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A CAR-responsive enhancer element locating approximately 31 kb upstream in the 5′-flanking region of rat cytochrome P450 (CYP) 3A1 gene

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ABSTRACT

Constitutive androstane receptor (CAR) is one of the principal regulators of hepatic cytochrome P450s (CYPs) 3A (CYP3A). cDNA-mediated expression of a mature rat CAR (*rCAR*) into rat hepatoma cells induced *CYP3A1* and *CYP2B* mRNAs. Aberrant *rCAR* failed in these inductions. Three important human *CYP3A4* regulatory elements (REs), proximal ER6 (proER6), xenobiotic responsive enhancer module (XREM) and constitutive liver enhancer module (CLEM), support constitutive and inducible expression of CYP3As mediated by CAR and pregnane X receptor (PXR). NHR-scan software predicted proER6, XREM and CLEM at −255 b, −8 kb and −11.5 kb, respectively of *CYP3A4*, but neither XREM nor CLEM was predicted in rat *CYP3A*. A luciferase reporter construct carrying a 5′-flanking sequence of *CYP3A1* (−31,739 to −31,585 from its transcription initiation site) revealed important for the *rCAR*-dependent transactivation of *CYP3A1*. This region includes two putative binding motifs of nuclear receptors (DR4 and DR2), a putative hepatocyte nuclear factor-1 binding motif (HNF1), nuclear factor-kappa B binding motif (NFκB), activator protein 1 binding motif (AP-1), and ecotropic viral integration site 1 binding motif (Evi1). We hereby conclude DR4 and/or DR2 motifs being primarily responsible and HNF1 being synergistically functioning elements for the *rCAR*-mediated transcription of *CYP3A1*.

1. Introduction

Constitutive androstane receptor (CAR) is a nuclear receptor (NR) and transcription factor that is activated by various xenobiotics [1] and steroids and thereby promotes drug metabolism in the liver. CAR is normally retained in the cytoplasm and translocates into the nucleus after being activated [2]. Once CAR translocates into the nucleus, it binds to specific DNA sites, called responsive elements (REs), with its heterodimeric partner, the retinoid X-receptor (RXR), and thus transactivates the transcription of various hepatic genes that encode cytochrome P450s and conjugation enzymes [1]. CAR, pregnane X receptor (PXR), and other NRs have overlapping roles in the drug-induced expression of these genes [3,4]. CAR is also found to play an essential role in nongenotoxic carcinogenesis. For example, drug activation of CAR by therapeutic drugs such as phenobarbital (PB) results in the promotion of hepato-cellular carcinoma (HCC) development in rodents [5], while no such promotion occurs in the absence of CAR [6]. Thus, it has been suggested that CAR regulates a cellular signal leading to cell growth and carcinogenesis.

CYP3A4 is a major drug-metabolizing enzyme in humans. It is highly expressed in the liver and small intestine, and it contributes to the disposition of about half of the clinically used pharmaceutical drugs [7]. Xenobiotic-responsive nuclear receptors PXR and CAR play central roles in the induction of CYP3A4. Nuclear receptors regulate gene expression by binding specific DNA sequences consisting of AG[G/T]TCA or AGAACA half-site motifs in a mutual direct (DR), everted (ER), or inverted orientation (IR), separated by a gap of 0–8 bp [8–10]. Human *CYP3A4* expression is chiefly mediated by PXR, and, to a smaller degree, CAR [7], which binds to and transactivates appropriate REs in human *CYP3A4* upstream sequences [8,10]. In humans, the three most important and well characterized regulatory elements are proER6, and XREM and CLEM modules [11].

Studies have revealed that cross-talk between PXR and CAR results in reciprocal activation of the human *CYP3A4* gene. Human CAR binds to proximal and distal response elements and can transcriptionally regulate *CYP3A4* gene expression [8]. Hepatocyte nuclear factor-4α (HNF-4α) recognizes CLEM and XREM, and it increases the activity of XREM, but decreases the activity of CLEM [11]. Sterol-sensitive liver X receptor α (LXRα) positively regulates

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the basal expression of *CYP3A4* but suppresses the xenobiotic/PXR-dependent *CYP3A4* expression in human hepatocyte [12].

Human PXR and human CAR can also bind to and activate the NR1 site (DR4) in the *CYP2B6* PBREM [13] and the DR3 and ER6 sites in the *CYP3A4* XREM [8]. Additionally, CAR-mediated activation of *CYP2B6* interacts synergistically with transcription factor early growth response 1 (EGR1) [13,14], HNF4α and CCAAT/enhancer binding protein (CEBP) [15]. Thus, the mechanisms of CAR activation have been well characterized in human CYPs.

XREM and CLEM are absent from most murine *Cyp3a* genes except for *Cyp3a25*, *Cyp3a57* and *Cyp3a59* [16]. But in mice, expression of *Cyp3a11*, which lacks XREM in its promoter, is induced by PB and 1,4-bis(2-(3,5-dichloropyridyloxy))benzene (TCPOBOP) only in the presence of CAR [17]. Activated sterol regulatory element-binding protein 2 (SREBP-2) interacts with proliferator-activated receptor α coactivator-1α (PGC-1α), resulting in reduced PGC-1α recruitment to HNF-4α on the *Cyp3a11* promoter and the subsequent down-regulation of *Cyp3a11* expression [18]. In mice, typical REs have not been identified yet, but the expression of *Cyp3a* is controlled by CAR as it is in humans [4]. Furthermore, although the human *CYP3A5* promoter lacks both XREM and CLEM, Qiu et al. showed that an approximately −4.4 kb DR4 was a second cis-acting element regulating *CYP3A5*. The *CYP3A5* DR4 is responsive to CAR, but not to PXR [16]. These findings prompted us to identify functional CAR-dependent REs in the rodent CYP3a. In the present study, *rCAR* cDNA-mediated expression in rat hepatoma FAA-HTC1 cells resulted in an extremely large degree of hepatic *rCYP3A1* induction. *rCYP3a2*, *rCYP2B1*, and *r*CYP2B2 were also clearly induced. As *rCYP3a* and *rCYP2B* forms are considered to be target genes among those in a non-genotoxic PB response in rat livers, it is of great significance to elucidate functional CAR-responsive elements in the flanking region of various rCAR target genes not only for CYPs but also for the investigation of non-genotoxic carcinogenicity in rodents. Thus, in the present study, we assessed the distribution of REs within the upstream sequence of the rat *CYP3a* family. We have investigated the possible involvement of rCAR in rat hepatic *CYP3A* expression and we have elucidated promoter/enhancer regions for rCAR-dependent trans-criptional activation of rat *CYP3A1*.

2. Materials and methods

2.1. Materials

Zaragozic acid was purchased from Sigma-aldorich (St. Louis, MO). Dexamethasone and phenobarbital were purchased from WAKO (Tokyo, Japan).

2.2. Expression plasmids and reporter gene constructs

Expression plasmids for *rCAR* (pcDNA-rCAR) and the *rCAR* splicing variant SV5 were constructed as follows. The rat *CAR* and SV5 liver cDNA fragments were amplified with Phusion DNA polymerase with F primers 5′-CACCATGgaattcATGACAGCTACTCTAAC-3′, 5′-CACCATGctcgagATGACAGCTACTCTAAC-3′ and an R primer 5′-ggaattcccgggGGAGAAGAGAGCCATG-3′, and were subcloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). To construct expression plasmids, the *rCAR*-exon 6 fragment was artificially amplified as a truncated form lacking sequences from exon 6 to the C-terminus using PCR primers, 5′-CACCATGgaattcATGACAGCTACTCTAAC-3′ and 5′-ggaattcccgggATGGACCGCATCTTCCAT-3′ (the end of exon 6), and *rCAR*-SV5 was also amplified using a sequence of the splicing variant SV5 using PCR primers, 5′-CACCATGgaattcATGACAGCTACTCTAAC-3′, and 5′-ggaattcccgggGGAGAGAAGAGAGCCATG-3′. The resultant amplified DNA fragments were introduced into a pcDNA3.1/V5-His-TOPO vector (pcDNA-exon 6 and pcDNA-SV5, respectively). All these *rCAR* expression constructs were designed to express rCAR proteins with an attached V5 recognition region, $(His)_6$ and a termination codon (TGA) at their common C-termini.

For various reporter constructs, DNA fragments of *rCYP3A1* promoters were isolated from genomic PCR-amplified or restriction enzyme digested BAC RNB1-193K14 clone DNA (RIKEN BRC, Tsukuba, Japan), and were cloned into tk-pGL3 (pGL3-Basic (Promega, Madison, WI) having thymidine kinase promoter and firefly luciferase) plasmid vector.

Mutant *rCYP3A1* promoter-luciferase constructs were prepared using mutated PCR products which were created by a two-step PCR. Some mutated constructs were prepared using QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA) with mutated PCR primers. Mutated PCR products were prepared using primers shown in Supplementary Table 1.

All constructs were confirmed by sequencing.

2.3. Reporter assays

A rat hepatoma FAA-HTC1 cell line, which was obtained from the American Type Culture Collection (ATCC), was cultured in William's medium E (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone, South Logan, UT), glutamine (Gibco, Grand Island, NY) and antibiotic-antimycotic (Gibco). Cells were seeded in 96-well plates (BD Biosciences) at 1.5×10^4 cells/well, 16 h before transfection. Reporter constructs (50 ng/well) and *rCAR* expression plasmids (50 ng/well) were cotransfected using X-treme Gene HP (Roche, Mannheim, Germany). The control vectors, pGL4.74 (HSV-tk-*Renilla* luciferase) (Promega) (20 ng/well) or pRL-CMV (CMV-*Renilla* luciferase) (Promega) (4 ng/well) were also cotransfected to normalize transfection efficiency. After 24 h of transfection, cell lysates were prepared with Passive Lysis Buffer (Promega) for luciferase assays using Dual-luciferase Reporter Assay System (Promega). The activities of firefly luciferase were normalized to those of *Renilla* luciferase.

2.4. Measurement of mRNA levels in rat liver and rat hepatocytes

Total RNA was prepared from five male Fisher 344 rat livers (38 days old, purchased from Charles River Co. Ltd. (Tokyo, Japan)). Other sources included cultured rat hepatocyte FAA-HTC1 that were transfected with rCAR, SV5, exon 6 or control vector using X-treme Gene HP. For qRT-PCR analysis, cDNA was prepared from 2 μg of total RNA by reverse transcription using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's instructions. qRT-PCR with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was performed using ABI PRISM 7500 Real-time PCR System (Applied Biosystems) following the manufacturer's protocol supplied with the SYBR Green PCR Master Mix.

The mRNA levels of nuclear receptors (*CAR*, *PXR*, *RXRA* and *FXR*) and *rCYPs* (*CYP3A1*, *CYP3A2*, *CYP3A73*, *CYP2B1* and *CYP2B2*) were determined, using β-actin as an internal control. The mRNA levels were determined as described previously [19] using primers shown in Supplementary Table 2.

2.5. Western blotting analysis

Cytoplasmic and nuclear extracts from FAA-HTC1 transfectants were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL), and protein concentrations were determined using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Each extract (10 μg) was resolved on SDS-polyacrylamide gels and electrophoretically transferred onto