CESs hydrolyze CPT-11 to SN-38, which is a good substrate for UDP-glucuronosyltransferase (UGT) (Sanghani et al., 2004). Thus, CESs play an important role in determining the metabolic fate of many drugs.

Transcription is a key step in the expression of genes, including *CES* genes. However, little is known about the transcriptional machinery that regulates tissue-specific or inductive expression of *CES* genes. With respect to the distribution of CESs, they are ubiquitously expressed, particularly in the liver, small intestine, kidney and lung, in various mammals. Several types of CESs are known to have an endoplasmic reticulum retention tetrapeptide, His-Xxx-Glu-Leu (HXEL), at the carboxyl-terminus and to be localized in the endoplasmic reticulum of many tissues (Satoh and Hosokawa, 1998; Satoh et al., 2002). Tissue-specific expression of CESs has been reported. CES1 is mainly expressed in the liver and lung, whereas CES2 is mainly expressed in the small intestine and kidney in humans (Hosokawa, 2008). The molecular mechanisms by which the two CESs are specifically expressed in some tissues are not clear. On the other hand, a number of chemical compounds are known to induce the expression of CESs, yet there is little information about the molecular mechanisms of induction of CESs. Alteration of the expression of drug-metabolizing enzyme genes by chemical compounds can be associated with drug-drug interactions in medical practice. Therefore, the molecular mechanisms by which chemical compounds induce CESs should be investigated.

The first aim of studies conducted for this thesis was to investigate the molecular mechanisms of the tissue-specific expression of the gene of CES1A1, one of the CES1 isozymes, in the light of epigenetics (chapter 1). Subsequently, we investigated the hypothesis that dexamethasone, a synthetic glucocorticoid, alters the expression levels and DNA methylation patterns of the *CES1A1* gene in human fetal liver cells (chapter 2). Finally, we examined the molecular mechanisms by which dexamethasone induces rat *CES2* gene expression in the liver (chapter 3).

Chapter I

DNA methylation and its involvement in carboxylesterase 1A1 gene expression

Abstract

Carboxylesterase 1A1 (CES1A1) efficiently catalyzes the hydrolysis of a substrate containing ester, amide, or thioester bonds. It is expressed at a high level in the human liver, but at a low level in the human kidney. In this study, we found the cause of this tissue-specific expression of the *CES1A1* gene using 5-aza-2'-deoxycytidine (5-aza-dC) and bisulfite sequencing. Treatment of HEK293 cells, human embryonic kidney cells not expressing the *CES1A1* gene, with 5-aza-dC caused dramatic expression of the *CES1A1* gene. Bisulfite sequencing revealed that the region around the transcription start site (TSS) of the *CES1A1* gene was almost entirely methylated in HEK293 cells, whereas the region was almost entirely unmethylated in HepG2 cells, human hepatoma cells. The hypomethylated DNA molecules for the region were observed in HEK293 cells treated with 5-aza-dC. In the genomes obtained from the kidney, the region downstream of the TSS was methylated compared with those obtained from the liver. From these findings, it can be concluded that DNA methylation is involved in *CES1A1* gene expression and that the difference between *CES1A1* gene expression in the human kidney and that in the human liver may arise from the difference in DNA methylation levels in the region around the TSS.

I-I Introduction

Various CES isozymes are expressed tissue-specifically in mammals. CES1A1 is a human CES1A subfamily isozyme and is mainly expressed in the liver and lung. CES2A1 is a human CES2A subfamily isozyme and is mainly expressed in the small intestine and kidney (Hosokawa, 2008). The molecular mechanisms by which such tissue-specific expression of CESs have been unclear.

It is important to understand the tissue-specific expression of CESs for design of ideal prodrugs that are efficiently hydrolyzed in target tissues and are associated with sufficient drug efficacy and few side effects. The tissue-specific expression of CESs in the human kidney is involved in renal excretion, since CESs change the polarity of prodrugs. As mentioned above, CES2A1 is expressed in the human kidney, and therefore participates in renal excretion of prodrugs. Other drug-metabolizing enzymes that are expressed in the human kidney also play an important role in the metabolism of exogenous and endogenous compounds. For example, the glucuronidation of propofol is catalyzed by UGT in the kidney (McGurk et al., 1998) and the secosteroid hormone 1,25-dihydroxyvitamin D3 (Calcitriol) is catalyzed by 25-hydroxyvitamin D3 1-alpha-hydroxylase (CYP27B1) in the kidney (Zehnder et al., 1999). In this way, many drug-metabolizing enzymes expressed in the human kidney contribute to the metabolism of compounds. However, the *CES1A1*, one of the main CES isozyme in humans, gene is poorly expressed in the human kidney. We previously reported that the transcription factor specificity protein (Sp) 1, which is ubiquitously expressed, can bind to the promoter region of the *CES1A1* gene, leading to transactivation of the promoter (Hosokawa et al., 2008). Hence, we have thought that there is a mechanism repressing *CES1A1* gene expression in the human kidney.

On the other hand, gene regulation by DNA methylation has been reported for some drug-metabolizing enzymes (Anttila et al., 2003; Gagnon et al., 2006). In mammals, DNA methylation occurs predominantly on cytosine in 5'-CpG-3' dinucleotide and its methylation mark is propagated into both DNA strands after DNA replication. Approximately 40% of mammalian genes include CpG islands, regions with relatively high frequency of CpG nucleotides, in their promoters and exonic regions (Larsen et al., 1992). It is generally accepted that DNA methylation of a CpG island at a promoter region is closely associated with silencing of gene expression. It is also known that DNA methylation in the region downstream of the transcription start site (TSS) causes dramatic reduction in gene expression (Graessmann et al., 1994; Hisano et al., 2003). Recently, it was proposed that a DNA methylation-free region extending several hundred bases downstream of the TSS may be a prerequisite for efficient transcription initiation (Appanah et al., 2007). The tissue-specific expression of a number of genes has been revealed by studies on DNA methylation (Kikuchi et al., 2007; Aoki et al., 2008). The aim of the present study was to elucidate the cause of the tissue-specific expression of the *CES1A1* gene, particularly the cause of the difference in gene expression in the human kidney and liver.

I-II Materials and methods

Cell lines and human tissues

HEK293 cells, human embryonic kidney cells, and HuH-7 and HepG2 cells, human hepatoma cells, were used in this study. CES1A1 mRNA is not expressed in HEK293 cells but is expressed in HuH-7 and HepG2 cells. The cell lines were cultured at 37°C in air with 5% CO_2 and in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin, and streptomycin. Human kidney and liver tissues were obtained from the National Disease Research Interchange (Philadelphia, PA, USA) through the Human and Animal Bridging (HAB) Research Organization (Chiba, Japan).

Treatment with DNA methylation inhibitor and histone deacetylase inhibitor

HEK293, HuH-7, and HepG2 cells were precultured for 24 h and then cultured for 3 days in medium containing 2 μM of 5-aza-2'-deoxycytidine (5-aza-dC), a DNA methylation inhibitor (Sigma-Aldrich, St. Louis, MO, USA), diluted with phosphate-buffered saline (PBS)(−). Subsequently, total RNA was extracted. For histone deacetylase (HDAC) inhibition, 500 nM trichostatin A (TSA) (Wako, Osaka, Japan) diluted with ethanol was added to the culture medium with cells 24 h before extraction of RNA.

Relative quantification of mRNA by real-time polymerase chain reaction (PCR)

Total RNA was extracted from the cell lines and tissues using an ISOGEN (Nippon Gene, Toyama, Japan) and then treated with DNase I (Invitrogen). Subsequently, cDNA was synthesized with the RNA using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan). Finally, the expression level of CES1A1 mRNA was analyzed using a Realtime PCR Master Mix (Toyobo) with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster, CA). The specific primers were designed as follows: forward, 5'-GAGACCTCGCAGGCCCC-3'; reverse, 5'-GACGAACTTCCCCAGCACTT-3'. The fluorescent probe for CES1A1 was 5'-(FAM)-TCCGTGCCTTTATC-(MGB)-3'. Real-time PCR was performed under the following conditions: 50°C for 2 min, 95°C for 10 min, and 50 cycles of 95°C for 15 s and 56°C for 1 min. CES1A1 mRNA expression was normalized with 18S rRNA expression:

> Delta C_t (ΔC_t) = threshold cycle (C_t) for target amplification − C_t for reference amplification

In each cell line, the delta delta C_t ($\Delta\Delta C_t$) values were based on the average ΔC_t value of the cell line treated with 5-aza-dC alone, except that the $\Delta\Delta C_t$ value of HuH-7 cells for Figure 1.1 was based on the average ΔC_t value of HEK293 cells treated with 5-aza-dC. The average mRNA expression ratio of three liver specimens was indicated as the result for the liver. The mRNA expression ratio of a specimen in which CES1A1 mRNA was always detected was selected as the representative value for the kidney. All assays were performed in triplicate. For improvement of

the assay of CES1A1 mRNA used in our previous study (Hosokawa et al., 2008), the *CES1A1* wild-type gene and the *CES1A1* gene variant were distinguished by sequencing and tissues that have only the *CES1A1* wild-type gene were used in the present study. The *CES1A1* gene variant has a similar exon 1 to that of the *CES1A3* gene instead of that of the *CES1A1* wild-type gene (Tanimoto et al., 2007; Fukami et al., 2008).

Determination of the CpG island

A search for the CpG island of the *CES1A1* gene was carried out using GENETYX-Mac Ver.12.2.0 software. The CpG island was determined using MethPrimer online software (Li and Dahiya, 2002). The criteria used were island size > 200 bp, GC% > 50.0, and observed/expected CpG ratio > 0.6.

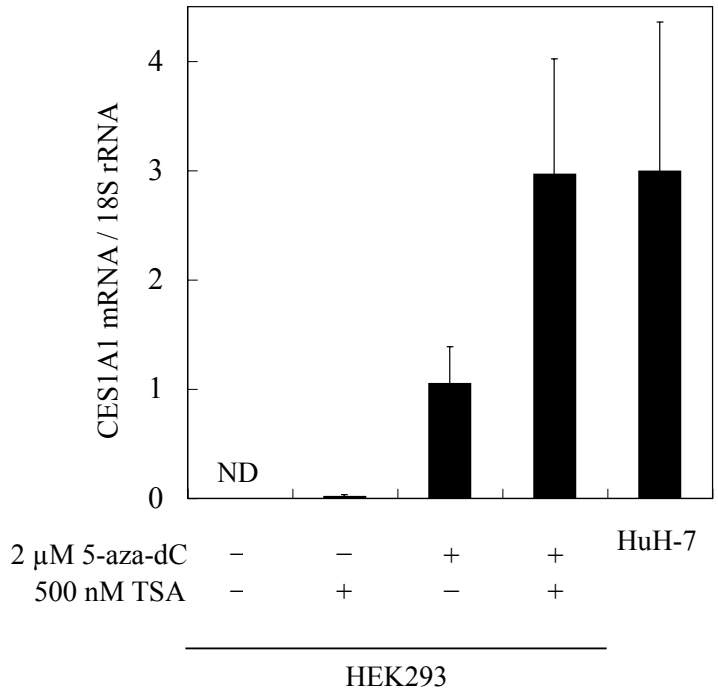

Figure 1.1. Effect of a single or combined treatment with 5-aza-dC and TSA in HEK293 cells. HEK293 cells were cultured for 3 days in medium containing PBS(−) as a mock or 2 μM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the culture medium with cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean ± standard deviation (SD) of three independent experiments, which were performed in triplicate. Results for HuH-7 cells are shown for comparison with results for HEK293 cells treated with 5-aza-dC. ND indicates that CES1A1 mRNA was not detected.

Bisulfite sequencing

Genomic DNA was extracted from the human kidney, human liver tissues, HEK293 cells, and HepG2 cells using a FastPure DNA Kit (Takara Bio, Shiga, Japan). After purification, the genomic DNA was treated with sodium bisulfite using an Epitect Bisulfite Kit (Qiagen, Hilden, Germany). The target region (−512 to +214) in the *CES1A1* gene was amplified from the genomic DNA by PCR using a GoTaq Green Master Mix (Promega, Madison, WI, USA) and primers designed as follows: forward, 5'-TTGTGAAGTTAATTTAGGTTTTAGAAAGG-3'; reverse, 5'-AACCTATTATATCTTTACCTTTCTAC-3'. PCR was performed under the following conditions: 95°C for 2 min; 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. To perform hot start PCR, the reverse primer was added to the reaction mix when the reaction mix first reached 95°C. After being purified, the PCR products were cloned into a pGEM-T vector (Promega). Approximately ten clones for each tissue specimen were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a Montage SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore, Billerica, MA, USA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). In addition, each of approximately 30 clones obtained from three independent experiments was sequenced for HEK293 cells and HEK293 cells treated with 5-aza-dC, and approximately 20 clones obtained from two independent experiments were sequenced for HepG2 cells. CpG methylation status was analyzed by the web-based tool QUMA (Kumaki et al., 2008). Three human kidney specimens (age/sex: 47 years/male, 54 years/male, and 75 years/female) and three human liver specimens (age/sex: 62 years/female, 68 years/male, and 70 years/female) were used. Three TSSs of the *CES1A1* gene are known (Hosokawa et al., 2008), and the most upstream TSS was selected as TSS (+1) in the present study.

Enzyme assay

The activity of the hydrolysis for 0.1 mM *p*-nitrophenyl acetate (PNPA) was determined colorimetrically according to the method of Hosokawa et al. (1987). The cell culture and the treatment with 5-aza-dC and TSA were performed at the same condition of mRNA experiment described above. Three independent experiments were performed.

Statistical analysis

All data of mRNA expression ratios were tested by the Smirnov-Grubbs test ($P < 0.01$) and two extreme values were rejected. The results of bisulfite sequencing at each CpG site for the human kidney and liver were tested by Fisher's exact test, and that of the entire set of CpG sites for the human kidney and liver were tested by the Mann-Whitney *U*-test using the web-based tool QUMA (Kumaki et al., 2008). The results of enzyme assay were tested by Student's *t*-test.

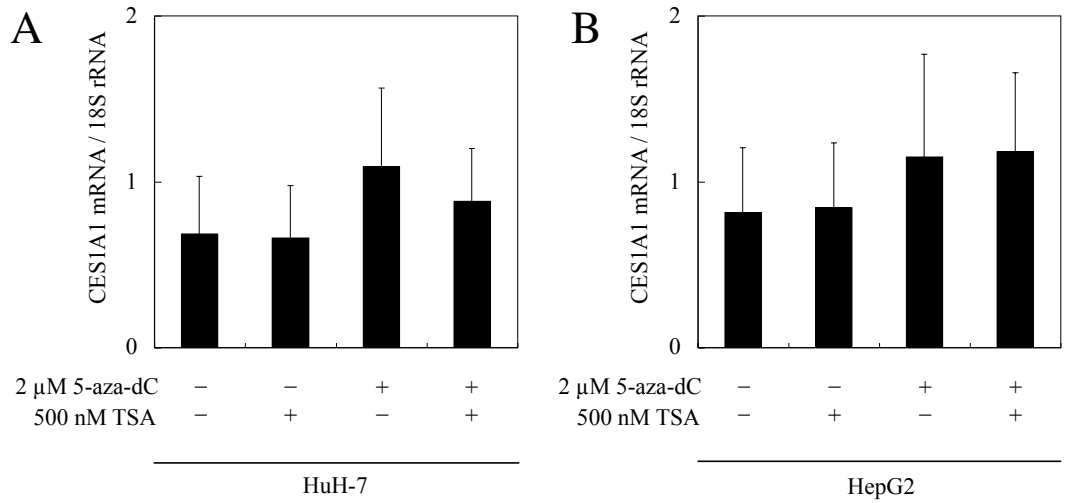

Figure 1.2. Effect of a single or combined treatment with 5-aza-dC and TSA in HuH-7 cells (A) and HepG2 cells (B). Each cell line were cultured for 3 days in medium containing PBS(−) as a mock or 2 μM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 500 nM TSA was added to the culture medium with cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean ± SD of three (for HuH-7 cells) or four (for HepG2 cells) independent experiments, which were performed in triplicate.

I-III Results

Comparison of CES1A1 mRNA expression levels in the human kidney, liver and the cell lines

We confirmed that the expression level of CES1A1 mRNA in the human liver is much higher than that in the kidney and that CES1A1 mRNA is detected in HuH-7 and HepG2 cells. When the $\Delta\Delta C_t$ values of the kidney, liver, and the cell lines were based on the average ΔC_t value of HuH-7 cells, the mRNA expression ratio (CES1A1 mRNA/18S rRNA) of the kidney, HuH-7, HepG2, and the liver was 0.106 ± 0.0223, 1.11 ± 0.508, 29.9 ± 14.4, and 76.2 ± 96.9 (mean ± standard deviation (SD)), respectively.

Activation of the *CES1A1* gene in HEK293 cells by 5-aza-dC

CES1A1 mRNA was not detected in HEK293 cells by real-time PCR (Figure 1.1). To investigate whether the *CES1A1* gene in HEK293 cells is silenced by DNA methylation, we treated HEK293 cells with 2 μM 5-aza-dC for 3 days. As a result, we detected dramatic expression of CES1A1 mRNA in HEK293 cells (Figure 1.1). In addition, 500nM TSA enhanced the expression in HEK293 cells treated with 5-aza-dC, but single treatment with TSA had little effect on the mRNA expression in HEK293 cells. The expression levels of CES1A1 mRNA in HEK293 cells treated with 5-aza-dC and TSA was almost the same as that in HuH-7 cells alone. Furthermore,

Figure 1.3. Positions of CpGs in the region of 726 bp (−512 to +214) around the TSS of the *CES1A1* gene. Positions of CpGs are shown in boxes. A broken arrow with a box indicates TSS (+1). Exon 1 is shown in a shaded box. A part of the CpG island is underlined with a heavy line. ATG is underlined with a thin line.

the dramatic enhancement of CES1A1 mRNA expression observed in HEK293 cells treated with 5-aza-dC was not observed in HuH-7 and HepG2 cells treated with 5-aza-dC (Figures 1.1-1.3).

Negative correlation between methylation and expression of the *CES1A1* gene

To determine DNA methylation status of the *CES1A1* gene in the human kidney and liver and the cell lines, we performed bisulfite sequencing for the region of 726 bp (−512 to +214) (Figure 1.3). The results revealed that the region around the TSS of the *CES1A1* gene is almost entirely methylated in HEK293 cells, whereas the region is almost entirely unmethylated in HepG2 cells (Figure 1.4). As a result of treatment with 2 μM 5-aza-dC for 3 days, hypomethylated DNA molecules for the region accounted for approximately 30% of the total molecules that were obtained from HEK293 cells treated with 5-aza-dC. In the genomes obtained from the kidney, the region downstream of the TSS was methylated compared with those obtained from the liver, although the difference in methylation levels between the kidney and liver was less clear than that between the cell lines (Figure 1.5). There was little difference in the methylation among individual liver or kidney specimens.

Activity of the hydrolysis of PNPA

As for the activity caused by a single or combined treatment with 5-aza-dC and TSA of HEK293 cells, the values (nmol/10^6cells/min) of control, TSA, 5-aza-dC, and TSA+5-aza-dC were 1.67 ± 0.425 (1.00-fold), 1.98 ± 0.377 (1.18-fold), 2.10 ± 0.0528 (1.26-fold), and 2.21 ± 0.680 (1.32-fold) (mean ± SD), respectively. However, statistically significant differences were not observed in the data of the activity of HEK293 cells. The result of the activity in HuH-7 cells was 13.3 ± 1.95 (nmol/10^6cells/min). Correlation coefficient between PNPA hydrolase activity and CES1A1 mRNA expression levels caused by a single or combined treatment with 5-aza-dC and TSA of

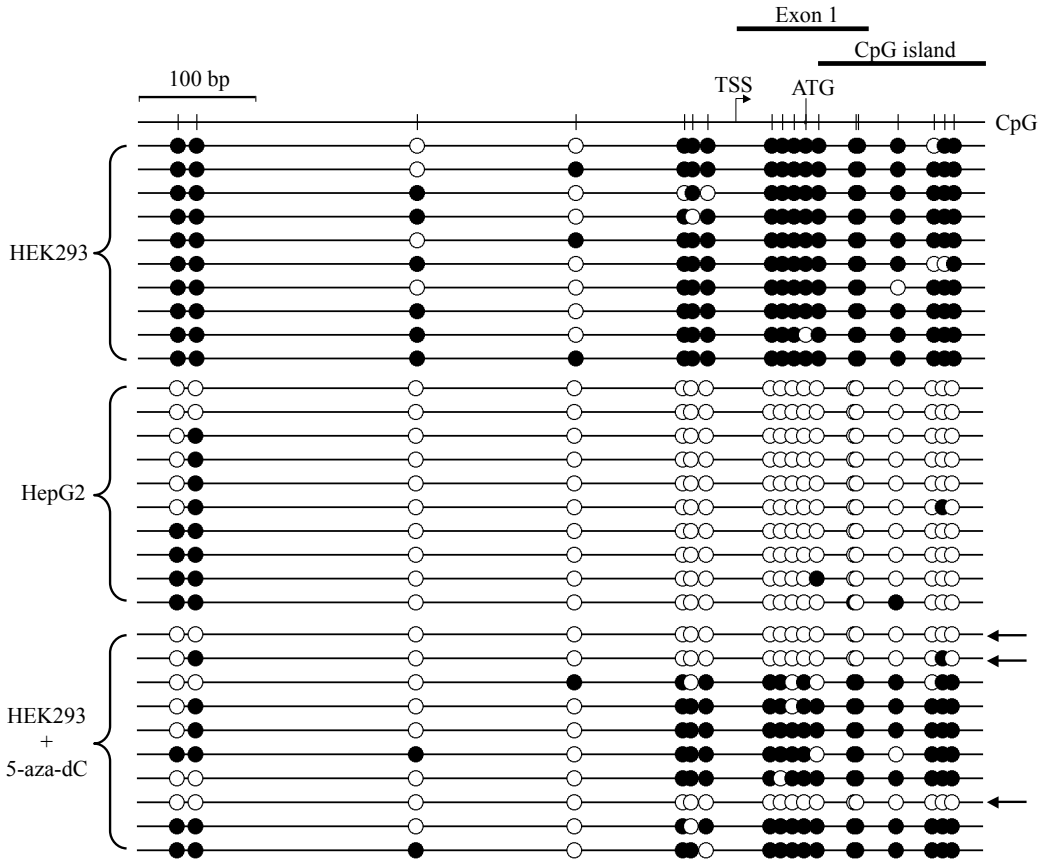

Figure 1.4. Appearance of hypomethylated molecules for the region around the TSS of the *CES1A1* gene in HEK293 cells treated with 5-aza-dC. Bisulfite sequencing was performed to determine the methylation pattern in the region from −512 to +214. A broken arrow indicates TSS (+1). Each CpG position is depicted as a vertical bar. The methylated and unmethylated CpGs are depicted as closed circles and open circles, respectively. Ten representative molecules of each cell line are shown. Horizontal arrows indicate hypomethylated molecules.

HEK293 cells was 0.789.

I-IV Discussion

DNA methylation is closely related to histone modifications such as deacetylation and methylation in mammals (Cameron et al., 1999). Histone proteins assemble into nucleosomes, and deacetylation of histone is important for silencing of the gene with the methylated promoter (Bird and Wolffe, 1999). DNA methylation in the promoter region causes histone deacetylation by mediating some proteins such as methyl-CpG binding protein and HDAC. As a result, the gene expression is repressed (Razin, 1998). However, once silencing of the gene with a methylated promoter is established, inhibition of histone deacetylation generally cannot cause active expression of the gene (Cameron et al., 1999; Coffee et al., 1999; El-Osta et al., 2002). The present results showing that single treatment with TSA had little effect on *CES1A1* gene expression in HEK293 cells are consistent with the finding described above (Figure 1.1). The reason why treatment with a HDAC inhibitor generally cannot cause active expression is related to histone methylation as described

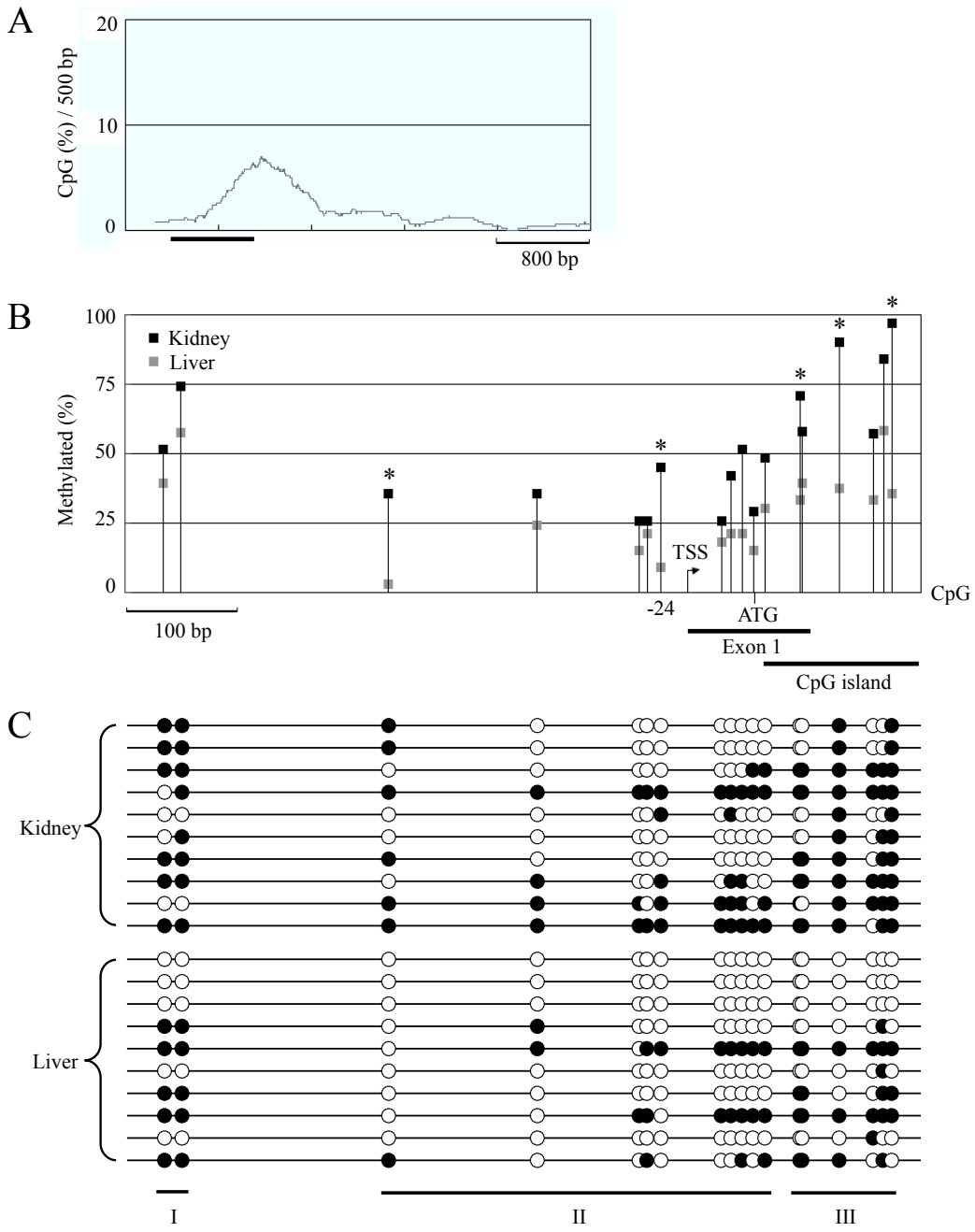

Figure 1.5. Comparison of methylation levels in the region around the TSS of the *CES1A1* gene in the genomes obtained from the human kidney and liver. (A) CpG island (+72 to +541) of the *CES1A1* gene. The CpG content (%) was computed in 500 bp overlapping segments across a 4-kb region. A heavy line indicates the amplified region of 726 bp. (B) Bisulfite sequencing was performed to determine the methylation patterns of the kidney and liver in the region of 726 bp (−512 to +214). The methylated levels (%) of the kidney and liver are shown with the TSS (broken arrow), ATG, exon 1, and a part of the CpG island (+72 to +214). The graph was made using approximately 30 molecules of each tissue. Each CpG position is depicted as a vertical bar. Asterisks indicate statistically significant differences in the methylation level between the human kidney and liver ($P < 0.01$, Fisher's exact test). There was the statistically significant difference in the methylation level of the entire set of CpG sites between the kidney and liver ($P < 0.01$, Mann-Whitney U-test). (C) The methylated and unmethylated CpGs are depicted as closed circles and open circles, respectively. Ten representative molecules of each tissue are shown.

below (Peters et al., 2002; Zegerman et al., 2002). According to Kondo et al. (2003), methylation on lysine 9 (Lys-9) in histone H3 causes a repressive folded chromatin structure, affects the access of regulatory factors to chromatin, and leads to silencing of *P16*, *MLH1*, and *MGMT* genes in colorectal cancer. Treatment with 5-aza-dC decreased DNA methylation and Lys-9 methylation dramatically, increased Lys-9 acetylation slightly and Lys-4 methylation moderately, and reactivated gene expression (Kondo et al., 2003). Consistent with these findings obtained by treatment with 5-aza-dC, our results showed that treatment with 5-aza-dC allows HEK293 cells to activate *CES1A1* gene expression (Figure 1.1). Combined treatment with 5-aza-dC and TSA decreased DNA methylation and Lys-9 methylation and increased Lys-9 acetylation markedly and Lys-4 methylation, while single treatment with TSA increased Lys-9 acetylation and had no effect on Lys-9 or Lys-4 methylation (Kondo et al., 2003). The acetylation level of Lys-9 in the case of combined treatment was higher than that in the case of treatment with 5-aza-dC alone. Consequently, a high expression level of the gene was observed. In agreement with these findings about combined treatment, the results showed that the level of *CES1A1* gene expression induced by combined treatment with 5-aza-dC and TSA was approximately three times higher than that induced by treatment with 5-aza-dC alone in HEK293 cells (Figure 1.1). The results of bisulfite sequencing showed that the region around the TSS of the *CES1A1* gene is entirely methylated in HEK293 cells and is entirely unmethylated in HepG2 cells (Figure 1.4). The hypomethylated DNA molecules for the region accounted for approximately 30% of the total molecules that were obtained from HEK293 cells treated with 5-aza-dC. Taken together with the previous findings, our results strongly suggest that *CES1A1* gene expression in HEK293 cells is silenced by DNA methylation. This is the first study demonstrating that DNA methylation is involved in *CES* gene expression.

Saxonov et al. (2006) found that promoters in the human genome segregate naturally into two classes by CpG content. One is a class with high CpG content and the other is a class with low CpG content. To date, gene silencing by DNA methylation has been studied mainly for the promoter region (Bird, 2002). Studies on DNA methylation for the promoter region seem to reflect the fact that approximately 70% of promoters in the human genome belong to the class with high CpG content. This class has a prominent peak in the frequency of CpG centered some 15 bp upstream of the TSS. It is also known that DNA methylation in the region downstream of TSS causes dramatic reduction in gene expression (Graessmann et al., 1994; Hisano et al., 2003). In addition, Appanah et al. (2007) proposed that methylation of the 3' promoter-proximal region, approximately 300 bp downstream of the TSS, may dramatically reduce transcription initiation efficiency by relating alteration of the promoter chromatin structure. The findings about the region downstream of the TSS explain why the expression levels of the *CES1A1* gene in the human kidney and liver are different. In the genomes obtained from the kidney, the region downstream of the TSS was methylated compared with those obtained from the liver (Figure 1.5). When the amplified region of 726 bp was segregated into three regions (I-III), there was no apparent difference between methylation in region I in the kidney and that in region I in the liver. In region II, CpGs in the kidney were slightly methylated compared with those in the liver. In region III, which is included

in the CpG island of the gene, CpGs in the kidney were more methylated than those in the liver. Hence, methylation in region III may play an important role in the difference between *CES1A1* gene expression in the kidney and that in the liver, and methylation in region II may also have a minor role in that difference. It was previously suggested that methylated and inactive promoters are occupied by nucleosomes in the silenced state of the *MLH1* gene (Lin et al., 2007). Methyl-binding proteins are involved in nucleosomal occupancy (Li et al., 2007). In such a mechanism, *CES1A1* gene expression may also be repressed possibly by methylation in the promoter region, particularly at position −24 immediately upstream of the TSS. There was partial discordance of the methylation pattern between cell lines and tissues. To explain this phenomenon, we focused on three points. The first is the point that the tissues consist of several kinds of cells while the cell lines consist of almost one kind of cell. The hypermethylated clones were observed in the genomes obtained from all liver tissues at almost the same rate. The hypermethylated clones may be obtained from hepatic non-parenchymal cells. The second is the possibility that a cell line is partially different from a normal tissue in DNA methylation pattern. The third is the point that the ages of tissue specimens and cell line samples used in this study were not the same, although DNA methylation pattern can change with age (Bjornsson et al., 2008).

Since CESs catalyze the hydrolysis of PNPA, we performed enzyme assay using PNPA. As the result of treatment with 5-aza-dC of HEK293 cells, the activity of hydrolysis of PNPA was increased approximately 25% compared with that of control. However, the increases of the activity were lower than expected. We thought that more time after transcription to observe the change caused by single or combined treatment with 5-aza-dC and TSA may be necessary, but after transcription how fast functional CESs are generated is unknown. It is known that the half-life of a rat liver CES isozyme is 42 h (Heymann et al., 1979). Although the half-life of CES1A1 protein is unknown, the finding of half-life for CES1A1 would be explain the difference between the activity of hydrolysis caused by treatment with 5-aza-dC of HEK293 cells and the activity of hydrolysis of HuH-7 cells.

The present study provides information on the metabolism and disposition of prodrugs associated with CES1A1. For example, CES1A1 converts oseltamivir, an inhibitor of viral neuraminidase, into the active metabolite Ro 64-0802, oseltamivir carboxylate, in the human liver (Shi et al., 2006). Oseltamivir can cross the blood-brain barrier (BBB), and its brain penetration at the BBB is limited by P-glycoprotein (P-gp) (Morimoto et al., 2008; Ose et al., 2008). The degree of penetration of Ro 64-0802 at the BBB is lower than that of oseltamivir. According to Ose et al. (2009), Ro 64-0802 in the brain is eliminated across the BBB by its active efflux by multidrug resistance-associated protein 4 (Mrp4, Abcc4) and organic anion transporter 3 (Oat3, Slc22a8) in mice, although Oat3 may not affect its brain distribution in a steady state. These findings indicate that if *CES1A1* gene expression in the human liver or brain capillary decreases, the distribution of oseltamivir to the brain may increase. It is known that oseltamivir and Ro 64-0802 affect neuronal excitability in rat hippocampal slices (Izumi et al., 2007). Recently, the relationship between abnormal behavior of children and oseltamivir medication has been studied in detail, but the relationship remains unclear. Yang et al. (2009) revealed that expression level of the human *CES1A*

gene in the liver of children is lower than that in the liver of adults and that liver microsomal samples pooled from children showed approximately 15% of the activity of the samples pooled from adults in hydrolyzing oseltamivir. Hence, these findings raise the possibility that the difference in *CES1A1* gene expression between children and adults is involved in the abnormal behavior of children. Considering that DNA methylation pattern can change with age (Bjornsson et al., 2008), we speculated that the difference in *CES1A1* gene expression between children and adults is probably due to the difference in DNA methylation levels in the region around the TSS. This point of view may help to understand the relationship between abnormal behavior and oseltamivir medication.

The present study has revealed that DNA methylation is involved in *CES1A1* gene expression. The difference between the gene expression in the human kidney and that in the human liver may arise from the difference in DNA methylation levels in the region around the TSS.

Chapter II

Dexamethasone-mediated transcriptional regulation of carboxylesterase 1A1 gene in human fetal liver cells

Abstract

Carboxylesterase 1A1 (CES1A1) is one of the main CES isozymes in humans and can convert many regular drugs to inactive forms and many prodrugs to active forms. Little attention has been paid to induction of CES1A1 in the human fetus. Here we show the effect of dexamethasone, a synthetic glucocorticoid, on the expression of the *CES1A1* gene in human fetal liver (HFL) cells. At 100 nM, dexamethasone increased the CES1A1 mRNA level in HFL cells in a time-dependent manner. The increase in CES1A1 mRNA caused by treatment with dexamethasone for 3 days was abolished by treatment with RU-486, an antagonist of glucocorticoid receptor (GR). To investigate the involvement of DNA methylation in the expression of the *CES1A1* gene, HFL cells were treated with 5-aza-2'-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, and expression levels of the *CES1A1* gene in HFL cells were measured by real-time PCR. Treatment with 5-aza-dC resulted in an increase in CES1A1 mRNA. The results of bisulfite sequencing showed that the region around a transcription start site (TSS) of the *CES1A1* gene was considerably hypermethylated in HFL cells. In addition, the results of DNA methylation analysis led us to propose that DNA methylation levels in the region around the TSS are decreased by treatment of HFL cells with dexamethasone for 1 and 18 days, although treatment with dexamethasone for 1 day did not increase CES1A1 mRNA in HFL cells. Taken together, these results suggest that dexamethasone increases CES1A1 mRNA through GR in HFL cells and that DNA demethylation caused by dexamethasone could be involved in *CES1A1* gene expression in HFL cells, although another subsequent type of alteration such as histone acetylation caused by dexamethasone is probably necessary for the increase in CES1A1 mRNA in HFL cells.

II-I Introduction

When there are two kinds of drugs in the body, if one induces a drug-metabolizing enzyme and the other is metabolized by the induced enzyme, the efficacy of the latter drug would decrease. This phenomenon is an example of drug-drug interactions, and medical workers have been taking care about combinations of dosing in clinical practice. Hence, an understanding of the induction of human drug-metabolizing enzymes including CESs is important for prediction of drug-drug interactions.

Human fetuses are highly susceptible to drugs and are generally protected from drugs by the placenta. There are drugs that can penetrate the human placenta and affect organs in the fetus. Some drugs can induce several kinds of drug-metabolizing enzymes in fetal hepatocytes (Matsunaga et al., 2004; Maruyama et al., 2007), and the induction of such enzymes might disturb fetal metabolism with potential adverse outcomes such as subsequent developmental disorders or transgenerational toxicities. However, there is limited information about induction of CESs caused by drugs in humans and there is no information about that in the unborn child.

Dexamethasone is a synthetic glucocorticoid and is used to treat many different conditions such as skin diseases, asthma, and rheumatoid arthritis. Dexamethasone alters expression levels of a large number of genes including tyrosine aminotransferase (TAT) (Schmid et al., 1987), glutamine synthetase (Gaunitz et al., 2002) and Na^+-K^+-ATPase (Celsi et al., 1991), and also many drug-metabolizing enzymes such as cytochrome P450 2C9 (CYP2C9) (Gerbal-Chaloin et al., 2002) and CYP3A4 (Pascussi et al., 2001). Postnatal exposure to dexamethasone is known to increase CES1 expression in human hepatocytes (Zhu et al., 2000). However, it is uncertain whether dexamethasone induces CES1 in fetal hepatocytes.

With respect to gene regulation by dexamethasone, some molecular mechanisms are known. In many cases, homodimers consisting of glucocorticoid receptors (GRs) activated by ligands such as dexamethasone directly bind to glucocorticoid response elements (GREs) and regulate gene expression. At high concentrations (more than 10 μM), dexamethasone can activate pregnane X receptor (PXR) as well as GR, leading to alteration of gene expression (Huss and Kasper, 2000; Pascussi et al., 2001). In addition, GR activated by dexamethasone contributes to induction of *CYP2A6* gene expression by interacting with hepatocyte nuclear factor-4 α (Onica et al., 2008). These findings prompted us to hypothesize that dexamethasone can increase CES1A1 mRNA expression via GR in prenatal human hepatocytes.

Several studies have shown a new mechanism of dexamethasone-mediated gene regulation. It has been reported that exposure to dexamethasone is linked to loss of DNA methylation in the rat *Tat* gene (Thomassin et al., 2001). Kress et al. (2006) suggested that DNA demethylation results from DNA strand breaks through GR. Yang et al. (2012) reported dexamethasone-mediated DNA demethylation in a part of the region of the mouse FK506 binding protein 5 (*Fkbp5*) gene. They proposed a passive DNA demethylation process based on results showing that the decreases in DNA methyltransferase (*Dnmt1*) mRNA occurred in AtT-20 cells treated with dexamethasone and in hippocampal tissue obtained from mice treated with corticosterone. It seems that dexamethasone can cause loss of DNA methylation in some genes, although the molecular mechanisms

remain unclear.

Human CES1A mRNA expression level and the level of hydrolase activity of CES1 in the liver of fetuses are lower than those in the liver of adults (Yang et al., 2009). Silenced or repressed expression of CES1A1 mRNA in HEK293 cells or the human kidney was ascribed to DNA hypermethylation (Hori and Hosokawa, 2010), and it has been shown that DNA methylation levels can change with aging (Bjornsson et al., 2008). From these findings, it can be hypothesized that the region around the TSS of the *CES1A1* gene in the fetal liver is more methylated than that in the adult liver. Results regarding the effect of dexamethasone on DNA demethylation let us further hypothesize that dexamethasone treatment decreases DNA methylation levels of the *CES1A1* gene in fetal hepatocytes. If this is so, dexamethasone-mediated decrease in DNA methylation levels can cause long-term induction of CES1A1 in fetal hepatocytes, since once DNA demethylation in CpG positions occurs in the presence of dexamethasone, the situation can continue to be propagated into DNA strands replicated at cell division even in the absence of dexamethasone. Hence, we need to pay attention to alteration in DNA methylation levels of the *CES1A1* gene in the human fetus.

In the study described in this chapter, we examined time-dependent changes in *CES1A1* gene expression in human fetal liver (HFL) cells treated with and not treated with dexamethasone. Our results showed that CES1A1 mRNA in HFL cells was increased after treatment with dexamethasone for 3 to 18 days and that GR is involved in the increases. In addition, we asked whether dexamethasone treatment alters DNA methylation levels in the *CES1A1* gene. The results of DNA methylation analysis have revealed that there was a trend toward decreased DNA methylation levels in the region around the TSS of the *CES1A1* gene in the presence of dexamethasone.

II-II Materials and methods

Human fetal liver cells and human liver tissues

HFL cells (Applied Cell Biology Research Institute, Kirkland, WA, USA) were kindly provided by Dr. Tamihide Matsunaga (Department of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University). The cell culture was initiated from a pool of six normal human liver tissues (average of 13 weeks of gestation) by elutriation following dispase digestion of tissue. After proliferation, HFL cells were suspended in Cell Banker and then cryopreserved at –150°C until use (Matsunaga et al., 2004; Maruyama et al., 2007).

In the present study, HFL cells were cultured at 37°C in air with 5% CO_2 and in Williams' medium E (Sigma-Aldrich) supplemented with 5% (v/v) FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. The culture medium was changed approximately every 24 h to maintain growth of cells.

Adult human liver tissues were obtained from the National Disease Research Interchange through the HAB Research Organization. Three human liver specimens (age/sex: 62 years/female, 68 years/male, and 70 years/female) were used.

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Treatment of HFL cells with dexamethasone

To examine time-dependent changes in mRNA expression levels or DNA methylation levels of the *CES1A1* gene in the presence or absence of dexamethasone, HFL cells were seeded at 1.6×10^5 cells/mL $\times$ 2 mL/well onto 6-well plates coated thinly with collagen and were precultured for 24 h. After preculture, HFL cells were cultured in medium containing 100 nM dexamethasone (Sigma-Aldrich) or Milli-Q (MQ) water (Millipore) as a control. The culture medium in each well was replaced with fresh medium containing 100 nM dexamethasone or MQ water approximately every 24 h. Total RNA was extracted 1, 3, 6, 12, and 18 days after treatment with medium containing dexamethasone or MQ water. Genomic DNA was extracted 1 and 18 days after treatment with medium containing dexamethasone or MQ water.

To assess the effect of RU-486 (mifepristone) (Sigma-Aldrich), a potent inhibitor of GR, on dexamethasone-mediated increase in CES1A1 mRNA, HFL cells were seeded at 1.6×10^5 cells/mL $\times$ 2 mL/well onto 6-well plates coated thinly with collagen. After 24 h, HFL cells were cultured in medium containing 100 nM dexamethasone or MQ water as a control in the presence or absence of 10 μM RU-486 dissolved in ethanol. The culture medium in each well was replaced with fresh medium containing 100 nM dexamethasone (or MQ water) and RU-486 (or ethanol) approximately every 24 h.

Treatment with DNA methylation inhibitor and HDAC inhibitor

To investigate the effects of 5-aza-dC and TSA, HFL cells were seeded at 0.8×10^5 cells/mL $\times$ 2 mL/well onto 6-well plates coated thinly with collagen. HFL cells were precultured for 24 h and then cultured for 3 days in medium containing 2 μM 5-aza-dC (Sigma-Aldrich) diluted with PBS(−). Subsequently, total RNA was extracted. For HDAC inhibition, HFL cells in some wells were exposed to 250 nM TSA (Wako) diluted with ethanol 24 h before extraction of RNA.

Relative quantification of CES1A1 mRNA by real-time PCR

CES1A1 mRNA expression levels were analyzed essentially as described previously (Hori and Hosokawa, 2010). Total RNA was extracted from HFL cells and human liver pieces using an ISOGEN II (Nippon Gene) and treated with DNase I (Invitrogen) to prevent contamination by DNA. First-strand cDNA was synthesized using the DNase I-treated RNA and a ReverTra Ace qPCR RT Kit (Toyobo). The expression levels of CES1A1 mRNA were analyzed using cDNA (1 μg/sample), a THUNDERBIRD Probe qPCR Mix (Toyobo), gene-specific TaqMan probe, and primer sets with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The specific primers were designed as follows: forward, 5'-GAGACCTCGCAGGCCCC-3'; reverse, 5'-GACGAACTTCCCCAGCACTT-3'. The fluorescent probe for CES1A1 was 5'- (FAM) -TCCGTGCCTTTATC- (MGB) -3'. The CES1A1 mRNA expression was normalized with 18S rRNA expression. The $\Delta\Delta C_t$ method was used for analysis of data on CES1A1 mRNA expression. The conditions of real-time PCR were as follows: 95°C for 1 min and 50 cycles of 95°C for 15 s

and 60°C for 1 min. All assays were performed in triplicate.

DNA methylation analysis

Bisulfite sequencing was performed essentially as described previously (Hori and Hosokawa, 2010). After preincubation for 24 h, HFL cells were cultured in medium containing 100 nM dexamethasone or MQ water for 1 day or 18 days. Genomic DNA was extracted from HFL cells treated with dexamethasone or MQ water using a FastPure DNA Kit (Takara Bio). After purification, the genomic DNA was treated with sodium bisulfite using an Epitect Bisulfite Kit (Qiagen). The region from −512 to +214 in the *CES1A1* gene was amplified from the genomic DNA by PCR using a GoTaq Hot Start Green Master Mix (Promega) and primers designed as follows: forward, 5'-TTGTGAAGTTAATTTAGGTTTTAGAAAGG-3'; reverse, 5'-AACCTATTATATCTTTACCTTTCTAC-3'. PCR was performed under the following conditions: 95°C for 2 min; 40 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 1 min; and finally 72°C for 5 min. After purificaiton, PCR products were cloned into a pGEM-T vector (Promega). Clones were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a Montage SEQ_{96} Sequencing Reaction Cleanup Kit (Millipore) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Twenty-four clones were sequenced for each of 4 groups of HFL cells, i.e., HFL cells cultured in medium containing 100 nM dexamethasone or MQ water for 1 day or 18 days. CpG methylation status was analyzed by the web-based tool QUMA (Kumaki et al., 2008). The position of the TSS (+1) is shown in Figure 1.3 on page 15.

HFL cells were obtained from a pool of six normal human liver tissues and thereby genetically have 12 kinds of genomes. Eventually we obtained approximately 100 plasmid clones to analyze DNA methylation status in HFL cells. There was one DNA molecule derived from the *CES1A1* gene variant in all clones. In other words, six liver tissues are not distributed equally in HFL cells. This result indicates that the number of DNA molecules derived from the *CES1A1* gene variant is extremely small compared to that derived from the *CES1A1* wild-type gene and that DNA molecules stemmed from the *CES1A1* gene variant can be eliminated from this experimental system.

II-III Results

Expression levels of CES1A1 mRNA in HFL cells and adult human liver

When HFL cells were cultured for up to 18 days, CES1A1 mRNA expression increased in a time-dependent manner (Figure 2.1). After 18 days of culture, the expression levels of CES1A1 mRNA were increased by approximately 8.6-fold compared with the expression levels in HFL cells cultured for 1 day.

When the expression levels of CES1A1 mRNA in HFL cells and the human liver were compared based on the average ΔC_t value of HFL cells cultured for 1 day in the absent of dexametha-

sone, CES1A1 mRNA expression ratios (CES1A1 mRNA/18S rRNA) of HFL cells cultured for 1 day, HFL cells cultured for 18 days, and adult human liver tissues were 1.05 ± 0.341, 8.59 ± 2.26, and 225 ± 319 (mean ± SD), respectively.

Dexamethasone treatment elevated *CES1A1* gene expression in HFL cells

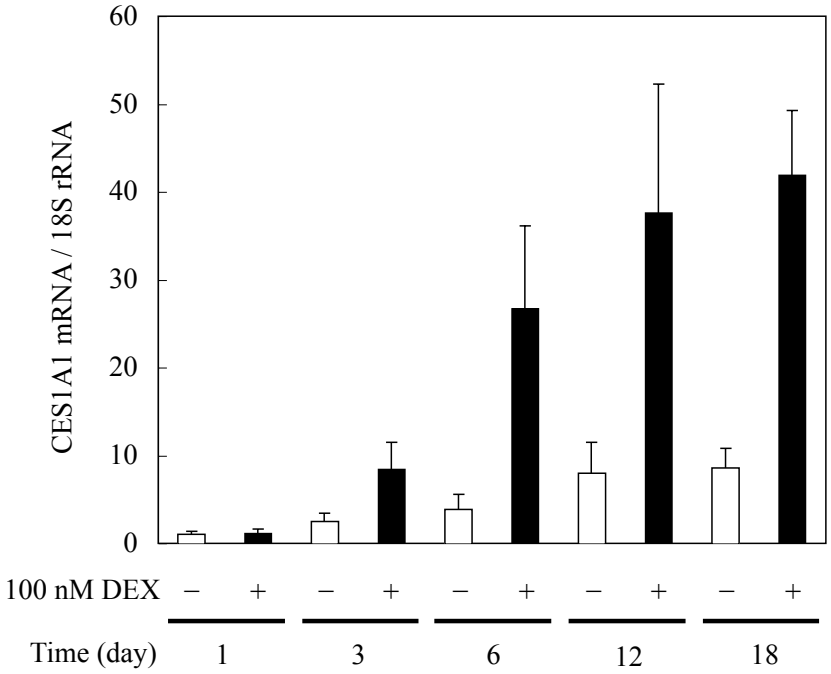

Figure 2.1. *CES1A1* gene expression in HFL cells and the effect of dexamethasone on the expression. HFL cells were cultured in medium containing 100 nM dexamethasone or MQ water for 1, 3, 6, 12, or 18 days, and the expression levels of the *CES1A1* gene were analyzed by real-time PCR. DEX indicates dexamethasone. Each value is shown as the mean ± SD of two independent experiments, which were performed in triplicate.

HFL cells were cultured in medium containing 100 nM dexamethasone or MQ water for 1, 3, 6, 12, or 18 days. As a result, approximately 3- to 7-fold increases in CES1A1 mRNA expression were observed from 3 to 18 days after exposure to dexamethasone (Figure 2.1).

GR antagonist inhibited the increase in CES1A1 mRNA expression caused by dexamethasone

When HFL cells were treated with 100 nM dexamethasone in the presence of 10 μM RU-486, a GR antagonist, for 3 days, the increase in CES1A1 mRNA expression caused by dexamethasone was not observed (Figure 2.2).

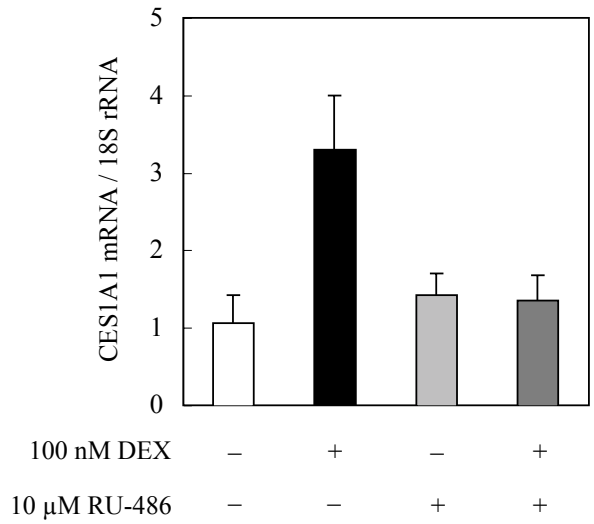

Figure 2.2. Effect of RU-486 on the increase in CES1A1 mRNA caused by dexamethasone. HFL cells were treated with 100 nM dexamethasone in the presence or absence of RU-486 for 3 days, and the expression levels of the *CES1A1* gene were analyzed by real-time PCR. DEX indicates dexamethasone. Each value is shown as the mean ± SD of two independent experiments, which were performed in triplicate.

Treatment with 5-aza-dC or TSA resulted in an increase in CES1A1 mRNA expression

HFL cells were treated with 5-aza-dC or TSA, and expression levels of the *CES1A1* gene in HFL cells were measured by real-time PCR. Treatment with 5-aza-dC increased CES1A1 mRNA expression by approximately 1.7 fold compared with the control (no treatment) (Figure 2.3). Similarly, treatment with TSA increased CES1A1 mRNA expression by approximately 1.8 fold. A synergistic effect of 5-aza-dC and TSA on *CES1A1* gene expression was observed, and CES1A1 mRNA expression ratio was increased by 3.2 fold.

DNA methylation levels in the *CES1A1* gene in HFL cells and effect of dexamethasone on the levels

DNA methylation levels were examined in the region around the TSS of the *CES1A1* gene in the genomes obtained from HFL cells cultured for 1 and 18 days in the presence or absence of 100 nM dexamethasone. The region around the TSS was hypermethylated in HFL cells cultured for 1 day compared with the region in HepG2 cells (see page 16). When HFL cells were cultured for 18 days in medium containing MQ water, DNA methylation levels in the region around the TSS were lower than those in HFL cells cultured for 1 day.

To examine whether dexamethasone treatment decreases DNA methylation levels in the *CES1A1* gene, HFL cells were treated with 100 nM dexamethasone for 1 or 18 days. To make the effect of dexamethasone on DNA demethylation easier to assess, hypomethylated DNA molecules were arbitrarily defined as molecules containing fewer than 3 methylated cytosines in 14 CpG sites in

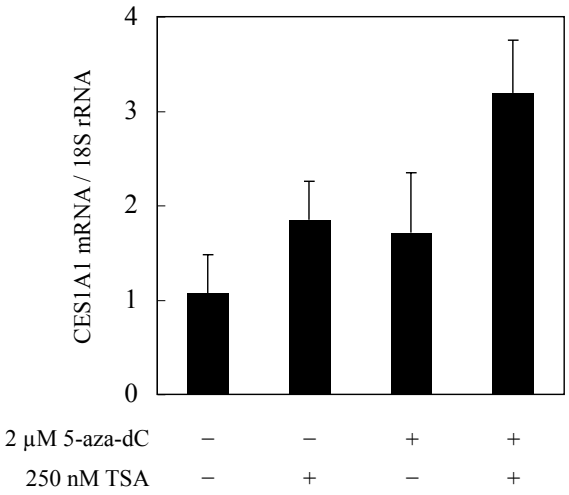

Figure 2.3. Effect of a single or combined treatment with 5-aza-dC and TSA on *CES1A1* gene expression in HFL cells. HFL cells were cultured for 3 days in medium containing PBS(−) as a mock or 2 μM 5-aza-dC and then total RNA was extracted for real-time PCR. Ethanol as a mock or 250 nM TSA was added to the culture medium with cells 24 h before extraction of total RNA. CES1A1 mRNA expression was normalized with 18S rRNA. Each value is shown as the mean ± SD of two independent experiments, which were performed in triplicate.

an arbitrarily defined region named region A (Figure 2.5). Dexamethasone treatment for 1 day decreased DNA methylation levels in the region around the TSS (Figures 2.4A, B, and 2.5), while dexamethasone treatment for 18 days very slightly decreased DNA methylation levels in the region (Figures 2.4A, B, and 2.5).

II-IV Discussion

In this study, we examined the effect of dexamethasone on *CES1A1* gene expression. We found that dexamethasone increases CES1A1 mRNA in HFL cells and that GR is involved in the increase. In addition, we investigated whether dexamethasone causes DNA demethylation in the *CES1A1* gene in light of the recent findings that dexamethasone treatment decreased DNA methylation levels in a part of the rat *Tat* gene (Thomassin et al., 2001; Kress et al., 2006). Our results showed that dexamethasone treatment decreased, in part, DNA methylation levels in the region around the TSS of the *CES1A1* gene in HFL cells. These results raise the possibility that dexamethasone-mediated DNA demethylation in the region is involved in dexamethasone-mediated increase in CES1A1 mRNA expression in the human fetus.

As a first step, we performed real-time PCR analysis to examine time-dependent changes in *CES1A1* gene expression in the presence or absence of dexamethasone in HFL cells. At 100 nM, dexamethasone increased CES1A1 mRNA expression levels from 3 to 18 days after treatment of HFL cells (Figure 2.1), although treatment with dexamethasone for 1 day did not increase CES1A1 mRNA in the present study. This result raises the possibility that the dexamethasone-mediated increases in CES1A1 mRNA involve GR, but not PXR, since 100 nM of dexamethasone is sufficient to activate GR but not sufficient to activate PXR (Huss and Kasper, 2000; Pascussi

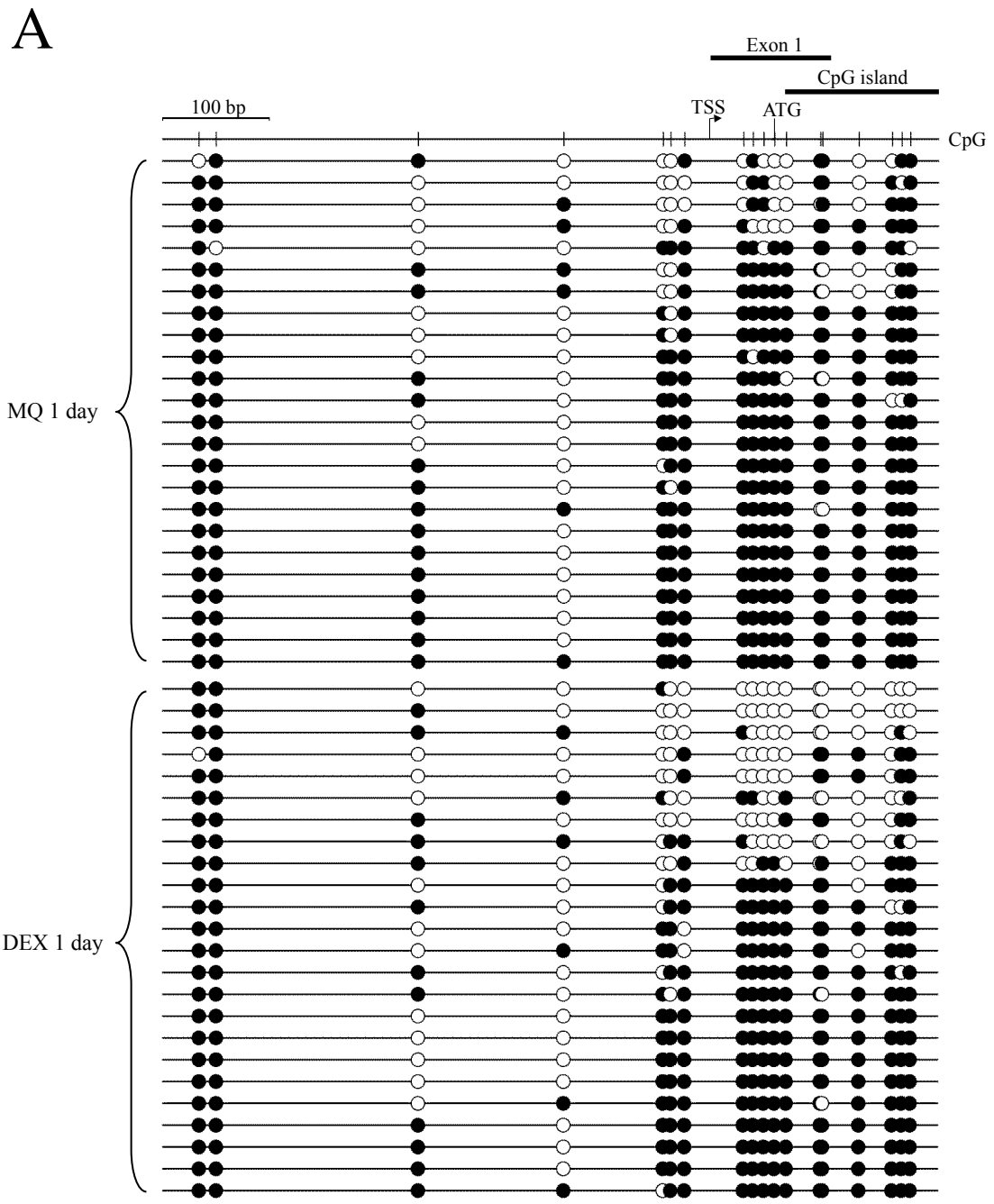

Figure 2.4. Effect of dexamethasone on DNA methylation levels in the *CES1A1* gene. (A) HFL cells were cultured in medium containing 100 nM dexamethasone or MQ water for 1 day, and DNA methylation levels in the region around the TSS of the *CES1A1* gene were analyzed by bisulfite sequencing. Twenty-four molecules were assessed for each group.

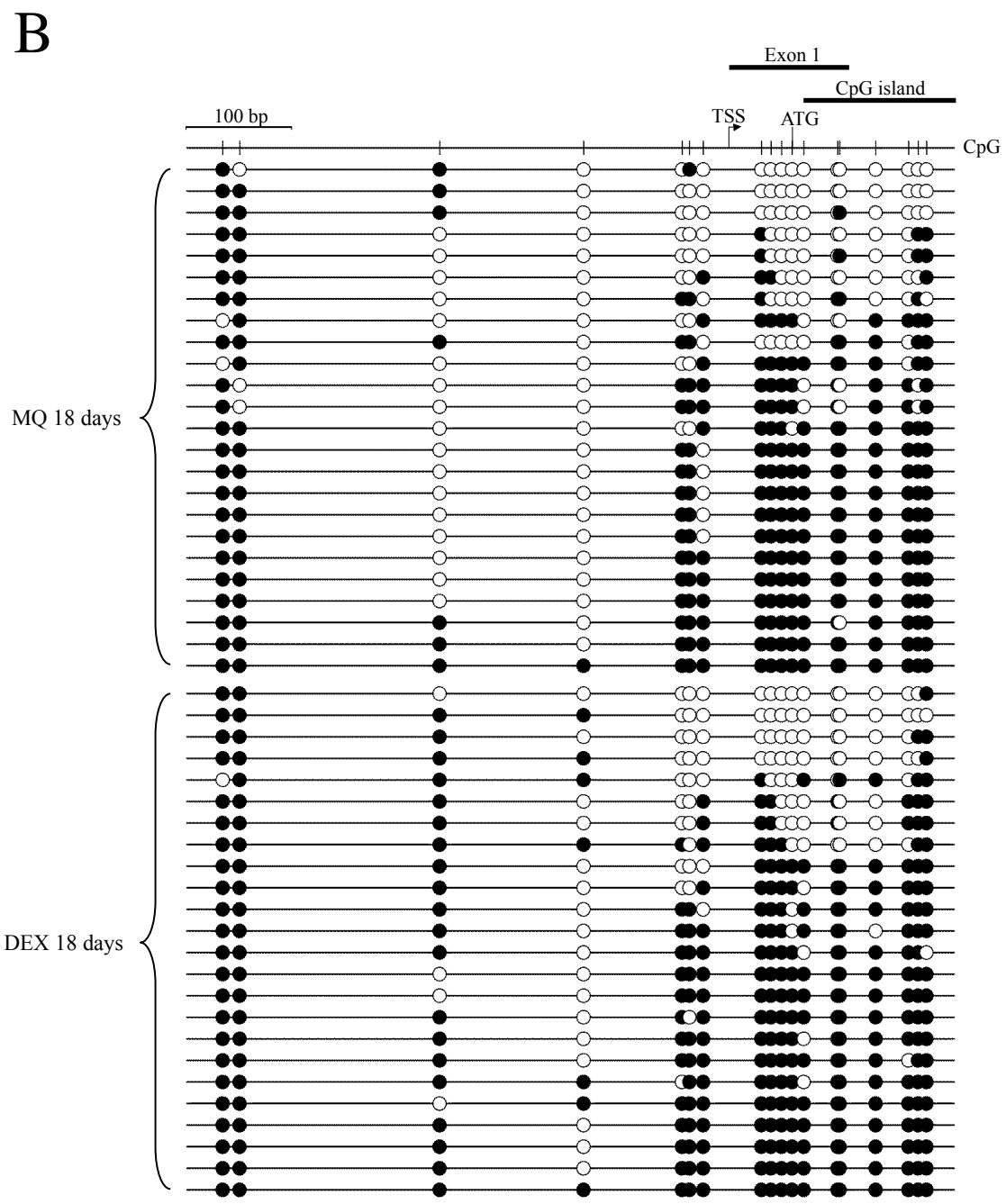

Figure 2.4. (continued) (B) HFL cells were cultured in medium containing 100 nM dexamethasone or MQ water for 18 days, and DNA methylation levels in the region around the TSS of the *CES1A1* gene were analyzed by bisulfite sequencing. Twenty-four molecules were assessed for each group.

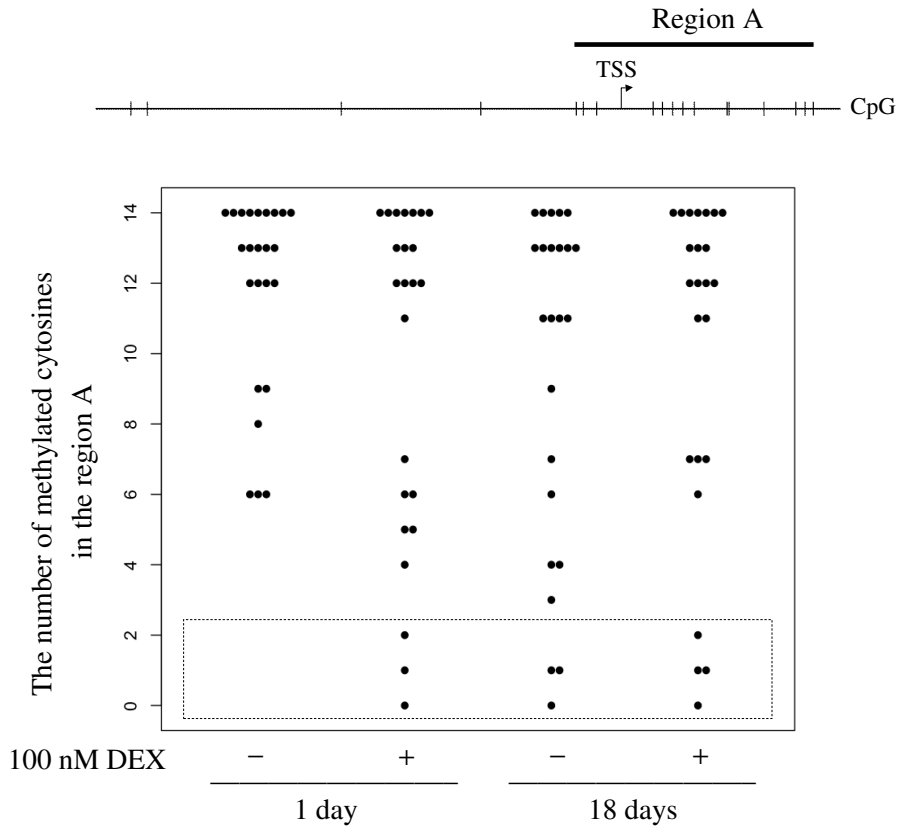

Figure 2.5. Summary of the results of DNA methylation analysis. The results of DNA methylation levels in Figures 2.4A and B are summarized according to the number of methylated cytosines in 14 CpG sites in an arbitrarily defined region named region A. Dots for hypomethylated DNA molecules are enclosed by a broken line. DEX indicates dexamethasone.

et al., 2001).

To understand how dexamethasone increases CES1A1 mRNA, we treated HFL cells with 100 nM dexamethasone for 3 days in the presence of 10 μM RU-486. As a result, the increase in CES1A1 mRNA caused by dexamethasone was completely inhibited (Figure 2.2). The results shown in Figures 2.1 and 2.2 indicated that the dexamethasone-mediated increase in CES1A1 mRNA occurred through GR.

Our study described in chapter 1 showed that suppression of *CES1A1* gene expression in HEK293 cells is due to DNA methylation and histone modification (Hori and Hosokawa, 2010). Since *CES1A1* gene expression level in HFL cells was much lower than that in the human liver tissues in the present study, we asked whether *CES1A1* gene expression in HFL cells is repressed by DNA methylation. After treatment of HFL cells with 5-aza-dC or TSA, the amount of CES1A1 mRNA was slightly increased. Moreover, it appears that the addition of both 5-aza-dC and TSA had a synergistic effect on the expression of the *CES1A1* gene. These results indicated that *CES1A1* gene expression in HFL cells may be, in part, repressed by DNA methylation and histone modification and that the region around the TSS of the *CES1A1* gene in HFL cells may be more methylated than that in the liver of adults.

To investigate DNA methylation patterns, bisulfite sequencing was performed using the genomes obtained from HFL cells cultured for 1 and 18 days. As expected, the region around the TSS of the *CES1A1* gene in HFL cells was mostly hypermethylated (Figures 2.4A and B). DNA methylation levels in the region around the TSS in the genomes obtained from HFL cells cultured for 1 day were higher than those in the genomes obtained from HFL cells cultured for 18 days (Figures 2.4A and B). Alterations in DNA methylation have been reported to occur during development and aging. The time-dependent changes in DNA methylation levels of the *CES1A1* gene might explain why CES1A1 mRNA expression increased in a time-dependent manner in HFL cells (Figures 2.1, and 2.4A and B).

There was a trend toward an association between dexamethasone treatment and loss of DNA methylation. Genomes obtained from HFL cells cultured with dexamethasone for 1 day were more hypomethylated than those obtained from control HFL cells cultured with MQ water for 1 day (Figures 2.4A, 2.4B, and 2.5). Taken together with the results showing that treatment with dexamethasone for 1 day did not increase CES1A1 mRNA, it follows that other alterations such as subsequent histone modification mediated by GR may be necessary for an increase in CES1A1 mRNA caused by dexamethasone and that treatment with dexamethasone for 1 day might be sufficient to lead to DNA demethylation but insufficient to lead to other alterations.

Although it is unclear how dexamethasone caused DNA demethylation in the present study, the molecular mechanisms of DNA demethylation have recently been investigated. Mechanisms of loss of DNA methylation can be divided into two types. One is passive DNA demethylation and the other is positive DNA demethylation. Passive DNA demethylation is achieved by a deficit of DNA methylation mediated by DNMTs, particularly DNMT1. When the amount of DNMT1 is depleted or the activity of DNMT1 is reduced for some reason, cells will be unable to maintain DNA methylation patterns. As for active DNA demethylation, three enzyme families for active DNA demethylation via DNA repair have been intensively investigated (Bhutani et al., 2011). The first family is the ten-eleven-translocation (TET) family, and enzymes of this family can catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine. 5-Hydroxymethylcytosine is considered to an intermediate form potentially involved in DNA demethylation, and recent studies have revealed that 5-hydroxymethylcytosine is involved in gene regulation. The second family is the activation-induced deaminase (AID)/apolipoprotein B mRNA-editing enzyme catalytic polypeptide (APOBEC) family of cytidine deaminases, and enzymes of this family can deaminate 5-methylcytosine or 5-hydroxymethylcytosine. The third family is the uracil-DNA glycosylase (UDG) family of base excision repair (BER) glycosylases, and enzymes of this family can replace several kinds of bases to cytosine. Since it remains to be determined how dexamethasone decreased DNA methylation levels in the present study, further studies are necessary in the future. Recent studies have suggested that decreases in the expression of the *Dnmt1* gene (Yang et al., 2012) or increases in DNA strand breaks (Kress et al., 2006) may participate in dexamethasone-mediated DNA demethylation. These findings raise the possibility that such mechanisms are involved in loss of DNA methylation in the region around the TSS of the *CES1A1* gene.

In summary, one of the major findings presented in this chapter is that the dexamethasone-

mediated increase in CES1A1 mRNA in HFL cells occurs through GR. Our results implied that dexamethasone decreases DNA methylation levels in the region around the TSS of the *CES1A1* gene. Although alteration in DNA methylation levels after removal of dexamethasone from the culture medium should be investigated in the future, it is tempting to speculate the presence of a potential long-lasting induction of CES1A1 caused by treatment of fetuses in pregnant women with dexamethasone in medical practice. It would be interesting to examine whether toxic action in childhood and adulthood takes place by long-lasting CES1A1 induction that could be caused by DNA demethylation during the fetal stage.

More data are required to support the hypothesis that dexamethasone induces DNA demethylation through GR in the *CES1A1* gene. In that sense, we will investigate whether RU-486 inhibits dexamethasone-mediated DNA demethylation in the region around the TSS of the *CES1A1* gene.

The analysis of DNA demethylation in the present study was based on the method of bisulfite sequencing. However, recent studies have revealed that 5-hydroxymethylcytosine is indistinguishable from 5-methylcytosine in results of bisulfite sequencing (Huang et al., 2010). Since 5-methylcytosine-binding proteins such as methyl-CpG binding protein 2 (MeCP2) and DNMTs do not bind to hydroxymethylated DNA (Munzel et al., 2011), it has been thought that 5-hydroxymethylcytosine does not behave in the same manner as that of 5-methylcytosine in gene regulation. Accordingly, bisulfite sequencing could not correctly reveal 5-hydroxymethylcytosine-mediated DNA demethylation in the present study, although it has been found that the amounts of 5-hydroxymethylcytosine in the liver and testes are very small in the mammalian body, while the amount in the central nervous system is large (Munzel et al., 2011).

Chapter III

Dexamethasone-mediated transcriptional regulation of rat carboxylesterase 2 gene

Abstract

Rat carboxylesterase 2 (rCES2), which was previously identified as a methylprednisolone 21-hemisuccinate hydrolase, is highly inducible by dexamethasone in the liver. In the present study, we investigated the molecular mechanisms by which this induction occurs. Injection of dexamethasone (1 mg/kg weight) into rats resulted in increases in the expression of rCES2 mRNA in a time-dependent manner with a peak at 12 h after injection. In primary rat hepatocytes, the expression level of rCES2 mRNA was increased by treatment with 100 nM dexamethasone, and the increase was completely blocked in the presence of 10 μM mifepristone (RU-486), a potent inhibitor of glucocorticoid receptor (GR), or 10 μg/mL cycloheximide, a translation inhibitor. Luciferase assays revealed that 100 nM dexamethasone increased *rCES2* promoter activities, although the effect of dexamethasone on the promoter activity was smaller than that on rCES2 mRNA expression. The increased activities were completely inhibited by treatment of the hepatocytes with 10 μM RU-486. Based on these results, it is concluded that dexamethasone enhances transcription of the *rCES2* gene via GR in the rat liver and that the dexamethasone-mediated induction of rCES2 mRNA may be dependent on *de novo* protein synthesis. Our results provide clues to understanding what compounds induce rCES2.

III-I Introduction

The expression levels of several rat *CES* genes are altered by dexamethasone treatment. Zhu et al. (2000) reported that the expression of rat CES1 (hydrolase A, B, and S) in the liver was suppressed after intraperitoneal injection of dexamethasone. GR is involved in the molecular mechanisms of the suppression (Shi et al., 2008). In contrast, rat CES2 (rCES2) (GenBank ID, AB191005), previously called CES RL4, is markedly induced by dexamethasone in the liver (Furihata et al., 2005). However, the molecular mechanisms underlying the induction of rCES2 have remained unknown.

Increased or decreased drug-metabolizing enzymes can change drug potency *in vivo*. Dexamethasone-mediated induction of rCES2 is known to cause a drug-drug interaction. Since rCES2 hydrolyzes methylprednisolone 21-hemisuccinate (MPHS), which is prescribed for various conditions including systemic lupus erythematosus, hemorrhagic shock and rejection episodes in renal transplant recipients, to the active metabolite methylprednisolone, treatment with dexamethasone increases MPHS hydrolase activity in the rat liver. Drug-mediated induction or repression of rat *CES* gene expression should be noted as long as rats continue to be used commonly in non-clinical studies.

The goal of this study was to determine the molecular mechanisms underlying dexamethasone-mediated induction of rCES2. In the present study, treatment of primary rat hepatocytes with RU-486, a potent antagonist of GR, resulted in inhibition of both increase in rCES2 mRNA and elevation of *rCES2* promoter activity caused by dexamethasone. These results suggested that GR plays a critical role in dexamethasone-mediated transcriptional activation of the *rCES2* gene. Moreover, results obtained by using cycloheximide indicated the possibility that *de novo* protein synthesis is necessary for the induction of rCES2 mRNA by dexamethasone.

III-II Materials and methods

Materials

Dexamethasone-water soluble, triamcinolone acetonide, cycloheximide, RU-486, MPHS, and methylprednisolone were purchased from Sigma-Aldrich. Dexamethasone (used with corn oil), corn oil, collagenase, a Ligation-Convenience Kit, and prednisolone were purchased from Wako Pure Chemical Industries. Trypsin inhibitor from soybean (>7000 BAEE units/mg), Williams' medium E without phenol red, Opti-MEM I, DNase I, and a Zero Blunt TOPO PCR Cloning Kit were purchased from Invitrogen Life Technologies. Pentobarbital sodium (somnopentyl) was purchased from Schering-Plough Corp. (Kenilworth, NJ, USA). Human recombinant insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals Inc. (Princeton, NJ, USA). A nylon mesh filter was purchased from Sefar Inc. (Heiden, Switzerland). AteloCell (native collagen bovine dermis) was purchased from Koken Co. Ltd. (Tokyo, Japan). FuGENE HD transfection reagent was purchased from Roche Diagnostics Corp. (Indianapolis, IN, USA). BD Matrigel (phenol red-free) was purchased from BD Biosciences (Bedford, MA, USA). ISOGEN was purchased from

Nippon Gene. A ReverTra Ace qPCR RT Kit, THUNDERBIRD Probe qPCR Mix, KOD -plus- DNA Polymerase, and *Kpn* I were purchased from Toyobo. *Xho* I was purchased from Takara Shuzo (Kyoto, Japan). TaqMan Gene Expression Assays (probe and primer sets) for rCES2 (AssayID: Rn00592205_m1) (FAM) and rat TAT (Rn01431532_m1) (FAM) and Pre-Developed TaqMan Assay Reagents for eukaryotic 18S rRNA (VIC) were purchased from Applied Biosystems. Dual-Luciferase Reporter Assay System was purchased from Promega. *Dpn* I was purchased from New England BioLabs (Hitchin, Hertfordshire, UK).

Intraperitoneal injection of dexamethasone

Male Sprague-Dawley (SD) rats (Japan SLC Inc., Shizuoka, Japan) of 5–6 weeks (150–200 g) of age were used in this experiment. Dexamethasone in corn oil (0.25 mg/mL) was intraperitoneally injected into rats at a dose of 1 mg/kg body weight, or only corn oil was injected as a control in the same way. Rats were put under anesthesia with diethyl ether at 3, 6, 12, or 24 h after injection of dexamethasone or at 24 h after injection of only corn oil for the control, and the livers were removed for relative quantification of mRNA expression and protein expression. One rat per experiment was used at each time point and the experiment was repeated thrice (n=3/time point).

Isolation of hepatocytes

Rat hepatocytes were isolated using a collagenase two-step perfusion method described by Seglen (1976) with some modifications. Rats were anesthetized by intraperitoneal injections of pentobarbital (100 mg/kg body weight) and the abdominal cavity was incised. An indwelling needle consisting of an inner needle and a flexible cover needle was inserted into the portal vein, and the inner needle was removed from the indwelling needle. The flexible cover needle and portal vein were immediately bound using a clamp, and the cover needle was connected to a tube with a pump. A pre-perfusate (137 mM NaCl, 5.37 mM KCl, 1.05 mM $MgCl_2{\cdot}6H_2O$, 0.832 mM $MgSO_4{\cdot}7H_2O$, 0.500 mM $NaH_2PO_4{\cdot}2H_2O$, 0.423 mM Na_2HPO_4, 9.98 mM HEPES, 0.500 mM EGTA, 4.17 mM $NaHCO_3$, and 5.00 mM D-glucose; pH 7.2 and 37°C) was flowed through the portal vein at a flow rate of ~24 mL/min. The inferior vena cava was immediately cut to make an exit site for the pre-perfusate. About 6 min later, the pre-perfusate was replaced by flowing a collagenase solution (137 mM NaCl, 5.37 mM KCl, 5.05 mM $CaCl_2$, 0.500 mM $NaH_2PO_4{\cdot}2H_2O$, 0.423 mM Na_2HPO_4, 9.98 mM HEPES, 4.17 mM $NaHCO_3$, 1 g/L collagenase, and 100 mg/L trypsin inhibitor; pH 7.5 and 37°C). The collagenase solution was flowed for ~6 min at the same flow rate. After perfusion of the collagenase solution, the digested liver was resected and washed briefly with ice-cold Hanks' balanced salt solution, HBSS (137 mM NaCl, 5.37 mM KCl, 1.26 mM $CaCl_2$, 0.812 mM $MgSO_4$, 0.336 mM Na_2HPO_4, 0.441 mM KH_2PO_4, 5.55 mM D-glucose, and 4.17 mM $NaHCO_3$; pH ~7.3). Cells were dispersed from the digested liver in 50 mL of ice-cold HBSS and the cellular suspension was filtrated through a nylon mesh filter (pore size, 150 μm). The filtrated cells were centrifuged for 2 min at 4°C and the supernatant was aspirated. For removal of non-parenchymal hepatocytes, the cells containing hepatocytes were suspended in 15

mL of cold HBSS and centrifuged for 2 min at 4°C, and the supernatant was removed (This series of steps for removal was performed twice.). Finally, the obtained hepatocytes were suspended in 20 mL of an ice-cold standard culture medium (Williams' medium E without phenol red, containing 0.25 U/mL insulin, 100 U/mL penicillin, and 100 μg/mL streptomycin) and the viability of hepatocytes was assessed by 0.4% trypan blue exclusion. Hepatocytes for which viability exceeded 85% were used in the present study.

Primary cultures of rat hepatocytes for mRNA expression analysis

Primary cultures of rat hepatocytes were performed using the method previously described by Kocarek and Reddy (1996) with some modifications to extract total RNA and perform the ensuing real-time PCR. Hepatocytes were isolated from SD rats of 5–7 weeks (~160–250 g) of age by the method described above. The cells were suspended in the standard culture medium and were seeded at 7.5×10^5 cells/mL $\times$ 2 mL/well onto 6-well plates coated with 0.5 mg/well (for example, 3.57 mg/mL $\times$ 140 μL/well) of matrigel. Hepatocytes then were incubated at 37°C in air with 5% CO_2. Twenty-four hours after seeding, the culture medium in the plates was replaced with 2 mL fresh medium. Dexamethasone (water-soluble) was dissolved in MQ water to make a 100-μM dexamethasone solution as a stock solution. RU-486 or cycloheximide was dissolved in ethanol to make a 10-mM RU-486 solution or 1-mM and 10-mg/mL cycloheximide solutions as stock solutions. The culture medium in plates was again replaced with 2 mL fresh medium 48 h after seeding, and reagents (drugs and vehicles) were added to the culture medium. The drug solutions or solvents were added at 0.1% (v/v). Extraction of total RNA from hepatocytes in each well was performed 24 h after treatment with reagents.

Relative quantification of mRNA expression

Relative quantification of target transcripts was performed essentially as described previously (Hori and Hosokawa, 2010). Total RNA was extracted from rat liver pieces and primary hepatocytes using an ISOGEN and treated with DNase I to prevent contamination by DNA. The treated RNA and a ReverTra Ace qPCR RT Kit were used to synthesize first-strand cDNA. The expression level of rCES2 mRNA was analyzed using cDNA (1 μg/sample), a THUNDERBIRD Probe qPCR Mix, gene-specific TaqMan probe and primer sets, and an Applied Biosystems 7500 Real-Time PCR System. Rat CES2 mRNA expression was normalized with 18S rRNA expression. The $\Delta\Delta C_t$ method was used for analysis of data on rCES2 mRNA expression. The conditions of real-time PCR were as follows: 95°C for 1 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of rat TAT mRNA was analyzed in the same way as that for rCES2 mRNA.

Western blot analysis

The expression level of rCES2 protein in the rat liver was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot essentially as previously described (Furihata et al.,

2005). Rat liver pieces obtained from rats that were injected with or without dexamethasone were homogenized with 1.15% KCl. The 20% (w/v) homogenates were centrifuged at 9,000 × *g* for 20 min, and the supernatants were centrifuged at 105,000 × *g* for 1 h. After replacement of the supernatants with fresh KCl buffer, the samples were again centrifuged at 105,000 × *g* for 1 h. The microsomal pellets were suspended in SET buffer (0.25 M sucrose, 1 mM EDTA·2Na, and 10 mM Tris-HCl; pH 7.4). SDS-PAGE was performed using 10% polyacrylamide gels and 10 μg/well of microsomal protein, and Western blot was performed using polyclonal anti-rCES2 antibodies, which were previously prepared (Derbel et al., 1996). The density of bands was measured using ImageJ 1.44o software (National Institutes of Health, Bethesda, MD, USA).

MPHS hydrolase activity assay

MPHS hydrolase activity was determined essentially according to the method described previously (Furihata et al., 2005). Rat liver microsomes were preincubated in citrate-phosphate buffer (pH 5.5) for 5 min at 37°C. The reaction was initiated by adding MPHS dissolved in 1% dimethylformamide water to the mixture. The mixture (50 mM citrate-phosphate buffer, 0.04–0.3 mg/mL microsomal protein, and 400 μM MPHS) was incubated for 15 min at 37°C. The reaction was terminated by adding 100 μL of acetonitrile containing 40 μM prednisolone, which was used as an internal standard, to 100 μL of the mixture. After the removal of protein, the amount of methylprednisolone formed from MPHS was determined by high performance liquid chromatography (HPLC). Methylprednisolone was detected at a wavelength of 254 nm. HPLC system consisted of a LC-20AD pump unit (Shimadzu Corp., Kyoto, Japan), a SIL-20A autosampler (Shimadzu), a CTO-10AS VP column oven (Shimadzu), a SPD-20A UV/VIS detector (Shimadzu), a SCL-10A VP system controller (Shimadzu), and a Mightysil RP-18 GP 150 mm × 4.6 mm column (Kanto Chemical Co., Inc., Tokyo, Japan). The mobile phase consisted of 50 mM phosphate buffer (pH 7.0)/acetonitrile (65:35, v/v) and was delivered at a flow rate of 0.9 mL/min.

Reporter constructs

The 5'-flanking region from −2957 to +51 (−2957/+51), when the TSS of the *rCES2* gene is +1, was amplified by PCR with KOD -plus- DNA Polymerase, rat genomic DNA as a template, and primers listed in Table 1. The primers were designed on the basis of the sequence obtained from a search by basic local alignment search tool (BLAST) with the sequence of AB191005. The amplified fragment was cloned into pCR -Blunt II-TOPO vector provided in the Zero Blunt TOPO PCR Cloning Kit following the manufacturer's instructions, and this plasmid was designated pCR-rCES2−2957/+51. After sequencing, pCR-rCES2−2957/+51 was digested by the two restriction enzymes, *Kpn* I and *Xho* I. After purification, the digested fragment containing the sequence of −2957/+51 was ligated using a Ligation-Convenience Kit to pGL3-Basic vector, a luciferase reporter vector, digested by the same restriction enzymes. After amplification using competent *E. coli* JM109 and the ensuing purification, this plasmid was designated pGL3-rCES2−2957/+51 for luciferase assays. A variety of deletion plasmids (pGL3-rCES2−1955/+51,

Table 1. Specific primers in the 5'-flanking region of the *rCES2* gene for genomic cloning and luciferase assays

Position	Sequence
Forward primer	
−2957/−2933	5'-GATGGCTGCGTGATACTTCTTCTGG-3'
Reverse primer	
+23/+51	5'-AGTTCTGAGTCTGTGCTGCTAGAATGACC-3'

Table 2. Specific primers in the 5'-flanking region of the *rCES2* gene for luciferase assays

Position	Sequence
Forward primer	
−1955/−1936	5'-ATCTTGGTGCCTTCTAACTG-3'
−1569/−1549	5'-TCTTTGACTAGCGAAATGGTG-3'
−991/−968	5'-TTTTTTTTTCTGGTGATGGATTCG-3'
−662/−642	5'-CCTCTGGAGACACTTCAGACA-3'
−195/−173	5'-AGTCCACACTGTGCCTTTCCAGG-3'
−73/−51	5'-TTCACCCACGACATCATGTTCCC-3'
Reverse primer	
+23/+51	5'-AGTTCTGAGTCTGTGCTGCTAGAATGACC-3'

−1569/+51, −991/+51, −662/+51, −195/+51, and −73/+51) were made in the same manner as that described above, except that these deletion plasmids were made using primers listed in Table 2 and pCR-rCES2−2957/+51 as a template. The plasmid of pGL3-rCES2−6/+51 was made by a site-directed mutagenesis method described below. First, PCR was performed using pGL3-rCES2−195/+51 as a template and primers that were designed to anneal to the same sequence on opposite strands and to carry a *Kpn* I site (Table 3). The PCR product, plasmid, was electrophoresed and the target plasmid was extracted. The plasmid was treated with *Dpn* I to digest template plasmid, pGL3-rCES2−195/+51. Following transformation of JM109 and purification of the plasmid, sequencing was performed to confirm the existence of the *Kpn* I site in the plasmid. Then the plasmid was digested by *Kpn* I and the fragment containing the sequence of −6/+51 was self-ligated using a Ligation-Convenience Kit. In this way, pGL3-rCES2−6/+51 plasmid was made.

Transient transfection and luciferase assay

Transient transfection into primary rat hepatocytes was performed using the method previously described by Runge-Morris et al. (1999) with some modifications. Hepatocytes were isolated from SD rats of 5–7 weeks (~150–250 g) of age by the method described above. Hepatocytes were suspended in a cold standard culture medium which was supplemented with 100 nM triamcinolone acetonide (TA). The cells were seeded at 3×10^5 cells/mL × 0.5 mL/well onto 24-well

Table 3. Mutant primers for site-directed mutagenesis to make *Kpn* I site (small letter)

Position	Sequence
Forward primer	
−25/+10	5'-CCTGCCTGGGCAAggtaccCGGTTTATTCTTCCTG-3'
Reverse primer	
−25/+10	5'-CAGGAAGAATAAACCGggtaccTTGCCCAGGCAGG-3'

plates coated thinly with collagen. Hepatocytes then were incubated at 37°C in air with 5% CO_2. Following ~6 h of seeding, the medium was replaced with 0.5 mL of Opti-MEM I, and plasmids were transfected to the cells as follows: 500 ng/well pGL3 plasmid (12.5 ng/μL), 50 ng/well phRL-TK plasmid (12.5 ng/μL), and 1.4 μL/well FuGENE HD. Five hours after transfection, the medium was replaced with 0.5 mL of the standard culture medium without TA, and hepatocytes were overlaid with 0.05 mg/well (for example, 3.57 mg/mL × 14 μL/well) of matrigel. Thirty hours after addition of matrigel, hepatocytes were treated with either 100 nM dexamethasone or MQ water in 0.5 mL of fresh medium without TA and again overlaid with 0.05 mg/well of matrigel. Simultaneously with dexamethasone or MQ water, in an experiment for which results are shown in Figure 3.5C, hepatocytes were exposed to RU-486 or ethanol. Twenty-four hours later, hepatocytes were rinsed once with PBS(−), and dual-luciferase reporter assays were performed according to the manufacturer's instructions.

Statistical Analysis

Multiple groups were compared by one-way analysis of variance (one-way ANOVA) followed by Dunnett's (Figure 3.1A and 3.1C) or Tukey's (Figures 3.2, 3.3, and 3.5C) multiple comparison test. Two groups were compared by Student's *t*-test (Figures 3.4 and 3.5A). These statistical analyses were performed using the free software R version 2.13.0 (R Development Core Team, 2011). A value of $P < 0.05$ was considered statistically significant.

III-III Results

Dexamethasone-mediated increases in TAT and rCES2 mRNA expression and alterations of rCES2 protein expression in the rat liver

Dexamethasone was injected into rats to investigate temporal changes in TAT and rCES2 mRNA expression. The expression levels of rCES2 mRNA peaked at 12 h after injection of dexamethasone and the maximum level was approximately 400-fold higher than that of the control (dexamethasone 0 h) (Figure 3.1A). The level of TAT mRNA, which is well known to be induced by dexamethasone, was also increased by dexamethasone. The increased level of TAT mRNA was highest at 6 h after injection, and this change was in agreement with the results of a previous

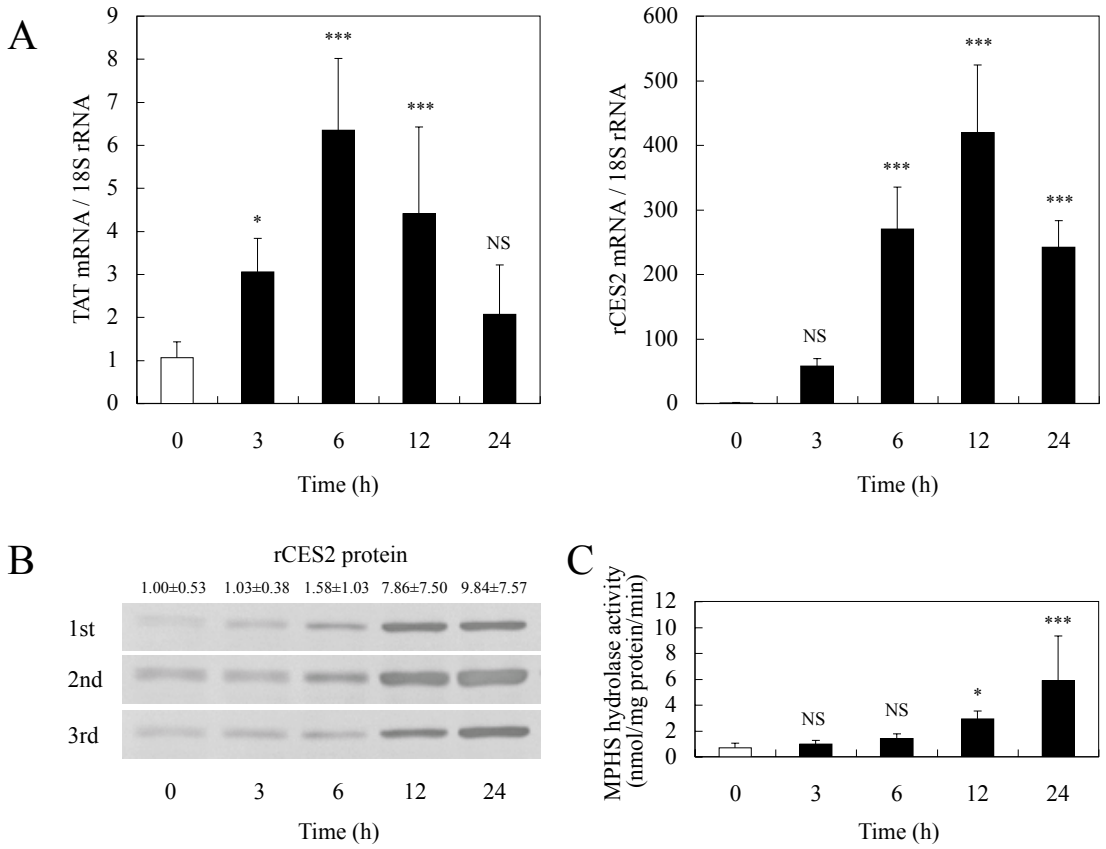

Figure 3.1. (A) Time course of increases in TAT and rCES2 mRNA by dexamethasone. Rats were sacrificed at 3, 6, 12, and 24 h after injection of dexamethasone, and liver pieces were used to determine the expression levels of TAT and rCES2 mRNA by real-time PCR. Each value is shown as the mean ± standard deviation (SD) of three independent experiments (n = 3/group), which were performed in triplicate. In analysis of TAT mRNA, the C_t (threshold cycles) values (mean ± SD) of a control (dexamethasone 0 h) were 23.1 ± 1.04 (TAT) and 12.6 ± 0.813 (18S rRNA). In analysis of rCES2 mRNA, the C_t values of the control were 27.8 ± 0.905 (rCES2) and 12.5 ± 0.535 (18S rRNA). NS indicates not statistically significant. Statistically significant difference; *P < 0.05 and ***P < 0.001. (B) Alterations of rCES2 protein expression by dexamethasone. Relative expression levels of rCES2 protein (mean ± SD of three independent experiments) were estimated using a standard curve that generated by a microsomal sample obtained from a rat injected with dexamethasone. (C) Temporal changes in MPHS hydrolase activity in liver microsomes after injection of dexamethasone. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Statistically significant differences; *P < 0.05 and ***P < 0.001.

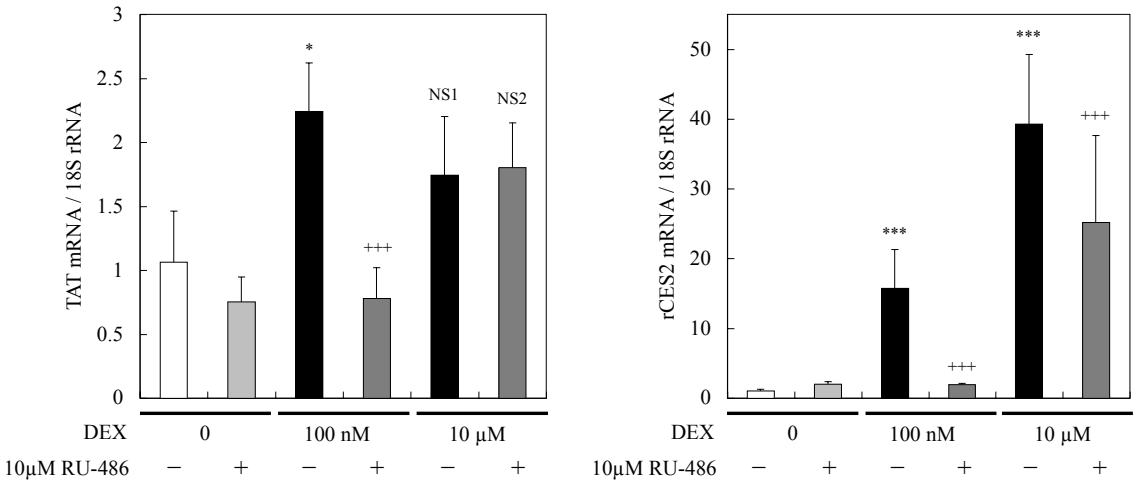

Figure 3.2. Effects of dexamethasone and RU-486 on the expression of TAT and rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone or water in the presence of RU-486 or ethanol. The expression levels of TAT and rCES2 mRNA were analyzed by real-time PCR. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Hepatocytes as a control (white bars) were treated with both water and ethanol. In analysis of TAT mRNA, the average C_t values (mean ± SD) of the control were 21.9 ± 0.410 (TAT) and 11.5 ± 0.189 (18S rRNA). In analysis of rCES2 mRNA, the average C_t values of the control were 23.9 ± 0.433 (rCES2) and 11.7 ± 0.247 (18S rRNA). DEX indicates dexamethasone. NS1 indicates not statistically significant (control versus DEX treatment). NS2 indicates not statistically significant (DEX treatment versus DEX+RU-486 treatment). Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P < 0.05$ and $^{***}P < 0.001$. Statistically significant differences (DEX treatment versus DEX+RU-486 treatment within each corresponding group); $^{+++}P < 0.001$.

study (Shi et al., 2008). Significant increases of rCES2 protein and MPHS hydrolase activity were observed at 12 and 24 h after injection (Figure 3.1B and 3.1C).

Comparison between rat primary hepatocytes and livers in expression of TAT and rCES2 mRNA

The expression levels of TAT or rCES2 mRNA in rat hepatocytes and livers were compared. Hepatocytes as a control were treated with both water and ethanol. When based on the average ΔC_t value obtained from the control hepatocytes, rCES2 mRNA expression ratio (rCES2 mRNA/18S rRNA) of the control hepatocytes and that of livers without dexamethasone were 1.02 ± 0.235 and 0.126 ± 0.0491, respectively. Likewise, TAT mRNA expression ratio (TAT mRNA/18S rRNA) of the control hepatocytes and that of livers without dexamethasone were 1.06 ± 0.400 and 0.946 ± 0.328, respectively.

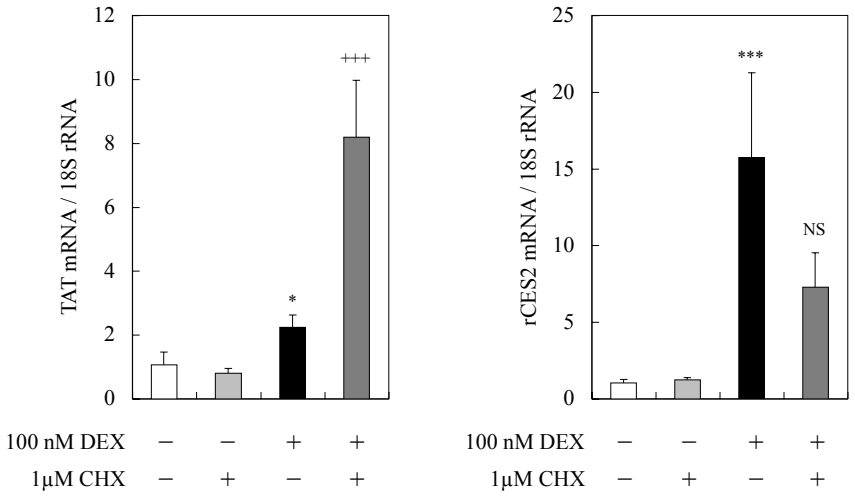

Figure 3.3. Effect of 1 μM cycloheximide on the expression of TAT and rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone (100 nM) or water in the presence of cycloheximide (1 μM) or ethanol. The expression levels of TAT and rCES2 mRNA were analyzed by real-time PCR. Each value is shown as the mean ± SD of three independent experiments, which were performed in triplicate. Data analysis was performed in combination with data used to make Figure 3.2. DEX and CHX indicate dexamethasone and cycloheximide, respectively. NS indicates not statistically significant (DEX treatment versus DEX+CHX treatment) and we retain the null hypothesis because P value is 0.0748. Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P < 0.05$ and $^{***}P < 0.001$. Statistically significant differences (DEX treatment versus DEX+CHX treatment); $^{+++}P < 0.001$.

Alterations of TAT and rCES2 mRNA expression by dexamethasone, RU-486, and cycloheximide in primary rat hepatocytes

The effects of dexamethasone, RU-486, and cycloheximide on TAT and rCES2 mRNA expression were investigated. Dexamethasone (100 nM and 10 μM) dose-dependently caused an elevation of rCES2 mRNA in primary rat hepatocytes (Figure 3.2). The increased rCES2 mRNA by 100 nM dexamethasone was completely inhibited by treatment with 10 μM RU-486 (Figure 3.2). Treatment with 1 μM cycloheximide for 24 h resulted in repression, in part, of the increase in rCES2 mRNA by 100 nM dexamethasone (Figure 3.3), while the elevation of rCES2 mRNA was completely inhibited by treatment with 10 μg/mL (~36 μM) cycloheximide for 24 h (Figure 3.4). In the case of TAT mRNA, 100 nM dexamethasone increased the expression, and the effect of 10 μM dexamethasone was weaker than that of 100 nM dexamethasone (Figure 3.2). Note that the expression of 18S rRNA was decreased by only approximately 2- to 3-fold at 10 μg/mL cycloheximide and that the values of the ratio (rCES2 mRNA/18S rRNA) were seemingly increased by treatment with 10 μg/mL cycloheximide. Therefore, we separated data obtained from samples with 10 μg/mL cycloheximide from the other data on mRNA expression and performed analysis as described in Statistical Analysis and the legend to Figure 3.4.

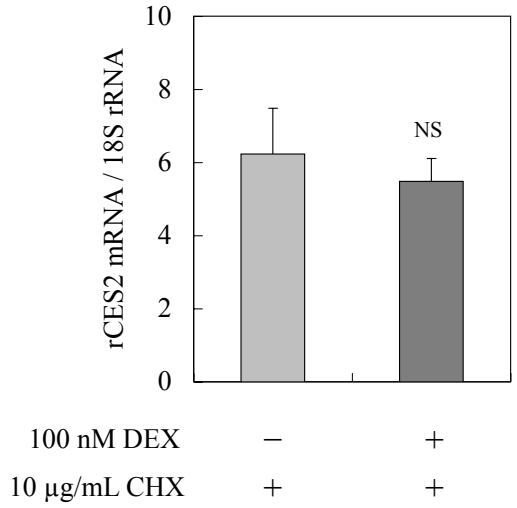

Figure 3.4. Effect of 10 μg/mL cycloheximide on the expression of rCES2 mRNA in primary rat hepatocytes. Primary rat hepatocytes were treated for 24 h with medium containing dexamethasone (100 nM) or water in the presence of cycloheximide (10 μg/mL) or ethanol. The expression levels of rCES2 mRNA were analyzed by real-time PCR. The vertical axis indicates the ratio (rCES2 mRNA/18S rRNA) based on the same control as shown in Figures 3.2 and 3.3. Each value is shown as the mean ± SD of two independent experiments, which were performed in triplicate. The average C_t values (mean ± SD) of the samples obtained from the hepatocytes treated with 10 μg/mL cycloheximide without dexamethasone were 22.7 ± 0.422 (rCES2) and 13.1 ± 0.227 (18S rRNA). NS indicates not statistically significant.

Activation of rCES2 promoters by dexamethasone and inhibition of the activation by RU-486

Nucleotide sequences necessary for basal transcription of the *rCES2* gene and for response to dexamethasone were investigated using transient transfections of deletion plasmids consisting of a variety of lengths of the *rCES2* promoter and dual-luciferase assays. Dexamethasone treatment, however, tended to decrease values of *Renilla* luciferase activity (Figure 3.5A and C). The decreased levels appeared to vary among cell populations that have pGL3 plasmids harboring different *rCES2* promoter regions, implying that phRL-TK plasmid is unsuitable for an internal control in the present study. Therefore, to determine the effect of dexamethasone on *rCES2* promoter activity, we analyzed data on *Firefly* luciferase activity instead of data on relative luciferase activity (*Firefly*/*Renilla*). Basal transcriptional activities were almost the same among the plasmids including the region of −195/+51 (Figure 3.5A). The basal transcriptional activity of the plasmid including the region of −195/+51 was decreased by more than half by deletion of the sequence from −195 to −74 and was abolished by additional deletion of the sequence from −73 to −7. Dexamethasone treatment resulted in an approximately 2- to 3-fold increase in promoter activity in the region of −2957/+51 (Figure 3.5A and C). Ten micromolars of RU-486 repressed the elevation of the promoter activation by 100 nM dexamethasone in the region of −2957/+51 (Figure 3.5C). The *rCES2* promoter activity tended to be increased by dexamethasone associating with the sequences of −73/−7 and −991/−663 (Figure 3.5B). The dexamethasone-mediated promoter activation was abolished by RU-486 treatment in the region of −73/+51 as well as in the region of −2957/+51

(Figure 3.5C).

III-IV Discussion

MPHS is hydrolyzed to methylprednisolone in rat hepatic microsomes (Hattori et al., 1981). MPHS hydrolase activity is increased following each injection of various glucocorticoids including dexamethasone and also methylprednisolone (Hattori et al., 1992a; Hattori et al., 1992b). We previously identified an MPHS hydrolase as rCES2 that is strongly induced by dexamethasone in the liver (Furihata et al., 2005). However, the molecular mechanisms by which the induction occurs have remained unknown. In the present study, we demonstrated that GR contributes to dexamethasone-mediated transcriptional activation of the *rCES2* gene.

First, we confirmed an increase in rCES2 mRNA at a lower concentration of dexamethasone than that employed in our previous study. Our previous study using reverse transcription-PCR showed that the expression of rCES2 mRNA was markedly increased when rats were injected with dexamethasone at a dose of 5 mg/kg body weight for 4 consecutive days (Furihata et al., 2005). Consistent with this observation, when rats were injected with dexamethasone at a single does of 1 mg/kg body weight in the present study, more than 200-fold increases in rCES2 mRNA were observed in the liver from 6 to 24 h after injection (Figure 3.1A). Hattori et al. (1992b) previously showed that when a suspension of dexamethasone in sesame oil was intraperitoneally administered to rats as a single dose of 60 μmol/kg (~23.5 mg/kg), MPHS hydrolase activity in rat microsomes rapidly increased and plateaued between 20 and 40 h after a 4-h lag period. Consistent with their results for hydrolase activity, the expression level of rCES2 mRNA increased with time until 12 h after injection of dexamethasone and the increase in rCES2 mRNA was followed by strong increases in rCES2 protein and MPHS hydrolase activity (Figure 3.1B and 3.1C). Hansen et al. (1999) reported that the mean of the maximum concentrations ($C_{\max}$) in plasma was 682 ng/mL (~1.7 μM) after a single subcutaneous injection of 0.8 mg/kg dexamethasone into pregnant rats. Thus, our results indicate that treatment with ~2 μM of dexamethasone for 6 to 24 h is probably sufficient for a significant increase in rCES2 mRNA in the liver.

In rat primary hepatocytes, the level of rCES2 mRNA was increased by treatment with 100 nM dexamethasone (Figure 3.2). This result implies that the responsiveness of the primary hepatocytes used in the present study to dexamethasone was restored and that the elevation of rCES2 mRNA occurred in hepatic parenchymal cells. Dexamethasone-mediated increase in TAT mRNA, which serves as a positive control for GR-mediated up-regulation (Courtois et al., 1999), was also observed in primary hepatocytes. In addition, 10 μM RU-486, which is known to antagonize the effect of dexamethasone on TAT mRNA expression (Runge-Morris et al., 1996), completely repressed the increase in rCES2 mRNA by 100 nM dexamethasone, similar to TAT mRNA (Figure 3.2). Since 100 nM dexamethasone can activate GR (Runge-Morris et al., 1999), these results suggest that dexamethasone-mediated increase in rCES2 mRNA occurs through GR. Stress-induced glucocorticoid hormones would increase the expression level of rCES2 mRNA in the rat liver, since 10 μM dexamethasone was more effective than 100 nM dexamethasone.

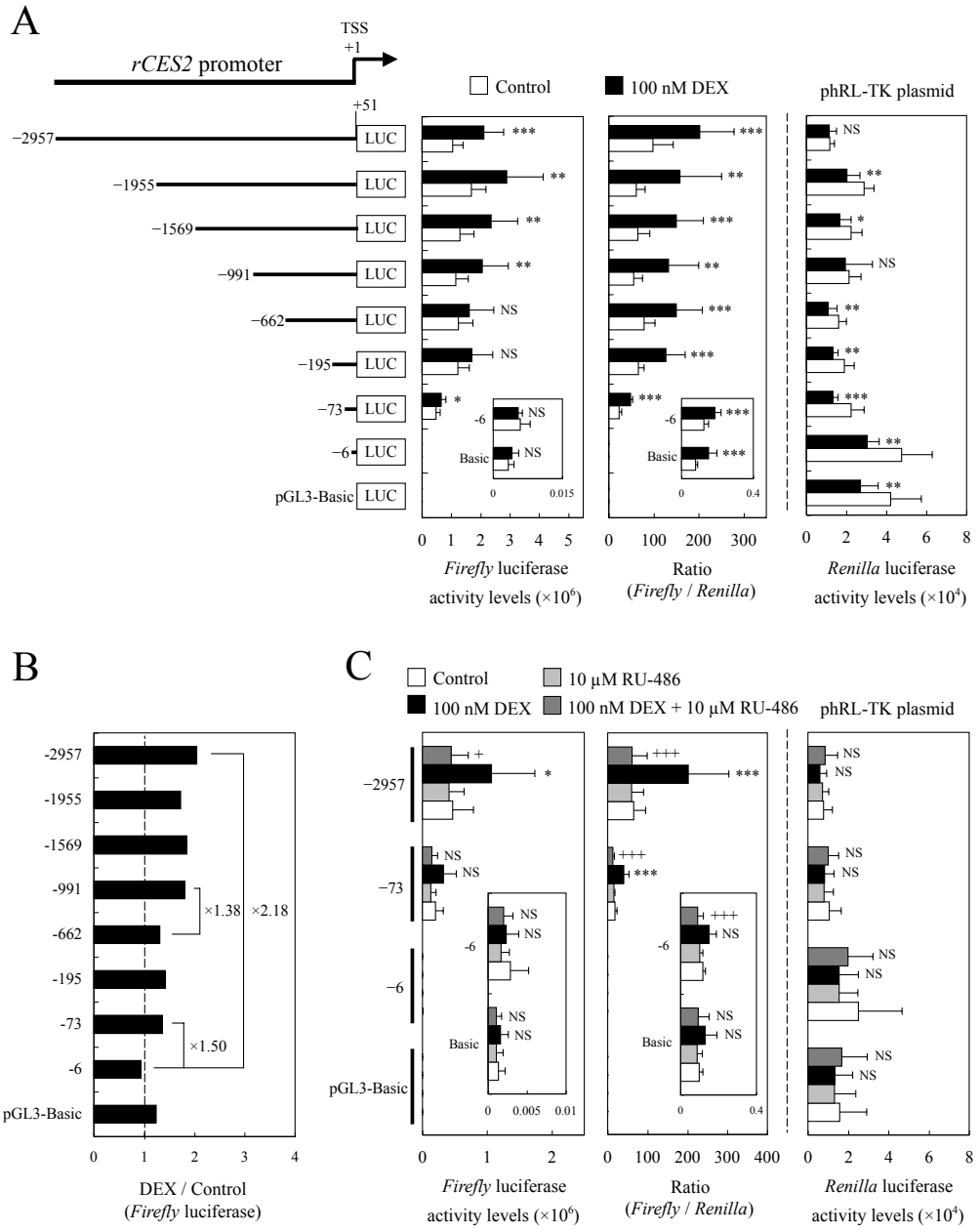

Figure 3.5. Analysis of the 5'-flanking region of the *rCES2* gene. (A) Deletion analysis of the 5'-flanking region of the *rCES2* gene. Primary rat hepatocytes transfected with 500 ng/well pGL3 plasmid (*Firefly* luciferase) and 50 ng/well phRL-TK plasmid (*Renilla* luciferase) were treated for 24 h with dexamethasone (100 nM) or water, and dual-luciferase assays were performed. TSS indicates the transcription start site of the *rCES2* gene. Hepatocytes as a control were treated with water (white bars). DEX indicates dexamethasone. Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. NS indicates not statistically significant. Four independent experiments were performed in triplicate. (B) Promoter regions necessary for response to dexamethasone. The ratios (DEX/control) were based on the results of *Firefly* luciferase activity levels shown in Figure 3.5A. (C) Effect of RU-486 on dexamethasone-mediated activation of *rCES2* promoters. Primary rat hepatocytes transfected with 500 ng/well pGL3 plasmid and 50 ng/well phRL-TK plasmid were treated for 24 h with dexamethasone (100 nM) or water in the presence of RU-486 (10 μM) or ethanol, and dual-luciferase assays were performed. Three independent experiments were performed in triplicate. Statistically significant differences (control versus DEX treatment within each corresponding group); $^{*}P < 0.05$ and $^{***}P < 0.001$. Statistically significant differences (DEX treatment versus DEX+RU-486 treatment within each corresponding group); $^{+}P < 0.05$ and $^{+++}P < 0.001$.

Promoter regions necessary for basal transcription of the *rCES2* gene and for response to dexamethasone were examined on the basis of data from luciferase assays. The results of luciferase assays showed that basal transcriptional activity was almost lost by truncation of the sequence of −73/−7, indicating that the region of −73/−7 is essential for binding of general transcription factors (Figure 3.5A). In the region of −2957/+51, dexamethasone treatment resulted in activation of the *rCES2* promoter to a level approximately 2- to 3-fold higher than that of the controls (Figure 3.5A and C), although the effect of dexamethasone on the promoter activity was smaller than that on rCES2 mRNA expression. RU-486 treatment was highly effective in repressing the promoter activation by dexamethasone in the region of −2957/+51 (Figure 3.5C). Taken together with the results of real-time PCR, these results strongly suggest that GR-mediated transcriptional activation participates in the dexamethasone-mediated increase in rCES2 mRNA in rat hepatocytes. The results of luciferase assays showed that responsivity to dexamethasone was altered in the regions of −73/−7 and −991/−663 (Figure 3.5B). The response in the region of −73/−7 appeared to be inhibited by RU-486, although the response to dexamethasone was not confirmed by statistical analysis in the experiment using both dexamethasone and RU-486 (Figure 3.5C). Therefore, nucleotide sequences necessary for dexamethasone-mediated activation of the *rCES2* promoter may be located in the regions of −73/−7 and −991/−663.

It appears that dexamethasone-mediated induction of rCES2 mRNA requires ongoing protein synthesis. When hepatocytes were exposed to 100 nM dexamethasone in the presence of 1 μM cycloheximide, an inhibitor of protein synthesis, the expression level of rCES2 mRNA was repressed by approximately 50% of that in cells exposed to 100 nM dexamethasone in the absence of cycloheximide, although the difference was not supported by statistical analysis (Figure 3.3). In contrast, the expression of TAT mRNA was markedly increased by cycloheximide in combination with dexamethasone, in accordance with results of previous studies using cortisol or hydrocortisone acetate (Hofer and Sekeris, 1978; Chesnokov et al., 1990). Although the augmentation of TAT mRNA is not attributed to the inhibition of protein synthesis *per se* (Ernest, 1982), it is conceivable that protein synthesis in rat hepatocytes was somewhat inhibited by 1 μM cycloheximide in the present study, considering that 1 μM cycloheximide treatment for 2 h decreased *de novo* protein synthesis by approximately 50% in primary rat hepatocytes (Sidhu and Omiecinski, 1998). At 10 μg/mL, cycloheximide completely inhibited dexamethasone-mediated induction of rCES2 mRNA (Figure 3.4). Cycloheximide at this concentration is known to inhibit *de novo* protein synthesis by more than 90% in human hepatocytes (Gerbal-Chaloin et al., 2002). Therefore these findings suggest that the striking induction of rCES2 mRNA by dexamethasone may require the presence of a protein(s) produced by *de novo* synthesis. A protein(s) necessary for dexamethasone-mediated induction of rCES2 mRNA may be induced in response to dexamethasone if the protein(s) is not short-lived.

In the present study, we demonstrated that dexamethasone enhances transcription of the *rCES2* gene and that GR contributes significantly to dexamethasone-mediated induction of rCES2 mRNA. We also found that another as-yet-unidentified factor(s) may play an essential role in the induction. Thus, we propose that therapeutic compounds that can activate GR induce rCES2.

Summary and general discussion

In this thesis, the molecular mechanisms of the transcriptional regulation of *CES1A1* and *rCES2* genes were described in terms of GR-mediated regulation or epigenetic regulation. Epigenetic modifications involved in the expression of a *CES* gene are shown for the first time in chapter 1, and dexamethasone-mediated transcriptional activation of two *CES* genes is described in chapters 2 and 3.

Tissue-specific expression of the *CES1A1* gene has been known for a long time. In brief, the amount of CES1A1 is very large in the liver and lung and is very small in the kidney and small intestine. The human liver plays important roles for metabolism of chemical agents including drugs, and therefore the fact that CES1A1, one of the most effective drug-metabolizing enzymes, is highly expressed in the liver seems to make sense. However, the biological significance and molecular mechanisms by which CES1A1 is distributed in this manner have been an open question. Our previous studies on transcription factors that can enhance transcription of the *CES1A1* gene cannot fully explain the tissue-specific expression of the *CES1A1* gene. In chapter 1, it is shown that *CES1A1* gene expression may be repressed by DNA methylation in the human kidney. This study sheds light on the mechanisms of the tissue-specific expression of CES1A1.

In chapter 2, discussion of the study of epigenetics in chapter 1 is extended to the study on prenatal gene regulation. It was stated in chapter 2 that CES1A1 mRNA was expressed at low levels and the region around the TSS of the *CES1A1* gene was methylated at high levels in HFL cells compared with levels in the adult liver, and it was also stated that the number of hypomethylated DNA molecules was greater after incubation for 18 days than after incubation for 1 day. These findings imply that DNA methylation levels in the *CES1A1* gene decrease with aging. If such decreases in DNA methylation levels in the *CES1A1* gene play important roles for metabolism related to human physiology, alteration in DNA methylation levels of the *CES1A1* gene in the fetus by chemical compounds during pregnancy might predispose the newborn or progeny to disease.

Since dexamethasone treatment increased CES1A1 mRNA via GR and tended to decrease DNA methylation levels in the region around the TSS of the *CES1A1* gene in HFL cells, we speculated that *CES1A1* gene expression can be induced, in part, by dexamethasone-mediated DNA demethylation of the *CES1A1* gene through GR in HFL cells. Dexamethasone can cause developmental toxicities such as cleft palate in fetuses of various mammals including mice (Pinsky and Digeorge, 1965; Ballard et al., 1977) and rats (Hansen et al., 1999). Much attention should be paid to both metabolic disorders attributed to induction of CESs and their biological consequences in the human fetus.

It is extremely important to answer the question of whether a combination of selected chemical compounds including pharmaceutical agents will lead to drug-drug interactions in clinical and non-clinical trials or clinical practice. However, this is not an easy question. Drugs such as dexamethasone are known to induce or repress a variety of drug-metabolizing enzymes in mammals. Therefore drug-drug interactions attributed to such drugs are more complicated. With respect to human and rat CESs, treatment of postnatal hepatocytes with dexamethasone caused a slight increase in human CES1 and CES2 (Zhu et al., 2000). In the rat liver, treatment with dexamethasone decreased the amounts of several kinds of CES1 isozymes (Zhu et al., 2000; Shi et al., 2008) but increased the amount of rCES2 (Furihata et al., 2005). Furthermore, dexamethasone is thought to moderately inhibit rat CES1 activity in the liver, according to inhibitory profiles reported by Takahashi et al. (2009). In this manner, dexamethasone has effects on the transcriptional activity of *CES* genes and the enzymatic activity of CESs. In such circumstances, analysis of the mode of action of dexamethasone would offer a clue to predict drug-drug interactions. It is shown in chapters 2 and 3 that dexamethasone-mediated increases in CES1A1 and rCES2 mRNA in the human fetal liver and rat liver, respectively, were mediated by GR. Our results may help to predict drug-drug interactions related to dexamethasone in drug development and medical practice.

We hope that the results of the present study on transcriptional regulation of *CES* genes will contribute to a better understanding of the expression of *CES* genes and to more precise assessment of therapeutic and toxic actions of drugs in the future.

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Lists of publications

Major parts of this doctoral thesis have been published in the following articles.

[1] Takeshi Hori, Liangjing Jin, Ayako Fujii, Tomomi Furihata, Yuko Nagahara, Kan Chiba, and Masakiyo Hosokawa. Dexamethasone-mediated transcriptional regulation of rat carboxylesterase 2 gene. *Xenobiotica*. 2012 Jul;42(7):614-23.

[2] Takeshi Hori and Masakiyo Hosokawa. DNA methylation and its involvement in carboxylesterase 1A1 (CES1A1) gene expression. *Xenobiotica*. 2010 Feb;40(2):119-28.

Reviewers

This doctoral thesis was reviewed by the professors listed below.

Dr. Masakiyo Hosokawa, Professor of Chiba Institute of Science, Faculty of Pharmaceutical Sciences. Chief examiner.

Dr. Koozi Matuoka, Professor of Chiba Institute of Science, Faculty of Pharmaceutical Sciences. Second reader.

Dr. Yasuhiro Masubuchi, Professor of Chiba Institute of Science, Faculty of Pharmaceutical Sciences. Second reader.

Dr. Masahiro Mori, Professor of Chiba Institute of Science, Faculty of Pharmaceutical Sciences. Second reader.

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