The Basis for Strain-Dependent Rat Aldehyde Dehydrogenase 1A7 (ALDH1A7) Gene Expression

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ABSTRACT

Aldehyde dehydrogenases (ALDHs) belong to a large gene family involved in oxidation of both endogenous and exogenous compounds in mammalian tissues. Among ALDHs, the rat ALDH1A7 gene displays a curious strain dependence in phenobarbital (PB)-induced hepatic expression: the responsive RR strains exhibit induction of both ALDH1A7 and CYP2B mRNAs and activities, whereas the nonresponsive rr strains show induction of CYP2B only. Here, we investigated the responsiveness of ALDH1A1, ALDH1A7, CYP2B1, and CYP3A23 genes to prototypical P450 inducers, expression of nuclear receptors CAR and pregnane X receptor, and structure of the ALDH1A7 promoter in both rat strains. ALDH1A7 mRNA, associated protein and activity were strongly induced by PB and modestly induced by pregnenolone 16α-carbonitrile in the RR strain but negligibly in the rr strain, whereas induction of ALDH1A1 and P450 mRNAs was similar between the strains. Reporter gene and chromatin immunoprecipitation assays indicated that the loss of ALDH1A7 inducibility in the rr strain is profoundly linked with a 16-base pair deletion in the proximal promoter and inability of the upstream DNA sequences to recruit constitutive androstane receptor-retinoid X receptor heterodimers.

SIGNIFICANCE STATEMENT

Genetic variation in rat ALDH1A7 promoter sequences underlie the large strain-dependent differences in expression and inducibility by phenobarbital of the aldehyde dehydrogenase activity. This finding has implications for the design and interpretation of pharmacological and toxicological studies on the effects and disposition of aldehydes.

Introduction

The aldehyde dehydrogenases (ALDHs) are present in all taxonomic groups, and they are involved in the irreversible oxidation of endogenous and exogenous aldehydes (Sophos et al., 2001). Their functionality extends far beyond enzymatic detoxification processes because they participate in the growth, differentiation, and survival of cells and in the synthesis of biomolecules, such as retinoic acid, betaine, and γ-aminobutyric acid. ALDHs may also serve as biomarkers for certain cancer types and Parkinson disease (Westerlund et al., 2005; Jackson et al., 2011). Among the 21 genes of the rat ALDH gene superfamily (Jackson et al., 2011), the cytosolic isoforms ALDH1A1, 2, 3, and 7 are involved in the metabolism of, for example, aliphatic aldehydes and retinal and cyclophosphamide (Kathmann et al., 2000; Alnouti and Klaassen, 2008). Among these isoforms, rat ALDH1A1 and ALDH1A7 are highly homologous and display similar tissue-expression patterns, with high mRNA levels reported in the liver, lung, and kidneys (Marselos et al., 1987; Hsu et al., 1999; Kathmann et al., 2000). Moreover, hepatic expression of rat ALDH1A1 and 1A7 isoforms is induced by phenobarbital (PB), a prototypical inducer of several cytochrome P450 (P450) enzymes.

In rodents, PB is known to induce many P450s and other drug-metabolizing enzymes and transporters via the nuclear receptor (NR) constitutive androstane receptor (CAR), whereas many drugs induce their expression by activation of...
the pregnane X receptor (PXR) (Honkasoki and Negishi, 2000; Chai et al., 2013). Others reported that selective CAR or PXR activators modestly increased (2- to 3.5-fold) Aldh1a1 and Aldh1a7 gene expression in the livers of wild-type mice but not in mice with disrupted Car or Pxr genes, whereas the expression of other Aldh family members was not appreciably affected by NR activation (Alexsunes and Klaassen, 2012). These findings indicate that the key NRs regulating drug-metabolizing enzymes are involved in the regulation of cytosolic ALDH1A isoforms. The induction of cytosolic ALDH by PB has been investigated because of genetic strain differences in specific Long-Evans rat strains (Deitrich, 1971; Dunn et al., 1989; Kathmann et al., 2000). The extent of PB-elicited induction of rat liver cytosolic ALDH activity appears to be regulated by two alleles of a single autosomal locus. The dominant allele R confers responsiveness to PB, whereas the recessive allele r is nonresponsive. Homozygous responders (RR) exhibit maximal ALDH induction by PB, heterozygous rats (Rr) display a moderate induction and homozygous nonresponders (rr) show only a small, if any, increase of ALDH activity. Constitutive ALDH mRNA expression (initially termed ALDH-PB, now recognized as ALDH1A7) is detectable in both strains, but it is strongly inducible in only the RR rats. Interestingly, the expression of other PB-inducible enzymes, such as CYP2B1 and CYP2B2, reached similar levels in these strains after exposure to PB (Dunn et al., 1989; Pappas et al., 2001); however, the molecular basis for this ALDH-selective disparity in induction by PB remains unexplored to this day. To investigate this problem, we used the Wistar/Af/Han/Mol/Kuo/Io rat strain that is currently bred at the University of Ioannina, creating substrains that are responsive (Rnor 5.0; Chr1: ALDH1A7 -actin mRNA levels and ~2-fold greater, and its PB inducibility is markedly higher in the RR strain (>15-fold vs. 2.5-fold) compared with the rr strain. Here, we cloned and characterized ALDH1A7 from both Wistar rat strains to identify the defect in PB induction and investigated its regulation by CAR and PXR.

Materials and Methods

Chemicals. Phenobarbital (PB) and mifepristone (RU486) were from Sigma-Aldrich (St. Louis, MO). Pregnenolone 16a-carbonitrite (PCN) was bought from SantaCruz Biotechnology (Heidelberg, Germany). Synthesis of the mouse CAR agonist 1,4-bis[(3,5-dichloropyridyloxy)]benzene (TCPOBOP) has been previously described (Honkasoki et al., 1996). All other chemicals were of the highest analytical grade.

Experimental Animal Model and Treatments. Wistar/ Af/Han/Mol/Kuo/Io male rats (aged 3–5 months and weighing 170–230 g) were isolated and reproduced by inbreeding at the University of Ioannina, creating substrains that are responsive (RR) and nonresponsive (rr) to PB (Marselos, 1976). All animals were treated according to the European Communities Council Directive (86/609/EEC) and housed in groups of two or three in plastic cages under constant environmental conditions on a 12-hour light/dark cycle at ambient temperature and provided with chow and tap water ad libitum. All in vivo experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Medical School at the University of Ioannina (license number EL33BIO-exp01). For the multiple-dose study, 3-month old male rats (four per group) were injected intraperitoneally with selective activators of CAR and PXR (Stanley et al., 2006): three doses of PB (80 mg/kg, dissolved in saline), four doses of PCN (50 mg/kg, dissolved in olive oil), or the corresponding vehicle. Animals were sacrificed by carbon asphyxiation 6 hours after the PB injection. For the time-response study, 3-month-old male rats were divided into groups of four and treated with a single dose of PB (80 mg/kg), PCN (50 mg/kg), or vehicle. Animals were sacrificed by carbon asphyxiation after 6, 12, or 24 hours of PB or PCN injection.

Total RNA Isolation, cDNA Synthesis, and Quantitative Reverse Transcription-Polymerase Chain Reaction. After decapitation, small liver samples were stored in RNAlater at −80°C until use. Total RNA was isolated with the Nucleospin RNA II total RNA isolation kit (Macherey-Nagel, Düren, Germany). RNA samples (1 μg) served as templates for cDNA synthesis using Quanti-Tect Reverse Transcription kit (Qiagen, Hilden, Germany). Analysis of liver mRNA levels was performed with real-time reverse transcription polymerase chain reaction (RT-PCR), based on the TaqMan protocol, by use of KAPA Probe Fast qPCR Master Mix (KAPA Biosystems, Wilmington, MA) and TaqMan Gene Expression Assay primers for rat ALDH1A1, ALDH1A7, CAR, PXR, CYP2B1, CYP3A23, and β-actin genes (Applied Biosystems, Foster City, CA). Amplification reactions (two replicate reactions per sample) were performed on the CFX96 real-time system and the C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA). Gene-expression analysis was performed by the ΔΔCt method (Müller et al., 2002), normalized for β-actin mRNA levels and expressed as mean ± S.D. (n = 4) relative to the value of control RR strain sample set as 1.

Protein Extraction and Western Blotting. Liver samples were placed into ice-cold PBS and then homogenized either in hypotonic buffer (10 mM Hepes pH 7.9, 1.5 mM MgCl2, 5 mM KCl with 0.1% NP-40) for cytosolic proteins, in high-salt extraction buffer (20 mM Hepes pH 7.9, 250 mM NaCl, 1 mM EDTA) for nuclear proteins or in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS) for total proteins. Protease and phosphatase inhibitors [1 μg/ml aprotinin, 1 μg/ml leupeptin, 50 μg/ml phenylmethylsulfonyl fluoride, 50 μg/ml Na3VO4, 1 mM dithiothreitol] were added to these buffers just before use. Proteins (50–100 μg) were separated in 7.5–12% SDS-PAGE and transferred onto a nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). Membranes were first blocked in 5% milk in Tris-buffered saline and then incubated with anti-rabbit ALDH1/2 antibody (1:5000; Santa Cruz, Santa Cruz, CA) likely to detect at least ALDH1A1 and ALDH isoforms that have similar molecular masses of 54 kDa), anti-mouse CAR (1:1000; PP-N4111-09; Perseus Proteomics; Tokyo, Japan), anti-mouse PXR (1:1000; PP-H417-00; Perseus Proteomics). Goat anti-rabbit (sc-2004; Santa Cruz) and goat anti-mouse (sc-2005; Santa Cruz) horseradish peroxidase-conjugated antibodies were used as secondary antibodies at 1:5000 dilution. Immuno-detection was based on enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, UK). Three independent sets of control, PB- or PCN-exposed rats were analyzed. Typical results from one set are shown in Figs. 1 and 2, and results of the remaining two sets are shown in the Supplementary Figs. 2 and 3.

ALDH1 Activity Assay. Liver samples were homogenized in ice-cold sucrose and centrifuged at 20,000g for 30 minutes. Supernatants were used for the kinetic measurement of ALDH activity with propionaldehyde as substrate as described (Vasiliou and Marselos, 1999). This activity cannot distinguish between the cytosolic ALDH isoforms 1A1 and 1A7. Determination of the protein concentration was carried out according to the Lowry method (Lowry et al., 1951). The specific activities are expressed as mean ± S.D. of four animals per group.

Cloning of the ALDH1A7 Promoter and Reporter Constructs. Genomic DNA was isolated from liver tissue (25 mg) from 3-month-old male RR and rr rats according to the protocol of NucleoSpin tissue kit (Macherey-Nagel). The amplification of ~10 kilobase pair (kb) fragments of ALDH1A7 promoters (Rnor 5.0; Chr1:...
247, 897, 931-247, 887, 931; reverse complement) from genomic DNA, and sequencing was done by Epoch Life Science Inc. (Missouri City, TX). The RR and rr promoter sequences have been submitted to the NCBI GenBank with accession numbers MK814117 and MK814118, respectively. The fragments were inserted into pGL3-Basic plasmid vectors (Promega, Madison, WI) at KpnI and XhoI restriction sites. Sequential deletion fragments of ALDH1A7 promoters were generated by amplification with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) or KAPA HiFi (hot start) DNA polymerase (KAPA Biosystems) and forward and reverse primers (Oligomer, Helsinki, Finland) shown in Supplemental Table 1. The deletion fragments were cloned into pGL3-Basic plasmid vectors and competent Escherichia coli bacteria (XL-10 or DH5α) were transformed with the constructs. Colonies were screened by colony PCR using DreamTaq DNA polymerase (ThermoScientific, Vantaa, Finland) or KAPA HiFi (hot start) DNA polymerase (KAPA Biosystems) and forward and reverse primers (Oligomer, Helsinki, Finland) shown in Supplemental Table 1. The deletion fragments were cloned into pGL3-Basic plasmid vectors and competent Escherichia coli bacteria (XL-10 or DH5α) were transformed with the constructs. Colonies were screened by colony PCR using DreamTaq DNA polymerase (ThermoScientific, Vantaa, Finland) or KAPA HiFi (hot start) DNA polymerase (KAPA Biosystems) and forward and reverse primers (Oligomer, Helsinki, Finland) shown in Supplemental Table 1. The deletion fragments were cloned into pGL3-Basic plasmid vectors and competent Escherichia coli bacteria (XL-10 or DH5α) were transformed with the constructs. Colonies were screened by colony PCR using DreamTaq DNA polymerase (ThermoScientific, Vantaa, Finland) or KAPA HiFi (hot start) DNA polymerase (KAPA Biosystems) and forward and reverse primers (Oligomer, Helsinki, Finland) shown in Supplemental Table 1. The deletion fragments were cloned into pGL3-Basic plasmid vectors and competent Escherichia coli bacteria (XL-10 or DH5α) were transformed with the constructs. Colonies were screened by colony PCR using DreamTaq DNA polymerase (ThermoScientific, Vantaa, Finland) or KAPA HiFi (hot start) DNA polymerase (KAPA Biosystems) and forward and reverse primers (Oligomer, Helsinki, Finland) shown in Supplemental Table 1.

**Fig. 1.** Strain-dependent expression and inducibility of ALDH1A isoforms. Normalized expression of ALDH1A7 (A) and ALDH1A1 (B) mRNA in control, PB-, or PCN-treated RR and rr rat livers. Levels are expressed as scatter plots and means ± S.D. from four animals, each sample with two technical replicates. Cytosolic and total protein levels of ALDH1 protein (C) and activity (D) in control, PB-, or PCN-treated RR and rr rat livers. Statistically significant differences (P < 0.05) compared with the respective control group or between the strains are marked by * and # signs, respectively. The data in (A, B, and D) are also shown relative to the control RR values, set at 1.0, and expressed as means ± S.D. of fold change below the x-axis.
samples (80–100 mg) were fixed in 1% formaldehyde solution, and the cross-linking reaction was quenched by incubating the samples in 0.125 M glycine. The samples were then homogenized in sucrose buffer (0.3 M sucrose, 15 mM NaCl, 5 mM MgCl₂, 0.1 mM EDTA, 15 mM Tris-HCl, 0.5 mM dithiothreitol, 0.1 mM phenylmethyl sulfonyl fluoride) and centrifuged. The pelleted homogenates were resuspended in lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS) and incubated on ice for 10 minutes. Lysates were centrifuged and resuspended in sonication buffer (50 mM Hepes pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X, 0.1% Na-deoxycholate, 0.1% SDS). After sonication (80% power, 4 × 20 seconds with 1-minute cooling intervals; Misonix sonicator, Farmingdale, NY), the DNA was sheared to an average length of 300–800 bp. The supernatants were diluted in ChIP dilution buffer (1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl pH 8, 1% Triton X-100, 0.01% SDS) and precleared with Protein A/G PLUS-agarose beads (sc-2003; Santa Cruz) at 4°C. Then, the beads were removed and 1/10 of the genomic DNA was retained as input control for quantitative PCR analysis. Precleared chromatin samples were incubated at 4°C overnight, with the addition of 5 μg polymerase II antibody (sc-899; Santa Cruz) or 5 μg of retinoid X receptor-α (RXRα) antibody (sc-553; Santa Cruz).

On the following day, protein-DNA complexes were precipitated after a 3-hour incubation with A/G beads. The beads were washed successively with low-salt buffer (2 mM EDTA, 20 mM Tris-HCl pH 8, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), high-salt buffer (low-salt buffer with 500 mM NaCl), LiCl wash buffer (1 mM EDTA, 10 mM Tris-HCl pH 8, 1% Nonidet P-40, 1% Na-deoxycholate, 0.25 M LiCl) and twice with TE buffer (1 mM EDTA, 10 mM Tris-HCl pH 8). All buffers contained 1/20 protease inhibitor cocktail (P-27140; Sigma). After the two elution steps, the eluates and inputs were incubated with 5 M NaCl in 65°C overnight to reverse the DNA/protein crosslinks. After treatment with proteinase K (2 hours at 55°C), DNA was purified by phenol–chloroform-isooamyl alcohol extraction and ethanol precipitation. The DNA samples were analyzed by quantitative PCR in duplicate using KAPA SYBR FAST qPCR Master Mix (KK4601) and calculated by the 2^[-ΔΔCt] method after correcting for the IgG-negative control. The PCR primers used were synthesized by Eurofins Genomics (Ebersberg, Germany) and listed in the Supplemental Table 2.

**Statistical Analysis.** Differences between groups were evaluated by analysis of variance and Bonferroni’s multiple comparison test. The main comparisons were between the strains and between the control
and inducer groups. Probability values $P < 0.05$ were considered statistically significant, but they are shown for descriptive purposes, not for hypothesis testing.

**Results**

**Differential Expression of ALDH1A7 in RR and rr Rat Strains.** Hepatic expression of ALDH1A1 and ALDH1A7 genes was studied after multiple doses of PB and PCN. Figure 1A shows that constitutive expression of rat ALDH1A1 mRNA was higher in the RR but still detectable in the rr rat strain. ALDH1A7 mRNA levels were induced more than 100-fold by PB and almost 20-fold by PCN only in the responsive RR strain, whereas no equivalent induction was detected in the rr strain. On the other hand, constitutive rat ALDH1A1 mRNA expression was only slightly higher in the rr strain whereas the induction of ALDH1A1 mRNA was comparable, reaching 4- to 10-fold by PB and 4- to 6-fold by PCN in both strains (Fig. 1B). Western blotting of cytosolic and total protein extracts (Fig. 1C; Supplemental Fig. 2) indicated low expression of ALDH1 proteins in control animals, whereas high expression levels were seen in PB-treated and moderate induction in PCN-treated RR rats only. It should be noted that the antibody used in Western blotting was the only one commercially available, and it does not distinguish between various ALDH1A isoforms. The ALDH1 (propionaldehyde/NAD$^+$) activity (Fig. 1D), catalyzed by all ALDH1A isoforms (Lindahl, 1992; Alnouti and Klaassen, 2008), displayed similar basal levels between the strains. It was elevated 3.5- and 4.5-fold by PB and PCN in the responsive RR rats, but it was only marginally elevated in the rr strain. These findings confirmed earlier reports on PB induction of both ALDH1A1 and 1A7 isoforms and the strain difference in ALDH1A7 inducibility by PB (Dunn et al., 1989; Pappas et al., 2001).

**Expression of Inducible P450s and NRs in RR and rr Rats.** Similar analyses revealed that hepatic transcripts of the main rat PB- and PCN-inducible CYP2B1 and CYP3A23 genes (Honkasoki and Negishi, 2000; Stanley et al., 2006) are detected in the untreated RR and rr animals at similar levels and are similarly induced by PB and PCN (Fig. 2A and B). PB, an activator of rodent CAR, preferentially induced CYP2B1 over CYP3A23 mRNA. PCN, a ligand of rodent PXR, induced CYP3A23 over CYP2B1 mRNA expression, as expected. Furthermore, there was maximally only about a 2-fold change in the Ct values of $\beta$-actin mRNA between the control and induced groups (Supplemental Table 5), indicating that the observed strain differences and induction responses are not due to changes in the expression of the control gene used in normalization.

Furthermore, the expression of rat CAR and PXR mRNA (Fig. 2C) and the protein levels in total liver extracts (Fig. 2D; Supplemental Fig. 3) were also comparable. In time-course experiments, induction of ALDH1A1, ALDH1A7 (RR strain only), CYP2B1, and CYP3A23 mRNAs was clearly detectable 6 hours after PB or PCN injection, indicating a rapid transcriptional response (Supplemental Figs. 4 and 5). These results show that CAR and PXR receptors are present in both rat strains and function appropriately as regulators of ALDH1A1, CYP2B1, and CYP3A genes.

**Comparison of ALDH1A7 Promoter Sequences in RR and rr Rats.** The remarkable absence of ALDH1A7 induction by PB, but normal expression and functionality of NRs in rr rats, suggested that the genetic difference in inducibility between the RR and rr strains may be related to changes in DNA sequences of their respective ALDH1A7 genes. To study this hypothesis, genomic DNA from both strains was isolated, and ~10 kbp of ALDH1A7 promoter fragments were amplified and sequenced. Alignment of the two promoter sequences showed that they are 97.6% identical (Supplemental Fig. 1), with the ALDH1A7 sequence from the rr strain matching completely the available rat ALDH1A7 sequence for Rattus norvegicus (ALDH1A7; Chr1:247, 847, 960-247, 887, 931 in the Rnor 5.0 assembly). Detailed comparison of the two ALDH1A7 promoter sequences indicated that there were two larger deletions in the rr strain compared with the RR strain: one was a 165-bp region at ~2200 bp upstream of the transcription start site (TSS), missing in the rr strain. Another deletion (16 bp) occurred just upstream of the TATA box at ~50/34 bp. In addition, several small deletions and point mutations were present (Supplemental Fig. 1). With regard to transcription factor (TF) sites that could influence PB or PCN inducibility and/or hepatic expression of ALDH1A7 mRNA, we identified binding sites for CAR/RXR, sites, CCAAT box and DR3, DR4 and ER6 elements, which are shown in Supplemental Tables 3 and 4, respectively. None, however, showed a major difference between the RR and rr strains, and none was present in the above two deletion regions.

**Activity of the Full-Length ALDH1A7 Promoters from RR and rr Rats.** The 10-kbp ALDH1A7 gene promoters were inserted into the pGL3-Basic reporter plasmid, co-transfected with mouse NR expression vectors into C3A hepatoma cells, and cells were treated with selective NR-activating ligands (Fig. 3). CAR and PXR activated the
fragments. Denotes a statistically significant different from the pGL3-
shorter RR of 12.5-fold over the promoterless control construct. Deletion to
(2 bp) fragments from both strains. Figure 4A shows that the longer
activity and responsiveness to NRs of two proximal promoter
30% of the longer fragment. The corresponding
deletion in the ALDH1A7 rr RR and rr Rats.

PXR-responsive elements, whereas the
10-kbp ALDH1A7 induction of ALDH1A7 mRNA (Fig. 1A) and indicate that the
PBREM- and XREM-driven positive control reporters as
expected (Mäkinen et al., 2002). The constitutively active
CAR enhanced the activity of ALDH1A7 promoter from the RR
strain by ~55-fold, and its ligand TCPOBO further
ligated CAR or PXR. These findings correlate well with
induction of ALDH1A7 mRNA (Fig. 1A) and indicate that the
10-kbp ALDH1A7 RR promoter sequence harbors CAR- and
PXR-responsive elements, whereas the rr promoter cannot be
activated by these NRs.

Activity of the Proximal ALDH1A7 Promoters from
RR and rr Rats. Because of the low ALDH1A7 expression in the
rr strain, we turned our attention next to the proximal
ALDH1A7 promoter that contained several TF-binding sites,
likely important for the constitutive activity, and the 16-bp
deletion in the rr promoter. We measured the reporter activity
and responsiveness to NRs of two proximal promoter
fragments from both strains. Figure 4A shows that the longer
(1571/-5 bp) RR fragment conferred a reporter activity of
12.5-fold over the promoterless control construct. Deletion to
shorter RR fragment (461/-5 bp) yielded an activity of about
30% of the longer fragment. The corresponding ALDH1A7
fragments from the rr strain had much weaker activities that
tended to be on par or marginally above the pGL3-Basic construct. This finding indicates that the proximal ALDH1A7
promoter (~444/-5 bp) in the rr strain contains defects that
reduced the promoter activity and presumably decreased the
expression of ALDH1A7 gene.

Figure 4B shows results from the cotransfection assays with
NR expression vectors and the proximal ALDH1A7 fragments.
In contrast to the 10-kbp RR promoter (Fig. 3), the more
proximal fragments (RR -1571/-5 bp; rr -1556/-5 bp, or shorter)
from either rat strain were not activated by ligand-
activated CAR or PXR. This result indicates that the CAR/
PXR-responsive element(s) lie in the far-upstream region of
ALDH1A7.

Binding of Transcriptional Regulators to the ALDH1A7
Promoter Sequences. To shed more light on the differential
expression of ALDH1A7 between RR and rr rats, we studied
the binding of RNA polymerase II, an essential factor for RNA
transcription, to the proximal ALDH1A7 promoter and binding
of NRs to the upstream regions. To this end, we used the
ChIP assay with chromatin samples extracted from both
strains exposed to vehicle or PB.

We detected significant RNA polymerase II binding (>6-fold
over background) to the proximal promoter region (~209/-12 bp)
in samples from control RR rats. This binding was clearly
 elevated by PB (Fig. 5). In contrast, RNA polymerase II
showed weak or no binding to the corresponding fragment
(~194/-12 bp) from rr rats, regardless of treatment. This
finding provides strong support to our hypothesis that the
ALDH1A7 gene has an active and PB-responsive promoter in
RR rats, whereas its activity is low and noninducible in the
rr strain.

Next, we sought to identify the locations of the CAR binding
sites in the ALDH1A7 far-upstream region. Because the
available CAR antibody was not suitable for ChIP assays,
we used instead an antibody to RXRα, the obligate hetero-
dimeric partner of CAR and PXR for DNA binding (Honkakoski
and Negishi, 2000). A similar approach of using RXRα as
a surrogate for CAR binding was taken in previous studies
(Saito et al., 2013; Ohno et al., 2014). Inspection of NR binding
sites with direct or everted repeats of AGGTCA-like motifs
with the NUBIScan algorithm (Podvinec et al., 2002) revealed
three putative CAR/RXRα binding sites at −3075 (named

Fig. 5. Polymerase II binding to RR-ALDH1A7 and rr-ALDH1A7 proximal
promoters. Both strains were dosed with saline (C) or PB for 3 hours;

liver nuclear fractions were isolated and crosslinked, and RNA polymerase-
associated DNA was analyzed by ChIP assay as detailed in Materials and
Methods. The amount of recovered DNA is shown relative to the negative
control (nc) region (set at 1.0). Data are scatter plots from two independent
experiments, each with two technical replicates.
CAR1), at −3331 (named CAR2) and at −5202 (named CAR3) for the RR strain (the respective locations are −2907, −3167, and −5046 for the rr strain). Sequence comparison of these sites between the RR and rr strains showed no major differences, apart from a single point mutation in the CAR-RXR binding CAR1 site (a DR4 element). There were also three other putative CAR/RXR binding sites (upstream from −8300 in RR); however, they have shown no significant ChIP binding (data not shown).

In Fig. 6A, binding of RXRα was detectable to all three CAR/RXRα binding sites in chromatin samples isolated from control RR rats. Exposure to PB substantially enhanced RXRα binding to CAR1 and CAR2 sites (5- and 10-fold, respectively), whereas the increase was lower at the CAR3 site (2-fold). Experiments performed with chromatin from the rr rats (Fig. 6B) showed low binding both in control and PB-treated samples.

**Discussion**

The present study provides evidence for the molecular basis underlying the differences in PB inducibility of ALDH activity observed in RR and rr rat strains. We showed that the low ALDH activity in the nonresponsive rr strain is associated with the lack of ALDH protein and mRNA expression. This difference cannot be attributed to defects in the PB induction process itself because expression of the CAR regulator and inducibility of the ALDH1A1, CYP2B1, and CYP3A23 mRNAs were comparable in the two strains. We also showed, for the first time, that the rat ALDH1A7 gene is regulated by PXR and its ligand, albeit to a lesser extent than by CAR.

Reporter gene assays using ALDH1A7 promoter fragments indicated that whereas the largest (10-kbp) fragment from the RR rats was strongly activated by CAR and PXR, the corresponding rr fragment was practically inactive. Other experiments indicated that the proximal rr promoter is clearly weak and cannot recruit RNA polymerase as efficiently as the RR promoter. Apart from a few dispersed point mutations and deletions at positions that did not seem to affect any predicted TF binding site, there is a 16-bp RR sequence at −50/−34 bp, very close to the predicted TATA box, that is lacking in rr rats. This 16-bp sequence is quite similar to a recognition element of the general transcription factor TFIIB (Littlefield et al., 1999), and this site is essential for recruitment of the RNA polymerase II. Accordingly, RNA polymerase II failed to bind to the fragment −194/−12 bp in chromatin samples from the rr rats. Based on the present findings, we suggest that the 16-bp deletion is the ultimate reason for the low ALDH1A7 activity and inducibility by PB in the nonresponsive rr rats.

Upstream regions at −3050 and −3300 bp harbored sites capable of NR binding as indicated by efficient recruitment of RXRα, which is essential for the CAR-dependent transcription. Whereas robust RXRα binding to the RR gene took place especially after PB administration, binding to the rr gene was detected only at low levels and not affected by PB. This is quite surprising given the fact that the NR binding sites in the rr gene were intact. This finding suggests that CAR binding to these distal sites may require priming, that is, appropriate recruitment of factors binding to the proximal ALDH1A7 promoter before CAR binding can occur. This implies that the inability of the proximal ALDH1A7 rr promoter to recruit general TFs may also result in reversion of distal CAR binding sites into a transcriptionally inactive state in the rr rats. There is similar evidence that mutations in a single regulatory sequence can affect the accessibility to TFs of the entire gene locus (Kumasaka et al., 2016).

The genome-wide chromatin accessibility and inducibility by TCPOBOP have recently been investigated in mice. The Cyp2b10 and Aldha1a7 genes belong to a group termed pattern I, where TCPOBOP produces stronger induction than PCN (Cui and Klaassen, 2016). In the Cyp2b10 gene upstream sequence, there is low but detectable CAR/RXR binding in control animals that is enhanced by 6- to 8-fold after TCPOBOP exposure (Tian et al., 2018). These data correlate well with our findings on the RR ALDH1A7 gene. The murine Aldha1a7 locus contains an active promoter, as evidenced by activating histone H3K4me3 and H3K27Ac marks (Sugathan and Waxman, 2013) and a TCPOBOP-responsive DNase I hypersensitive site (Lodato et al., 2018) overlapping the TSS, a finding that matches well with the active rat ALDH1A7 proximal promoter in RR rats. The far-upstream DNase I hypersensitive sites are located at
~17 and ~29 kbp upstream of the Aldh1a7 TSS but still within the same topologically associated domain (Lodato et al., 2018).

In contrast, information on genome chromatin accessibility or epigenetics in the rat liver is quite sparse. The available data [https://www.ebi.ac.uk/arrayexpress: accession number E-MTAB-2663] show that the rat ALDH1A1 gene possesses strong activating histone marks (H3K4me3 and H3K27Ac) that overlap the ALDH1A1 TSS and the first exon (Villar et al., 2015). The co-occurrence of these two histone modifications is indicative of an active promoter, in line with a robust ALDH1A1 mRNA expression in both rr and RR strains; however, in the same data set, coinciding H3K4me3 and H3K27Ac marks closest to the ALDH1A7 gene are more than 200 kbp away from its TSS, which correlates with the low expression of ALDH1A7 mRNA. It should be noted again that the rat reference genome (Rnor 6.0) used in these studies contains the rr ALDH1A7 sequence.

Collectively, our results show that PB induces ALDH1A7 expression at activity, protein, and mRNA level in only the responsive RR rats via binding of CAR to upstream elements in ALDH1A7, which is inherently associated with enhanced recruitment of RNA polymerase II to the proximal promoter. Because of sequence variation between these rat strains, the expression and accumulation of ALDH1A7 mRNA are disrupted in the nonresponsive rr rats, which likely also affects ALDH1A7-inducing signals other than PB that act via transcriptional activation.

The human relevance of our findings is still unclear. ALDH1A7 is not present in the human genome as it is thought to be a rodent-specific duplication of the ancestral ALDH1A1 gene. There are no direct publications on PB inducibility of human ALDH1A1, whereas rodent ALDH1A1 and 1A7 genes are both activated. We found an RNA sequencing data set of human HepaRG cells exposed to human CAR- and PXR-activating CITCO and PB (Li et al., 2015; GeoExpress GSE71446) that showed a substantial 3.5- to 16-fold increase of human CYP2B6 and CYP3A4 mRNA expression with the Folin phenol reagent.

In conclusion, we consider that the present data have shed light on the 40-year conundrum of strain differences in ALDH inducibility by PB. They also suggest that metabolism of endogenous and exogenous aldehydes may be attenuated in rat strains harboring this genetic defect, which could have consequences for the design and interpretation of biologic, pharmacological and toxicological studies on functions and disposition of aldehydes.

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References


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