Role of the Defective Splicing of mRNA in the Lack of Pulmonary Expression of Constitutively Active Receptor in Rat

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The mammalian constitutively active receptor (CAR) is a ligand-activated transcription factor that participates in controlling the expression of cytochrome P450 2B (CYP2B) genes in response to xenobiotics in an organ-specific manner. In the presence of phenobarbital (PB) or PB-type agents, hepatic CYP2B forms are highly inducible. In contrast, PB-dependent increases in the expression of CYP2B activities are rarely observed in the lung. Mature CAR mRNA could be detected in the liver of 7-week-old Wistar rats by RT-PCR, while lung CAR mRNA had a 91 bp extra nucleotide sequence inserted in a coding region of hepatic CAR mRNA. By comparing the full- length sequence of hepatic CAR mRNA, including 5′- and 3′-untranslated region (3′-UTR), with the genomic sequence completed in the present study, the genomic structure was clarified to consist of 9 exons and 8 introns, in which the coding region expanded from exon 2 in close to its 5′-end to the first one-third of exon 9 after 159 bp of 5′-UTR in the most frequently obtained cDNA clones. In pulmonary CAR mRNA, intron 6 was not spliced out, implying that the lack of CAR in the lung might in part result from the incomplete splicing of precursor mRNA during its maturation.

Key words —— constitutively active receptor, rat, lung, genome structure

INTRODUCTION

Constitutively active receptor (CAR) is predominantly expressed in the liver and intestine,[1] and mediates the phenobarbital induction of multidrug resistance-associated protein 2 (MRP2), a drug efflux pump found at the biliary pole of hepatocytes in *in vitro* systems, and UDP-glucuronosyltransferase (UGT1A1), 2) CYP2B1/2, CYP2B6 , CYP3A1/2 and CYP3A4 3, 4) in both *in vivo* and *in vitro* situations. CAR interacts with its cognate response element in the 5′-flanking region of target genes through DR4 motifs such as the phenobarbital (PB)-responsive enhancer module (PBREM) in the far upstream promoter regions of mouse, rat and human CYP2B genes, by forming a heterodimer with the 9-*cis*-retinoic acid receptor alpha (RXRα). In our previous paper, simultaneous expressions of both CYP2B1 and CYP2B2 were observed at low levels in the rat liver, and the exclusive expression of CYP2B1 among those xenobiotic-metabolizing CYPs was seen at a rather higher level in the rat lung. The hepatic expression of both CYP2B forms could be induced when the animal was treated with PB, whereas no increase in the pulmonary expression of CYP2B forms was observed.[5] Although the sequence of the coding region of rat hepatic CAR mRNA has been reported (Yoshinari *et al*., 2001, accession number AF133095),[6] details concerning the genomic structure of CAR and the expression of pulmonary CAR mRNA, on which the induction of CYP2B forms is dependent, remain to be disclosed.

In order to elucidate the mechanisms of organ specificity involved in the PB-dependent induction of rat CYP2B forms, the CAR transcripts in both the liver and lung were sequenced (both were deposited with accession numbers AB104736 and AB105072, respectively) in comparison with the genomic sequence completed in the present study (deposited with accession number AB105071) on the basis of the published data (Muzny *et al*., accession number AC099236).

MATERIALS AND METHODS

Animals and Treatment —— Seven-week-old male Wistar rats (Clea Japan) were kept under a 12-hr light-dark cycle and provided food and water *ad*

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libitum. The livers and lungs were used for the isolation of total RNA and genomic DNA.

RT-PCR Analysis of the Hepatic and Pulmonary CAR mRNA —— Total RNA was extracted from each homogenate of 25 mg of rat liver and lung using the RNeasy Kit (Qiagen, Hilden, Germany). After incubation at 65°C for 10 min, the total RNA extracts were quickly placed in an ice-cold water bath. The total RNA extract thus obtained and oligo-dT primer were added to RTG You-Prime First-Strand Beads (Amersham Biosciences, NJ, U.S.A.), and after being left at room temperature for 1 min, the reverse transcription proceeded at 37°C for 1 hr to obtain cDNA, to which a primer pair, Pyrobest DNA polymerase (Takara, Japan), 10 × Pyrobest Buffer II and dNTP Mixture were added. After adjusting the total volume to 25 μl, the cDNA was amplified by 25 or 30 cycles of denaturation at 95°C for 15 sec, followed by annealing at 58°C for 30 sec and extension at 72°C for 3 min in a thermal cycler. The following oligonucleotides were used as sense and antisense primers, respectively: 5′-CGCGAATTCATGACAGCTACTC-3′ (S-1) and 5′-AAGCAGCGGCATCATAGCAG-3′ (A-1). The reaction products were separated by agarose gel electrophoresis and analyzed using a Fluor Imager (Amersham Biosciences) after staining with ethidium bromide.

Subcloning of PCR Products —— The PCR-amplified hepatic and pulmonary CAR cDNAs were recovered from agarose gel using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosiences), then ligated to the Sma I site of pBluescript II after treatment with the Blunting Kination Ligation Kit (Takara) to blunt and dephosphorlylate their ends. *Escherichia coli* (*E. coli*) competent cells (XL-1 Blue) transformed with 100 ng–1 μg of plasmid DNA were plated on LB/Amp plates and incubated at 37°C overnight. White single colonies were selected from the plates and incubated in Luria Broth supplemented with ampicillin and tetracycline overnight. Plasmids harboring the RT-PCR products were purified using the GFX™ Micro Plasmid Prep Kit (Amersham Biosciences).

Sequencing —— The nucleotide sequencing of PCR products was carried out using the Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit and GeneRapid (Amersham Biosciences, Model Seq4×4). The conditions for PCR were as follows: 30 cycles of denaturation at 95°C for 30 sec, followed by annealing at the individual annealing temperatures for 30 sec and extension at 72°C for 90 sec. The electrophoresis was run under conditions of 1250 V and 30 mA, at 50°C for 60 min using Tris-Borate-EDTA as a running buffer. The pBluescript II-specific sequence primers and CAR-specific sequence primers are as follows, with the annealing temperatures in parentheses:

T3 HT sense primer (54°C), 5′-AATTAACCCTCACTAAAGGGAAC-3′;

S-2 sense primer (56°C), 5′-CGAACAGTCAGCAAGACCA-3′;

S-3 sense primer (61°C) , 5′-ACCAGTTTGTGCAGTTCAGG-3′;

A-2 antisense primer (58°C), 5′-CTTGAGAAGGGAGATCTGGT-3′;

T7 HT antisense primer (58°C), 5′-GTAATACGACTCACTATAGGGCGA-3′.

Structure Analysis of the Rat CAR Gene —— The extraction of genomic DNA from the rat liver was conducted using the Genomic Prep Cells and Tissue DNA Isolation Kit (Amersham Biosciences). In order to fill the gap in the reported genomic sequence corresponding to the CAR mRNA, a pair of sense primer S-1 and antisense primer A-3 were used for PCR amplification, and the fragment was subcloned to pBluescript II.

5′-Rapid Amplification of cDNA Ends (5′-RACE) and 3′-RACE —— The untranslated regions of hepatic and pulmonary CAR mRNA were sequenced using the Gene Racer™ PLM-Kit (Invitrogen).

Primers for 5′-RACE analysis were as follows:

Gene Racer™ 5′-primer, 5′-CGACTGGAGCACGAGGACACTG-3′;

reverse gene-specific primer (A-3), 5′-TCTGCTGCTGACTCAGTTGCA-3′;

Gene Racer™ 5¢-nested primer, 5′-GGACACTGACATGGACTGAAGGAGTA-3′;

reverse gene-specific nested primer (A-4), 5′-CGCGGATCCACCACACAGTTCCTT-3′.

Primers for 3¢-RACE analysis were as follows:

Gene Racer™ 3′-primer, 5′-GCTGTCAACGATACGATACGCTACGTAA-3′;

gene-specific primer (S-4), 5′-ACCAGATCTCCCTTCTCAAG-3′;

Gene Racer™ 3′-nested primer, 5′-CGCTACGTAACGGCATGACAGTG-3′;

gene-specific nested primer (S-5), 5′-GGAGTTGATCATCCACTTCC-3′.

An annealing temperature of 61°C was employed.

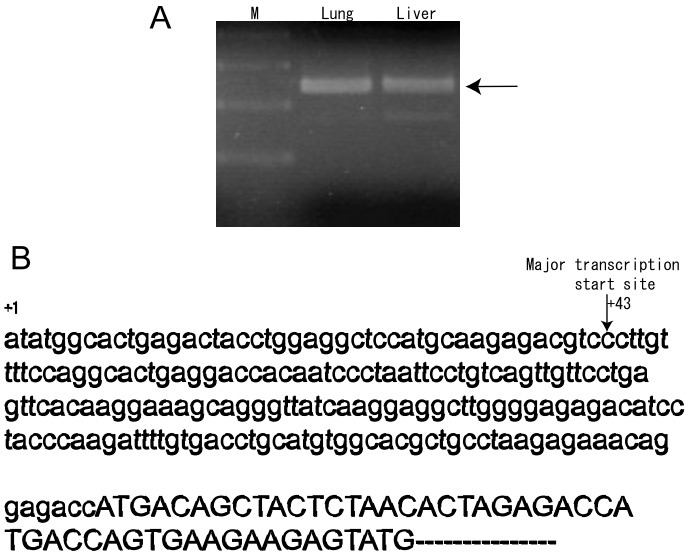

Fig. 1. Determination of the Transcriptional Start Sites of Hepatic CAR mRNA by 5′-RACE Analysis

(A) The transcriptional start site was analyzed by the Gene Racer™ Kit. The 5′-end sequence of hepatic and pulmonary CAR mRNA was amplified using a primer pair of A-3 and Gene Racer 5′-primer. Nested PCR was carried out using a primer pair of A-4 and Gene Racer 5′-nested primer. Lane M: molecular markers of 100 bp DNA ladders. (B) The 5′-UTR is shown in lowercase letters. The coding region is shown in uppercase letters. The 5′-end of the longest product is labeled as + 1. The major transcriptional start site is shown by the arrow (+ 43).

RESULTS

Analysis of the CAR Genome Structure

A homology search for CAR cDNA using BLAST2 identified a rat genome sequence with accession number AC099236 (Muzny *et al.*), in which an approximately 50 bp nucleotide sequence in intron 3 remained to be determined. The full-length CAR mRNA of the liver was sequenced by 5′- and 3′-RACE methods, showing a 158 bp major 5′-UTR sequence. (Fig. 1) Furthermore, the most upstream transcriptional start site in the liver mRNA was located 201 bp upstream of the translational start site. The gap-filled nucleotide sequence of CAR genomic DNA was 5329 bp-long, consisting of a 201 bp 5′-flanking region (158 bp in the major transcript), 9 exons and 8 introns, with the translation start site at the 7th nucleotide in the 143 bp-long exon 2, and a stop site approximately one-third of the way along the 412 bp-long exon 9 (Fig. 2). All 8 introns were surrounded with GT at the 5′-terminal and AG at the 3′-terminal (Table 1).

Comparison of the Nucleotide Sequences between Hepatic and Pulmonary CAR mRNAs

CAR mRNA isoforms expressed in the liver and lung are comparatively shown in Fig. 3. Three PCR bands were observed in the liver, whereas the lung showed a single band. The hepatic cDNA with the lowest molecular weight (Fig. 3, liver I) was estimated to correspond to a mature CAR mRNA based on the putative translated peptide structure. The sequence of the mature CAR cDNA was completely identical to the reported one (Yoshinari *et al.*, 2001; 1077 bp; AF133095)[6] (Fig. 4, I). The 91 bp-long intron 6 remained unspliced in the pulmonary CAR mRNA, as well as in the hepatic middle-sized isoform (Fig. 3, lung & liver II; Fig. 4, II), whereas the 195 bp-long intron 7 instead of intron 6 was not spliced in the largest hepatic CAR mRNA isoform (Fig. 3, liver III; Fig. 4, III). The 159 bp 5′-UTR (shown in Fig. 1) and 282 bp 3′-UTR (data not shown) sequences were shared in major hepatic and pulmonary mRNA isoforms.

DISCUSSION

The PB-dependent induction of CYP2B forms was observed in the rat liver, whereas the CYP2B1 constitutively expressed in the lung lacked the PB-responsiveness. The nuclear receptor CAR is reportedly sequestered in the cytoplasm in unstimulated hepatocytes, and the nuclear translocation of CAR in the presence of PB or PB-type agonists activates the transcription of target genes by interacting with *cis*-element referred to as the PBREM located in the upstream promoter region.[7] However, this type of

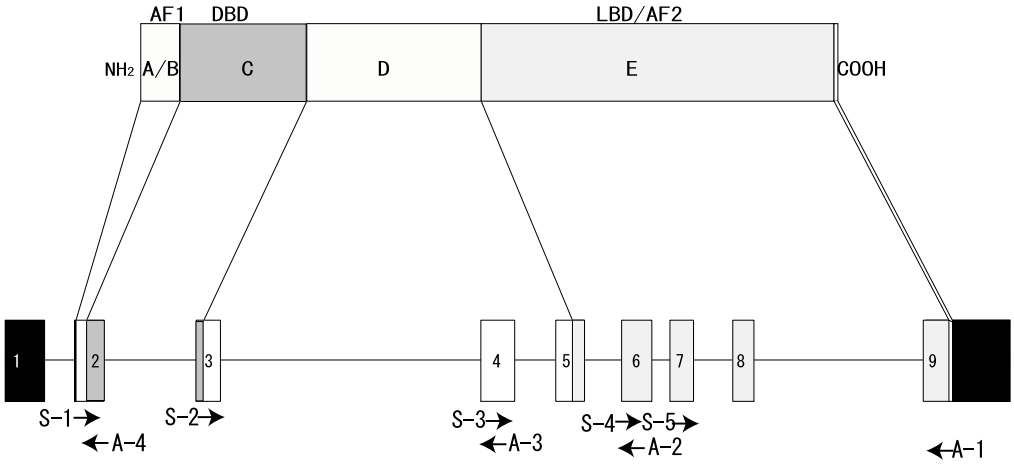

Fig. 2. Structure of the CAR Gene

The distribution of the exons of mature CAR mRNA is schematically presented. Exons are numbered from 1 to 9. Non-coding regions are shown as black boxes. DNA binding domains (DBD) are represented as dark gray boxes. Ligand binding domains (LBD) are represented as light gray boxes. (Q9QUS1) The primers used are summarized under the figures.

Table 1. Genomic Structure of the Rat CAR Gene

Exons			Introns				Splicing sites	
No.	Position	Size	No.	Position	Size	GC content (%)	Donor site	Acceptor site
1	291–486	196	1	487–635	149	49.0	CAGgta	cagGAG
2	636–778	143	2	779–1238	460	47.0	CAGgtg	cagACG
3	1239–1369	131	3	1370–2664	1295	47.7	ACAgtg	cagTGA
4	2665–2834	170	4	2835–3036	202	58.9	AGGgtg	cagCCT
5	3037–3176	140	5	3177–3362	186	47.8	CCGgtg	cagTTC
6	3363–3508	146	6	3509–3599	91	53.8	ATGgtg	cagTAG
7	3600–3716	217	7	3717–3911	195	57.4	CTGgtg	cagACA
8	3912–4017	106	8	4018–4872	855	48.9	TCGgta	aagGTT
9	4873–5285	413						

All splicing sites observe the GT-AG rule. Polyadenylation signal is located at 5268–5273. The 5′-untranslated region consists of positions 291–486 and 636–641. Major transcription start site is located at 334. The 3′-untranslated region covers position 5003–5285. Coding seqence start at 642. Stop codon is located at 5000–5002.

CAR-dependent gene activation was specific to such organs as the liver and intestine, but not the lung, although CYP2B1 was constitutively expressed to a greater extent in the lung. Therefore, it is interesting to clarify the mechanisms underlying the pulmonary defect of a CAR-dependent induction of CYP2B forms.

The full-length mRNAs detected by RT-PCR in both the lung and liver were divergent in their molecular weights (Fig. 3). Rat CAR mRNA isoforms of both hepatic and pulmonary origins share a common promoter, although another putative promoter which is rich in GC-boxes resides upstream of the adopted proximal promoter (Fig. 1). In good accordance with the murine CAR genome structure, the rat CAR genome was found to consist of 9 exons and 8 introns, in which the coding sequence expanded from the second exon to the last one. (Fig. 2) By comparing the CAR cDNA sequences with the genome structure, the pulmonary CAR mRNA was found to retain intron 6 in addition to the sequence of mature hepatic CAR mRNA, probably due to a failure in the mRNA processing, resulting in a lack of ligand binding domain (LBD) in the CAR protein (Fig. 4, I *vs* II). Many of the genes that encode the nuclear receptor superfamily members express more than a single protein as a consequence of either alternative promoter utilization or alternative mRNA processing. In the mouse liver, for example, the CAR gene encodes two proteins, mCAR1 and mCAR2, as a consequence of alternative splicing. mCAR2 is a truncated variant of intact mCAR1,

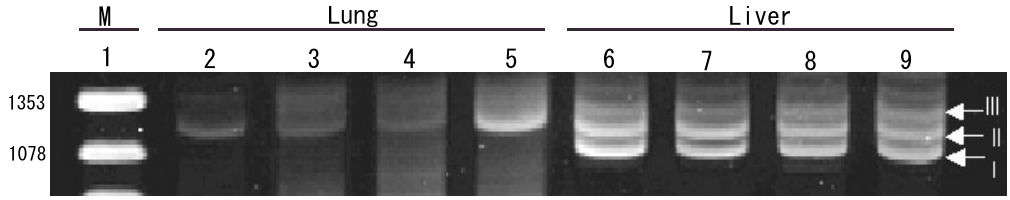

Fig. 3. Expression of the CAR mRNA Isoforms in the Rat Liver and Lung

RT-PCR amplifications of poly(A)-containing RNA extracts were performed using a primer pair of S-1 and A-1. Reaction products were separated by agarose gel electrophoresis and stained with ethidium bromide. lane 1: ϕX174/HaeIII size marker, lane 2–5: lung(35 cycle), lane 6–9: liver(30 cycle).

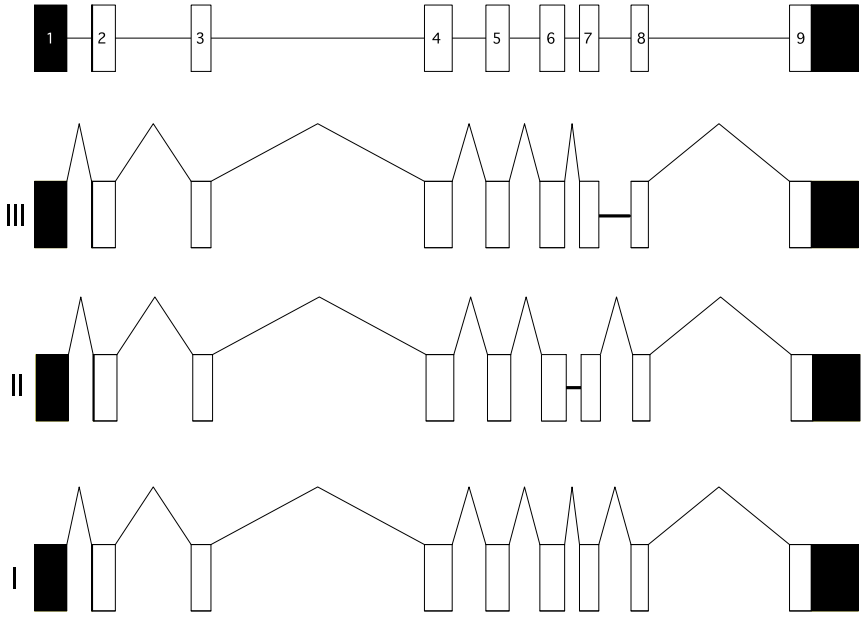

Fig. 4. Structural Organization of the CAR cDNA in Comparison with the Genomic Structure

The structural relationship among the mature (Fig. 1, band I) and immature (II, III) CAR mRNA isoforms is depicted. All three isoforms can be detected in the liver, while II is the sole isoform detected in the lung.

lacking exon 8 coding for the LBD/dimerization domain, and it neither transactivates by itself nor inhibits transactivation by mCAR1.[8)]

There are three CAR mRNA isoforms detected in the rat liver, referred to as I, II and III in ascending order of molecular weight. Among them, isoform II was exclusively expressed in the lung. These experimental results were explained as follows: During the course of CAR mRNA maturation, two pathways were proposed to exist in the liver. Isoform III could be processed to give isoform I in the first pathway, whereas isoform II was terminal in the second pathway. In the liver, the coexistence of both pathways resulted in the accumulation of the three isoforms. However, the second pathway could solely exist in the lung, leaving isoform II as a single isoform. In another explanation, the three isoforms were individually terminal. One of three independent pathways worked in the lung, in marked contrast to the case for the liver, where three pathways could be working simultaneously. In conclusion, we propose that the lack of expression of CAR function in the lung might be attributable to improper splicing during the course of CAR mRNA maturation.

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