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Transactivation of a DR-1 PPRE by a human constitutive androstane receptor variant expressed from internal protein translation start sites

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ABSTRACT

Downstream in-frame start codons produce amino-terminal-truncated human constitutive androstane receptor protein isoforms (ΔNCARs). The ΔNCARs are expressed in liver and *in vitro* cell systems following translation from in-frame methionine AUG start codons at positions 76, 80, 125, 128, 168 and 265 within the full-length CAR mRNA. The resulting CAR proteins lack the N-terminal DNA-binding domain (DBD) of the receptor, yielding ΔNCAR variants with unique biological function. Although the ΔNCARs maintain full retinoid X receptor alpha (RXRα) heterodimerization capacity, the ΔNCARs are inactive on classical CAR-inducible direct repeat (DR)-4 elements, yet efficiently transactivate a DR-1 element derived from the endogenous PPAR-inducible acyl-CoA oxidase gene promoter. RXRα heterodimerization with CAR1, CAR76 and CAR80 isoforms is necessary for the DR-1 PPRE activation, a function that exhibits absolute dependence on both the respective RXRα DBD and CAR activation (AF)-2 domains, but not the AF-1 or AF-2 domain of RXRα, nor CAR's DBD. A new model of CAR DBD-independent transactivation is proposed, such that in the context of a DR-1 peroxisome proliferator-activated response element, only the RXRα portion of the CAR-RXRα heterodimer binds directly to DNA, with the AF-2 domain of tethered CAR mediating transcriptional activation of the receptor complex.

INTRODUCTION

CAR (NR1I3) is a member of the nuclear receptor superfamily, comprised of 48 members in humans (1). The structural features of CAR include a clearly identifiable DNA-binding domain (DBD) but the lack of a conventional amino-terminal AF-1 motif found in most other nuclear receptors (2). While nuclear receptors are typically activated by chemical ligands, CAR maintains a high constitutive activity, limited by the cytosolic subcellular localization of the unactivated protein (3). CAR appears to be retained in a cytoplasmic complex with phosphatase 2A (PP2A), heat shock protein 90 (Hsp90) (4) and a cytosolic CAR retention protein, termed CCRP (5). Ligand and non-ligand activators, such as 6-(4-chlorophenyl)imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) (6) and phenobarbital, respectively (3), induce CAR cytoplasmic-to-nuclear translocation through a pathway involving an okadaic-acid-sensitive phosphatase (3,7).

Early studies with CAR revealed that certain androstane derivatives bind directly to the receptor and function to inhibit its transactivation. These repressive ligands were termed 'inverse agonists' (8). Furthermore, inverse agonist-bound CAR can be reactivated by exposing the receptor complex to specific inducer chemicals (9,10), such as, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), a ligand activator of mouse CAR (11); and CITCO, the human CAR-specific agonist ligand (6).

CAR targets include genes encoding phase I and phase II drug metabolizing functions as well as drug transport genes [reviewed in (12)]. The target gene promoters often contain consensus DNA response elements including direct repeat (DR)-2 (13), DR-3, DR-4 (14), DR-5 (2) and everted repeat (ER)-6 or ER-8 (15) motifs. CAR may bind the DNA response elements as a monomer or as a heterodimer with retinoid X receptor alpha (RXR) (15,16). In some cases, at least two separate elements may cooperate in a single gene promoter to further enhance CAR/RXR-mediated transcriptional activation (17,18). Like many nuclear receptors, CAR-mediated transactivation also depends on its association with

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nuclear co-regulator proteins, including the co-activators, GRIP-1 (19), SRC-1 (20) and PGC-1 (21).

In addition to the reference form of human CAR, termed CAR1, we and others have characterized alternatively spliced CAR transcripts that exist in human tissues (22–24). At least one of these CAR variants, CAR3, possesses the unique biological property of being a ligand-activated receptor, in contrast to the constitutively active nature of CAR1 (14). The results presented in the current investigation extend the complexity of CAR regulation by demonstrating that the receptor's expression is post-transcriptionally modulated through the use of internal translation start sites, resulting in the generation of truncated CAR proteins with altered biological function. Our data also suggest a new model for CAR–DNA interaction, whereby RXRα binds directly to a DR-1 peroxisome-proliferator-activated response element (PPRE) as a heterodimer with tethered CAR, such that only RXRα makes direct DNA contact, with the tethered CAR AF-2 allowing for transcriptional activation.

EXPERIMENTAL PROCEDURES

Cell lines, chemicals and biochemicals

COS-1 African green monkey kidney epithelial cell line (ATCC, Manassas, VA) was cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Gaithersburg, MD) plus 10% fetal bovine serum, 1.5 g/l sodium bicarbonate, HEPES, L-glutamine and penicillin–streptomycin. 5α-Androstan-3α-ol (androstanol) and 9-*cis*-retinoic acid (RA) (Sigma, St. Louis, MO), CITCO (BioMol, Plymouth Meeting, PA), and Wyeth 14643 were dissolved in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO). Enriched primary human hepatocyte cultures plated on collagen were obtained through the Liver Tissue Procurement and Distribution System, Pittsburgh, funded by NIH Contract #NO1-DK-9-2310. Cells were placed in fresh William's E media containing: 1% penicillin–streptomycin, 1% HEPES, 20 μM glutamine, 25 nM dexamethasone, 10 nM insulin, 1% linoleic acid/BSA, 5 ng/ml selenious acid and 5 μg/ml transferrin and overlayed mL BD Matrigel™/mL BD Matrigel™ Basement Membrane Matrix (BD Biosciences, San Jose, CA), as described previously (25). Adenovirus expressing human CAR was produced using a commercially available system AdEasy (Stratagene, Cedar Creek, TX). Primary hepatocytes from one individual were infected with Adenovirus-CAR (Adv-CAR) for 24 h, cells were scraped in Laemmli's loading buffer and a small aliquot was resolved by SDS-PAGE, transferred to PVDF membrane and CAR detected by immunoblotting. Lentiviral expression plasmids, pCDH1-MCS1-EF1-copGFP and pSIH1-H1-copGFP, and packaging plasmid mix, pPACKH1, were purchased from System Biosciences (Mountain View, CA). Production of lentiviral particles and subsequent infection of target cells were performed essentially according to the manufacturer's protocols, except that Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection of HEK-293T packaging cells. Pseudoviral supernatant harvested from the packaging cells was either used directly for target cell infection or was concentrated prior to infection by 10% PEG-8000 (Sigma, St. Louis, MO) precipitation. Primary human hepatocytes were infected with equal volumes of appropriate lentiviral supernatant in the presence of 6 μg/ml polybrene (Sigma, St. Louis, MO), and media was replaced the following day.

Oligonucleotides and plasmids

Oligonucleotides and PCR primers were synthesized by Integrated DNA Technologies (IDT) (Coralville, IA). PCR primers and EMSA oligonucleotides used are listed in Supplementary Data. A consensus DR-4 probe used in EMSA competition experiments contained the NR1 sequence from CYP2B6 PBREM and the sense strand contained the classical DR-4 element AGTTCA$(N)_4$AGTTCA. Reporter plasmids contained four copies of the rat acyl-CoA-oxidase (rAox) PPRE (26), a PCR fragment from rat cellular retinol-binding protein II promoter (rCRBPII) (27) and three copies of an optimized AGTTCA DR-4(14). Complementary oligonucleotides were annealed, phosphorylated and inserted in the Sma*I* site of ptk-Luc, derived from pGL3-Basic vector (Promega, Madison, WI), which was modified by insertion of the minimal thymidine kinase (tk) element from pBLCAT. prAox-1198/-463-tk-Luc contains a single copy of the PPRE (DR-1: tgacctttgtcct) in its endogenous context, encompassed by the region between −1198 and −463 bp upstream of the rAox gene transcription start site and was amplified using rat genomic DNA as a template. Primer sequences employed were as previously described (28), but with slight modifications; the sense primer (5′-GGT ACC GGT ACC CCA GTA GAA CCT TGT TCA GG-3′) and antisense primer (5′-GGT ACC GCT AGC CAG GGT CTC GGG CGG AGT GAA G-3′) contained the underlined KpnI and NheI restriction enzyme sites, respectively. The 754-bp amplicon was gel-purified, restriction enzyme digested, gel-purified and ligated into the ptk-Luc vector (Promega, Madison, WI). Plasmids were prepared using QIAfilter Plasmid Maxi Kit (Qiagen, Valencia, CA) and sequenced.

Coupled *in vitro* transcription translation

Rabbit reticulocyte lysate and wheat germ expression systems were used essentially according to the manufacturer's (Promega, Madison, WI) protocols. DNA templates were in the form of plasmids containing CAR-coding sequence downstream of T7 promoter, or PCR products of T7 promoter-tagged CAR fragments. In some experiments, [^{35}S]methionine (ICN) was incorporated, while in other experiments a polyclonal antibody against human CAR (23) was used to perform western blotting.

Western-immunoblotting analysis

Cells were harvested directly in 1× Laemmli's loading dye and boiled for 5 min. Alternately, cells were scraped in radioimmunoprecipitation assay (RIPA) buffer, protein concentrations were measured by Bradford method,

and then samples were mixed with Laemmli's buffer (BioRad, Hercules, CA). For western blots examining expression of endogenous CAR variants in primary human hepatocytes, the cells from four different human donors were cultured as described above, harvested by scraping in Trizol reagent (Invitrogen, Carlsbad, CA) and protein samples were isolated according to the extended manufacturer's protocol. One-hundred and fifty micrograms of total protein were dissolved in loading buffer and were resolved by SDS-PAGE and separated proteins were transferred to PVDF membrane (BioRad, Hercules, CA), then probed with primary antibodies against human CAR (generated in our laboratory) or RXRα (D-20) (Santa Cruz). Secondary antibody goat–anti-rabbit-IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:5000 dilution. Blots were exposed to LumiLight chemiluminescent substrate (Roche Applied Science, Indianapolis, IN) and placed on autoradiography film (American X-Ray & Medical Supply, Sacramento, CA). PVDF membrane was stained with Ponceau S in dilute acetic acid and briefly washed in de-ionized water until distinct protein bands could be distinguished. Pink-stained blots were scanned and images were changed to grayscale for publication.

Transient transfections

p(NR1)$_5$-Tk-Luc and pCMV-Renilla expression plasmid (Invitrogen, Carlsbad, CA) (for normalization of transfection efficiency) and pCDNA3.1-CAR expression plasmids were transiently transfected into COS-1 cells using Fugene6 (Roche Applied Science) for 12–24 h. Transfection cocktails were prepared essentially according to the manufacturer's recommendations, except that 1× phosphate-buffered saline (PBS) was substituted for serum-free medium in the transfection cocktail. Cells were harvested with 50 µl 1× passive lysis buffer (Promega, Madison, WI) and soluble protein was extracted and assayed for luciferase activity using Stop-'N'-Glow system (Promega) and dual auto-inject luminometer (Turner Biosystems, Sunnyvale, CA). Assay reagents were diluted 1:1 with Tris-buffered saline and 25 µl of each reagent was used to measure Firefly and *Renilla* luciferase activity in 40 µl of cell lysate. Experiments were repeated at least two times and representative results are shown as mean ± standard error for three or four replicates per treatment group.

Preparation of COS-1 cell nuclear extracts

Nuclear extracts were prepared as described (29). Briefly, transfected COS-1 cells were harvested in 1× Promega lysis buffer and allowed to swell on ice for 15 min, centrifuged at 14000 × *g* for 1 min at room temperature, and a pellet containing crude nuclei was isolated. Approximately five pellet volumes of 1× lysis buffer supplemented with 500 mM KCl (high-salt buffer) were added to the pellet and further incubated on ice for 30 min to 1 h with frequent vortexing. Nuclei in high-salt buffer were centrifuged at 14000 × *g* for 1 min at room temperature, and aliquots of nuclear proteins in supernatant were stored at −80°C for use in EMSA.

Electrophoretic mobility shift assays (EMSAs)

A 20–30 µl EMSA reaction mixture contained ~75–150 mM KCl (a balance of low-salt and high-salt buffers used in the nuclear extraction protocol), ~5 µg of crude nuclear protein, 1 µg poly(dI-dC) (Roche Molecular Biochemicals, Indianapolis, IN), with or without unlabeled competitor oligonucleotide and 10–100-fmol-labeled probe. Oligonucleotides were end labeled by incubation with T4-polynucleotide kinase (Promega, Madison, WI) and [γ^{32}P]-ATP (ICN/MP Biomedicals, Solon, OH). Reactions were incubated at room temperature for 15 min and protein–DNA complexes were resolved by 5% PAGE and specific DNA–protein complexes were observed as more slowly migrating complexes in the gel. Gels were dried under vacuum and exposed to X-ray film.

CAR immunofluorescence microscopy

Cells were grown on Lab-Tek 4-well chamber slides (Nunc, Naperville, IL) and fixed in −20°C methanol, air-dried, then permeabilized and rehydrated in PBS supplemented with 0.3% Tween-20 (PBS-Tween). Cells were blocked in goat serum, washed and incubated with rabbit polyclonal CAR antibody (1:50 dilution). Primary antibody was washed from cells and fluorescein isothiocyanate (FITC)-conjugated goat–anti-rabbit IgG (CALTAG Laboratories, Burlingame, CA) diluted 1:200 in antibody dilution buffer was placed on the cells for 2 h at room temperature. Finally, slides were extensively washed in PBS-Tween, blotted dry, covered with Prolong Anti-fade mounting solution with 4′,6-diamidino-2-phenylindole (DAPI) DNA-labeling reagent (Molecular Probes, Eugene, OR) and sealed with a coverglass. Immunofluorescence of CAR protein was observed with a Nikon inverted fluorescence microscope (Nikon USA, Melville, NY). Image capture was performed with SpotRT software and digital camera (Diagnostic Instruments, Sterling Heights, MI).

Real-time PCR methods and data analysis

RNA was isolated from primary human hepatocytes using TRIzol Reagent (Invitrogen, Carlsbad, CA) and converted to cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), both according to the manufacturer's protocols. Real-time PCR was performed using Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions, with minor modifications and run on an ABI 7300 Real-time PCR System (Applied Biosystems). Real-time PCR data were analyzed using the $\Delta\Delta C_T$ method (30).

RESULTS

Initial identification of truncated CAR proteins

Primary human hepatocytes express multiple nuclear receptors. In Figure 1A, we present results from western immunoblot assays performed on whole-cell extracts derived from primary hepatocytes of four individual human donors, using rabbit polyclonal antibodies directed

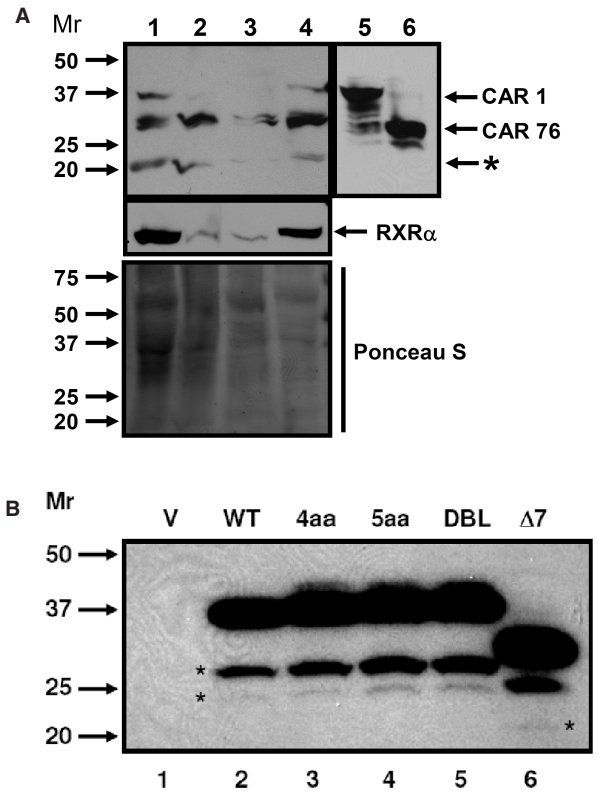

Figure 1. Western immunoblot analyses. (**A**) Expression of CAR-variant proteins and RXRα protein in human primary hepatocytes from four donors. Lanes 1–4, 150 μg whole-cell protein extract from each of four donors; lanes 5 and 6 are positive control whole-cell protein extracts from HEK-293T human embryonic kidney cell lines transfected with expression plasmids for CAR 1 or CAR 76 and treated with 1 μM MG-132 proteasome inhibitor to augment CAR expression. Ponceau S staining was performed to indicate total protein loading in each lane. (**B**) CAR protein expression in COS-1 cells transiently transfected with CAR and CAR splice variant expression plasmids. Lane 1, empty vector transfection (V); lane 2, CAR wild-type (WT); lane 3, CAR4aa (SPTV amino acids inserted between exons 6/7); lane 4, CAR5aa (APYLT amino acids inserted between exons 7/8); CAR-DBL (four and five amino acid insertion); and CARΔ7 (partial deletion of exon 7 coding for 39 amino acids). Embedded asterisks denote unexpected lower molecular weight CAR products.

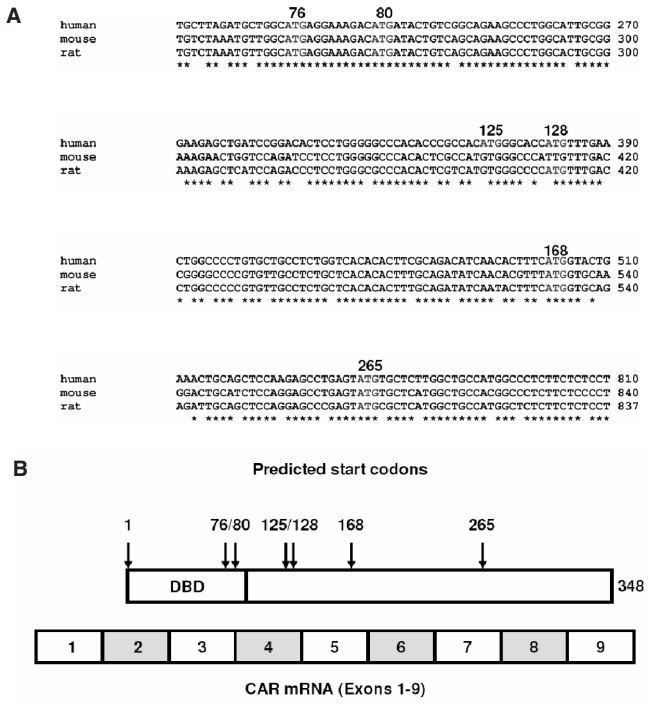

Figure 2. ClustalW alignment of human, mouse and rat CAR mRNA sequences. (**A**) Numbers above sequences denote NetStart1.0-predicted in-frame internal protein translation start sites. Numbers above sequences represent codon numbering based on human sequence. Numbers listed to the side indicate relative position of nucleotides within coding sequence, asterisks denote sequence conservation across species. (**B**) CAR protein and mRNA sequences alignment. Predicted CAR translation start sites are denoted by arrows at 1, 76, 80, 125, 128, 168 and 265. For full-length CAR protein, translation begins from a start codon 1 in exon 2 and continues to codon 348 in exon 9.

against the carboxyl-terminus of CAR and RXRα. Multiple protein bands were detected for CAR. At least three immunoreactive CAR bands were observed. One CAR product migrated at the predicted molecular weight of the full-length protein (~37 kDa). A second prominent immunoreactive band displayed an apparent molecular weight in excess of 25 kDa and a third band migrated at ~20 kDa. Similar results were obtained with protein extracts analyzed from liver tissues of other individuals (23). The CAR immunoreactive bands detected co-migrated with positive control extracts from HEK-293T cells transfected with expression plasmids for CAR1 and CAR76 that were run concurrently. Known splice variants of CAR include CAR2, containing a four amino acid (4 aa) insertion (SPTV) between exons 6 and 7; CAR3, which possesses a 5 amino acid (5 aa) insertion (APYLT) between exons 8 and 9; a transcript including insertion of both SPTV and APYLT (DBL); and a variant possessing a partial deletion of exon 7 (Δ7), that results in the loss of 39 aa from the ligand-binding domain of CAR (23). Each of these variant proteins is therefore predicted to migrate well above the ~25 kDa range. In COS-1 cells transiently transfected with expression plasmids for CAR and CAR splice variants, multiple immunoreactive CAR products of similar molecular mass were also observed in western immunoblot assays, conducted using an antibody directed against the carboxyl-terminus of CAR (Figure 1B).

We performed computer alignments using the ClustalW tool (http://www.ebi.ac.uk/clustalw/) (31) to demonstrate the conservation of human, mouse and rat CAR mRNA sequences (Figure 2A). The numbers indicated above the sequences are relative to the predicted initial start of translation at codon 1 in the human sequence. Numbers to the right denote relative sequence within the coding exons of CAR. It should be noted that mouse and rat CAR include 30 extra nucleotides, coding for 10 additional amino-terminal amino acids as compared with human CAR, such that codon 76 in human CAR equates to codon 86, etc., in mouse/rat CAR, respectively. The alignments of (human) codons 76 and 80 were most highly conserved across species, while more variability existed for

codons 125 and 128. The human CAR-coding sequence was analyzed with the web tool, NetStart 1.0 (http://www.cbs.dtu.dkservices/NetStart) (32) to predict internal protein translation start sites on a 0–1.0 scale. A call of 0.5 or higher was assigned by the program as a high-probability translation start site. Codon 125 is included in the figure as well, as it scored close to 0.5, within the 0.4–0.5 range. In Figure 2B, a simple alignment of human CAR protein and exons was performed and revealed that all potential internal translation start sites remove the amino-terminal (N-terminal) DBD of the receptor, and that most fall in the exon 3–5 region. Therefore, subsequent analyses were focused on the less severely deleted variants, arising from potential translation start sites at codons 76, 80, 125, 128 and 168.

Truncated CAR proteins transactivate a DR-1 PPRE

To quickly test whether predicted downstream and in-frame AUG start codons in CAR would drive translation of shorter protein products, the first AUG was mutated to AAG, to produce a lysine (K) instead of methionine (M) at position 1, and the derived construct was termed CAR M1K. Assuming CAR mRNA is translated to protein by a 'cap-dependent' scanning ribosome process, there is an expectation that increased translation would then occur from the use of downstream AUG start sites in the CAR M1K template. Results presented in Figure 3A clearly show that overexpression of CAR1 by Adv-CAR-mediated infection of primary cultures of human hepatocytes, or expression of CAR1 and CAR M1K mutant in wheat germ lysates, produced multiple protein products as detected by immunoblotting with an antibody designed against the C-terminus of CAR. In lanes 1 and 3, major CAR products were observed in close alignment with the 37 kDa, and between the 25 and 37 kDa markers, respectively. In lane 4, introduction of the M1K mutation resulted in a dramatic increase in the expression of truncated CAR protein, likely from initiation at one or both of the codons 76 and 80. The very low level of apparent full-length CAR in the CAR M1K lane was not observed in subsequent experiments, but in this overexposed film the band may have arisen from use of the less common internal translation start at leucine/codon 8, or more likely from slight spill-over from the CAR1 lane during gel loading. Once it was established that CAR M1K expression resulted in expression of truncated protein products, the potential transcriptional activities of the variant proteins were tested in transient transfection assays, using assorted, previously identified nuclear receptor consensus response elements. We hypothesized that the truncated forms of CAR may exert dominant negative or enhanced activity on specific elements. Previous studies in this and other laboratories demonstrated that CAR1 primarily activates reporter genes driven by DR-4 or DR-5 elements (2,14), and exhibits particularly strong activation of a DR-4 that contains the perfect half-site AGTTCA (15). In our initial transient transfection assays, we screened a number of potential response elements for activity of CAR1 and CAR M1K in COS-1 cells. Surprisingly, CAR1 and CAR

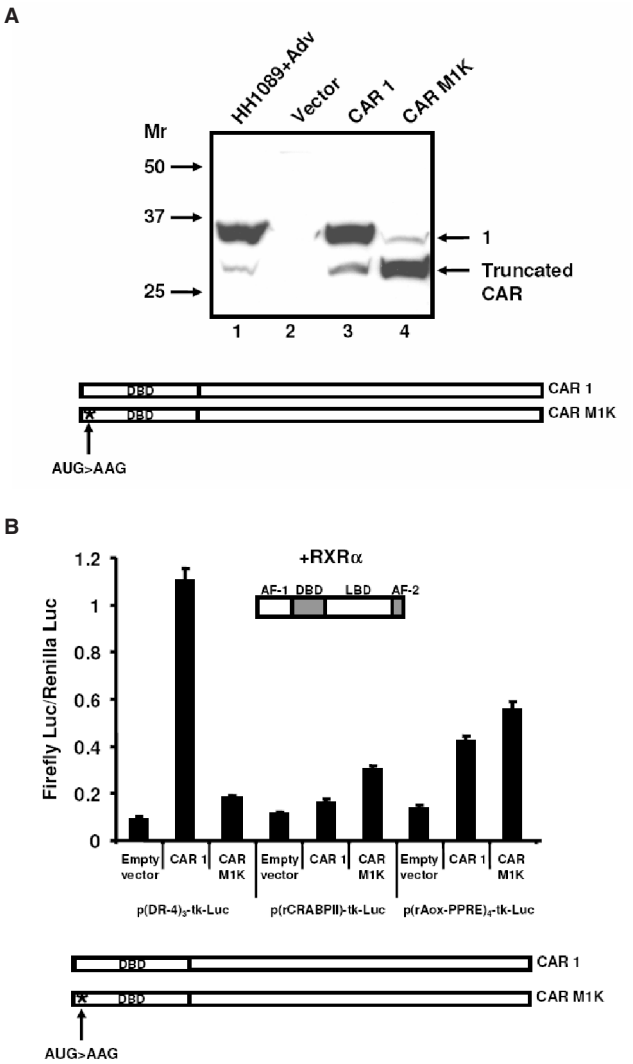

Figure 3. Western immunoblot assay. (**A**) CAR full-length protein and translational variants are expressed in Adv-CAR-infected human hepatocytes and in *in vitro* wheat germ expression system. Lane 1, human primary hepatocytes infected with adenovirus expressing wild-type CAR mRNA; (lanes 2–4, wheat germ lysates); lane 2, empty vector control; lane 3, pCDNA3.1(−)CAR 1; lane 4, pCDNA3.1(−)CAR M1K—start methionine mutated to lysine. Numbers to the left of figure indicate predicted internal translation variants' relative molecular weights (Mr). (**B**) CAR 1 and CAR M1K differentially transactivate DR-4 and DR-1 elements. COS-1 cells were transiently transfected with 100 ng of reporters p(DR-4)3-tk-Luc, p(rCRBPII)-tk-Luc or p(rAox-PPRE)$_4$-tk-Luc, 50 ng RXRα and CAR expression plasmids and 10 ng pRL-CMV.

M1K similarly activated the special DR-1 element, rAox PPRE, containing the sequence (TGACCT(N)$_1$TGT CCT), as well as an element containing a series of DR-1 motifs derived from the rCBPII promoter, where CAR M1K exhibited greater responsiveness than did CAR1 (Figure 3B). These marked transcriptional activation responses were dependent on the presence of co-transfected RXRα and also exhibited apparent cell line dependency, as they were retained in HepG2 and COS-1 cells but not readily detected in HuH7, A549, Hepa1c1c7, HEK-293A or MCF-7 cells (data not shown).

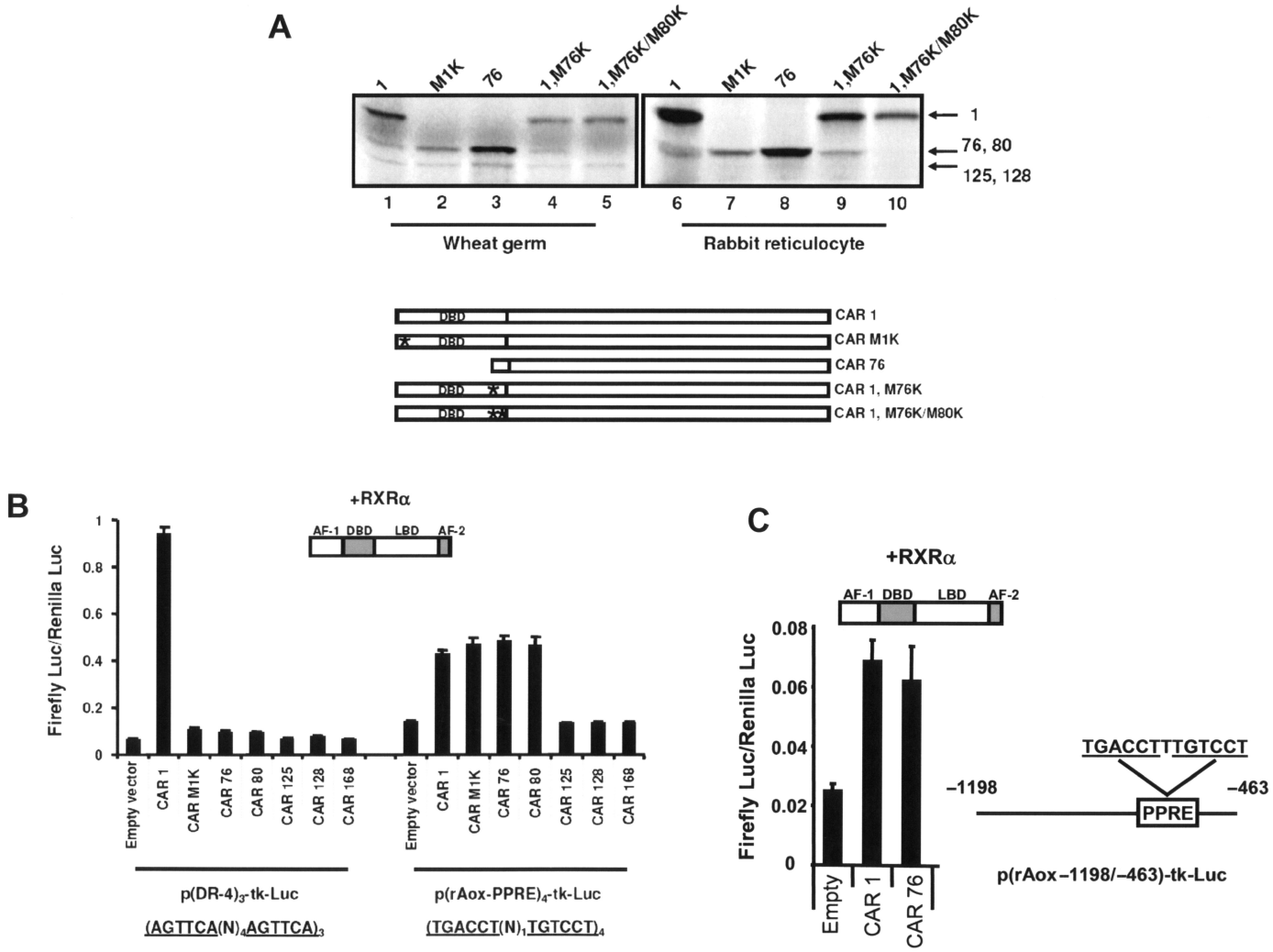

Figure 4. Truncated forms of CAR are expressed and activate transcription through a special DR-1 element. (**A**) SDS-PAGE of coupled *in vitro* transcription/translation of [^{35}S]-methionine-labeled CAR in wheat germ lysate (lanes 1–5) and rabbit reticulocyte lysate (lanes 6–10). Lanes 1 and 6, pCDNA3.1(−)CAR 1; lanes 2 and 7, pCDNA3.1(−)CAR M1K; lanes 3 and 8, pCDNA3.1(−)CAR 76; lanes 4 and 9, pCDNA3.1(−)CAR 1, M76K; and lanes 5 and 10, pCDNA3.1(−)CAR 1, M76K/M80K. (**B**) Differential activation of p(DR-4)$_3$-tk-Luc and p(rAox-PPRE)$_4$-tk-Luc by amino-terminally truncated forms of CAR. (**C**) Activation of p(rAox-1198/-463)-tk-Luc, a genomic promoter fragment containing a single copy of the PPRE element, by CAR 1 and CAR 76. COS-1 cells were transiently transfected with 100 ng of reporter construct, 50 ng pCDNA3.1(−) empty vector or pCDNA3.1(−)CAR plasmids, 50 ng pCDNA3.1(+)RXRα and 10 ng pRL-CMV (for transfection normalization) and assayed for luciferase activity 24–48 h post-transfection.

The basis for the cell specificity of the transcriptional responses is not yet understood, however the cell selective presence/absence of specific co-regulator proteins is likely responsible. ΔNCAR functional characterization studies were continued in COS-1 cells since these cells were markedly activated in a highly reproducible manner and exhibited the highest transfection efficiencies of all the cell lines tested.

ΔNCARs activate transcription in the absence of a DBD

Given the initial success with the CAR M1K mutation and the expression of truncated CAR proteins, we tested whether expression of the lower molecular weight CAR products could be ablated by mutating additional putative downstream start codons at positions 76 and 80. Indeed, expression of CAR M1K in wheat germ and rabbit reticulocyte lysate systems in the presence of [^{35}S]-methionine (Figure 4A) (lanes 2 and 7) resulted in the predominant production of a lower molecular weight species. When CAR76 template was overexpressed (lanes 3 and 8), the product co-migrated with the protein produced by CAR M1K, suggesting that codon 76 may be a biologically relevant internal translation start site in CAR. However, expression of CAR 1,M76K (lanes 4 and 9) revealed that mutation of codon 76 alone, in the context of the full-length template, could not abolish the lower molecular weight product. Subsequent dual mutation of both codons 76 and 80 in the form of CAR 1,M76K/M80K (lanes 5 and 10) completely eliminated expression of the truncated form of CAR, supporting a model of ribosome scanning from the 5′- 'cap' structure of CAR mRNA, resulting in the use of codon 80 as an

initiation site only when codon 76 was mutated. In wheat germ lysate and in longer exposures of rabbit reticulocyte lysate, even lower molecular weight CAR products were detected, likely attributable to further alternative translation from codons 125 and 128 (data not shown).

To test the transcriptional activity of the various CAR constructs, COS-1 cells were transiently transfected with $p(DR\text{-}4)_3$-tk-Luc and $p(rAox\text{-}PPRE)_4$-tk-Luc. CAR1 and translational variants of CAR1 including, CAR M1K, CAR76, CAR80, CAR125, CAR128 or CAR168, were examined, all in the presence of co-transfected RXRα expression plasmid (Figure 4B). In Figure 4C, CAR1 and CAR76 activated transcription of a non-oligomerized PPRE from the rAox gene promoter, contained in a 750-bp DNA fragment of rat genomic DNA and placed upstream of tk-Luc. The magnitude of the response and pattern of activation closely resembled the activation obtained when using four copies of the PPRE upstream of tk-Luc. To summarize the results obtained, CAR1 was active on DR-4 and DR-1 elements, but successive deletion of CAR protein to position 80 redirected full activation to only the DR-1 element. When CAR expression was forced from downstream codons, at 125, 128 and 168, the yielded proteins were without demonstrable transactivation potential on either DR-4 or DR-1 elements.

Results detailed in Figure 5 demonstrate that CAR1 transcriptional activation of the DR-4 element is RXRα independent and that CAR76 and CAR80 were completely inactive on this element, regardless of RXRα co-transfection. To ascertain the extent to which CAR could be deleted on its N-terminus and still retain transcriptional activity on a DR-4 motif, a number of other deletion mutants of CAR were tested (Figure 5A). Deletion of 10 aa from the N-terminus of CAR (CAR11) resulted in complete loss of activity, however co-transfection of RXRα restored transactivation of CAR11 on the DR-4 element, near to the levels seen with CAR1. Further truncation of CAR (CAR14 to CAR125) produced constructs that were incapable of activating DR-4 response element-driven luciferase gene expression. CAR11 includes the first cysteine residue within the first zinc finger of the receptor; predicted to be an important determinant for DNA binding. Surprisingly, when the same successively deleted CAR constructs were assayed for activity on the DR-1 element, in the presence of excess RXRα, all the constructs—through CAR103, maintained full transcriptional activity, while CAR113 and CAR125 were inactive (Figure 5B). Recently, the crystal structure of CAR:RXRα was reported, derived from a minimal CAR:RXRα dimerization interface that included amino acids 103–348 of CAR (33). Our results similarly suggest that proper heterodimer formation between RXRα and CAR residues 103–348 is important for CAR transcriptional activity on a DR-1 element.

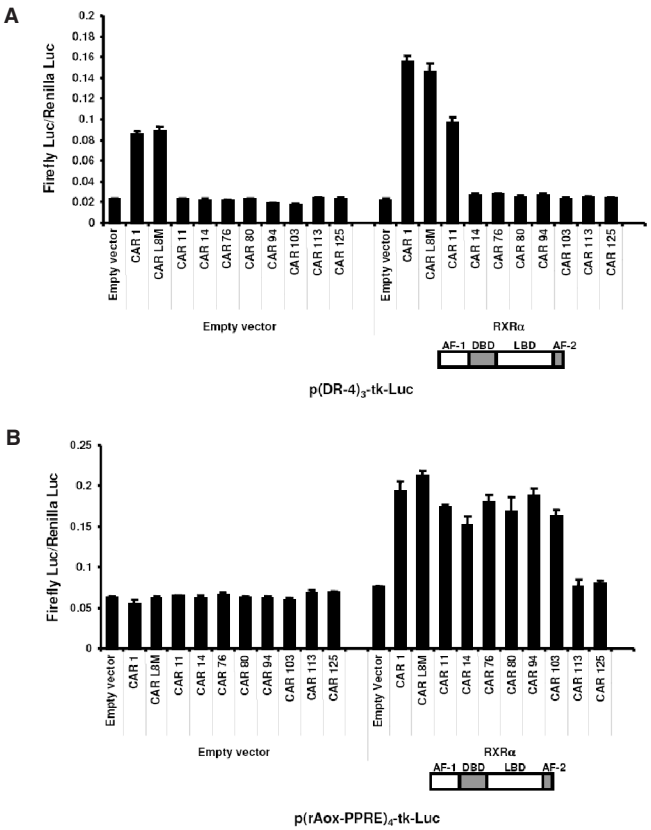

Figure 5. Transient transfection assays. (**A**) An intact DNA-binding domain is necessary for CAR transcriptional activity on a DR-4 element. COS-1 cells were transfected with 100 ng $p(DR\text{-}4)_3$-tk-Luc, 50 ng empty vector or RXRα, 50 ng empty vector or CAR constructs and 10 ng pRL-CMV. (**B**) CAR amino acids 103–348 and co-transfected RXRα are required for full transcriptional activity on a DR-1 element. COS-1 cells were transfected with 100 ng $p(rAox\text{-}PPRE)_4$-tk-Luc, 50 ng empty vector or RXRα, 50 ng empty vector or CAR constructs and 10 ng pRL-CMV.

CAR activity on DR-1 PPRE is RXR-dependent

Since the first 102 aa of CAR were dispensable for transcriptional activity on a DR-1, we probed the importance of other domains for this activity. We deleted a portion of the CAR C-terminus to remove 8 aa that contained the receptor's AF-2 domain. The results indicated that, even in the presence of RXRα, CAR 1△AF-2 was devoid of transcriptional activity on the DR-1 element (Figure 6A). Although CAR activity on the DR-1 did not require the first third of the protein, it was dependent on an intact AF-2 domain, as well as co-transfected RXRα. To further delineate the RXRα-dependent transactivation of the DR-1 by CAR and the ΔNCARs, a series of RXRα domain deletion constructs were co-transfected into COS-1 cells (Figure 6B). Deletion of RXRαDBD amino acids 135–161 resulted in a loss of CAR1 and ΔNCAR transcriptional activity, likely due to the loss of RXRα's ability to directly bind DNA. However, deletion of the AF-1 and AF-2 functions of RXRα, alone or in combination, had little effect on the transactivation ability of CAR1, CAR76 or CAR80 to activate the DR-1 element (Figure 6C). These results provided additional support for the concept that direct binding of RXRα to DNA was a necessary component for CAR's activity at the DR-1 element,

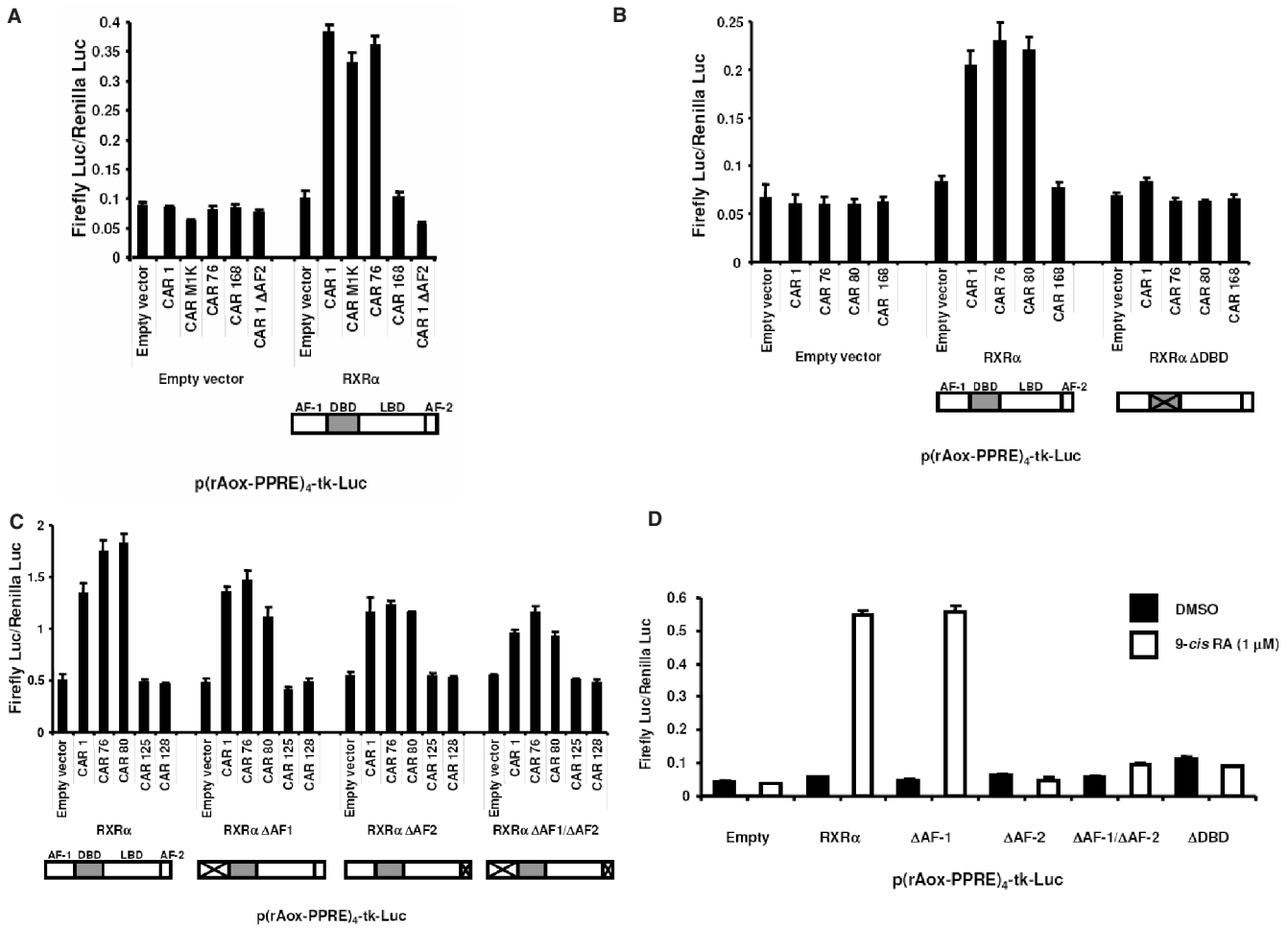

Figure 6. Transient transfection assays. (**A**) CAR AF-2 is required for transactivation through a DR-1 element. COS-1 cells were transfected with 100 ng p(rAox-PPRE)$_4$-tk-Luc, 50 ng empty vector or RXRα, 50 ng empty vector or CAR constructs and 10 ng pRL-CMV. (**B**) CAR activation of p(rAox-PPRE)$_4$-tk-Luc is RXRα DNA-binding domain dependent. COS-1 cells were transiently transfected with 100 ng of reporter construct, 50 ng pCDNA3.1(−) empty vector or pCDNA3.1(−)CAR plasmids, 50 ng pCDNA3.1(−), pCDNA3.1(+)RXRα or pCDNA3.1(+)RXRαΔDBD and 10 ng pRL-CMV (for transfection normalization) and assayed for luciferase activity 24–48 h post-transfection. (**C**) CAR transactivation of p(rAox-PPRE)$_4$-tk-Luc is independent of RXRα AF-1 and RXRα AF-2. COS-1 cells were transiently transfected with 100 ng of reporter construct, 50 ng pCDNA3.1(−) empty vector or pCDNA3.1(−)CAR plasmids, 50 ng pCDNA3.1(−), pCDNA3.1(+)RXRα, pCDNA3.1(+)RXRαΔAF-1 or pCDNA3.1(+)RXRαΔAF-2 and 10 ng pRL-CMV (for transfection normalization) and assayed for luciferase activity 24–48 h post-transfection. (**D**) RXRα deletion constructs DBD- and AF-2-dependently transactivate p(rAox-PPRE)$_4$-tk-Luc in response to RXR ligand 9-*cis*-retinoic acid (RA). COS-1 cells were transiently transfected with 100 ng of reporter construct, 50 ng pCDNA3.1(+) or pCDNA3.1(+)RXRα or RXRα domain deletion variants and 10 ng pRL-CMV (for transfection normalization) for 24 h, then treated with dimethyl sulfoxide (DMSO) solvent control or 1 μM 9-*cis* RA for 24 h and assayed for luciferase activity.

but that CAR's AF-2 domain provides the requisite interaction with co-regulators.

Since RXRα homodimers bind well to DR-1 elements, we also tested whether RXRα deletion mutants were themselves transcriptionally active on p(rAox-PPRE)$_4$-tk-Luc in the presence of the RXRα ligand RA (Figure 6D). The results obtained showed that wild-type and AF-1-deleted RXRα were fully activated by ligand, while any deletion of AF-2 or DBD yielded transcriptionally inactive proteins.

Our initial data indicated that CAR or ΔNCAR interaction with RXRα was necessary for CAR-mediated transactivation through the DR-1 PPRE. Co-transfection experiments were performed with two different point-mutated RXRα plasmids to demonstrate that CAR:RXRα dimerization was absolutely required for transcriptional activity (Figure 7A). Use of RXRα Y397A, a mutation in the human receptor corresponding to a well-characterized heterodimerization-deficient Y402A mutation in mouse RXRα (34), completely ablated CAR-mediated transactivation on the PPRE. However, this heterodimerization-mutant RXRα was still fully functional as a homodimer after addition of RA. An additional mutation of RXRα yielded L430F, a mutant defective in homodimerization (35), as demonstrated here by the lack of induction of luciferase activity in the presence of RA. However, the L430F mutant mediated nearly full induction of reporter activity when CAR1 was co-transfected. Further, immunofluorescence microscopy demonstrated transfected CAR1 was expressed primarily

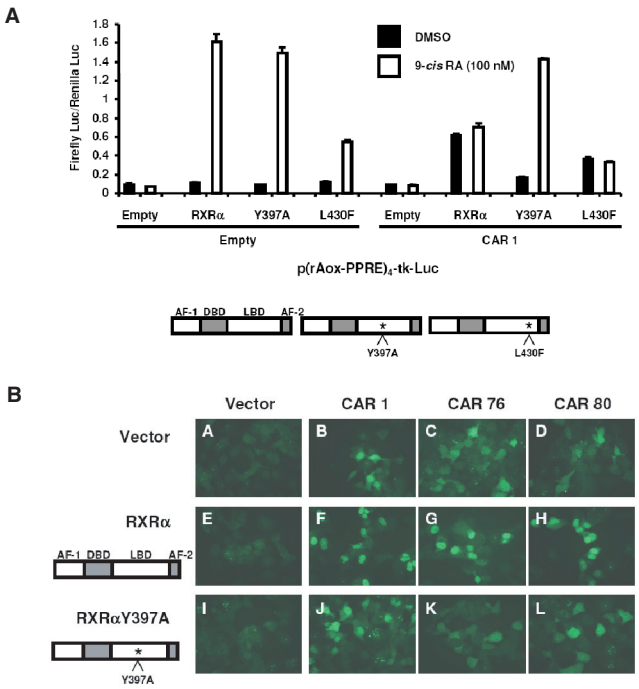

Figure 7. Transient transfection assays. (**A**) Interplay of RXRα homodimerization or heterodimerization with CAR in transactivation of p(rAox-PPRE)$_4$-tk-Luc in the absence or presence of RXRα ligand 9-*cis*-RA. COS-1 cells were transiently transfected with 100 ng of reporter construct, 50 ng pCDNA3.1(−) or pCDNA3.1(−)CAR 1, 50 ng pCDNA3.1(+) or pCDNA3.1(+)RXRα or RXRα point mutants (Y397A heterodimerization mutant; L430F homodimerization mutant) and 10 ng pRL-CMV (for transfection normalization) for 24 h, then treated with dimethyl sulfoxide (DMSO) solvent control or 100 nM 9-*cis*-retinoic acid (RA) for 24 h and assayed for luciferase activity. (**B**) RXRα heterodimerization-dependent nuclear localization of CAR 1, 76, 80 in COS-1 cells. COS-1 cells were transfected with 200 ng pCDNA3.1(−) empty vector, CAR 1, 76 or 80 and 200 ng pCDNA3.1(−), RXRα, RXRαY397A (heterodimerization mutant) for 24–48 h. Immunofluorescent detection of CAR was performed as described in the Experimental procedures section.

in the nucleus of COS-1 cells, either in the absence or presence of co-transfected RXRα (Figure 7B). In contrast, CAR76 and CAR80 expression was evenly distributed between the cytoplasmic and nuclear compartments in the absence of co-expressed RXRα, but became primarily nuclear in the presence of excess RXRα. When the RXRαY397A heterodimerization mutant was co-expressed with CAR1, CAR76 or CAR80, the distribution of the CAR proteins was similar to results obtained with co-transfected empty vector alone.

CAR ligands modulate variant protein activity

CAR exhibits high constitutive activity in transfected cells, presumably because proteins involved in cytoplasmic sequestration of the protein in primary hepatocytes are missing from cell lines (5). Consequently, early studies identified so-called inverse agonists of CAR, such as androstanol, which could repress the constitutive transactivation potential of CAR (8). When CAR1 was transfected with RXRα, p(DR-4)$_3$-tk-Luc activity was highly induced, as predicted, while CAR76 and PPARα were inactive on this element (Figure 8A). A titration of androstanol from 0.5 to 8 micromolar (μM) indicated that a maximal inhibition of CAR1 constitutive activity was achieved at ~2 μM. A similar androstanol treatment was repeated on cells transfected with CAR1, CAR76, and PPARα with p(rAox-PPRE)$_4$-tk-Luc. A 2 μM concentration of reagent produced a maximal inhibition of CAR1 and CAR76 activity and modestly raised PPARα activity at higher concentrations (Figure 8B). Although inhibition of PPARα activation by androstanol was not anticipated, the effect provided an internal control for the experiments and indicated that androstanol was not simply decreasing luciferase activity through a non-specific mechanism of transcriptional repression. Since the truncated CAR76 and CAR80 isoforms behaved like CAR1 in the presence of androstanol, it appears that ligand binding and dimerization properties of CAR are maintained in the least truncated ΔNCARs. CAR1, 76 and 80 activities that were decreased by androstanol treatment on the p(rAox-PPRE)$_4$-tk-Luc construct were restored by co-treatment with the human CAR agonist ligand, CITCO (Figure 8C). Titration of CITCO from 0.3 to 2.5 μM revealed that 0.3 μM was sufficient to completely antagonize the effect of 2 μM androstanol treatment, and lower concentrations of CITCO, in the range of 3–30 nanomolar (nM), significantly activated androstanol-repressed CAR1, CAR76 and CAR80 transcription on the DR-1 PPRE (data not shown).

No correlation between CAR/RXRα DNA binding and transactivation

Nuclear extracts from COS-1 cells enriched for CAR1 or CAR76 expression (by neomycin selection of a CAR-expressing cell population) were used to perform gel shift assays with a [^{32}P]-labeled, CYP2B6-derived DR-4 element (NR1) or a [^{32}P]rAoxPPRE (Figure 9A). These results demonstrated that CAR1 and CAR76 bind to both elements, but that the interaction of CAR1 was the most robust. Since CAR76 was not active on a DR-4 in transient transfection assays, and CAR1 and 76 were equally active on the rAox-PPRE DR-1 in the luciferase assays, no obvious correlation was apparent between DNA-binding affinity in the EMSAs and the respective reporter gene activation levels. When CAR 1 or PPARα were expressed with RXRα in COS-1 cells, and nuclear extracts from those cells were incubated with [^{32}P]NR1 DR-4 or [^{32}P]rAox-PPRE DR-1, CAR1 and PPARα interacted with both DR-4 and DR-1 elements more strongly than did RXRα alone (Figure 9B). Again, even in the presence of excess RXRα, no direct correlation was manifested between receptor–DNA complex formation and transactivation of DR-4- or DR-1- driven luciferase reporter activity.

Transcriptional activation in primary human hepatocytes

Although the rAoxPPRE promoter construct used in the COS-1 transfection studies presented in Figure 4B was derived from a natural mammalian DR-1 promoter element, and was activated by CAR76, we further tested the relative biological activities associated with the CAR1

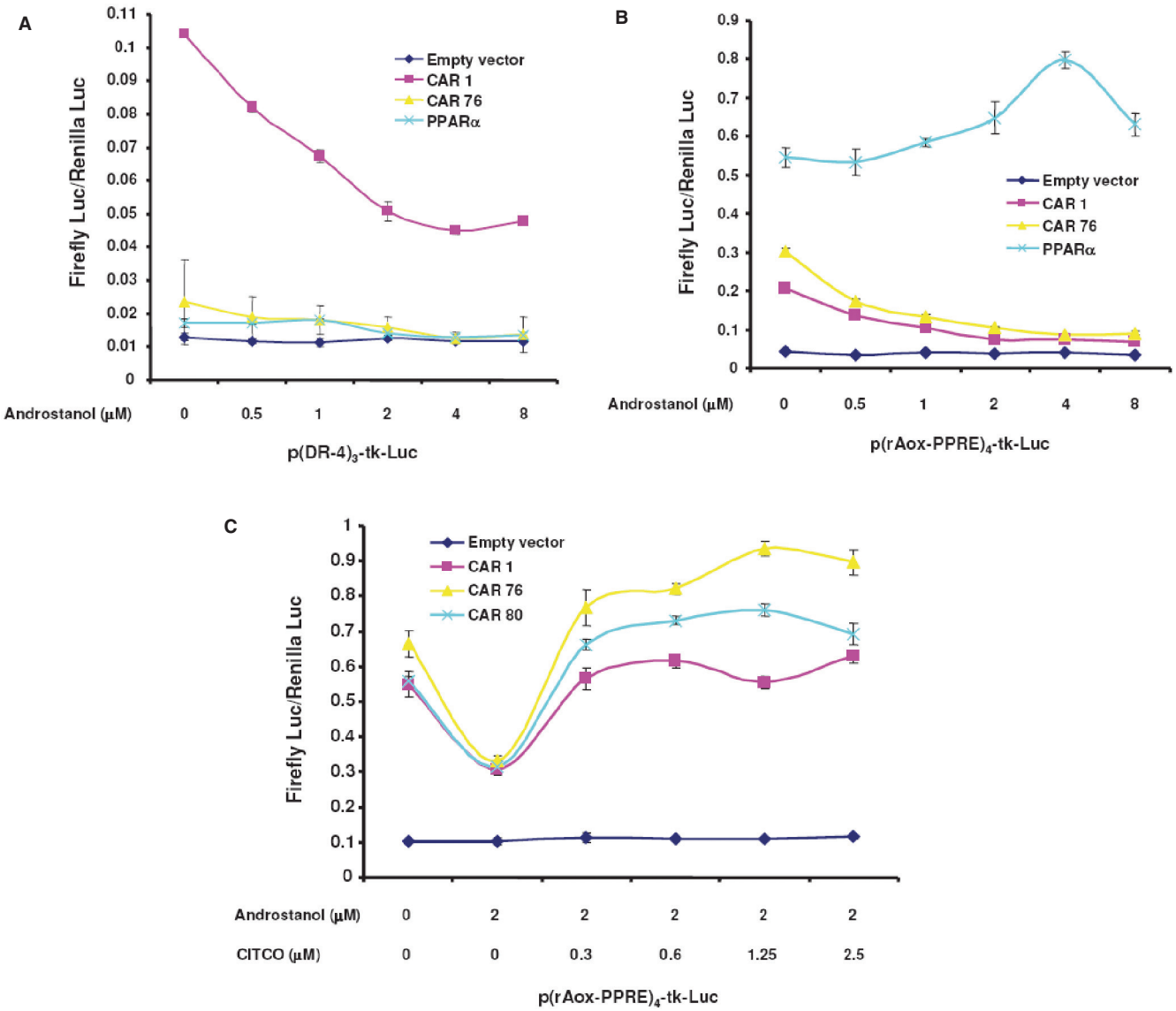

Figure 8. Transient transfection assays. (**A**) 5α-Androstan-3α-ol (androstanol) dose–response to optimize downregulation of CAR 1-dependent transactivation of $p(DR\text{-}4)_3$-tk-Luc. COS-1 cells were transiently transfected with 100 ng of reporter construct, 50 ng pCDNA3.1(−), pCDNA3.1(−)CAR 1 or CAR76, 50 ng pCDNA3.1(−)PPARα and pCDNA3.1(+)RXRα and 10 ng pRL-CMV (for transfection normalization) for 24 h. Cells were then treated with dimethyl sulfoxide (DMSO) solvent control or 0.5, 1, 2, 4 or 8 μM androstanol for 24 h and assayed for luciferase activity. (**B**) 5α-Androstan-3α-ol (androstanol) dose–response to optimize downregulation of CAR 1 and ΔNCAR-dependent transactivation of $p(rAox\text{-}PPRE)_4$-tk-Luc. COS-1 cells were transiently transfected with 100 ng of reporter construct, 50 ng pCDNA3.1(−), pCDNA3.1(−)CAR 1 or CAR76, 50 ng pCDNA3.1(−)PPARα and pCDNA3.1(+)RXRα and 10 ng pRL-CMV (for transfection normalization) for 24 h. Cells were then treated with DMSO solvent control or 0.5, 1, 2, 4 or 8 μM androstanol for 24 h and assayed for luciferase activity. (**C**) CITCO dose–response to optimize re-activation of androstanol-antagonized CAR 1 and ΔNCAR activity on $p(rAox\text{-}PPRE)_4$-tk-Luc. COS-1 cells were transiently transfected with 100 ng of reporter construct 50 ng pCDNA3.1(−), pCDNA3.1(−)CAR 1, CAR 76, CAR 80 or pCDNA3.1(−)PPARα and pCDNA3.1(+)RXRα and 10 ng pRL-CMV (for transfection normalization) for 24 h. Cells were then treated with DMSO solvent control, 2 μM androstanol, or co-treated with androstanol and increasing CITCO (0.3, 0.6, 1.25, 2.5 μM) for 24 h and assayed for luciferase activity.

and CAR76 receptor isoforms in a more *in vivo* context. In these latter assays, we infected primary cultures of human hepatocytes with CAR lentiviral vectors that stably integrate in the genome and direct cellular expression of their respective receptor proteins. The results, from two different donors, are presented in Figure 10 and recapitulate the data derived from the transfected COS-1 cell experiments, indicating that CAR76 expression in hepatocytes selectively activated expression of the endogenous human PEPCK and HMGCS2 genes, both of which contain DR-1 elements in their respective promoter regions.

DISCUSSION

Previous studies of CAR variants demonstrated that not all of the detected protein products were likely attributed to alternatively spliced mRNA species (23). For examples,

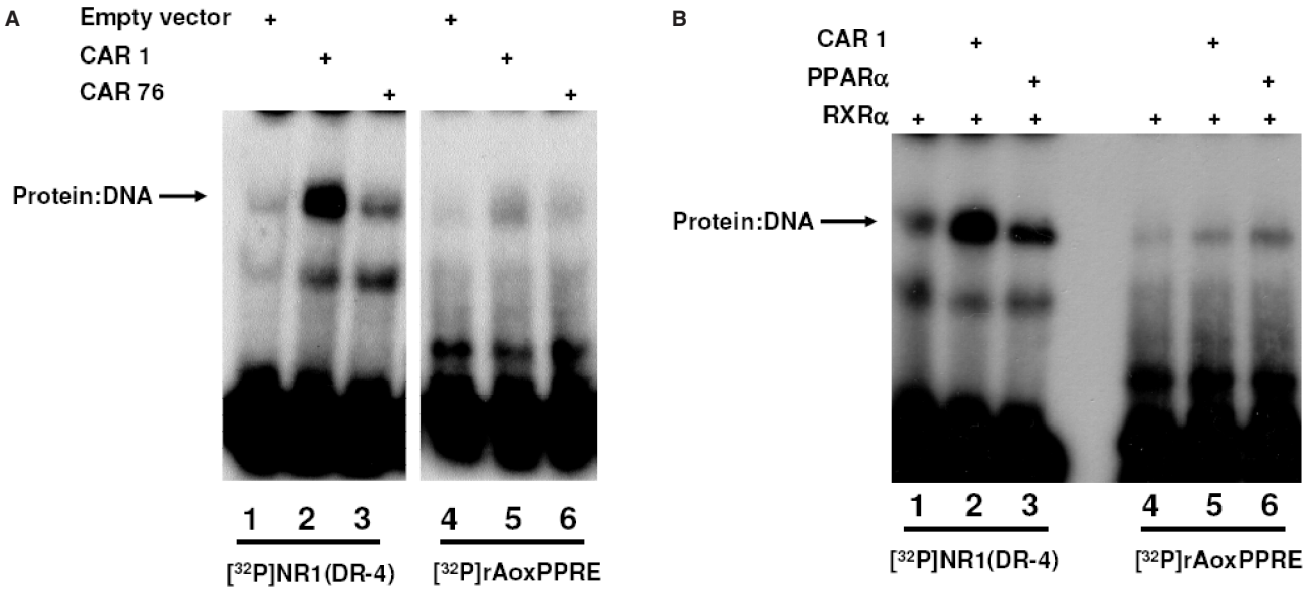

Figure 9. Electrophoretic mobility shift assays. (**A**) CAR 1 and CAR 76 binding to [^{32}P]NR1(DR-4) or [^{32}P]rAox-PPRE. COS-1 cells were transiently transfected with pCDNA3.1(−), CAR 1 or CAR 76 and subjected to selection with 500 μg/ml G418 to enrich a population of cells overexpressing CAR 1 and CAR 76. Nuclear extracts were obtained from the CAR-expressing cells and used in EMSA as described in the Experimental procedures section. (**B**) CAR 1 and PPARα binding to [^{32}P]NR1(DR-4) or [^{32}P]rAox-PPRE. COS-1 cells were transiently transfected with pCDNA3.1(−) CAR 1 or pCDNA3.1(−)PPARα for 24 h, nuclear extracts were obtained and EMSA performed as described in the Experimental procedures section.

when COS-1 cells were transfected with plasmids expressing various CAR mRNA splice variants, lower molecular weight bands were consistently observed in SDS-PAGE gels. Even upon expressing a CAR splice variant possessing a partial deletion of exon 7, a proportionally lower molecular weight band was detected. From these observations, we hypothesized that isoforms of CAR may arise from the use of internal translation start sites. In the present study, we identify new protein translational variants of human CAR that differ from those reported previously. Specifically, we demonstrate that downstream in-frame start codons produce N-terminally truncated CAR variants (ΔNCAR) from wild-type as well as alternatively spliced mRNA transcripts. We determined that CAR1, CAR76 and CAR80 proteins all functionally activate luciferase reporter activity when driven by a DR-1-type PPRE promoter element.

Specifically, since strong Kozak sequences (36,37) and translational start sites were predicted from the use of methionines at positions 1, 76, 80, 125, 128, 168 and 265, we generated plasmid constructs with optimized Kozak sequences to enable overexpression of CAR proteins from each putative internal start site, and expressed these constructs in transiently transfected COS-1 cells, in rabbit reticulocyte lysates and in wheat germ *in vitro* translation systems. In each case, protein bands were observed that migrated with the expected molecular weights of the truncated CAR76 and CAR80 proteins, and also co-migrated with the more rapidly migrating bands detectable upon co-expression of the full-length protein encoded by the CAR1 template. A methionine to lysine mutation at codon 1 (CAR M1K) abolished expression of full-length CAR, but yielded prominent bands resulting from CAR76 and CAR80 initiation. Similarly, when the codons for amino acids 76 and 80 were mutated from methionine to lysine in CAR1, the CAR76 and CAR80 bands were abolished, with proteins predicted to initiate at amino acids 125 and 128 becoming readily detectable. Dramatic sequential mutations of upstream methionines always led to increases of protein expression from downstream internal translation start sites, supporting a model of 'cap-dependent' translation of CAR mRNA, whereas internal ribosome entry site (IRES)-dependent levels of protein translation of ΔNCAR would be expected to proceed independently of mutations of upstream initiator codons (38).

Others have reported that full-length mouse CAR can activate a DR-2-type PPRE, but failed to show any significant transactivation on a DR-1 (13). Rather, mouse CAR repressed ligand-dependent PPAR α/RXRα activity on a DR-1 (13). A more recent study suggested that full-length CAR binds to the promoters of the CYP7A1 and PEPCK genes, decreasing their expression by competing with the transcription factor HNF4 for binding to DR-1 elements (39). Perhaps the specific sequence context of a DR-1 element may influence whether CAR exerts a positive or negative effect on transcription activity. The reported discrepancies may also have resulted from differences in the cell lines used or from the inclusion/exclusion of RXRα co-transfection. It is interesting that another laboratory reported that alternative splicing of human CAR may result in an mRNA variant that lacks the usual protein translational start site in exon 2, instead enabling translation from an AUG start codon in exon 1 (24). In this latter case, the use of an alternative exon 1 translation initiation codon predicts the inclusion of unique N-terminal amino acids that are in-frame with amino acids encoded by exon 3. These investigators also predicted CAR translation from codons 125 and 128, rather than codons 76 and 80

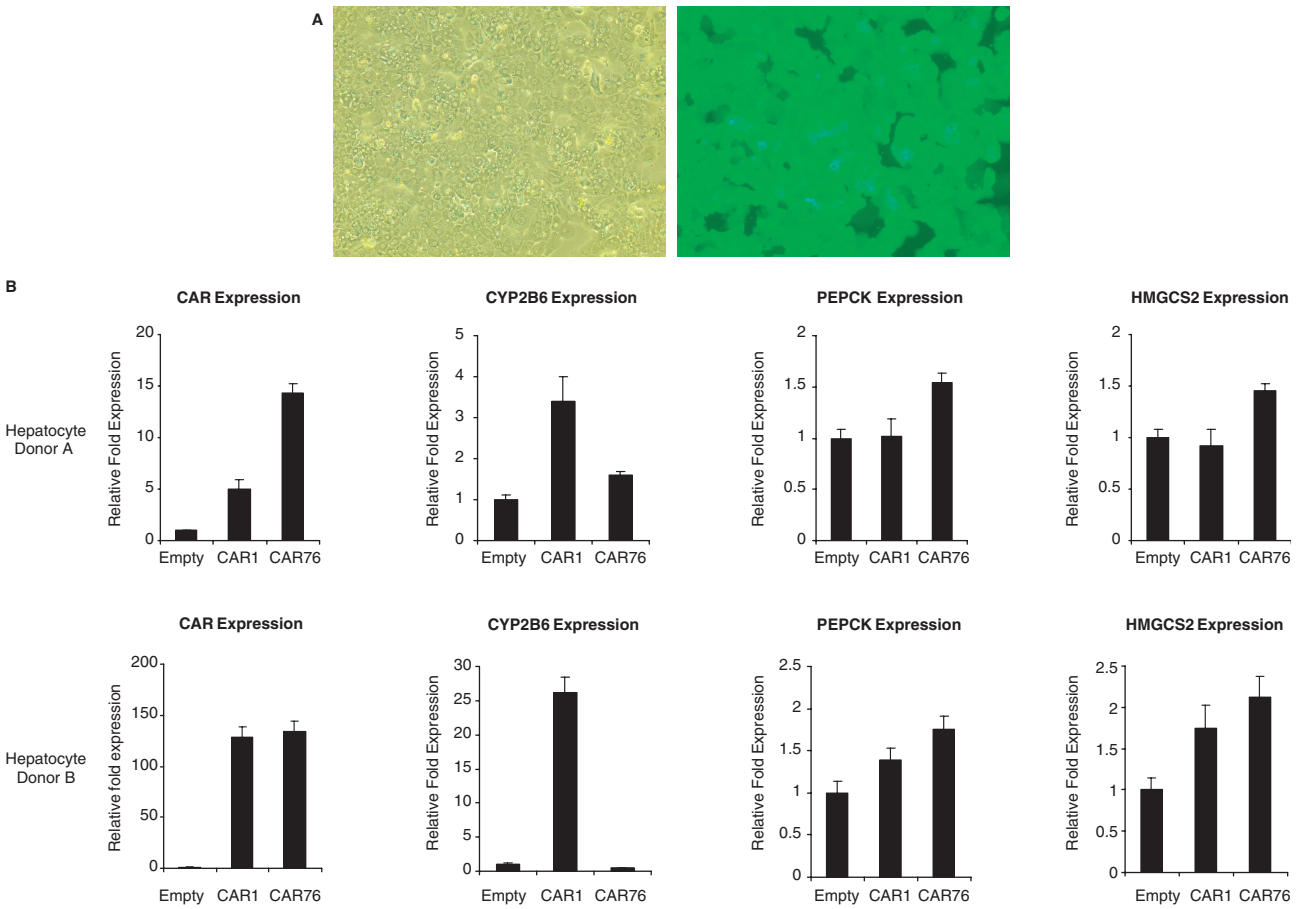

Figure 10. Lentiviral infections and real-time PCR analysis. (**A**) GFP expression in primary human hepatocytes infected with GFP-expressing lentivirus. Pseudoviral particles were produced using pSIH1-H1-copGFP lentiviral vector, virus was concentrated and infections were conducted as described in the Experimental procedures section. Images depict hepatocytes at 5 days post-infection in phase-contrast (100×), and fluorescence microscopy (100×). (**B**) Real-time PCR analysis of CAR, CYP2B6, PEPCK and HMGCS2 expression levels in primary human hepatocytes from two different donors infected with CAR1- and CAR76-expressing lentivirus. Pseudoviral particles expressing CAR1 and CAR76 in pCDH1-MCS1-EF1-copGFP lentiviral vectors were produced and infections were performed as described in the Experimental procedures section. Four days post-infection hepatocytes were harvested and RNA was isolated, converted to cDNA, subjected to real-time PCR and the data analyzed as described in the Experimental procedures section. Each bar depicts the average relative fold expression of two PCR reactions from a single hepatocyte donor.

that we identify here as the actual start sites used to generate ΔNCARs possessing unique transcriptional activities (24).

In our experiments, full-length CAR and the ΔNCARs, CAR76 and CAR80, exhibited constitutive activity on a special DR-1 PPRE, and the receptor activities were downregulated by treatment with the CAR inverse agonist 5α-androstan-3α-ol (androstanol). Co-treatment with androstanol and the human CAR-specific agonist, CITCO, completely restored CAR and ΔNCAR activities to constitutive levels. Transient co-expression of RXRα variants and CAR or ΔNCARs revealed that receptor heterodimerization was the most important criterion for gene promoter activation. In this respect, the RXRα AF-1 domain was not required for transactivation by the ΔNCARs, nor was RXRα's AF-2 domain. When point-mutated RXRα expression constructs were co-transfected with the ΔNCARs, the RXRα heterodimer-defective variant, Y397A, completely ablated the ability of the ΔNCARs to activate a PPRE response element, yet the Y397A RXRα could still homodimerize and function in reporter transactivation. Conversely, a mutation of RXRα that abolishes RXRα homodimerization, L430F, still allowed for efficient heterodimer formation with CAR and the ΔNCARs, reflected by subsequent reporter gene activation, but effectively inhibited RXRα homodimer transactivation in the presence of chemical agonist.

Therefore, our results demonstrate that the lack of a CAR-DNA-binding domain in a CAR-RXRα receptor heterodimer does not inactivate the dimer's transactivation function on a DR-1 or in a PPRE promoter context. In fact, examples of classical nuclear receptors have been reported previously that retain transactivation ability on gene promoters without direct DNA-binding interactions, including ER (40), the retinoic acid receptor (RAR) (41) and the glucocorticoid receptor (42). In the latter case, transgenic mice possessing DNA-binding defective GRs were still viable and the modified receptors retained functional ability to cross-talk with other transcription factors, thereby participating in processes such

as transrepression. For CAR, and perhaps for other nuclear receptors as well, we predict that truncated forms of the receptor may exhibit full or partial activation function, or even repression, and that the spectrum of these activities may manifest on an altered repertoire of response elements. Specifically, we propose a new model of CAR DBD-independent transactivation, whereby only the RXRα component of the heterodimer binds directly to a DR-1-containing PPRE, tethered to CAR or ΔNCARs (CAR76 or CAR80), with functional contributions from the CAR AF-2 domain providing the basis for co-activator recruitment and subsequent gene transactivation function. This type of heterodimer–DNA interaction is in striking contrast to that reported previously for the APYLT-CAR splice variant, a variant that possesses a 5 aa insertion in the receptor's dimerization interface (14). For the latter receptor, the RXRα DBD, rather than the APYLT-CAR DBD, is expendable for transcriptional activation, implying a model invoking direct contact of only the CAR portion of the dimer with DNA, not the RXRα component (14). Taken together, along with the recently identified phosphorylation modifications that impact the ability of CAR to translocate (43), it is becoming clear that the post-transcriptional regulation of CAR expression is an important means of generating a diversity of receptor modalities that likely function to enhance its complex role as a receptor integrator of xenobiotic and endobiotic sensing in mammalian cells.

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Conflict of interest statement. None declared.

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