Expression of Constitutive Androstane Receptor Splice Variants in Rat Liver and Lung and Their Functional Properties

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The mammalian constitutive androstane receptor (CAR) is a transcription factor that participates in controlling the expression of xenobiotic metabolizing and transporting genes in response to xenobiotics in an organ-specific manner. In addition to the wild-type CAR (CAR WT) mRNA, mRNAs for five splice variants (SVs) could be detected in the liver of 7-week-old male Wistar rats by RT-PCR using primer pairs covering a full-length mRNA derived from 9 exons; insertion of 18 bp at the 5′-end of intron 8 with or without deletion of 3 bp from the 5′-end of exon 7 (CAR SV1 or SV2), deletion of 4 bp from the 5′-end of exon 8 (CAR SV3), insertion of 195 bp intron 7 (CAR SV4), and insertion of 91 bp intron 6 (CAR SV5). In contrast, only CAR SV5 was detected in lung. Due to the introduction of novel stop codons, all the SVs were considered to code for premature proteins. The liver homogenate gave two protein bands in the vicinity of 37 kDa on Western blotting. They were attributable to CAR WT and SV-complex, respectively, based on their putative molecular weights in descending order. Upon co-transfection with the reporter plasmid, only the cells transfected with the CAR SV4-expression plasmid showed enhanced luciferase activity similar to the WT-transfected cells, for which the further splicing of the remaining intron 7 seemed to be responsible. The transactivation-defective SVs downregulated CAR WT-induced luciferase activity to some extent in the cotransfection experiments.

Key words rat constitutive androstane receptor; splice variants; mRNA; reporter assay

Mammalian constitutive androstane/active receptor (CAR) is predominantly expressed in the liver and intestine[1] and mediates the production of various drug-metabolizing and drug-transporting proteins.[2—4] CAR interacts with its cognate response element in the 5′-flanking region of targets such as the mouse, rat and human cytochrome P450 2B (CYP2B) genes, by forming a heterodimer with the 9-*cis*-retinoic acid receptor alpha (RXRα).

Four alternatively spliced variants of CAR have been reported in human liver[5—7]; isoforms with a 12-bp (4 amino acids) insertion from intron 6 and/or a 15-bp (5 amino acids) insertion from intron 7, and an isoform with exon 7 deleted. Recently, 16 additional splice variants (SVs) were detected in human liver as well as 4 brain-specific isoforms and a prostate-specific one: the deletion of exon 2, exon 4, exon 7, and partial deletion of exon 9 as well as the previously identified insertions of 12-bp from intron 6 and 15-bp from intron 7 were commonly observed in various combinations. The translation initiation codon ATG in exon 1, evolutionally conserved in baboon, chimp and cow, but not mouse or rat, took the place of the wild-type CAR initiation codon in the exon 2-skipped isoforms (13 isoforms including 2 brain-specific ones). The loss of exon 2 also results in the loss of one of the zinc fingers in DNA binding domain (DBD). In contrast to liver where multiple SVs were detected, only wild-type CAR mRNA was observed in small intestine and single unique CAR SVs were individually detected in prostate, spleen and heart. There were no SVs in lung, colon, muscle, stomach, thyroid, ovary and uterus.

Like human CAR (hCAR), murine CAR (mCAR) is expressed at highest levels in liver. Of nine independent clones isolated from a mouse liver cDNA library using an hCAR probe, 4 were wild-type clones with an intact ligand binding domain (LBD) (mCAR1), and 3 had an out-of-frame internal deletion of 107 bp corresponding to exon 8 (mCAR2).[8] In addition to mCAR1 and mCAR2, there was one additional clone containing 188 bp of unspecified intron-derived sequence. However, it is unclear whether this clone corresponds to an authentic variant or a contaminating nuclear precursor. None of the human and murine CAR SVs identified to date transactivate CAR response element (CRE)-driven reporter genes either alone or in the presence of activators except for the hCAR SV with a 12-bp (4 amino acids) insertion from intron 6 which showed marginal activity.[5,7]

In order to elucidate the mechanisms of organ specificity involved in the phenobarbital (PB)-dependent induction of rat CYP2B forms under the control of promoters consisting of phenobarbital-responsive enhancer module (PBREM), the CAR transcripts in both liver and lung were sequenced. Wild-type CAR (CAR WT) mRNA was detected only in liver. Two CAR SVs (CAR SV4 and SV5) transcribed from the rat CAR (rCAR) gene were simultaneously detected in liver, while CAR SV5 was solely detected in lung (deposited with accession numbers AB104736 and AB105072, respectively).[9] It has been estimated that many mammalian genes generate divergent RNA transcripts by alternative promoter usage and/or exon splicing. As for human, mouse and rat CARs, only the latter has been reported. An extensive search for rCAR isoforms resulted in the novel discovery of three more CAR SVs (CAR SV1, SV2 and SV3). In this paper, so far detected CAR SVs and novel SVs in the rat liver and lung were studied for their structural and functional properties in comparison with those of CAR WT.

MATERIALS AND METHODS

Animals and Treatment Seven-week-old male Wistar rats (Clea Japan, Tokyo, Japan) were kept under a 12-h light–dark cycle and provided food and water *ad libitum*. The livers and lungs were used for the isolation of total RNA.

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Cell Culture Conditions HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Corp., Carlsbad, CA, U.S.A.) containing 10% fetal bovine serum and penicillin–streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

RT-PCR and Amplification of Hepatic and Pulmonary CAR cDNAs Total RNA was extracted from each homogenate of rat liver and lung using the RNeasy Midi Kit (Qiagen, Hilden, Germany). After incubation at 65 °C for 10 min, the total RNA extract was 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 1 min at room temperature the reverse transcription proceeded at 37 °C for 1 h to obtain cDNA, to which a primer pair, *Pyrobest* DNA polymerase (TAKARA, Japan), 10×Pyrobest Buffer II and a dNTP Mixture were added. After the total volume was adjusted to 25 μl, the cDNA was amplified for 30 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s and extension at 72 °C for 1.5 min in a thermal cycler. The following oligonucleotides were used as sense and antisense primers, respectively: 5′-CGC GGA TCC ATG ACA GCT ACT CTA A-3′ and 5′-ATC CTC GAG TCA GCT GCA AAT CTC C-3′, where shaded boxes represent *BamH*I and *Xho*I sites, respectively.

The reaction products were separated by agarose gel electrophoresis and analyzed using a Fluor Imager (Amersham Biosciences) after staining with ethidium bromide.

Subcloning and Sequencing of PCR Products The PCR-amplified hepatic and pulmonary CAR cDNAs were ligated to Zero Blunt TOPO Vectors (Invitrogen Corp.). Plasmid DNA was transformed into competent *E. coli* cells (XL-1 Blue), and plasmids harboring the RT-PCR products were purified using the Quantum Prep® Plasmid Miniprep Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The nucleotide sequencing of resulting plasmids was carried out using a Thermo Sequenase Cy5.5 Dye Terminator Cycle Sequencing Kit and GeneRapid (Amersham Biosciences, Model Seq4x4). The Zero Blunt TOPO Vector-specific sequence primers are as follows with the annealing temperatures in parentheses: T3 primer (58 °C), 5′-ATT AAC CCT CAC TAA AGG GA-3′; T7 primer (54 °C), 5′-TAA TAC GAC TCA CTA TAG GG-3′.

Western Blot Analysis Liver and lung homogenates were centrifuged at 600×g for 10 min to precipitate the nuclear fraction. The supernatant was further centrifuged at 10500×g for 60 min to precipitate the microsomal fraction. The supernatant was the cytosolic fraction. The protein concentration of the cytosolic fraction was quantitated using a 2-D Quant Kit (Amersham Biosciences). One hundred micrograms of cytosolic proteins and 25 μg of nuclear proteins were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. The membrane was treated with goat anti-rat CAR (Santa Cruz, U.S.A.) and peroxidase-labeled anti-goat IgG. Chemiluminescence was determined using the Western blotting detection reagent, ECL-Plus (Amersham Biosciences). The protein band-images were read with a Storm TM 830 (Amersham Biosciences) and analyzed with Image Quant software.

Rat CAR Expression Plasmids and the Reporter Plasmid The CAR SVs were digested from TOPO vector by *BamH*I and *Xho*I, and cloned into pcDNA 3.1 (Invitrogen Corp.).

The PBREM-pGL3 reporter plasmid was constructed by ligating the PBREM sequence into the *Sac*I and *Bgl*II sites of the pGL3-Promoter Vector (Promega, U.S.A.). A double stranded PBREM nucleotide sequence was prepared by annealing the following synthetic oligomers: 5′-GAA TTC TCT GTA CTT TCC TGA CCT TGG CAC AGT GCC ACC A TC AAC TTG ACT GAC ACC A-3′ and GAT CTG GTG TCA GTC AAG TTG ATG GTG GCA CTG TGC CAA GGT CAG GAA AGT ACA GAG AAT TCA GCT-3′.

Site-Directed Mutagenesis of CAR SV4 Site-directed mutagenesis was performed on the pcDNA-CAR SV4 expression plasmid using a QuickChange Site-directed Mutagenesis Kit (Stratagene, CA, U.S.A.). The forward version of the mutagenic primer and reverse primer are 5′ ATG GCT CTC TTC TCT CCT GGA GAG AAC CAT-3′ (the altered nucleotide, which introduces a silent mutation, is underlined) and 5′-CCA GGA GAG AAG AGA GCC ATG GCA GCC-3′, respectively. The sequence of the mutant CAR SV4 DNA was confirmed by sequencing.

Expression Profiles of pcDNA-CAR WT, SVs and SV4mut Plasmids in Transfected HepG2 Cells HepG2 cells were subcultured on 6-well plates for 24 h and transfected with 500 ng of individual pcDNA-CAR expression plasmids with the help of a mixture of TransFast (Promega) and OPTI-MEM (Invitrogen Corp.) for 2 h until DMEM was added to the culture. After 24 h, total RNA was extracted from transfected cells using a SV Total RNA Isolation System (Promega). After reverse-transcription, cDNA was amplified using Blend Taq DNA Polymerase (TOYOBO, Japan) and CAR-specific primers, including exon 5 through 9.

Transient Transfection and Reporter Gene Assay HepG2 cells were subcultured on 48-well plates. Twenty-four hours later, the cells were transfected in each well with 100 ng of PBREM-pGL3 reporter plasmid, 25 ng of phRL-SV40 control plasmid (Promega) and pcDNA-CAR WT and/or individual SV expression plasmids according to the same procedures. The total DNA content was adjusted to 325 ng with pcDNA empty plasmid. After overnight incubation, the reporter assay was performed using the Dual-Luciferase Reporter Assay System (Promega) and a Turner Designs Luminometer (Model TD-20/20, Promega).

RESULTS AND DISCUSSION

Identification of Hepatic CAR Splice Variants (SVs) To identify novel isoforms of rCAR, we amplified rCAR cDNA using primer pairs covering exon 1 through 9. PCR was performed with the total RNA extract of Wistar rat liver and the products were subcloned into the Zero Blunt TOPO Vector. Twenty-seven random clones were removed and sequenced. The distribution of the clones was as follows: sixteen clones coding for wild-type rCAR (CAR WT), two with a 3-bp deletion and 18-bp insertion (CAR SV1), four with an 18-bp insertion (CAR SV2), two with a 4-bp deletion (CAR SV3), one with an intron 7-insertion (CAR SV4), and one with an intron 8-insertion (CAR SV5) (Figs. 1A, B). CAR SV4 and SV5 identical with those reported previously[9] were also detected in smal intestine (data not shown).

CAR WT is a protein of 358 amino acids (a.a.) with a the-

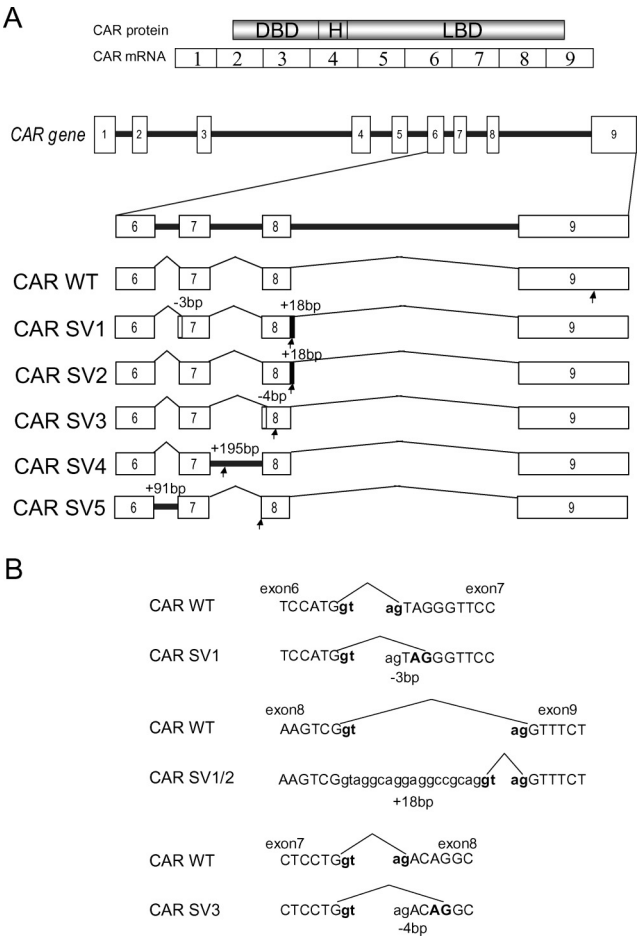

Fig. 1. Structural Organization of cDNAs of Mature and Splice Variants of rCAR in Comparison with the Genomic Structure

(A) The structural relationship among the mature and splice variants of rat liver CAR mRNA. (B) Nucleotide sequences in the vicinity of splice junctions of CAR SVs' mRNA.

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1   MTATLTLETMTSEEEYGPRNCVVCGDRATGYHFHALTCEGCKGFFRRTVSKTIGPICPFA
61  GRCEVSKAQRRHCPACRLQKCLNVGMRKDMILSAEALALRRARQARRRAQKASLQLSQQQ
121 KELIQTLLGAHTRHVGPMFDQFVQFRPPAYLFSHHRPFQPLAPVLPLLTHFADINTFMVQ
181 QIIKFTKDLPLFRSLTMEDQISLLKGAAVEILHISLNTTFCLQTQNFFCGPLCYKMEDAV

CAR WT   241 HVGFQYEFLELIIHFHKTLKRLQLQEPEYALMAAMALFSPDRPGVTQREEIDQLQEEVAL
CAR-SV1      H-GFQYEFLELIIHFHKTLKRLQLQEPEYALMAAMALFSPDRPGVTQREEIDQLQEEVAL
CAR-SV2      HVGFQYEFLELIIHFHKTLKRLQLQEPEYALMAAMALFSPDRPGVTQREEIDQLQEEVAL
CAR-SV3      HVGFQYEFLELIIHFHKTLKRLQLQEPEYALMAAMALFSPGLGLPKEKRLISCRKRSR--
CAR-SV4      HVGFQYEFLELIIHFHKTLKRLQLQEPEYALMAAMALFSPGENHPRAEELWPHHLYPNSQ
CAR-SV5      HGETVVQNTVCTLWYEVWSNGQEAAQPTISHSRVPVRVFGVDHPLPQNPEKIAAPGARVC

CAR      301 ILNNHIMEQQSRLQSRFLYAKLMGLLAELRSINSAYSYEIHRIQGLSAMMPLLGEICS 358
CAR-SV1      ILNNHIMEQQSRLQSR----------
CAR-SV2      ILNNHIMEQQSRLQSR----------
CAR-SV3      ----------
CAR-SV4      LPPLIHP----------
CAR-SV5      AHGCHGSLLS----------
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Fig. 2. Amino Acid Sequence Alignments of Mature and Splice Variants of rCAR

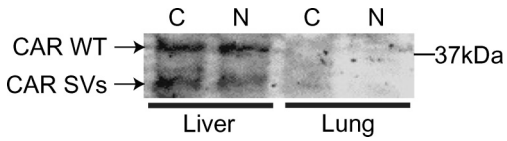

Fig. 3. Expression Levels of Various CAR Proteins in the Cytoplasmic and Nuclear Fractions of Rat Liver and Lung

Nuclear (N) and cytoplasmic fractions of liver and lung from Wistar rats were subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane, which was subsequently incubated with polyclonal anti-CAR antibodies. The membrane was further incubated with anti-goat IgG conjugated to horseradish peroxidase and the signals were visualized using Storm TM 830.

oretical molecular weight of 39 kDa (Fig. 2). The genomic structure of rCAR consists of nine exons and eight introns, with the translation start site in exon 2, and the stop codon in exon 9. CAR SV1 with 3 bp deleted from the 5′-end of exon 7 in frame and 18 bp inserted at the 5′-end of intron 8 with a premature termination codon, gives a 315 a.a. protein with a theoretical molecular weight of 34 kDa (Fig. 2). CAR SV2, with 18 bp inserted at the 5′-end of intron 8 containing a novel termination codon, gives a 316 a.a. protein with a theoretical molecular weight of 34 kDa (Fig. 2). CAR SV3, with 4 bp deleted from the 5′-end of exon 8, gives a truncated isoform of 298 a.a. with a theoretical molecular weight of 32 kDa (Fig. 2). In CAR SV4, intron 7 (195 bp) was not completely spliced out, introducing a novel termination codon to give a protein of 307 a.a. with a theoretical molecular weight of 33 kDa (Fig. 2). A complete intron 6 (91 bp) remained in CAR SV5, resulting in a 310 a.a. protein with a theoretical molecular weight of 34 kDa due to a novel stop codon in exon 8 (Fig. 2). The amino acid sequences of CAR SVs are shown in Fig. 3 in comparison with the sequence of CAR WT.

Western immunoblot analyses were conducted using the nuclear and cytoplasmic fractions of rat liver and lung samples to study whether CAR SVs would be expressed as protein isoforms. The polyclonal antibodies recognizing the N-terminal regions shared by all the rCAR protein isoforms were employed. There can be seen two distinct immunoreactive bands in both subcellular fractions of liver homogenate but not in the lung counterparts (Fig. 3). The upper band migrates to *ca.* 39 kDa, corresponding to the estimated size of the wild-type CAR, and the broad band in the range of 32 to 34 kDa could be attributed to CAR SVs. Comparing the band intensities of top (CAR WT) and bottom (CAR SVs), and the numbers of PCR-amplified cDNA colonies of CAR WT and SVs, CAR WT was expressed dominantly in liver in comparison with CAR SVs.

Transcription of Reporter Gene by CAR SVs and Their Effects on Transcription by CAR WT To determine whether CAR SVs were transcriptionally active like CAR WT, a reporter gene assay was performed by transfecting a luciferase reporter construct carrying PBREM derived from the CYP2B1 gene promoter and individual CAR SV-expression plasmids. CAR WT has been shown to translocate to the nucleus and transactivate the PBREM-driven reporter genes in the absence of any ligands or activators in immortalized cell lines.[10,11] CAR WT and SV4 activated PBREM-Luc transcription, while the other CAR SVs did not transactivate the reporter gene (Fig. 4). Next, we determined whether CAR SVs could affect transactivation caused by cotransfected CAR WT. As shown in Fig. 5, none of the transactivation-inactive CAR SVs affected CAR WT (50 ng)-dependent transactivation at up to 150 ng. In contrast, CAR SV4 had a

marginal but significant suppressive effect on the transactivation by CAR WT in a dose-dependent manner. We previously proposed two explanations concerning the correlation among CAR WT, SV4 and SV5. Both CAR SV4 and SV5 were detected in liver as well as CAR WT, whereas only CAR SV5 was detected in lung, where PB-dependent CYP2B induction was missing.[9] Therefore, CAR SV4 might be a hepatic intermediate of CAR WT, whereas CAR SV5 would be the terminal from in an alternative pathway. Alternatively, both CAR SV4 and SV5 are endoproducts not being processed into CAR WT. As the silent mutant of CAR SV4 lacking a splice donor in intron 7 (CAR SV4mu, Fig. 6A) was found to be transactivationally inert (Fig. 6B), the latter would not be the case. In fact, CAR SV4 should be further processed into CAR WT, which might be responsible for the transactivating potential of CAR SV4. The down-regulation of the transactivating activity of CAR WT by CAR SV4 (Fig. 5E) could be attributed to the nature of CAR WT as can be seen in Fig. 5A, because of CAR SV4mut without splicing to CAR WT were devoid transactivating activity. However, the RT-PCR analysis showed that CAR WT was less abundant than the unspliced CAR SV4 in HepG2 cells transfected with the CAR SV4 expression plasmid (Fig. 6B). Likewise, the CAR SV5 expression plasmid gave double bands corresponding to SV5 and the novel transcript by skipping intron 6 and exon 7, though this variant has never been detected in rat liver or lung. Taking all these findings into consideration, we propose that CAR SV4 and SV5 are not in the main pathway from the very precursor to mature CAR WT mRNA, though SV4 is partially processed to CAR WT by the splicing out of intron 7 (Fig. 7).

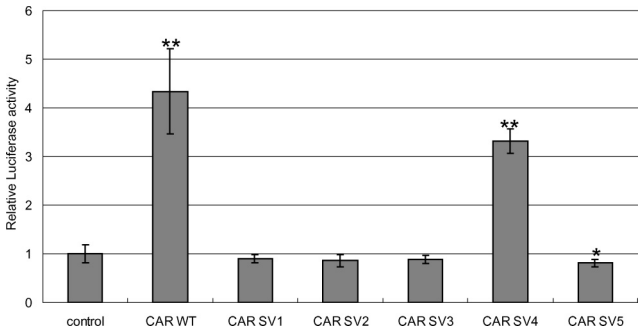

Fig. 4. Transactivation of PBREM-Luc by Mature and Splice Variants of rCAR

HepG2 cells were transfected with 100 ng of the expression construct of CAR SVs (empty vector in control), PBREM-Luc, and 10 ng of phRL-*Renilla* luciferase, as internal standard vector. The firefly luciferase activity measured after 24 h was normalized with *Renilla* luciferase activity of the same sample and the results are expressed as the mean±S.D. from three independent transfections. ＊Statistically significant from the empty vector-treated cells at the level of $p<0.05$. ＊＊$p<0.01$.

Recently, alternative splicing was proposed a new factor restricting the expression of functional CARs at relevant sites.[12] As for human CAR, alternative splicing within exon 9 was proposedly involved in the control of the tissue- and differentiation state-specific expression of the functional form. The ubiquitous but weak expression of non-functional CAR isoforms appears to be associated with the alternative splicing within exon 9, ensuring that no functional transcripts are produced ectopically, and compensating for inappropriate transcription of the CAR promoter. In the case of rat CAR, CAR SV4 might be a candidate for a defective variant, which

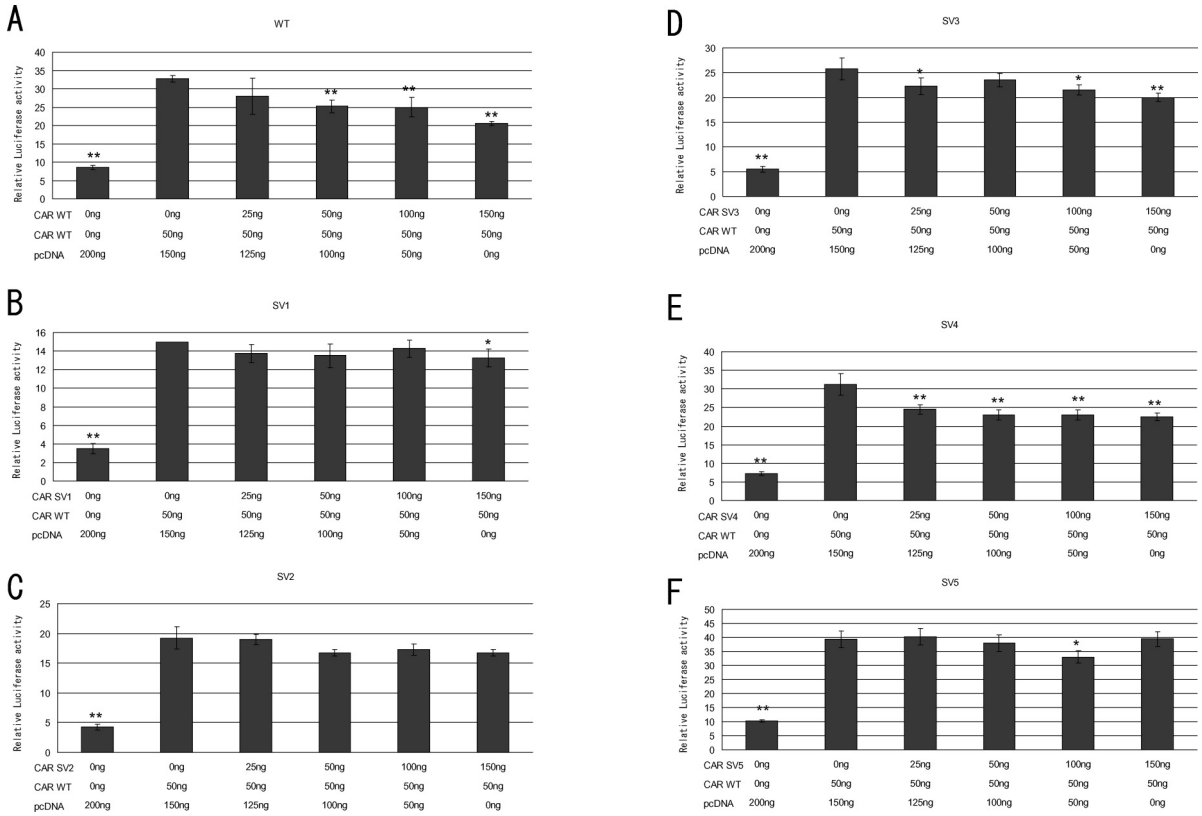

Fig. 5. Effects of Splice Variants of rCAR on the Transactivation of PBREM-Luc by CAR WT

HepG2 cells were transfected with 50 ng of CAR WT, 0—150 ng of respective CAR SV, 100 ng of PBREM-Luc, and 10 ng of phRL-*Renilla* luciferase. Further details are given in the legend to Fig. 4.

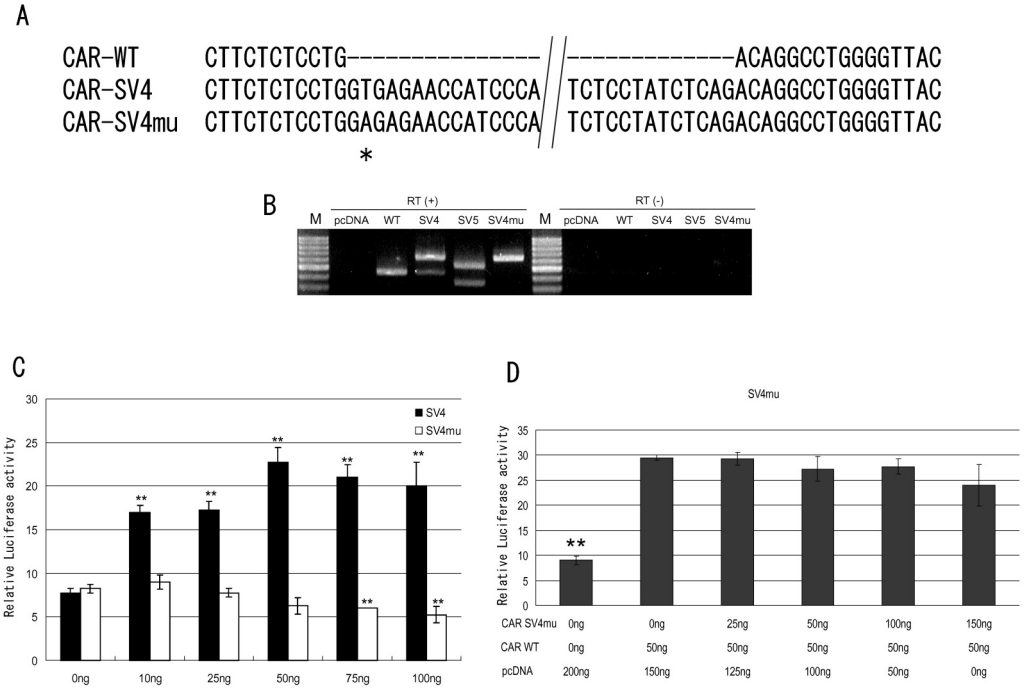

Fig. 6. Transactivation of PBREM-Luc and Interference with CAR WT-Induced PBREM-Luc Activity by Splicing-Defective CAR SV4 Mutant

(A) CAR SV4mut was constructed by single nucleotide replacement from CAR SV4. (B) HepG2 cells were transfected with 50 ng of CAR WT, SV4 or SV4mut expression plasmid. After 24 h, cells were analyzed for the expression of transcripts by PCR with or without reverse transcription. (C) HepG2 cells were transfected with 0—100 ng of CAR SV4 or SV4mut simultaneously with 100 ng of PBREM-Luc and 10 ng of phRL-*Renilla* luciferase. Further details are given in the legend to Fig. 4. (D) HepG2 cells were transfected with 50 ng of CAR WT, 0 (positive control)—150 ng of CAR SV4mut, PBREM-Luc, and 10 ng of phRL-*Renilla* luciferase. In the negative control, cells were transfected with empty vector instead of CAR WT. Further details are given in the legend to Fig. 4.

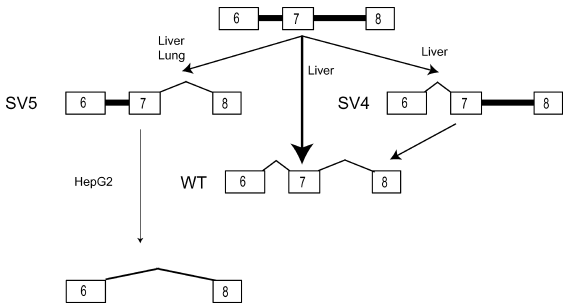

Fig. 7. Putative Biosynthetic Correlation among Mature and Splice Variants of Rat CAR mRNA

The sequences of CAR WT and SVs between exons 6 and 8 are presented schematically.

is ubiquitously expressed. Thus, alternative splicing contributes to the complex regulation of CAR expression and function.

In this study, we discovered novel rCAR splice variants, CAR SV1, SV2 and SV3, in liver in addition to the previously reported SV4 and SV5. In conclusion, SVs lacked both transactivation and dominant-negative potentials except for CAR SV4, in which the further splicing to CAR WT was expected. Tissue specific expression of CAR WT might be regulated by splicing events. The maturation of rCAR mRNA SV4 was confined into the CAR WT expressing tissues such as liver and small intestine, whereas lung the dead-ended route to SV5 was ubiquitously observed.

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