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Expression of CAR splice variants in human tissues and their functional consequences

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Abstract

The constitutive androstane receptor CAR (NR1I3) is a transcription factor that upon activation by xenobiotics induces transcription of drug metabolizing and drug transporter genes. Our goal was to identify whether alternative splicing of CAR makes an important contribution to the generation of novel CAR proteins. The wild-type CAR mRNA (CAR.1) and splice variants (CAR-SVs) were amplified from human liver cDNAs and in a panel of cDNAs from 36 human tissues, using exon 1 and 3'-UTR primers, cloned and sequenced. Twenty-two unique hCAR splice variants (CAR-SV) containing different combinations of splicing (deletion of exon 2, 4, 5, 7, partial deletion of exon 9 or insertion of 12 or 15 bp from Intron 6 or 7) were identified. CAR mRNAs were expressed in small intestine, kidney, adrenal gland, testis, adrenal and brain caudate nucleus. Intestine expressed only CAR.1 mRNA, while spleen, heart and prostate expressed only CAR-SVs. In vitro transcription and translation of CAR-SVs lacking exon 2 (missing ATG start site) generated CAR proteins that differed in M_r from CAR.1. These CAR-SVs used a translation initiation site in exon 1, generating CAR with a unique amino-terminal sequence. Transcripts lacking part of exon 9 altered the CAR reading frame generating CAR proteins with a unique carboxy-terminal end. CAR-SVs demonstrated compromised binding to CYP2B6 NR elements and transcriptional activation of a CYP2B6 luciferase reporter. The expression of CAR in additional human cell types increases the range of tissue specific transcriptional responses regulated by this receptor, suggesting additional biological roles for CAR and CAR-SV proteins in these tissues.

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Introduction

The constitutive androstane receptor CAR (NR1I3) is essential for regulating both constitutive expression and induction of some drug detoxification genes such as CYP2B in liver (Honkakoski et al., 1998; Wei et al., 2000). CAR is also highly conserved throughout evolution. CAR is activated either directly by ligands (TCPOBOP) or indirectly by some chemicals (phenobarbital) to a form that transcriptionally regulates gene expression (Yamamoto et al., 2003). Ultimately, CAR activators facilitate translocation of CAR from the cytosol to the nucleus and specific binding to CAR response elements in the promoters of a growing number of drug detoxification genes including CYP2B, CYP3A4 and UGT1A1 (Yamamoto et al., 2003). CAR is also required for liver hypertrophy caused by treatment with drugs such as phenobarbital (Wei et al., 2000). It is possible that CAR is also responsible for phenobarbital's ability to act as a liver tumor promoter (reviewed in (Schuetz, 2001)). Activation of CAR by drugs like phenobarbital may be a double-edged sword, enhancing the metabolism and removal of xenobiotics, and controlling liver tumor promotion by phenobarbital. Thus, it is important to understand factors regulating CAR expression.

A common paradigm for many genes, including nuclear hormone receptors, is the generation of additional spliced mRNA transcripts. It has been estimated that at least 59% of all human genes utilize alternative RNA processing to generate multiple mRNA products that can have different exon compositions (Venter et al., 2001). The inclusion or exclusion of exonic sequences enhances generation of protein isoforms that can vary in structure and functional properties. Nuclear hormone receptor alternative mRNAs arise by a combination of alternative promoter usage and exon splicing (Ding and Miller, 2002; Keightley, 1998). These nuclear hormone receptor splice

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variants can differ in their patterns of expression, gene targets, biological functions (gain-of and loss of function), DNA-binding, intracellular binding interactions with other proteins, and subcellular localization. Many receptors, both steroid and nuclear hormone, have at least one major splice variant form. This molecular diversity may allow for additional layers of signaling complexity from the nuclear hormone receptors.

Four alternatively spliced CAR splice variants have been reported in human liver (Auerbach et al., 2003; Savkur et al., 2003): an isoform with a 12 bp (4 amino acids) insertion from intron 6; an isoform with a 15 bp (5 amino acid) insertion from intron 7; an isoform with both the insertions; and an isoform with exon 7 (39 amino acids) deleted. All of these SVs alter the ligand binding domain of CAR. Compared with CAR.1, these CAR-SVs had lower affinity for CAR binding elements, decreased transactivation potential and decreased ability to recruit coactivators (Auerbach et al., 2003; Savkur et al., 2003).

Northern blot analysis has shown that CAR is highly expressed in human and rodent liver and intestine with lower expression in heart, muscle, kidneys and lung. The major CAR-SV identified by both groups contributed 6-8% of total CAR transcripts in liver, and expression of the SVs was detected in a variety of extrahepatic tissues including kidney, lung and adrenal (Savkur et al., 2003). However, the primers used to identify the splice variants in liver (Auerbach et al., 2003) were immediately flanking the translational start (in exon 2) and stop codons (in exon 9) and would not amplify CAR transcripts with deletions in exons 2 or 9 or that had alternative translational start sites further upstream. Similarly, the primers used to quantitate the amounts of CAR-SVs in extrahepatic tissues (Savkur et al., 2003) amplify only small fragments of CAR and would not distinguish whether additional CAR-SVs existed in these tissues. Thus, the objectives of this study

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were to identify all CAR mRNA transcripts in human tissues and to determine their functional consequences. The current report identifies a large number of CAR alternatively spliced mRNAs that are widely distributed in many human organs. The additional CAR isoforms and wider tissue distribution of CAR may increase the spectrum of gene targets regulated by CAR.

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Materials and Methods

RNA from Liver samples and Tissue Samples. Total RNA was prepared from liver samples (University of Pittsburgh/St Jude Liver Resource) from three different ethnic groups (African American n=30, Caucasians n=30 and Hispanics; n=25). The majority of the livers used in this study were from organ donors and their histories have been previously published (Lamba et al., 2003). Rapid-Scan Human multi-tissue and Human Brain Gene Expression Panels were purchased from OriGene Technologies, Inc. (Rockville, MD). OriGene isolated polyA+ RNA, synthesized first strand cDNA and the cDNAs were normalized such that they all contained equivalent concentrations of b-actin cDNA.

RT-PCR and Amplification of CAR cDNAs. Primers for amplification of full length CAR (Table 1) were designed with PRIMER3 (<http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>), and their sequence homology and specificity checked using BLASTn (<http://www.ncbi.nlm.nih.gov>). To amplify CAR from human livers, first-strand cDNA was synthesized from 3 µg of total RNA from human liver samples according to the manufacturer's instructions (Superscript Preamplification System for First-Strand cDNA Synthesis, InVitrogen Life Technologies, Carlsbad, CA, USA). Using CAR-F1 and CAR-R1 primers (Table 1), the CAR cDNA was amplified in a total volume of 50 µl consisting of 1 µl human liver cDNA, 10X PCR buffer, 1mM MgCl₂, 10 pmol of each primer, 0.2 mM dNTP (Life Technologies, Gaithersburg, MD, USA), and 2.5 U of *Taq* DNA polymerase (Expand High-Fidelity PCR system, Roche Diagnostics Corp., Indianapolis, IN) using conditions in Table 1. Using the same primers, CAR was also amplified in the Rapid-Scan Human multi-tissue and Human Brain Gene Expression

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Panels as per the manufacturer's instructions. The CAR cDNAs were analyzed by agarose gel electrophoresis. Because the amount of amplified cDNA in some samples was low, a second round of nested amplification was performed using the CAR-F2/R2 primers and appropriate dilutions of the first round PCR products. The conditions used were the same as the first round amplification with minor modifications (Table 1) and inclusion of 1.5 mM MgCl₂. CYP2B6 and MRP4 were amplified from the Human Brain Gene expression panel as described in Table 1.

Characterization and Cloning of hCAR variant isoforms. The amplified CAR cDNAs were analyzed by agarose (1%) gel electrophoresis. If a single band was observed, the amplification product was incubated with shrimp alkaline phosphatase and exonuclease I (USB Corporation, Cleveland, OH, USA) for 30 min at 37 °C to remove unincorporated nucleotides and primers; the enzymes were inactivated by incubation at 80 °C for 15 min before sequencing. If multiple bands were observed in the agarose gel, each band was cut from the gel using the Zymoclean Gel DNA recovery kit (Zymo Research, Orange, CA, USA), eluted in water, and sequenced by using the PCR primers. Alternately, the extracted cDNA was subcloned into PCR2.1 using the TOPO/TA cloning system (Invitrogen Life technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Ten clones were selected at random for each band that was cloned and after lysing the cells by repeated freezing and thawing, PCR amplification was performed using M13 reverse and M13 (-21) forward primers and the product sequenced using M13 primers. Sequencing was carried out on an ABI Prism 3700 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). GenBank accession numbers for the twenty-two CAR-SV nucleotide sequences are sequentially AY572806 through AY572827.

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Human CAR expression plasmids. Once the variants were characterized, the hCAR isoforms were digested from pCR2.1 by *EcoRI* and cloned into pCDNA3.1 (Invitrogen) and pMSCV-IRES-GFP. Mutagenesis of the [A/T]TGs in either exon 1 in the hCAR expression plasmid were achieved by site directed mutagenesis using a mutated primer (Table 1) and the hCAR expression plasmid to assess putative ATG start sites.

In vitro transcription and translation. In order to check if the variant isoforms of CAR encode a protein, in vitro coupled transcription and translation (TNT) was carried out using the rabbit reticulocyte lysate system (Promega Corporation, Madison, WI). One microgram of each pcDNA3.1-hCAR isoform was used for the TNT. Protein expression in rabbit reticulocyte lysate system was carried out in the presence of [³⁵S]Methionine as described by the manufacturer. The protein products were resolved by 7.5% SDS-PAGE.

Electrophoretic mobility shift assay (EMSA). Briefly, hCAR isoforms and RXRa protein were generated by TNT and incubated with ³²P-labeled double-stranded oligonucleotides (400,000 cpm/reaction) encoding the CYP2B6 NR1 or NR2 element (Table 1) with or without excess unlabeled oligonucleotides in the presence of DNA binding buffer (10 mM Tris pH 8.0, 4 mM KCl, 0.05% NP40, 6% glycerol, 0.2 μg polydI/dC, 6% glycerol, 50 μM ZnCl and 1 mM DTT) at 4°C. Complexes were resolved by electrophoresis through a non-denaturing 6% polyacrylamide gel and analyzed by using a phosphoimager (Molecular Dynamics/Amersham Biosciences,

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Piscataway, NJ).

Transient transfection and reporter gene assay. HepG2 cells were maintained in MEM medium supplemented with 10% fetal bovine serum. Cells were plated in 24-well plates at 3×10^5 cells per well. 24 hours later, they were transfected overnight by calcium phosphate precipitation with 500 ng of 2B6-LUC reporter plasmid (Tirona et al., 2003), in the presence or absence of either MSCV-hCAR.1 (200 or 400 ng) or MSCV-hCAR-SV (200 ng) expression plasmid or empty vector plasmid and 100 ng of pSV- β -galactosidase control vector to normalize for transfection efficiency. The next day, cells were washed once with medium and incubated with fresh medium containing 10 % charcoal-dextran treated fetal bovine serum (HyClone, Logan, UT) with or without 100 nM CITCO (Biomol, Plymouth Meeting, PA). Twenty four hours later, cells were harvested, lysed, and centrifuged at 2,000 x g, and luciferase activities were determined on an aliquot of supernatant according to the manufacturer's instructions (Luciferase Assay System, Promega Corporation) using an automated luminometer (model OPTOCOMP 1, MGM Instruments, Hamden, CT). LUC activities were normalized to β -galactosidase activity according to manufacturer's instructions (Beta-Galactosidase Enzyme Assay System with Lysis Buffer, Promega Corporation). All experiments were performed in triplicate and each experiment was repeated at least three times.

Preparation of Primary Cultures of Human Hepatocytes. Human livers were procured from donor organs that were not suitable for whole organ transplantation or from remaining tissue after

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reduced allograft transplantation. Donor livers were flushed, in situ, and maintained with Belzar's UW solution. Hepatocytes were isolated within 24 hrs of cross-clamp. Reasons for not using tissues for transplantation included traumatic damage, errors in organ harvest, brief anoxic periods or macro-or microsteatosis. Human hepatocytes were isolated essentially as described (Strom et al., 1996; Strom et al., 1998). Cells were plated on collagen-coated 6-well plates or 60 mm culture dishes and maintained in Modified Williams E for 48 hrs and then treated with drugs for 48 hr. Media was then aspirated, Trizol solution (Sigma, St. Louis, MO) added to lyse the cells, scraped from the plates and RNA isolated, first strand cDNAs synthesized as described above and targets amplified using primers in Table 1.

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Results

Identification of novel hCAR splice variants

A recent report identified a number of CAR splice variants in the livers of Caucasian persons (Auerbach et al., 2003) using primers immediately adjacent to the translation start and stop codons in CAR. To determine whether the same pattern of CAR splice variants was observed in persons from other ethnic populations, and in order to identify any longer alternative CAR transcripts, we designed primers residing in exon 1 and in the 3'-UTR of CAR. CAR was amplified from the livers of Caucasians, African Americans and Hispanics (Fig 1A) and the PCR products were analyzed by agarose gel electrophoresis (Fig. 1B). A CAR cDNA of the expected size was amplified in the livers of all ethnic groups along with several additional CAR transcripts. Multiple CAR splice variants were present in livers representing males and females of each ethnic group (Fig. 1B).

Using subcloning and sequencing, we characterized the additional CAR mRNA transcripts. Sixteen unique variants of hCAR were identified in the liver samples (Figure 2). The common splicing events were complete deletion of exon 2 (140 bp), exon 4 (170 bp), exon 7 (117 bp), and partial deletion of exon 9 (154 bp). Two previously identified insertions were also observed (insertion of 12 bp from intron 6 and insertion of 15 bp from intron 7) (Savkur et al., 2003; Auerbach et al., 2003). Each of the splicing events (4 deletions and 2 insertions) occurred in multiple combinations generating 16 isoforms of hCAR in human livers.

Several CAR splice variants were unlikely to encode functional proteins as they had premature termination codons and would be more likely to be rapidly degraded by nonsense mediated decay: SVs with an exon 4 deletion (SV4, SV9 and SV10) have a disrupted reading frame and introduce a

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premature termination codon. CAR-SV2 (derived from the use of a cryptic splice site in exon 2) had a deletion of 67 bp from exon 2 that introduced a premature termination codon in exon 3. SV15 had part of exon 4 deleted and also introduced a premature termination codon. In addition, SV16 had deletion of exons 3 through exon 9.

The other alternative mRNAs could encode unique CAR proteins. Deletion of exon 7 (Savkur et al., 2003; Auerbach et al., 2003) results in an in-frame loss of the 39 amino acids in the CAR ligand binding domain (LBD). Exon 9 is 258 bp in length and encodes 42 amino acids (the TGA stop codon is at nucleotide 128 in exon 9). Some SVs skipped the first 154 nucleotides of exon 9 resulting in the loss of the carboxy-terminal 42 amino acids. However, these same SVs utilize a cryptic AG splice site in exon 9 causing a frame-shift and encoding 29 unique carboxy-terminal amino acids. Although a splice variant of mouse CAR that skips exon 8 has been previously reported (Choi et al., 1997) (Fig 2), an orthologous hCAR-SV was not identified.

Expression profiling of hCAR isoforms in different tissues.

Because tissue specific splicing can lead to generation of unique alternatively spliced mRNAs, we screened multiple tissues for the presence of CAR and alternative CAR mRNAs. Following the first round of amplification, CAR.1 and alternative CAR mRNA transcripts were readily detected in adult and fetal liver, kidney, adrenal, small intestine and testis (Fig 3A). Lower CAR expression was also seen in brain. In contrast to liver where multiple mRNA transcripts were always amplified, heart, spleen, small intestine and prostate expressed a single CAR mRNA even after a second round of amplification using nested primers (Fig. 3A). Sequencing of the CAR

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mRNAs revealed a novel CAR-SV21 in heart with deletion of both exon 4 and 7, however, these deletions introduce a premature termination codon. CAR-SV3 present in spleen skips exon 2 and exon 7 generating a unique CAR mRNA. The single unique CAR transcript present in prostate, CAR-SV22, was characterized by deletion of exon 2. Only CAR.1 was observed in small intestine.

We next screened for CAR mRNAs in regions of the human brain (Fig 3B). CAR transcripts were only amplified in caudate nucleus and were distinct from the transcripts identified in other tissues. The caudate nucleus specific isoforms, CAR-SV17 to SV20 (Fig 2) all share deletion of exons 4, 5 and 7 with or without exon 2/exon 9 deletions. None of the caudate nucleus mRNAs would be expected to encode a functional protein as they have a premature termination codon. Although we detected CAR.1 in the total brain cDNAs (Fig 3A), CAR.1 was not amplified from the brain regions present in our analysis, including caudate nucleus, and must be present in other regions of the brain not represented in this panel of cDNAs.

Because some hCAR targets, CYP2B6 and MRP4, are present in human brain (Tyndale and Sellers, 2001; Assem et al., 2004), we screened these same samples for co-expression of these mRNAs. Although CYP2B6 was expressed in substantial amounts in caudate nucleus, it was also present in temporal lobe, cerebellum, hippocampus and spinal cord (Fig 3B). MRP4 was not present in caudate nucleus but was present in temporal lobe, cerebellum, hippocampus, medulla and spinal cord, with low expression in hypothalamus. Thus in most regions of the brain CYP2B6 was coexpressed with MRP4 but not with CAR.

Bioinformatic features of the alternative human CAR isoforms

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We identified a novel CAR SV with only deletion of exon 2 (SV22), or with exon 2 deleted in combination with other splicing changes (SV3, 4, 6, 7, 9, 10, 11, 12, 13, 15, 19, 20, and 22) (Fig. 2). Since the translation start site of hCAR is present in exon 2 it was not clear whether any alternative start site existed that could result in translation of a unique CAR protein in the absence of exon 2. We analyzed the sequence of hCAR cDNAs lacking exon 2 with an AUG evaluator program (<http://125.itba.mi.cnr.it/~webgene/wwwaug.html>) that predicted translation initiation sites. The ATG at position 220 (Genbank # Z30425) in exon 1 had a predicted initiation score of 0.83 compared to a score of 0.85 for the ATG in exon 2 (Fig 4A). This score is strongly in favor of translation initiating at the ATG in exon 1. However, if the ATG in exon 1 is used as an alternative translation initiation site, it would result in full length CAR protein only when there is deletion of exon 2. In the presence of exon 2 it encounters a premature termination codon after the first 9 amino acids. Translation initiation from the exon 1 ATG encodes six unique amino acids (Fig 4B). In the CAR-SVs that skip exon 2, the final two nucleotides in exon 1 and the first nucleotide in exon 3 form a codon encoding an arginine (R) exactly in-frame with the typical CAR protein (Fig 4A). Thus, compared to CAR.1 that encodes a 348 amino acid protein, all CAR-SV skipping exon 2 lack the usual amino terminal 35 amino acids but have instead six unique amino acids at the amino-terminus.

CAR cDNAs have now been cloned from a variety of model organisms. The availability of these genomic sequences from diverse species allowed us to align the nucleotides in exon 1 containing the alternative ATG initiator codon. There was striking conservation between species in the exon 1 sequence, including the ATG that was conserved in baboon, chimp and cow (Fig 4C). However, mouse and rat do not contain this ATG. Nevertheless, high evolutionary conservation

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among many species of the upstream ATG suggests they could also use this alternative translation start site.

Nuclear hormone receptors have a highly conserved DNA binding domain (DBD) that interacts in a very specific manner with cognate binding elements (Rastinejad et al., 1995). Splice variants with deletion of exon 2 would have a truncated DBD missing the first zinc finger (Fig 4D).

We next examined the features of the CAR-SVs that skip the first portion of exon 9. Although exon 9 typically encodes the 42 carboxy terminal amino acids of CAR, many of the CAR isoforms failed to use the canonical donor/acceptor splice site for exon 9 (Fig 5). These SVs instead used a cryptic AG splice site in exon 9 and encoded 29 unique carboxy terminal amino acids. Comparison of the CAR sequences available in GenBank revealed that this cryptic AG splice site is evolutionarily conserved in rat and mouse CAR (not shown). The alternative CAR transcripts lacking exon 9 are not a PCR artifact because there are two submissions in the AceView database (supporting clones AA887435 and AA918394) representing partial CAR cDNAs cloned from human kidney with the partial deletion of exon 9.

Function of CAR splice variants

To determine whether initiation occurred at the exon 1 ATG, we constructed expression plasmids for the CAR-SVs characterized by deletion of exon 2 alone (SV22), exon 2 deleted in combination with other splicing events (SV3, SV6, SV7, SV11, SV12), and CAR SVs with deletion of part of exon 9 in combination with other splicing events (e.g., SV1, SV14). Coupled in vitro transcription and translation experiments revealed that these CAR-SVs made a CAR protein (Fig 6). These CAR-SV isoforms also readily expressed the SV protein in both a bacterial (BL21 strain

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of *E coli*) and mammalian (Cos 7 cells) expression system (data not shown). A protein of the expected molecular weight consistent with translation initiation at the exon 1 ATG was observed for each CAR-SV skipping exon 2. An additional smaller protein was also observed for most of the CAR-SVs. Based on incorporation of radiolabeled methionine into the proteins, the higher molecular weight protein was synthesized to a greater extent as compared to the smaller molecular weight protein from each cDNA. To determine whether initiation did occur at the exon 1 ATG we mutated and eliminated the putative ATG start in SV6 and SV22. The mutated CAR-SV6 and SV22 failed to express the CAR protein with the higher molecular weight, consistent with translation from the exon 1 ATG. Thus, in CAR SV mRNAs skipping exon 2, translation can start at the exon 1 ATG and a full length CAR protein is made. However, the proteins still expressed a smaller molecular weight protein. Based on analysis of the CAR cDNA using the AUG predictor program it is most likely that the downstream ATGs, encoding Met 125 or Met 128 in exon 4 (Fig 6B) are the likely sites for alternative translation initiation.

hCAR isoforms demonstrate compromised binding to DNA and transactivation of the CYP2B6-LUC reporter

We used EMSAs to determine whether any of the SVs could bind to the CAR binding elements (NR1 or NR2) of the PBREM (phenobarbital response element module) in the CYP2B6 promoter (Sueyoshi et al., 1999). None of the SVs lacking exon 2 or exon 9 singly or in combination with other splicing events formed a complex with either the NR1 or NR2 in the presence or absence of RXR (Fig 7A). We next performed transfection experiments in HepG2 cells using a

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CYP2B6-LUC reporter and co-transfecting either CAR.1 or CAR-SV expression plasmids. Wild-type CAR dose-dependently activated CYP2B6-LUC reporter activity while all of the CAR-SVs failed to activate CYP2B6-LUC transcription either alone or in cells treated with the CAR ligand activator CITCO (Fig 7B) (Maglich et al., 2003).

Regulation of CAR expression

There is growing evidence that transcription and splicing are coincident events and that co-activator recruitment to nuclear hormone receptors can alter splice site selection (Auboeuf et al., 2002). To determine whether drug treatment could alter the expression of CAR and CAR-SV mRNAs we treated primary human hepatocytes with either a PXR (rifampin) or CAR (phenobarbital and CITCO) activator and examined expression of CAR mRNAs. Treatment with phenobarbital consistently induced the amount of CAR transcripts (Fig 8). However, CITCO, a ligand specific for human CAR did not induce expression of either CAR or PXR. Thus, CAR appears to be differentially regulated by its activators. The extensive number of alternatively spliced CAR transcripts that share splicing changes makes it impossible to generate primers that are unique to each CAR-SV and prevented us from determining whether there was any preferential induction of individual CAR isoforms by phenobarbital.

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DISCUSSION

Human Genomic diversity for any gene encompasses sequence variations as well as alternatively spliced mRNAs. Indeed, alternate splicing of mRNAs is thought to be one of the mechanisms generating protein diversity. According to recent estimates (Venter et al., 2001) between 22% and 59% of human genes are alternatively spliced. Alternative splicing can regulate the expression of the wild type mRNA, can show gain-of-function, loss-of-function or dominant negative activity (Christmas et al., 2001; Chandrasekharan et al., 2002; Rose et al., 2003). Polymorphic alternative splicing can cause human diseases and is a frequent mechanism of human variation in cytochrome P450 expression (<http://www.imm.ki.se/CYPalleles>). Bioinformatics approaches to identify splice variants exist that use a variety of statistical approaches to predict the probability that these are bonafide splice sites. The results are currently inadequate because the rules governing all factors that can influence splicing are still being identified (e.g., exon splice enhancers, exon splice silencers, levels of expression of SR proteins). The only definitive way to identify all alternatively spliced mRNAs is to PCR the transcripts and directly sequence them. Moreover, because tissue specific splicing is a common event that can further be influenced by race, development and gene transcription, it is necessary to identify the transcripts in multiple tissues and from persons representing different ethnic populations to capture the full range of alternative transcripts for any gene.

In this report we identified twenty two novel variant isoforms of hCAR, five of them uniquely expressed in extrahepatic tissues and 16 transcripts amplified from human liver. Of the 22 variants, thirteen had deletion of exon 2, eleven had deletion of exon 9, and seven had both exons 2 and 9 deleted in combination with other splicing events (in frame insertion of 12 bp from intron 6

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and/or insertion of 15 bp from intron 7, or deletion of exon 7). Our results are in contrast to recent reports that each identified the same three CAR-SVs with single alterations in the ligand binding domain (Auerbach et al., 2003; Savkur et al., 2003; Jinno et al., 2004) which have insertions of 12 bp and 15 bp singly or together and a transcript with deletion of exon 7. Several of the studies missed the variants that skip exon 2 and exon 9 because their amplification primers were located internal to the deleted fragment. In contrast, the primers used in the present study to amplify the cDNA of hCAR were designed such that the forward primers annealed in exon 1, 94 bp upstream of the exon 2 ATG and the reverse primers annealed downstream of the typical exon 9 stop codon. Nevertheless, it is highly likely that many of the CAR-SV identified are translated into protein in vivo as we saw in vitro. A recent report identified multiple proteins in human liver that immunoreacted with CAR antibody and that varied in molecular mass from ~28 to 39 kd (Auerbach et al., 2003). This polyclonal antibody was raised against a peptide that encodes the last 10 amino acids in exon 8 and the first 6 amino acids in exon 9. This antibody would likely recognize the majority of the CAR-SV we identified.

Our identification of the large number of CAR splice variants is consistent with an analysis of alternative splicing in the human genome that found that there are a higher number of alternatively spliced genes encoding receptors and other genes regulating signals, as compared with genes encoding metabolic functions (Modrek and Lee, 2002). The complexity of CAR alternative mRNAs exceeds that of many other nuclear hormone receptors. FXR variants have been identified with insertions in the hinge region (zhang et al., 2003), and PXR splice variants lack sequences in exon 5 encoding part of the ligand binding domain (Lamba et al., 2004). In contrast to the four previously identified CAR-SV that each had alterations in the ligand binding domain, some splice

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variants we identified skipped exon 2 resulting in usage of an alternative start site and yielding a unique amino terminal sequence (AF1 domain), but lacking a portion of the DNA binding domain. CAR-SVs that had partial deletions of exon 9 and instead utilized a cryptic AG splice site in exon 9 generate CAR proteins with a unique carboxy-terminal region (AF2 domain). Although some of the CAR-SVs had premature termination codons (25% of SV in liver and 40% in all tissues) and would not encode functional proteins, many of the CAR-SV would yield novel proteins (75% in liver and 60% in all tissues). This result is consistent with a recent survey of over 1000 alternatively spliced exons that reported that the majority (~78%) of alternative spliced variants were functional. Although some CAR mRNAs identified had a single splicing event, the majority of the CAR alternative mRNAs had multiple alternative splice events in a variety of combinations.

Our results also extend the list of tissues in which CAR is present. CAR has previously been reported in liver, intestine and adrenal gland. Using PCR we readily detected CAR in human kidney, liver, adrenal, testis, and fetal liver and at low levels in brain, heart, spleen, small intestine, placenta, prostate and skin. However, some of the CAR-SV were expressed in a tissue-specific fashion and the relative expression of the various CAR isoforms was significantly different between the tissues. For example, prostate does not express CAR.1, but exclusively expresses a CAR-SV that skips exon 2. The complexity of the CAR isoforms identified will make it very difficult, if not impossible, to quantitate individual transcripts. Although a recent study (Savkur et al., 2003) quantified by real-time PCR the amounts of CAR-SV with either the 12 and 15 bp insertions or with deletion of exon 7, our results now show that the primers utilized in that study would have quantitated multiple mRNA transcripts. Additionally, the primers used would not identify the unique CAR-SV isoforms present in some tissues.

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A majority of the CAR-SVs skipped exon 2 and thus lose the native ATG codon. These CAR-SVs encode novel isoforms of CAR that utilize an alternate start site in exon 1. In these variants 35 amino acids coded by exon 2 are replaced by 6 amino acids from exon 1. Interestingly, if the exon 1 ATG is used in the presence of exon 2, a premature termination codon is encountered after coding for 9 amino acids. Thus, translation initiating from the exon 1 ATG would never produce a full length CAR protein unless exon 2 is skipped. Upon skipping of exon 2, an in-frame full-length variant isoform is encoded with an altered NH₂ terminal end. The loss of exon 2 also deletes a portion of one of the zinc fingers in the DNA binding domain. Accordingly, transcripts skipping exon 2 had compromised ability to both bind DNA and transactivate CYP2B6 transcription. Nevertheless, the fact that the alternative ATG in exon 1 is evolutionarily conserved across species, and the fact that some nuclear receptors that lack the DNA binding domain, such as SHP (small heterodimer partner) can still heterodimerize with other receptors and negatively regulate their function (Seol et al., 1996), suggests that there may be an unidentified function for these CAR-SVs.

Eleven of the CAR SVs had partial deletion of exon 9. These CAR-SVs lose the normal 42 carboxy-terminal amino acids. However, these CAR-SVs utilize a cryptic splice site in exon 9 that is 23 bp downstream of the TGA stop codon and thus encode 29 unique carboxy-terminal amino acids. This deletion also results in the loss of helix 10, 11 and 12 in the ligand binding domain. Helix 12 contains a short transactivation function (AF-2) domain (Moras and Gronemeyer, 1998). The AF-2 domain is critical for the constitutive activity of CAR, mediating the ligand independent interaction of CAR with coactivators. It remains to be determined how the altered carboxy-terminus of these CAR-SVs affects interaction with co-activators or co-repressors.

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It is also possible that the CAR-SVs with partial deletion of exon 9 might have an altered cellular localization compared to CAR. CAR normally resides in the cytosol but upon treatment with phenobarbital translocates to the nucleus. Carboxy-terminal amino acids in CAR are required for this translocation because deletion of the 30 C-terminal amino acids abolishes the translocation (Zelko et al., 2001). In addition, carboxy-terminal amino acids in CAR are critical to ligand-dependent and –independent transactivation of CAR (Andersin et al., 2003). It remains to be determined whether the CAR-SVs with an altered C-terminus can translocate to the nucleus.

In conclusion, the present investigation identified 22 novel splice variants of hCAR that are expressed in a tissue-specific fashion. The identification of these cDNAs is the first step towards understanding their function and whether the transcripts are polymorphically expressed. hCAR isoforms with altered N-terminal and C-terminal ends have been identified thus providing increased complexity and diversity of CAR proteins. Some of the CAR-SVs had alterations in functional domains, such as loss of one of the zinc fingers necessary to dock at the CAR binding element in DNA and resulting in their compromised ability to bind to or to transactivate the CYP2B6 promoter. Nevertheless, some of these CAR-SVs may have unique functions as has been shown for SV isoforms of other genes. Indeed, despite loss by some SV proteins of what are considered required functional domains, these SVs can have unique functions (Rose et al., 2003). Nuclear hormone receptors lacking DNA binding domains, such as SHP, can still be functional (Seol et al., 1996), and nuclear hormone receptors can also have distinctive functions outside the nucleus (Li et al., 2000). Thus, it is possible and likely that some of the CAR-SVs have unique functions that remain to be identified.

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Footnotes

¹ Abbreviations used: CAR, constitutive androstane receptor; wild-type CAR (CAR.1); alternatively spliced CAR mRNAs (CAR-SV).

²Normal human liver (processed through Dr. Mary Relling's laboratory at St. Jude Children's Research Hospital) and hepatocytes were obtained through the Liver Tissue Procurement and Distribution System (Pittsburgh, PA), which was funded by NIH contract #N01-DK-9-2310 and by the Cooperative Human Tissue Network." This work is supported in part by NIH grant GM60346, by the NIH/NIGMS Pharmacogenetics Research Network and Database (U01GM61374, <http://pharmgkb.org>) under grant U01 GM61393, by NIH grant P30 CA21765 Cancer Center Support grant and by the American Lebanese Syrian Associated Charities (ALSAC).

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Legends to Figures

Figure 1. Alternatively spliced isoforms of CAR are present in the liver samples. A) A schematic representation of the CAR cDNA showing the position of the forward and reverse primers, and ATG and TGA codons. B) cDNAs obtained from liver samples of African American, Hispanic and Caucasian females and males were amplified using F1/R1 primers. An arrow indicates the wild-type CAR.1 band. All the liver samples showed multiple CAR-SV transcripts.

Figure 2. Schematic representation of alternatively spliced variants of CAR. All the exons are numbered, shaded boxes represent the insertions and the deletions are represented by gaps. The shaded area represents DNA and ligand binding domains. Stop codons are depicted by an asterisk (*) and brain specific isoforms are depicted by a double asterisk (**). The wild-type and alternatively spliced isoforms of mouse CAR are indicated. The right hand panel shows the size of the amplified product following F2/R2 amplification, the predicted molecular weight if the variant forms makes a full length protein, number of amino acids in different isoforms and the alternatively spliced events of each isoform. PTC, Premature termination codon; aa, amino acid; nt, nucleotide; MW, molecular weight; del, deletion; ins, insertion.

Figure 3. Tissue distribution of hCAR variant transcripts. A) Distribution of CAR transcripts in different tissues. CAR cDNA was amplified from first strand cDNA templates of 22 adult and 2 fetal tissues using first round primers (top panel) and second round nested primers (bottom panel). An arrow indicates the CAR WT band. Heart, spleen and prostate contained only a single CAR-SV transcript, and small intestine contained only CAR.1. B) Distribution of CAR

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transcripts in different regions of the brain. First strand cDNA from different regions of the brain was amplified as in Fig 3A. The top panel shows first round amplicons and the bottom panel shows 2nd round-nested amplicons. Amplification of two target genes of CAR: CYP2B6 (3rd panel) and MRP4 (4th panel) was also performed in different regions of the brain.

Figure 4. CAR-SVs with deletion of exon 2. A) Exon 1 ATG is a potential alternate start site. The nucleotide sequence of part of exon 1, exon 2 (shaded gray) and exon 3 and the amino acids encoded by them are shown. The normal start codon is in exon 2 (asterisk). A potential alternate start site was identified in exon 1 (asterisk). B) Alignment of the amino terminal ends of wild-type CAR.1 and CAR-SVs that skip exon 2. C) Conservation of the alternate start site in different species. Amino acids flanking the ATG in exon 1 from various species were aligned using ClustalW. The conserved ATG is shaded. The mouse contains a CTG instead of ATG. Genbank Accession numbers for different species are: Human: Z30425, Cow: BM433108, Mouse: NM_009803 and Rat: NM_0022941). D) Structure of the DNA binding domain of CAR.1 versus CAR-SV with deletion of exon 2. Amino acids in exon 2 and 3 in the DNA binding domain of CAR.1 were modeled based on the structure of the steroid hormone receptor DBDs (Rastinejad et al., 1995). Zinc fingers (ZF) are indicated along with the zinc-coordinating cysteines. The amino terminus of the CAR-SV lacking exon 2 and utilizing the alternative ATG in exon 1 is indicated in the gray box.

Figure 5. CAR-SVs with partial deletion of exon 9. A) The amino acid sequences of the C-termini of CAR.1 and CAR-SV with partial deletion of exon 9. B) Schematic representation of the

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alternative splicing in exon 9. The consensus splice junction at the boundary of exon 8 and exon 9 and the cryptic *AG* in exon 9 are shown. The splice site acceptor scores for these sites (http://www.fruitfly.org/seq_tools/splice.html) are indicated. Skipping of the consensus exon 9 splice site and utilizing the cryptic *AG* in exon 9 generates these CAR-SVs. Partial deletion of exon 9 deletes 42 C-terminal amino acids, and use of the cryptic splice site adds 29 unique C-terminal amino acids to the CAR-SV. The reading frame is shown below each sequence.

Figure 6. In vitro expression of human CAR isoforms. A) CAR.1 and CAR-SVs were expressed by in vitro coupled transcription and translation using a rabbit reticulocyte lysate system and ³⁵S-methionine. The radiolabeled products were resolved on acrylamide gels and analyzed using a phosphoimager. Above each lane is the alternate splicing event associated with the variant isoform. The last two lanes show the mt-SV22 and mt-SV6 variant isoforms with the ATG start site in exon 1 mutated to TTG. The molecular weights are indicated. B) The smaller molecular weight protein in panel A was analyzed by the ATG evaluator software program and the two probable start codons corresponding to Met125 and Met128 are indicated with the predicated initiation scores.

Figure 7. CAR-SVs demonstrate compromised binding to the CYP2B6 PBREM and transactivation of CYP2B6-LUC. A) EMSAs were performed using ³²P-labelled CYP2B6-NR1/2 response elements and in vitro translated CAR isoforms and hRXRa protein in the absence (no competitor) or presence of unlabeled oligonucleotide (50-100 fold excess). EMSAs with the in vitro translated backbone vectors pCDNA3.1 and pSG5 are represented in last two lanes. After

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electrophoresis, complex formation (indicated by arrow) was assessed by phosphoimager. B) HepG2 cells were transfected with 200 or 400 ng expression construct (empty vector, CAR.1 or CAR-SV), CYP2B6-LUC, and 100 ng of pSV- β -galactosidase control vector and treated with vehicle or 100 nM CITCO. Results are expressed as fold increase in CYP2B6-LUC by co-transfected CAR plasmids treated with and without CITCO. The experiment was run multiple times in triplicate and representative results are shown where each bar represents the mean \pm SD for three different measurements.

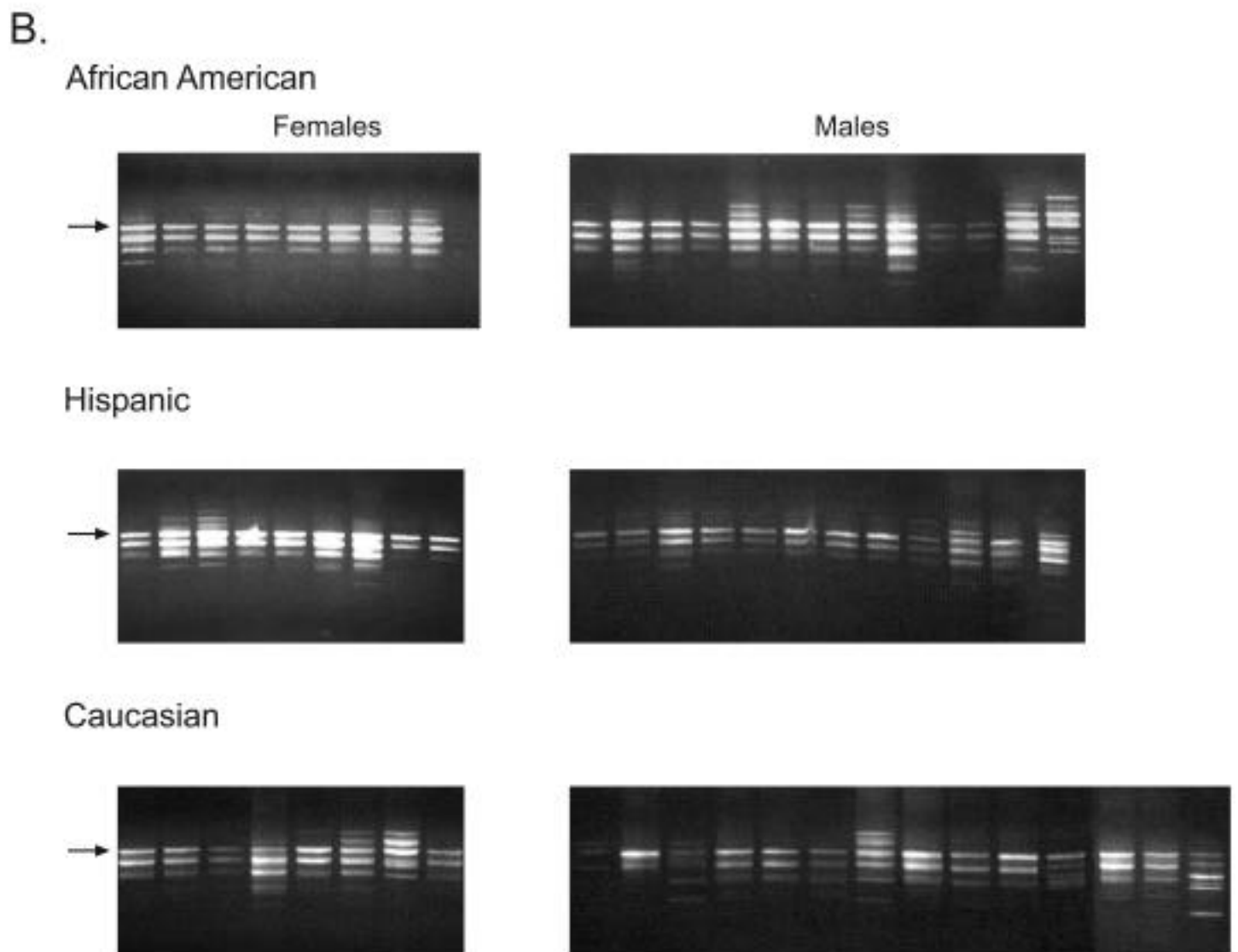
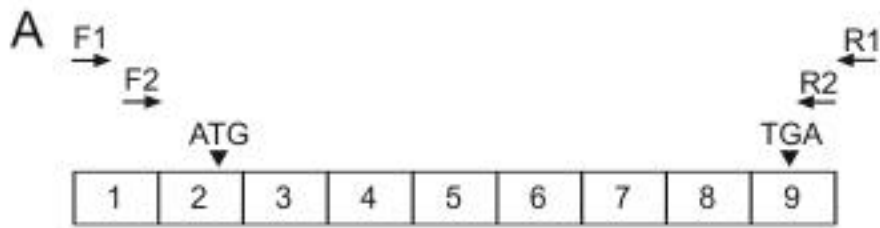
Figure 8. Effect of drug treatments on CAR expression in primary human hepatocytes.

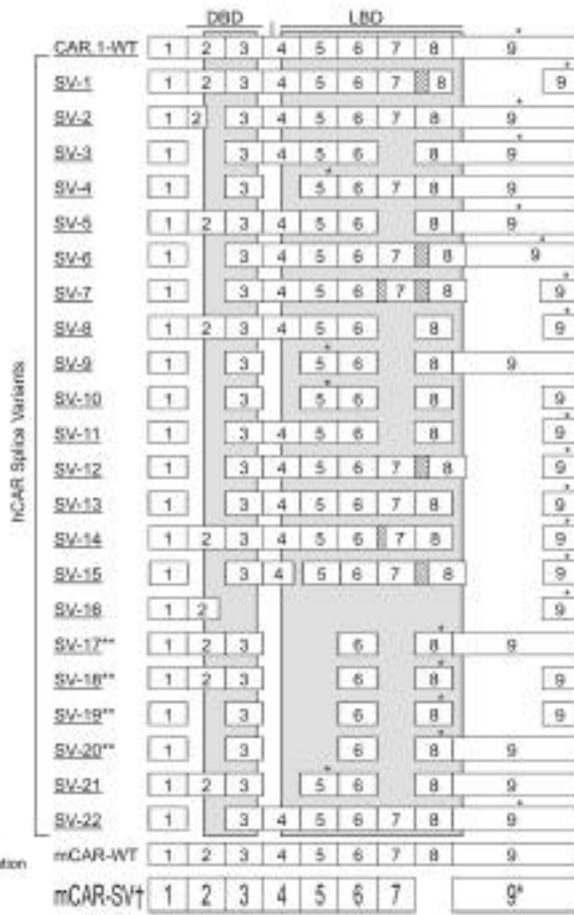
Hepatocytes from six donors (#73, 76, 87, 90, 92 and 95) were treated for 48 h with DMSO vehicle (D), 1 mM phenobarbital (P), 10 μ M rifampin (R), or 100 nM CITCO (C). RNA was isolated, reverse-transcribed and CYP2B6, CYP3A4, PXR.1 and PXR.2 (splice variant), CAR, and GAPDH mRNAs amplified from first-strand cDNAs and resolved on agarose gels.

Table-1. Sequence and conditions of oligos used for PCR amplification and EMSA

Primer name	Sequence (5'-3')	Conditions
Oligos for amplifying the cDNA		
CAR-F1	GGAGAGGCATTCCATACCAG	94°C x 30 sec, 58°C x 30 sec, 72°C x 90 sec, 32 cycles
CAR-R1	TTCCCACTCCAGTGTATCCAG	
CAR-F2	CAGGTGACATGCTGCCTAAG	94°C x 30 sec, 57.3°C x 30 sec, 72°C x 90 sec, 15 cycles
CAR-R2	TATCCAGGGTGTCCAGGTG	
CAR-Taq-F	CCAGCTCATCTGTTCATCCA	94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec, 30 cycles
CAR-Taq-R	GGTAACTCCAGGTCGGTCAG	
CYP2B6-F	CACTCATCAGCTCTGTATTTCG	94°C x 30 sec, 57°C x 30 sec, 72°C x 30 sec, 30 cycles
CYP2B6-R	GTAGACTCTCTCTGCAACATGAG	
MRP4-F	GCAAGATGCTGCCCGTGTAC	94°C x 30 sec, 60°C x 30 sec, 72°C x 30 sec, 30 cycles
MRP4-R	ACCCTTGCAACTCCTCTCCAAG	
Oligos for EMSA		
CYP2B6-NR1	GATCCTGTACTTTCTGACCCTGAGATC	
CYP2B6-NR2	GATCCATGGACTTTCCTGAACCAAGATC	
Oligos used for creating ATG mutants		
CAR-ATG-exon1-220-F	GTGGCCTGCAGGTGACTT G GCTGCCTAAGAGAAGC*	95°C x 30 sec, 55°C x 30 sec, 68°C x 12 min, 12 cycles
CAR-ATG-exon-220-R	GCTTCTCTTAGGCAGCAAGTCACCTGCAGGCCAC*	
Primers used to amplify the cDNA from Hepatocytes.		
CYP3A4-F	CCAAGCTATGCTCTTCACCG	95°C x 30 sec, 50°C x 30 sec, 72°C x 30 sec, 28 cycles
CYP3A4-R	TCAGGCTCCACTTACGGTGC	
CYP2B6-F	TCCTTTCTGAGGTTCCGAGA	95°C x 30 sec, 58°C x 30 sec, 72°C x 30 sec, 35 cycles
CYP2B6-R	TCCCGAAGTCCCTCATAGTG	
PXR-F	CAAGCGGAAGAAAAGTGAACG	95°C x 30 sec, 60°C x 30 sec, 72°C x 30 sec, 28 cycles
PXR-R	CTGGTCTCGATGGGCAAGTC	
GAPDH-F	ACCACAGTCCATGCCATCAC	95°C x 30 sec, 60°C x 30 sec, 72°C x 30 sec, 25 cycles
GAPDH-R	TCCACCACCCTGTGCTGTA	

* bolded nucleotide indicates the base mutated to change ATG to TTG

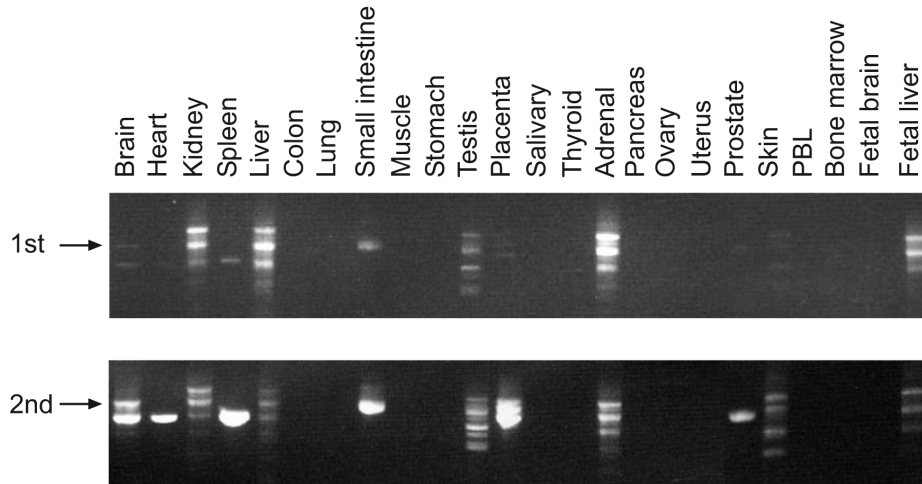




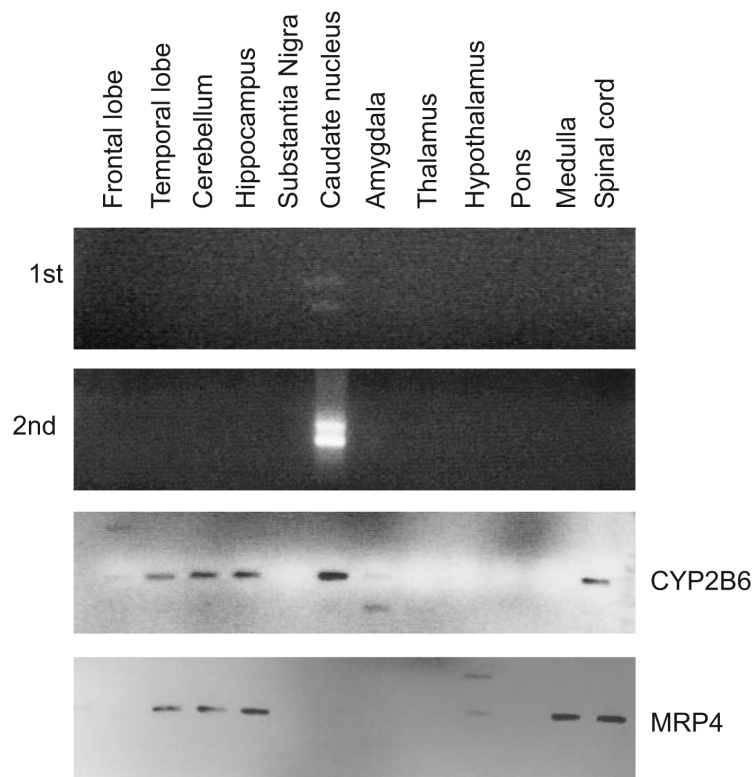
* = Stop codon
 ** = Brain specific
 PTC = Premature termination codon
 † = Choi et al, 1997.

Size (nt)	Predicted protein MW (Kda)	Amino acid	Changes
1153	~39.66	348aa	WT
1062	~38.5	346aa	ins15bp, partial del Ex9
1086	PTC		partial del Ex2
896	~31.8	280aa	del Ex2, del Ex7
843	PTC		del Ex2, del Ex4
1036	~35.0	309aa	del Ex7
1028	~36.96	324aa	del Ex2, ins 15bp
934	~35.7	315aa	del Ex2, ins 15bp, 12bp, partial del Ex9
930	~33.4	296aa	del Ex7, partial del Ex9
726	PTC		del Ex2, del Ex4, del Ex7
620	PTC		del Ex2, del Ex4, del Ex7, partial del Ex9
790	~30.3	267aa	del Ex2, del Ex7, partial del Ex9
822	~35.32	311aa	del Ex2, ins15bp, partial del Ex9
907	~34.77	305aa	del Ex2, partial del Ex9
1059	~38.3	339aa	ins 12bp, partial del Ex9
836	~31.7	360aa	del Ex2, partial del Ex4, ins15bp, partial del Ex8
120	~5.3	47aa	partial del Ex2 to partial del Ex9
726	PTC		del Ex4, del Ex5, del Ex7
620	PTC		del Ex4, del Ex5, del Ex7, partial del Ex9
480	PTC		del Ex2, del Ex4, del Ex5, del Ex7, partial del Ex9
586	PTC		del Ex2, del Ex4, del Ex5, del Ex7
866	PTC		del Ex4, del Ex7
1013	~36.4	319aa	del Ex2
	40.8	358aa	WT
	~33	284aa	del Ex8

A. Human Multitissue



B. Human Brain



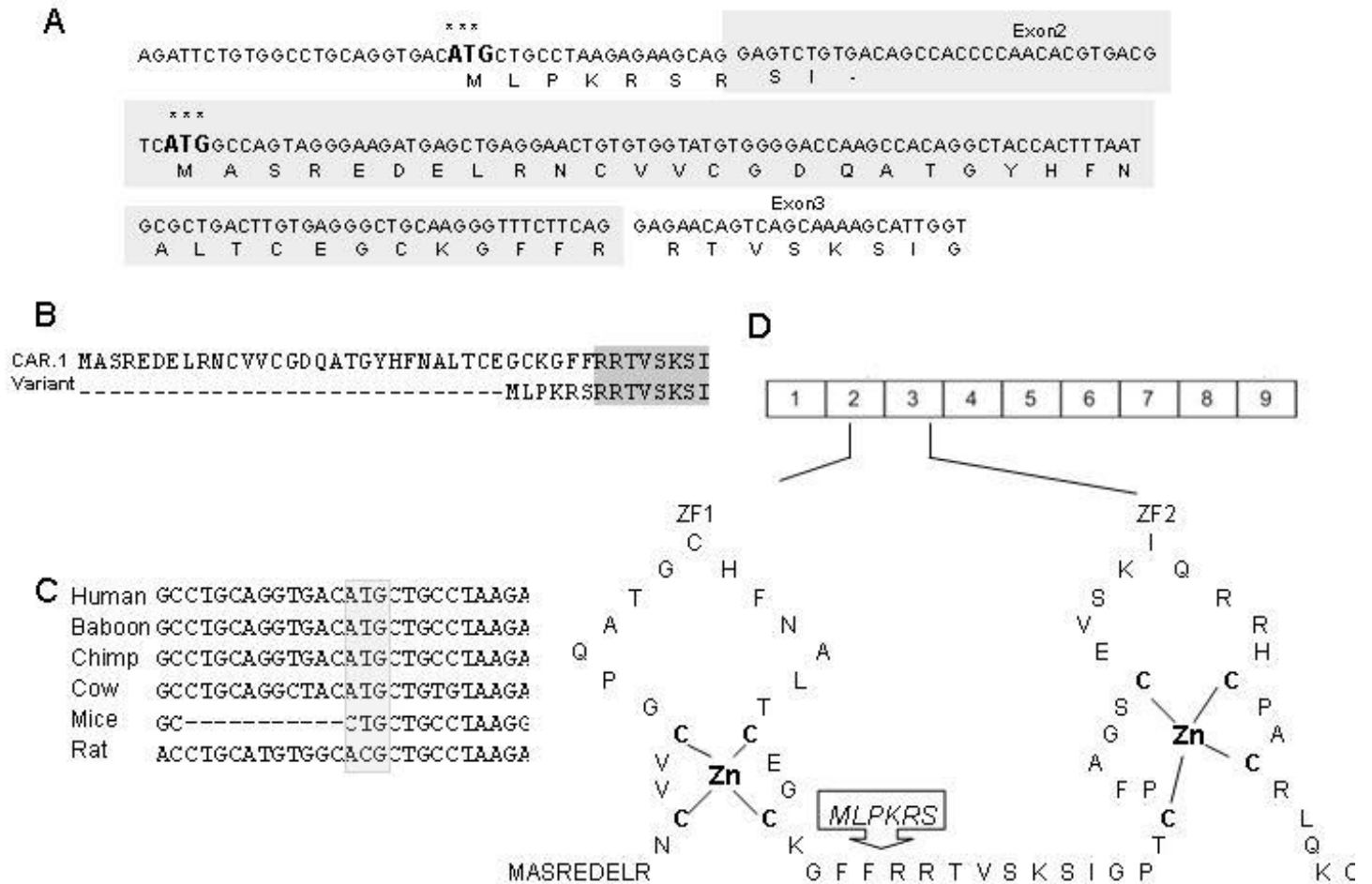


Figure 4

A

	Exon8		Exon9
CAR.1	GQQRPRDR	FLYAKLLGLLAELRSINEAYG-	YQIQHIQGLSAMMPLLQEICS
Variant	GQQRPRDR	SPGTPWIHWSGKMLGPKIGPGSKGAQWLQ	-----

B

	Score 0.87		Score 0.98
Exon8	Exon9		
GATCgt	ag	TTTCTG.....CAGGAGATCTGCAGCTGA	GGCCATGCTCACTTCCTTCCCCagCTCACCTGGACACCC
D R	F L	Q E I C S .	S P G T P
TGGATACACTGGAGTGGGAAAATGCTGGACCAAAGATTGGGCCGGGTTCAAAGGGAGCCCAGTGGTTGCAATGA			
W I H W S G K M L G P K I G P G S K G A Q W L Q .			

figure5

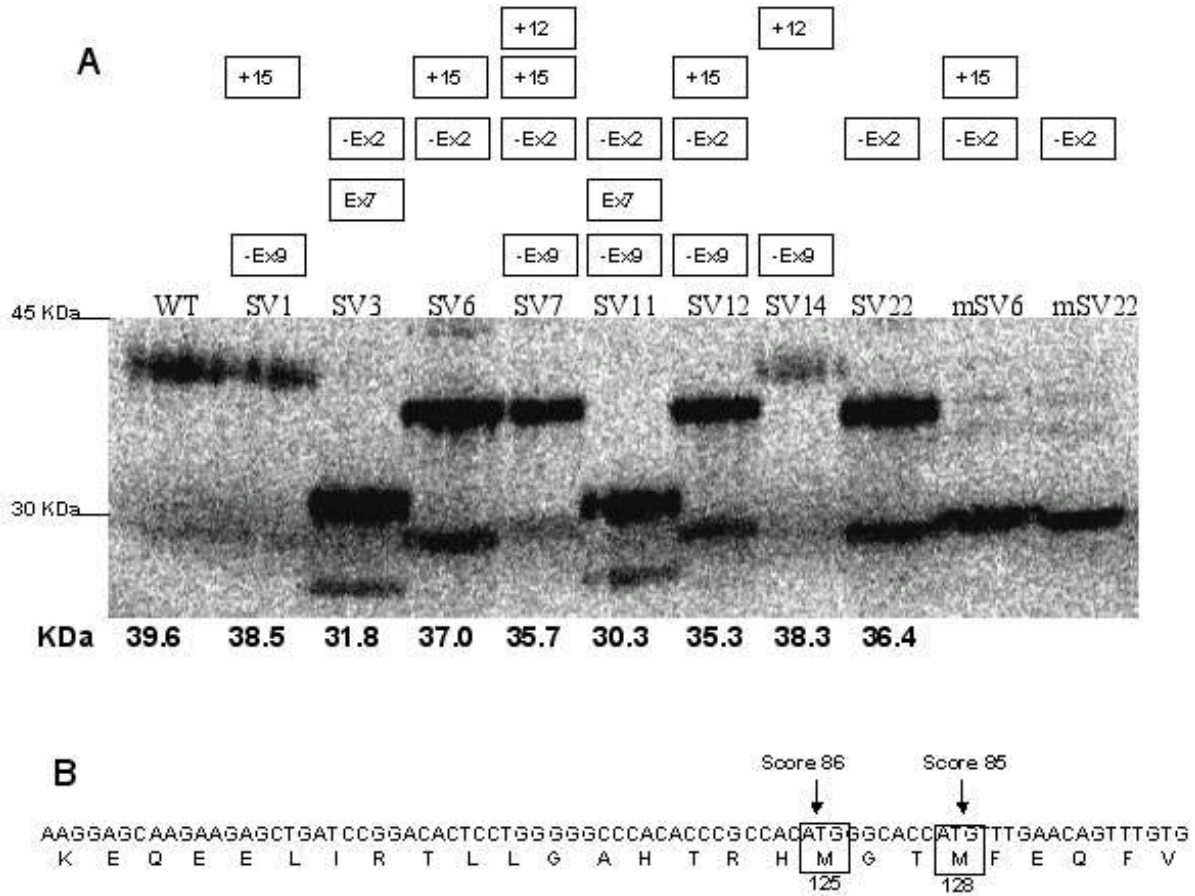


Fig 6

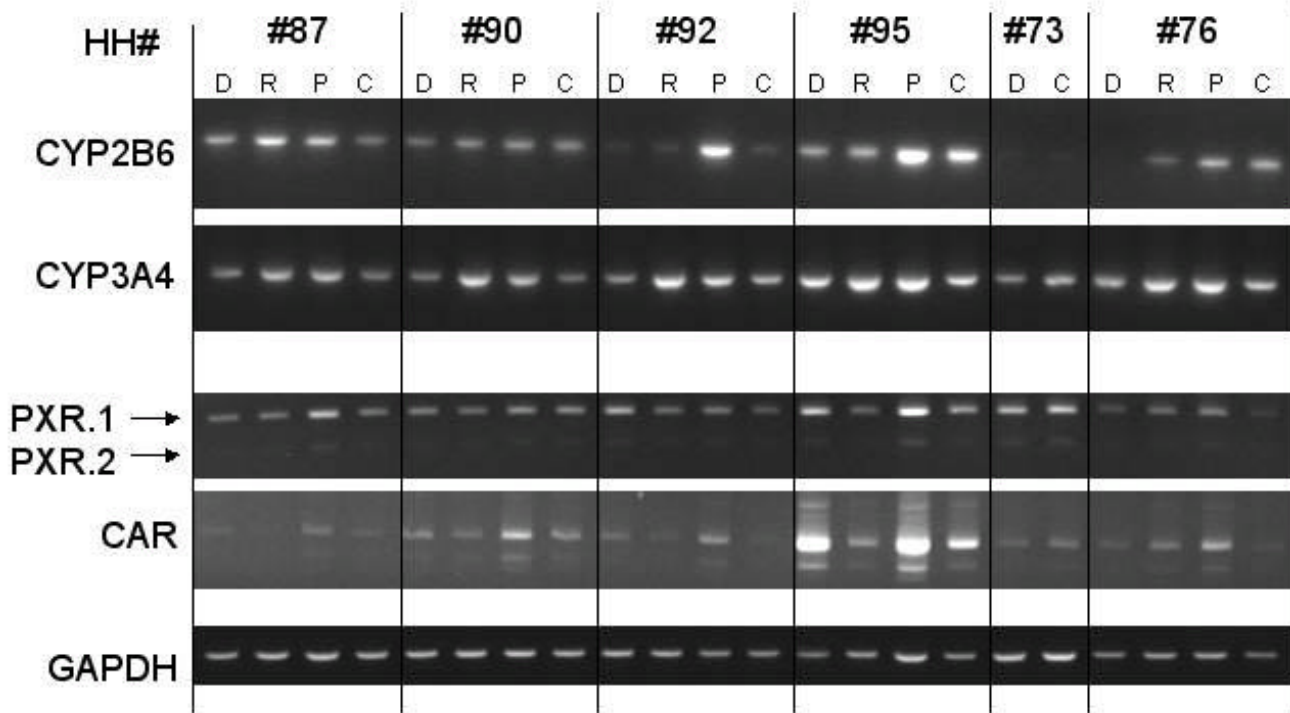


Fig 8