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Constitutive androstane/active receptor is a target of retinoic acid receptor in humans

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ABSTRACT

Nuclear receptor constitutive androstane/active receptor (CAR) is well known as a transcription factor regulating many genes that encode drug-metabolizing enzymes and factors modulating hepatic gluconeogenesis. However, there have been few studies on regulation of the *CAR* gene itself. In this study, we examined the involvement of retinoic acid receptor α (RARα) in transcriptional regulation of the *CAR* gene in the liver. The expression levels of CAR mRNA in human primary hepatocytes and HepG2 cells were increased by all-trans retinoic acid. Activities of the human *CAR* promoter containing a region (termed cRARE) located at +1453/+1469 within intron 1 were increased by co-expression of RARα in HepG2 cells. In addition, introduction of mutation into cRARE abolished transcriptional activation of the promoter by RARα. The results of gel mobility shift assay and chromatin immunoprecipitation assay showed that RARα was bound to cRARE. These results suggest that RARα transactivated the human *CAR* gene by binding to cRARE located at +1453/+1469 within intron 1 of the gene. In contrast, the rat *CAR* gene was not activated by exposure to all-trans retinoic acid, probably due to the lack of a region corresponding to cRARE in the human *CAR* gene. Although the physiological significance of RARα-dependent up-regulation of CAR in the human liver remains to be clarified, retinoid metabolism may be regulated by the up-regulation of CAR.

1. Introduction

CAR (constitutive androstane/active receptor, NR1I3), a member of the nuclear receptor superfamily, regulates transcription of many genes encoding cytochrome P450s (CYPs), UDP-glucuronosyltransferases, sulfotransferases and drug transporters [1,2]. CAR activity is modulated by many synthetic drugs and natural products, which can be classified as agonists, indirect activators or inverse agonists [3]. Most of CAR is normally retained in the cytoplasm by forming a complex with Hsp90 (heat shock protein 90) and the co-chaperone CCRP (cytoplasmic CAR retention protein) [4,5]. In response to activators such as phenobarbital, CAR translocates into the nucleus, where it forms a heterodimer with RXRα (retinoid X receptor α) and binds to response elements in the target gene promoters, resulting in transcriptional activation of genes such as mouse, rat and human *CYP2B* [2,6].

AtRA (all-trans retinoic acid) modulates the transcription of a set of genes associated with cellular apoptosis, growth and differentiation as a ligand of RARs. Although atRA is currently used as a chemotherapeutic agent against acute promyelocytic leukemia, breast cancer, Kaposi's sarcoma and glioma, excess intake of vitamin A may cause liver fibrosis or other pathological features such as retinoic acid syndrome [7,8]. A study has suggested that catabolic metabolism of atRA by CYP26 functions as a regulator preventing overshoot of atRA concentration in the liver [9]. CYP26 is a unique atRA-inducible enzyme that catalyzes oxidative metabolism of atRA to various polar inactive metabolites [10]. In addition to CYP26, various CYP isoforms (CYP3A4/5/7, CYP2C8/9, CYP1A1 and CYP4A11) have been reported to be responsible for the oxidative metabolism of atRA [11]. Among them, CYP2C8 and CYP3A4 are the major enzymes contributing to atRA metabolism in the human liver [12]. It has also been reported that expression of CYP2C8 and CYP3A4 is regulated by CAR at the transcriptional level [13,14].

While CAR is known to regulate many genes at the transcriptional level, there have been few studies on regulation of the *CAR* gene itself. Four receptors, glucocorticoid receptor, hepatocyte nuclear factor 4α, arylhydrocarbon receptor and proliferator-activated receptor α have been suggested to be involved in transcriptional regulation of the *CAR* gene [15–18].

Abbreviations: atRA, all-trans retinoic acid; CAR, constitutive androstane/active receptor; ChIP, chromatin immunoprecipitation; cRARE, CAR RARα response element; CYP, cytochrome P450; FoxO1, forkhead transcription factor 1; IRS, insulin response sequence; PEPCK, phosphoenolpyruvate carboxykinase; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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However, there is no report on whether RARα affects regulation of the *CAR* gene.

In this study, we examined the effects of atRA on the expression levels of CAR mRNAs in HepG2 cells and human primary hepatocytes. We report herein that CAR mRNA is induced by atRA and that this effect is caused by the activation of RARα by atRA. This activation of RARα enhances transcription of the human *CAR* gene through the cRARE (CAR RARα response element) located within intron 1 of the human *CAR* gene. Interestingly, the rat *CAR* promoter lacks cRARE and no induction of CAR mRNA is observed in primary rat hepatocytes in the presence of atRA.

2. Materials and methods

2.1. Expression vector for human RARα

The coding region of human RARα (GenBank accession no. NM_0029575) was cloned into the pCR-BluntII-TOPO vector (Invitrogen, Carlsbad, CA) after amplification by PCR using reverse-transcribed human liver cDNA as a template and the specific primers 5′-CCGCTTGGCATGGCCAGCAACAGCA-3′ and 5′-TGTCCATGTGGCGTGGGCGGTCACG-3′. After BamHI and NotI digestion, the coding region of human RARα was subcloned into the pTarget vector (Promega, Madison, WI) to generate an expression vector for human RARα.

2.2. Human CAR promoter cloning and reporter constructs

The sequence of the promoter region of the human *CAR* gene was provided by NCBI (NC_000001). Fragments containing an upstream region (−2312/+58, P1) and a downstream region (−317/+1579, P2) of the transcription start site were cloned into the pCR-BluntII-TOPO vector after amplification by PCR using human genomic DNA (Promega) as a template and the following specific primers: 5′-CTCTTTCAAAGTTTCCTGATGGACTC-3′ and 5′-CCAAACTCCCACGCTGTTGCTGGTTTTCC-3′ for P1 and 5′-CTCTCTCTCTCTTCCCAGCTTGTTCAG-3′ and 5′-GGTGGCTGTCACA-GACTCCTGAATGT-3′ for P2. After KpnI and XhoI digestion, the two human *CAR* promoter regions (P1 and P2) were subcloned into the pGL4.17 vector (Promega) to generate reporter constructs for the human *CAR* promoter. The fragments of three truncated reporter constructs of P2 human *CAR* promoter (+1009/+1579, +1386/+1579 and +1501/+1579) were digested by KpnI and XhoI after amplification by PCR using the reporter construct for P2 human *CAR* promoter as a template, the following specific forward primers: 5′-AAGGTACCATCCTGGTGTGGTGGT-3′ for +1009/+1579, 5′-GTGGTACCGCAAGAACTTGTGTCA-3′ for +1386/+1579, 5′-ACGGTACCGAAGGGACAGAAAAGG-3′ for +1501/+1579 and the common reverse primer 5′-CCATGGTGGCTTTACCAACAGT-3′. Then the digested fragments were subcloned into the pGL4.17 vector to generate three truncated reporter constructs of P2 human *CAR* promoter.

2.3. Site-directed mutagenesis

According to a previous report [19], site-directed mutagenesis of a DR5 element (+1453/+1469) in the promoter region of the human *CAR* gene was carried out from P2 human *CAR* promoter as a template using the primers 5′-TAACGCCAGGGAAGTAGTCATCGG-CATGGTTTGGT-3′ and 5′-TCCCTGGCGTTATCTTTTGTGGTTTTC-3′. Mutation sites are underlined.

2.4. Rat CAR promoter cloning and reporter construct

The sequence of the promoter region of the rat *CAR* gene was provided by NCBI (NC_005112). A fragment containing an upstream region of the transcription start site (−3000/+353) was cloned into the pCR-BluntII-TOPO vector after amplification by PCR using rat genomic DNA as a template and the primers 5′-AGAGGACCATTCCCAGTATGTACATGAT-3′ and 5′-ATGGTCTCCT-GAATGTGGGAGGAGTTA-3′. After SacI and XhoI digestion, the rat *CAR* promoter region (−3000/+353) was subcloned into the pGL4.17 vector to generate a reporter construct for the rat *CAR* promoter.

2.5. Human liver samples

Human liver samples from a 34-year-old African female (Donor 1) and a 35-year-old Hispanic female (Donor 2) were supplied by the National Disease Research Interchange (Philadelphia, PA) through the HAB Research Organization (Tokyo, Japan). The use of human liver samples in this study was approved by the Ethics Committee of Chiba University (Chiba, Japan).

2.6. HepG2 cells and primary hepatocytes

HepG2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Human primary hepatocytes from Donor 2 were prepared in HAB Research Organization. Rat primary hepatocytes were prepared from adult male Sprague–Dawley rats (5 weeks old, weighing 120–140 g; Japan SLC Inc., Shizuoka, Japan). The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Chiba University.

2.7. RT-PCR

HepG2 cells (6×10^5) were plated onto 6-well plates and maintained for 24 h. Rat primary hepatocytes (8×10^5) were plated onto collagen-coated 6-well plates and maintained in William's E medium supplemented with 10% fetal bovine serum, 10 nM dexamethasone, 100 × ITS-X (Invitrogen), 4 mM glutamine and 50 μg/ml gentamycin for 4 h. After cell attachment, the medium was changed to serum-free medium and maintained for 16 h. At 24 h after cell seeding, both lines of cells were treated with 1 or 10 μM of atRA (Wako, Osaka, Japan) or a solvent (0.1% DMSO) for 48 h. Total RNA was isolated using an RNeasy plus mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA (500 ng) was subjected to cDNA synthesis using an Exscript RT reagent kit (Takara, Shiga, Japan) according to the manufacturer's protocol. To determine mRNA levels, cDNAs were subjected to semi-quantitative RT-PCR using the following primers: 5′-TGGTACTG-CAAGTCATCAAGT-3′ and 5′-CTTCAATTGTGTAGCGAAGAG-3′ for human CAR, 5′-ATCTTTGCAACTGAGTCAGCAG-3′ and 5′-GTATTGA-TATCTGCAAAGTGTGT-3′ for rat CAR, 5′-CGAACTCCTCTTTGGAG-GACA-3′ and 5′-CCATGTCCAACTTGTTGTCTT-3′ for human CYP26A1, 5′-CTAGTGGAGGCTTTCGAGGA-3′ and 5′-ATGAGTGCTCAATCAA-GAGC-3′ for rat CYP26A1, and 5′-TGCACCACCAACTGCTTA-3′ and 5′-GGATGCAGGGATGATGTTC-3′ for GAPDH. The PCR cycling parameters were as follows: 94 °C for 2 min and indicated cycles of 94 °C for 25 s, 55 °C for 25 s and 72 °C for 25 s (34 cycles for human CAR and human CYP26A1, 30 cycles for rat CAR, 33 cycles for rat CYP26A1 and 24 cycles for GAPDH). Intensities (densitometric units) of bands were determined using ImageJ software ver.1.43 (http://rsb.info.nih.gov/ij).

2.8. Quantitative real-time PCR analyses

Human primary hepatocytes (8×10^5) were plated onto collagen-coated 12-well plates and maintained as described for rat primary hepatocytes. Cells were treated with 10 μM atRA or a

solvent (0.1% DMSO) for 48 h. To measure mRNA levels, cDNAs prepared from total RNA (800 ng) were subjected to quantitative real-time PCR using TaqMan Gene Expression Assays designed for human CAR (Hs_00901571_m1) by an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The mRNA levels were normalized against human β-glucronidase (hGUS) determined by predeveloped TaqMan Assay Reagents for hGUS (Applied Biosystems).

2.9. Transfection and luciferase assay

HepG2 cells (6×10^4) were plated onto 48-well plates and transfected with 50 ng/well of reporter vectors, 10 ng/well of expression vectors and 5 ng/well of an internal control *Renilla* luciferase plasmid, pGL4.74 vector (Promega), by the reverse transfection method. At 24 h after transfection, cells were treated with 1 μM atRA or a solvent (0.1% DMSO) for 24 h and luciferase activities were measured as described previously [20].

2.10. Gel mobility shift assay

Gel mobility shift assays were performed by using double-strand DNA labeled with [γ-^{32}P]ATP (GE Healthcare, Piscataway, NJ) and 40 ng of *in vitro* transcribed/translated human RARα and RXRα proteins synthesized using T_NT T7 Quick Coupled Transcription/Translation System (Promega) following the manufacturer's protocol as previously described [19]. The protein-coding region of human RXRα (GenBank accession no. NM_000964) was cloned into the pCR-BluntII-TOPO vector after amplification by PCR using reverse-transcribed human liver cDNA as a template and the primers 5′-CGCAGACATGGACACCAAACATTTCCTG-3′ and 5′-CAGAACGGGTGGGCACAAAGGATG-3′. Both the protein-coding regions of human RARα and human RXRα were digested by EcoRI from the pCR-BluntII-TOPO vector and subcloned into the pT_NT vector (Promega). Oligonucleotide sequences containing the RARα responsive element located in the RARβ promoter (βRARE) and cRARE found in the human *CAR* promoter and its mutant (cRARE MT) used as probes or competitors were designed as follows: 5′-tagGGTTCAccgaaAGTTCActc-3′ (βRARE), 5′-cagGGATCAagtcaAGGGCAtgt-3′ (cRARE) and 5′-cagGGAAGTagtcaTCGGCAtgt-3′ (cRARE MT). Only the sequences of the sense strands are displayed above, and mutated nucleotides are underlined. The nucleotide half-sites of putative RARα/RXRα responsive element are shown in the large letters. Unlabeled competitive double-strand DNA was added to the binding reaction mixture at 10-, 50- and 250-fold excess of the probe amount before addition of the probe. For supershift assays, *in vitro* transcribed/translated proteins and double-strand DNA were incubated with either 1 μg of anti-human RARα antibody (PP-H1920-00, Perseus Proteomics, Tokyo, Japan) or control mouse IgG (sc-2025, Santa Cruz Biotechnology, Santa Cruz, CA).

2.11. Chromatin immunoprecipitation (ChIP) assays

Chromatin shearing was carried out using fresh human liver tissues from Donor 1 as described previously [19]. ChIP assays were performed using ChIP-IT Express (Active Motif, Carlsbad, CA) according to the manufacturer's protocol with 15 μg of sheared chromatin and 2 μg of anti-human RARα antibody or control mouse IgG. Human *CAR* promoter regions (+1381/+1521 and −4027/−3790) were amplified by PCR using the following specific primers: 5′-ACAGAGCAAGAACTTGTGTCA-3′ and 5′-TTGACCCTTTTCTGTCCCTTC-3′ for +1381/+1521 and 5′-TAACCCTGAAAGCCTGTTCACCATGGATGC-3′ and 5′-TGGTCCAGGGATGCCCTTCAC-3′ for −4027/−3790. PCR reactions were performed at 94 °C for 2 min followed by 40 cycles of 98 °C for 15 s, 55 °C for 25 s and 72 °C for 25 s.

3. Results

3.1. Effects of atRA on expression levels of CAR mRNA in HepG2 cells and primary human hepatocytes

In initial experiments, HepG2 cells were treated with 1 and 10 μM of atRA and the amounts of CAR mRNA were determined (Fig. 1a). RT-PCR analysis showed that treatment with both concentrations of atRA increased the mRNA level of human CAR by about 3-fold. We also measured the amount of CYP26A1 mRNA as a positive control, which showed about 30-fold increases. To determine whether this effect of atRA on CAR mRNA in HepG2 cells could also be found in the human liver, we studied the effect of atRA on CAR mRNA using human primary hepatocytes. The hepatocytes were treated with 10 μM of atRA and the amounts of CAR mRNA were determined (Fig. 1b). Quantitative real-time PCR showed that treatment with atRA caused a 3.2-fold increase in CAR mRNA in human primary hepatocytes. The results suggest that expression of the *CAR* gene is induced by atRA in the human liver.

3.2. Effects of RARα on activities of the human CAR promoter and its deletion constructs

The effect of atRA on the transcriptional regulation of genes is mainly mediated by activation of RARs [21]. Therefore, we studied

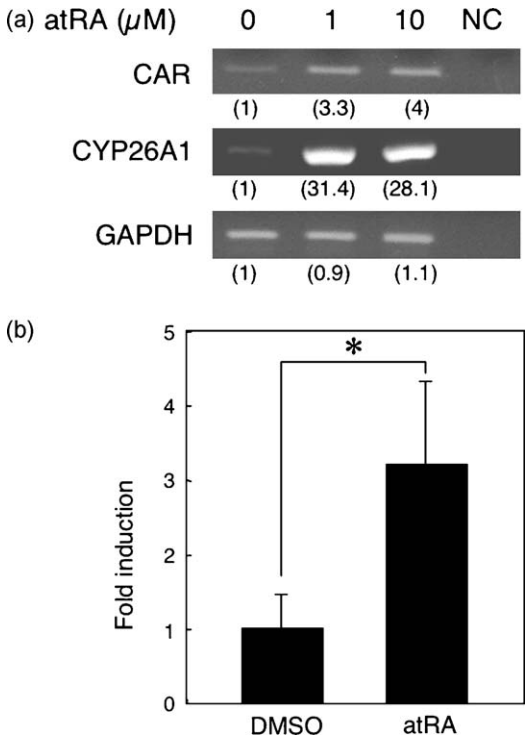

Fig. 1. Effects of atRA on the expression levels of CAR mRNA in HepG2 cells and human primary hepatocytes. (a) HepG2 cells were treated with 1 or 10 μM atRA or DMSO alone for 48 h. Total RNA isolated from HepG2 cells was reverse-transcribed and detected by semi-quantitative RT-PCR. The PCR products for human CAR, human CYP26A1 and GAPDH were amplified at 34, 34 and 24 cycles, respectively ($n = 2$–4). Parallel PCR reactions were performed without any DNA (NC). Values in parentheses are expressed as fold induction relative to DMSO-treated cells. (b) Human primary hepatocytes from Donor 2 were treated with 10 μM atRA or DMSO alone for 48 h. Total RNA isolated from primary human hepatocytes was reverse-transcribed and quantified by quantitative real-time PCR. The expression levels of CAR mRNA were normalized against the expression levels of hGUS mRNA. Data are expressed as fold induction of the normalized CAR mRNA measured in DMSO-treated primary human hepatocytes. Quantification of the expression levels of both human CAR and hGUS mRNA was performed in triplicate and each value is the mean ± S.D. from three independent quantifications. The p values were determined using Student's t test. $^*p < 0.05$ compared with DMSO-treated cells.

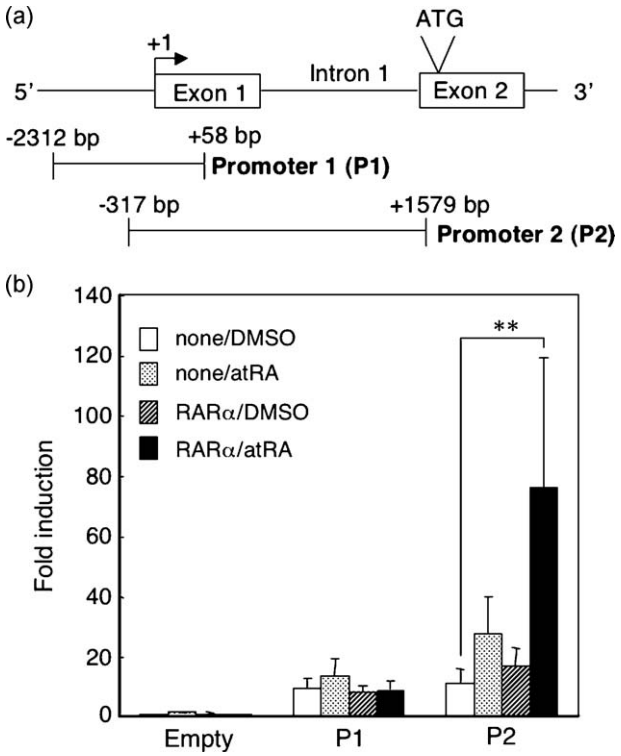

Fig. 2. Transcriptional activation of the human *CAR* promoter by RARα. (a) Positions of two human *CAR* promoters used in this study (P1 and P2). Nucleotides are numbered from the transcription start site as +1. (b) Transcriptional activation of the human *CAR* promoter by RARα. HepG2 cells were transfected with reporter constructs for the human *CAR* promoter (P1 or P2) or an empty vector (pGL4.17) with or without an expression vector for human RARα. Cells were treated with 1 μM atRA or DMSO alone for 24 h. Data are expressed as fold induction of the normalized luciferase activity measured in DMSO-treated cells transfected with an empty reporter construct (pGL4.17). Experiments were performed in triplicate and each value is the mean ± S.D. from three independent experiments. The *p* values for each experimental comparison were determined using Student's *t* test. $^{**}p < 0.01$ compared with DMSO-treated cells transfected with reporter constructs alone.

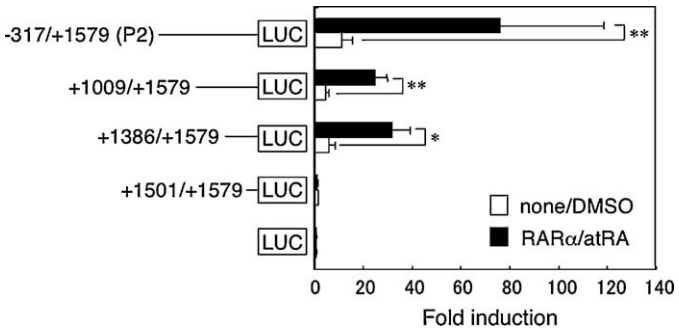

Fig. 3. Effect of deletion of the human *CAR* promoter in transcriptional activation by RARα. HepG2 cells were transfected with reporter constructs for truncated human *CAR* promoter (P2, +1009/+1579, +1386/+1579 and +1501/+1579) or an empty vector (pGL4.17) with or without an expression vector for human RARα. Cells were treated with DMSO alone or were transfected with an expression vector for human RARα in combination with 1 μM atRA treatment for 24 h. Data are expressed as fold induction of the normalized luciferase activity measured in DMSO-treated cells transfected with an empty reporter construct (pGL4.17). Experiments were performed in triplicate and each value is the mean ± S.D. from three independent experiments. The *p* values for each experimental comparison were determined using Student's *t* test. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with DMSO-treated cells transfected with reporter constructs alone.

the role of RARα in transcriptional regulation of the human *CAR* gene. To determine whether RARα activates the human *CAR* promoter, an expression plasmid containing RARα was cotransfected with a P1 human CAR luciferase promoter construct (from −2312 to +58 bp), a P2 human CAR luciferase promoter construct (from −317 to +1579) or an empty vector pGL4.17 into HepG2 cells (Fig. 2a). As shown in Fig. 2b, transfection of RARα in combination with atRA treatment activated P2 human CAR by 6.5-fold, whereas it did not activate P1 human CAR or the empty vector. Neither transfection of RARα nor treatment with atRA itself significantly activated any of the three constructs, although treatment with atRA activated P2 human CAR by 2.4-fold. Since HepG2 cells endogenously express RARα, it is thought that atRA activated endogenous RARα. These results suggested that the human *CAR* promoter was activated by RARα and RARα response element(s) that exist within the P2 promoter region of the human *CAR* gene.

To identify the RARα response region contributing to activation of the human *CAR* promoter by atRA, we examined deletions of a P2 *CAR* promoter luciferase promoter construct. As shown in Fig. 3, activation of human CAR by RARα in combination with atRA treatment was maintained upon deletion of P2 human CAR to a length of +1386 to +1579 bp (more than 5-fold). Further deletion to +1501 bp resulted in the loss of RARα-dependent activation by atRA. These results suggested that RARα mediated activation of the human *CAR* promoter by atRA through the +1386/+1501 region in the promoter.

3.3. Identification of RARα-binding sites that are required for full activation of the human CAR promoter by RARα

Using NUBIScan [22] findpatterns tool, we analyzed the +1386/+1501 region in the human *CAR* promoter sequence for potential RARα/RXRα-binding sites based on homology with established sites from the literature. We found a putative RARα/RXRα-binding site within the +1453/+1469 region in the human *CAR* promoter (cRARE) similar to the canonical RARα/RXRα-binding site located in the RARβ promoter (βRARE). To specifically examine the potential of cRARE in nuclear receptor-mediated regulation, we tested whether RARα could bind to cRARE by gel mobility shift assays with *in vitro* transcribed/translated RARα (Fig. 4a). βRARE sequence was used as a positive control, which also showed strong binding with RARα/RXRα (Fig. 4a, lane 3). This binding was completely supershifted by coincubation with anti-RARα antibody (Fig. 4a, lane 12). Similarly, cRARE also showed a strong binding with RARα/RXRα (Fig. 4a, lane 7). To determine the specificity of cRARE in binding with RARα/RXRα, cRARE was mutated. The mutated cRARE (cRARE MT) did not bind to RARα/RXRα (Fig. 4a, lane 9). In addition, the shifted band formed by cRARE and RARα/RXRα was completely supershifted by coincubation with anti-RARα antibody (Fig. 4a, lane 14). Furthermore, competition binding analysis of βRARE, cRARE and cRARE MT unlabeled oligonucleotides revealed that the shifted band formed by cRARE and RARα/RXRα was inhibited by molar excess of unlabeled βRARE and cRARE in a dose-dependent manner but not by cRARE MT (Fig. 4b). This result further supports the specificity of the binding of RARα/RXRα to cRARE. Taken together, these results suggested that RARα bound to cRARE as a heterodimer with RXRα.

Next, to clarify the potential role of cRARE in the regulation of human *CAR* promoter activity, we introduced the same mutation into the P2 human CAR luciferase promoter construct as shown in Fig. 4a. As shown in Fig. 5, transfection of RARα in combination with atRA treatment activated the P2 human CAR wild-type reporter by 5.2-folds. The mutated reporter had less response to atRA compared with the wild-type reporter and the difference was statistically significant. These results indicated that cRARE was required for activation of the human *CAR* promoter by RARα in the presence of atRA.

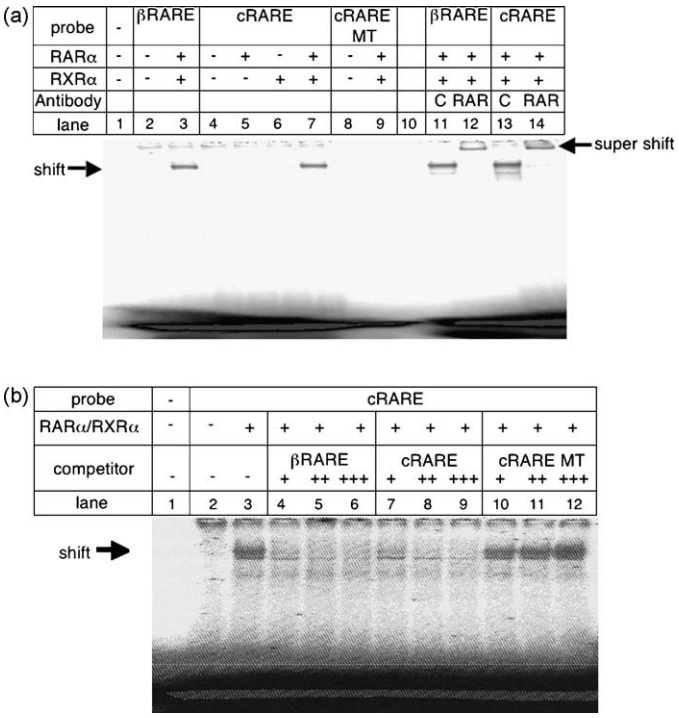

Fig. 4. Binding of RARα to its response element located in the human *CAR* promoter (cRARE) by gel mobility shift assays. (a) Binding and supershift assays of cRARE with RARα and RXRα. Radiolabeled double-stranded βRARE (lanes 2, 3, 11 and 12), cRARE WT (lanes 4–7, 13 and 14) and cRARE MT (lanes 8 and 9) were incubated with *in vitro* transcribed/translated human RARα and/or human RXRα. Control mouse IgG (C) or anti-human RARα antibody (RAR) was used for supershift assays. (b) Competition assay of cRARE with RARα and RXRα. Radiolabeled double-stranded cRARE was incubated with *in vitro* transcribed/translated human RARα and/or human RXRα. Unlabeled double-stranded βRARE (lanes 4–6), cRARE (lanes 7–9) and cRARE MT (lanes 10–12) were used as competitors. The amounts of competitors are indicated as follows: +, x10, ++, x50 and +++, x250.

3.4. Occupancy of RARα in the intron 1 region of the CAR gene in the human liver

The results of our experiments on the induction of CAR by atRA in human primary hepatocytes (Fig. 1b) and the binding of RARα to cRARE *in vitro* (Fig. 4) suggested that RARα could bind to the human *CAR* promoter and contribute to transcriptional activation of the *CAR* gene in the human liver. To confirm that RARα occupies cRARE within the human *CAR* promoter *in vivo*, we performed a ChIP assay using a human liver sample. As shown in Fig. 6, RARα occupancy was observed within the intron 1 region containing cRARE in the human *CAR* promoter and no signal was detected within the upstream region of the human *CAR* promoter. These results suggested that the human *CAR* gene was directly regulated by RARα in the human liver.

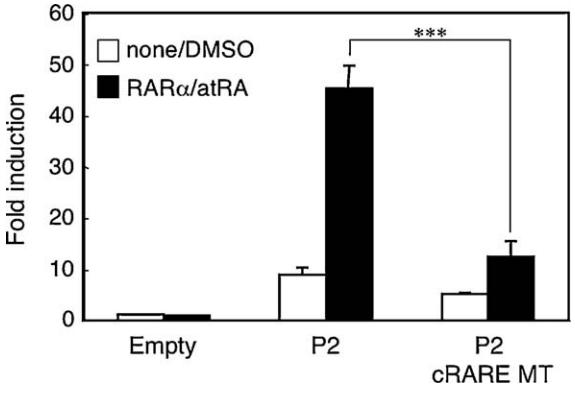

Fig. 5. Effect of mutation for the cRARE on the transcriptional activation by RARα. HepG2 cells were transfected with reporter constructs for wild-type or mutant P2 human *CAR* promoter (P2 or P2 cRARE MT) or an empty vector (pGL4.17) with or without an expression vector for human RARα. Cells transfected with an expression vector for human RARα were treated with 1 μM atRA or DMSO alone for 24 h. Data are expressed as fold induction of the normalized luciferase activity measured in DMSO-treated cells transfected with an empty reporter construct (pGL4.17). Experiments were performed in triplicate and each value is the mean ± S.D. from three independent experiments. The *p* values for each experimental comparison were determined using Student's *t* test. $^{***}p < 0.005$ compared with atRA-treated cells transfected with an expression vector for human RARα and reporter construct with or without mutation of cRARE.

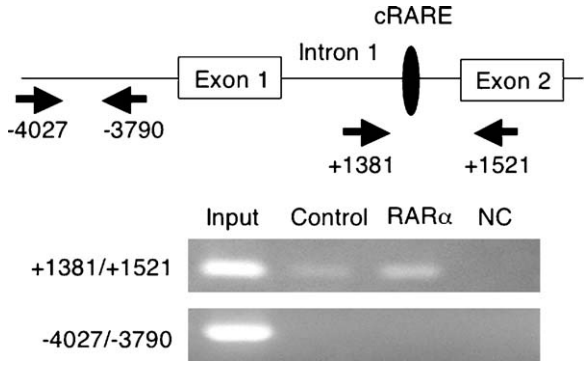

Fig. 6. Binding of RARα to cRARE in the human liver. The locations of priming sites of the specific primers used in the ChIP assay are indicated by arrows. Sheared genomic DNA (15 μg) form Donor 1 was incubated with 2 μg of anti-human RARα antibody (RARα) or control mouse IgG (control). Immunoprecipitated DNA fractions were amplified by PCR with specific primers for the cRARE-containing region of the human *CAR* promoter (+1381/+1521) and a randomly selected region of the human *CAR* promoter (−4027/−3790). Parallel PCR reactions were performed both with input DNA fraction (input) and without any DNA (NC).

3.5. Effect of atRA on the expression level of CAR in rat primary hepatocytes

We studied whether the inductive effect of atRA on the *CAR* gene found in the human liver is similar or different from that in the rodent liver. To determine whether atRA induces CAR in the rat liver, rat primary hepatocytes were treated with 1 and 10 μM of atRA and amounts of CAR mRNA were determined (Fig. 7). RT-PCR analysis showed that treatment with both concentrations of atRA did not cause any increase in rat CAR mRNA level. We also measured the amount of CYP26A1 mRNA as a positive control, which showed about 20-fold increases. This finding is in contrast to the results for human primary

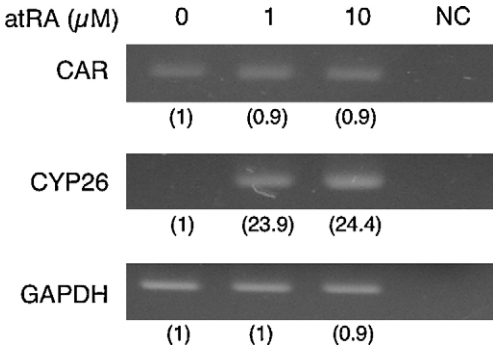

Fig. 7. Effect of atRA on the expression level of CAR mRNA in rat primary hepatocytes. Primary rat hepatocytes were treated with 1 or 10 μM atRA or DMSO alone for 48 h. Total RNA isolated from primary rat hepatocytes was reverse-transcribed and detected by semi-quantitative RT-PCR. The PCR products for rat CAR, rat CYP26A1 and GAPDH were amplified at 30, 33 and 24 cycles, respectively ($n = 2$). Parallel PCR reactions were performed without any DNA (NC). Values in parentheses are expressed as fold induction relative to DMSO-treated cells.

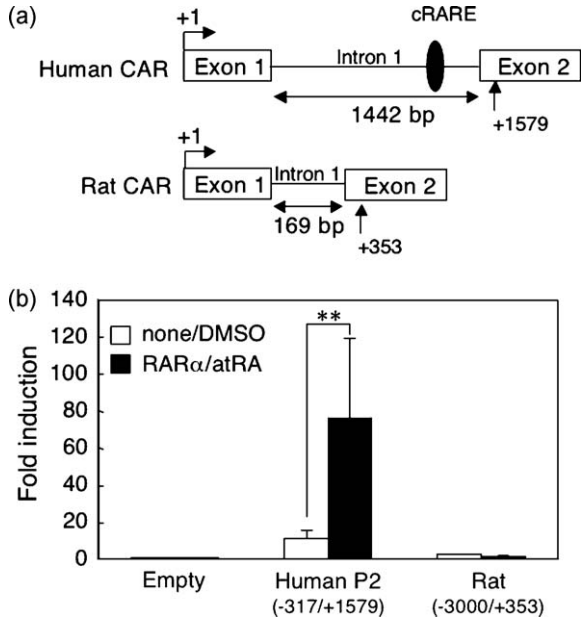

Fig. 8. Comparison of transcriptional activation of the *CAR* promoter by RARα between humans and rats. (a) Comparison of the length of intron 1 in the human and rat *CAR* promoters. The location of cRARE is indicated by a black circle. Nucleotides are numbered from the transcription start site predicted by NCBI as +1. (b) Comparison of transcriptional activation of the *CAR* promoter by RARα between humans and rats. HepG2 cells were transfected with reporter constructs for P2 human *CAR* promoter, rat *CAR* promoter or an empty vector (pGL4.17) with or without an expression vector for human RARα. Cells were treated with DMSO alone or were transfected with an expression vector for human RARα in combination with 1 μM atRA treatment for 24 h. Data are expressed as fold induction of the normalized luciferase activity measured in DMSO-treated cells transfected with an empty reporter construct (pGL4.17). Experiments were performed in triplicate and each value is the mean ± S.D. from three independent experiments. The *p* values for each experimental comparison were determined using Student's *t* test. $^{**}p < 0.01$ compared with DMSO-treated cells transfected with reporter constructs alone.

hepatocytes, which showed a significant increase in the level of human CAR mRNA with atRA treatment (Fig. 1b). These findings suggested that there was a species difference in the effect of atRA on transcriptional regulation of the *CAR* gene between humans and rats.

3.6. Comparison of intron 1 regions of the CAR gene in humans and rats and effect of RARα on activity of rat CAR promoters

Since it is possible that the different effects of atRA on the induction of CAR in humans and rats are due to the different nucleotide sequences of promoter regions in the *CAR* genes, we compared the promoter regions, especially intron 1 regions, which contain cRARE, in human and rat *CAR* genes. Fig. 8a shows that the intron 1 region in the rat *CAR* genes was much shorter than that in the human *CAR* gene (169 bp *vs.* 1442 bp). The intron 1 region in mouse *CAR* gene was similar to that in the rat *CAR* gene (149 bp). In addition, no corresponding sequence of cRARE existed in the intron 1 regions of these rodent *CAR* genes. These findings suggested that no effect of atRA on mRNA levels of CAR in rat primary hepatocytes was due to the lack of cRARE in the intron 1 region of the rat *CAR* gene. To clarify this possibility, expression plasmids containing RARα were cotransfected into HepG2 cells in combination with a rat CAR luciferase promoter construct (from −3000 to +353), a P2 human CAR luciferase promoter construct (from −317 to +1579) or the empty vector pGL4.17 (Fig. 8b). In contrast to the human P2 *CAR* promoter, the rat *CAR* promoter was not activated by cotransfection of RARα in the presence of atRA. These results suggest that the lack of effect by atRA on the expression level of CAR mRNA in the rat liver is due to the lack of cRARE in the intron 1 region of the rat *CAR* promoter.

4. Discussion

In the present study, we showed that atRA, a major ligand of RARα, significantly induced CAR mRNA expression in both human primary hepatocytes and HepG2 cells (Fig. 1). The cRARE located in intron 1 of the human *CAR* gene is thought to be important for this induction since: (1) RARα/RXRα could bind to cRARE *in vitro* (Fig. 4), (2) introduction of mutation into the element abolished the RARα-mediated activation of the promoter in HepG2 cells (Fig. 5), and (3) the occupancy of this element by RARα was detected in the human liver (Fig. 6). Therefore, the present study clearly showed that RARα could activate transcription of the *CAR* gene in the human liver through the direct binding of RARα to cRARE.

Results of the present study showed that the cRARE was located in the intron 1 region of the human *CAR* gene but not in the upstream of the exon 1. In addition, activated RARα could increase the activities of reporters deleted the *CAR* promoter before the exon 1 (Fig. 3). It would be appear that RARα-mediated transcription might not be dependent on the *CAR* promoter before the exon 1, although it remains unclear which position in the intron 1 was used as a transcription start site. Further study is needed to clarify whether a second promoter exists in the intron 1 of human *CAR* gene.

Since RARα is a ligand-dependent nuclear receptor [21], the concentration of atRA in the liver would be one of the important factors regulating *CAR* gene expression mediated by RARα. The concentration of atRA that caused up-regulation of the *CAR* gene in the present study was 1 or 10 μM (Fig. 1). On the other hand, it has been reported that the mean concentration of atRA in human livers is 53 nM [23]. Therefore, it was thought that up-regulation of the *CAR* gene through RARα occurs in the case of excess intake of vitamin A.

Excessive atRA coordinately enhances the induction of CYP26 by activation and up-regulation of RARα, resulting in prevention of overshoot of atRA concentration in the liver [9]. Not only CYP26 but also various CYP isoforms (CYP3A4/5/7, CYP2C8/9, CYP1A1 and CYP4A11) have been reported to be responsible for the oxidative metabolism of atRA [11]. Among them, CYP2C8 and CYP3A4 are the major enzymes contributing to atRA metabolism in the human liver [12]. Since these enzymes account for 50–60% of 4-hydroxylation of atRA, a major oxidative pathway of atRA in the adult liver, they are thought to function in a major detoxification process of atRA working in parallel with CYP26. Since CAR plays an important role in the transcriptional regulation of *CYP2C8* and *CYP3A4* genes [13,14], we are tempted to speculate that up-regulation of the *CAR* gene through RARα supplies adequate levels of CAR in order to be able to induce CYP2C8 and CYP3A4 when the liver is exposed to an excess amount of atRA, although further investigations are needed to clarify this possibility.

Although CAR regulates the transcription of many genes through direct binding to the responsible elements of their genes, it has also been reported that CAR directly binds insulin response transcription factor FoxO1 (forkhead transcription factor 1) and prevents binding of FoxO1 to the IRS (insulin response sequence) in the promoter region of the gluconeogenetic *PEPCK1* (phosphoenolpyruvate carboxykinase 1) gene, resulting in repression of the *PEPCK1* gene [24]. The repression of FoxO1 by CAR provides a molecular basis for understanding the long-standing question of why chronic treatment with phenobarbital reduces plasma glucose levels and improves insulin sensitivity in diabetic patients [25]. On the other hand, a few clinical studies have shown that retinoid therapy reduces glucose levels in response to a glucose load and improves insulin sensitivity [26,27]. This finding suggests that

atRA is also involved in down-regulation of *PEPCK1* gene expression. Since up-regulation of CAR by atRA in the human liver was indicated in the present study, it would be worth discussing the possible significance of crosstalk between CAR and RARα with focus on the regulation of gluconeogenesis. Considering atRA at physiological levels, it has been suggested that expression of the hepatic *PEPCK1* gene and gluconeogenesis are positively regulated by the activation of RARα [28,29]. This is supported by the findings that vitamin A deficiency in mice decreased hepatic *PEPCK1* gene expression and that the expression was restored by atRA supplementation [30]. On the other hand, it is possible that intake of excessive atRA induces up-regulation of CAR though RARα and prevents excessive expression of the hepatic *PEPCK1* gene by atRA, resulting in regulation of hepatic gluconeogenesis.

Finally, our results suggest that the regulation of *CAR* gene transcription by RARα does not occur in rodents (Fig. 7). Rat *CAR* gene transcription did not occur with atRA treatment, probably due to the lack of a region corresponding to cRARE in the human gene (Fig. 8). Therefore, care should be taken in extrapolating results obtained from experiments using rodents to humans because of a significant species difference in the regulation of *CAR* gene transcription by RARα. Since the biological significance of RARα-dependent regulation of CAR in the human liver remains elusive, further studies are required to integrate rodent and human data. Such a challenge could give us a novel insight into the orchestrated regulation of physiological functions of the "human" liver.

In conclusion, our results suggested that RARα regulated transcription of the human *CAR* gene through the cRARE located in the intronic region of the gene. The fact that cRARE does not exist in the rodent *CAR* gene likely explains why the rat *CAR* gene is not activated by exposure to atRA. Although the biological significance of RARα-dependent regulation of CAR in the human liver remains to be clarified, the challenge to explore the significance could have an impact on hepatology since CAR plays important roles in various metabolisms in the human liver.

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