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Title Page

Constitutive and 3-MC-induced Rat *ALDH3A1* Expression is Mediated by Multiple Xenobiotic Response Elements

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Running Title Page

Running title: XRE-Mediated Constitutive and Inducible *ALDH3A1* Expression

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Discussion: 1521

The abbreviations used are: *CYP1A1*, cytochrome p450 1A1 gene; *ALDH3A1*, rat class three aldehyde dehydrogenase gene; 3-MC, 3-methylcholanthrene; HIF-1α, hypoxia inducible factor-1 alpha; ARNT, aryl hydrocarbon nuclear translocator; AhR, aryl hydrocarbon receptor; TCDD, tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic response element; DMSO, dimethylsulfoxide; HNE, 4-hydroxy-2-nonenal; GSH, glutathione; CAT, chloramphenicol acetyl-transferase

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Abstract

The rat class three aldehyde dehydrogenase gene (*ALDH3A1*) is expressed constitutively or by xenobiotic induction depending on the tissue in which it occurs. While the mechanism that mediates inducible expression has been well characterized, relatively little is known about constitutive regulatory mechanisms. Previous *ALDH3A1* promoter analyses have indicated that primary regulatory regions within the *ALDH3A1* 5' flanking region exert similar effects on both constitutive and inducible *ALDH3A1* expression. However, promoter gene analyses that served as the basis of early work were limited by the lack of sufficient 5' flanking region sequence. To gain a more complete picture of how the 5' flanking region regulates both modes of expression, we have subcloned an 8.0 kb fragment from the 5' flanking region of the *ALDH3A1* gene and subjected it to reporter gene analyses. We found a region located between 4.8 and 7.8 kb upstream of the non-coding first exon that drives strong *ALDH3A1* reporter activity. This region contains xenobiotic response element (XRE) consensus sequences that mediate constitutive and inducible *ALDH3A1* reporter gene expression. Using the new generation of *ALDH3A1* reporter constructs we were unable to confirm the presence of a negative regulatory region that was apparent in previous studies using a shorter fragment of the 5' flanking region. We also demonstrate that 3-methyl cholanthrene induces *ALDH3A1* expression above high constitutive background in corneal epithelial cells.

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Class 3 aldehyde dehydrogenase (ALDH3A1) is a broad-substrate cytosolic detoxification enzyme that catalyzes the oxidation of medium-chain length aliphatic aldehydes to carboxylic acids (Lindahl, 1992). Aldehydes derived from lipid peroxidation, such as 4-hydroxy-2-nonenal (HNE), cause glutathione (GSH) depletion, protein damage, growth inhibition and apoptosis (reviewed in Yu, 1993). ALDH3A1 expression protects against HNE-induced protein adduct formation and growth inhibition, and enhances cell survival (Townsend et al., 2001).

ALDH3A1 enzyme activity occurs only in a subset of tissues and this distribution is determined by ALDH3A1 gene (*ALDH3A1*) expression regulatory mechanisms. These mechanisms confer a bimodal expression pattern such that *ALDH3A1* is expressed constitutively or by xenobiotic induction. Constitutive expression occurs in the lung, stomach, trachea, tongue, bronchial epithelium, urinary tract, skin and in corneal epithelium (Su et al., 2002; Boesch et al., 1996). In the latter tissue, ALDH3A1 protein may account for up to 40% of cytosolic protein in some species (Kinoshita et al., Piatigorsky, 1988). Constitutive expression also occurs in some rat hepatoma lines such as HTC, H4-II-EC3 and MH-7777 (Lin et al., 1988). In the normal liver *ALDH3A1* is not expressed. However hepatic expression is activated by polycyclic and halogenated aromatic hydrocarbons including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and 3-methylcholanthrene (3-MC) (Dunn et al., 1988). With the exception of hepatoma cell lines, xenobiotic

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inducible expression has not previously been demonstrated in constitutive *ALDH3A1* expression systems.

Xenobiotic induction of *ALDH3A1* expression is mediated by a transcription factor complex that includes a dimer formed by the aryl hydrocarbon nuclear translocator (ARNT) and the aryl hydrocarbon receptor (AhR) (Schmidt and Bradfield, 1996; Reisdorph and Lindahl, 2001). Xenobiotic ligands bind to AhR which then forms transcriptionally active dimers with ARNT. AhR/ARNT dimers transactivate target genes via xenobiotic response elements (XRE). In contrast, the mechanism that mediates constitutive *ALDH3A1* expression has not been elucidated. However, evidence indicates that constitutive and inducible mechanisms are fundamentally similar. For example, previous *ALDH3A1* chloramphenicol acetyl transferase (CAT) reporter gene deletion analyses indicate that both modes of expression are directed by the same regulatory regions; most notably, a region near -2.0 kb that contains a functional XRE (Takimoto et al., 1994; Boesch et al., 1999). Mutagenesis of the XRE ARNT half-site confirmed that it mediates both xenobiotic-inducible and constitutive ALDH3A1 reporter gene expression (Boesch et al., 1999). Interestingly, the pALDHCAT reporter containing the largest segment of *ALDH3A1* 5' flanking region (~5.0 kb) displays sharply reduced constitutive and xenobiotic-induced reporter activity compared to a reporter which contains ~3.2 kb of 5' flanking region. This observation led to the hypothesis that negative regulatory elements reside upstream of -3.2 kb (Takimoto et al., 1994; Boesch et al., 1999). The

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existence of negative elements in this region has not been confirmed, and the lack of sufficient *ALDH3A1* 5' flanking region has hindered more complete analyses.

Another commonality between constitutive and xenobiotic-induced expression is that both are downregulated by hypoxia (Reisdorph and Lindahl, 1998). This phenomenon has been demonstrated for P4501A1 (*CYP1A1*), another gene regulated by ARNT/AhR (Gradin et al., 1996). However, *ALDH3A1* is the only ARNT/AhR responsive gene that has a constitutive expression component downregulated by hypoxia. Although a definitive mechanism by which downregulation occurs has not been described, it may involve competition for limiting transcription factors, as ARNT is also a dimerization partner for hypoxia inducible factor-1a (HIF-1α). It has been proposed that competition for ARNT or a factor required for ARNT function during concomitant activation of hypoxia and xenobiotic pathways results in inhibition of ARNT/AhR-mediated transactivation (Gradin et al., 1996; Chan et al., 1999). However, there is also evidence indicating that hypoxia-induced downregulation is more complex than simple sequestration of a single transcription factor (Pollenz et al., 1999). Regardless of the specific mechanism of hypoxia-induced downregulation of AhR/ARNT regulated expression, in light of previous promoter analyses, the effects of hypoxia on both modes of *ALDH3A1* expression support the idea that the underlying transcriptional mechanisms are similar.

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In order to gain a more complete picture of how the 5' flanking region regulates *ALDH3A1* expression, we subcloned an 8.0 kb fragment of the ALDH3A1 gene and generated a battery of luciferase reporter gene deletion constructs. This sequence corresponds to the rat ALDH3A1 gene sequence with a chromosomal location of 10:47365155-47374873. The 8.0 kb fragment includes the 5.0 kb used in previous CAT reporter gene studies plus an additional ~3.0 kb located directly upstream. The *ALDH3A1* luciferase deletion reporters were used to determine *ALDH3A1* expression patterns in both inducible and constitutive *ALDH3A1* expression cell culture systems, represented by the rat hepatoma line H4-II-EC3 and rat corneal epithelial cells respectively. Our results show that a 3.0 kb segment of the 5' flanking region located between 4.8 kb and 7.8 kb upstream of the non-coding first exon drives robust *ALDH3A1* reporter expression. Deletion studies indicate that XRE consensus sequences located within this region cooperate with other XREs in the 5' flanking region to drive both constitutive and xenobiotic-induced *ALDH3A1* expression. In the course of these studies we tested for effects of 3-MC on constitutive expression in rat corneal epithelial cells. We show for the first time that 3-MC strongly induces *ALDH3A1* expression above constitutive levels, and that hypoxia downregulates 3-MC-induced expression in corneal epithelial cells.

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Methods

Cell culture and treatment. H4-II-EC3 rat hepatoma cells were cultured in Ham's F-12 medium supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml amphotericin B (all from Sigma). Corneal epithelial cells were cultured as described previously (Boesch et al., 1996) with modifications; explants were evenly spaced and anchored in groups of four per 60 X 15 mm culture plate (Falcon). Explants were fed for four days with serum free Dulbecco's modified Eagle's medium Ham's F-12 supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, 0.5% DMSO, 0.1 μg/ml cholera toxin, and 10 ng/ml epidermal growth factor (all from Sigma). Insulin was omitted. After four days cells were fed with medium described above supplemented with 10% fetal bovine serum until harvest between 10 and 18 days in culture (or at about 90% confluence). For xenobiotic induction, 3-methylcholanthrene (3-MC) (Sigma) in dimethylsulfoxide (DMSO) was added to the culture medium at a final concentration of 1 mM. For hypoxic induction, tissue culture plates were placed in a modular incubator chamber (Billups-Rothenberg) that was flushed with a gas mix comprised of 1% O_2, 5% CO_2, balance N_2. The chamber was then placed in an incubator at 37^o for the duration of hypoxic treatment.

DNA constructs. The *ALDH3A1* CAT reporters were described previously (Takimoto et al., 1994). The 8.0 kb SacI/BglII fragment from UTR-1 consisting of 7,651 bp of *ALDH3A1* 5' flanking region, the entire 40 bp non-coding first exon, and 198 bp of the first intron was subcloned into pGL3basic (Promega) to

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generate the *ALDH3A1*-luciferase reporter, pALDH8.0. Deletion constructs were then generated using the following restriction enzymes; SacI/SphI (pALDH4.6), KpnI (pALDH3.2 and pALDH3.2ΔXRE2), SacI/SfiI (pALDHluc0.8), and KpnI/PstI for pALDHluc0.6. pALDH8.0ΔBglI was generated by deletion of a 2.7 kb BglI fragment from pALDHluc8.0. The 730 bp ApaI/HincII fragment containing XRE3 and XRE4 was subcloned into pALDH0.6 to create pALDHXRE3/4. pALDHXRE3 was created by removing a KpnI fragment from the 5' end of pALDHXRE3/4. pALDH8.0ΔXRE2 was a result of a cloning artifact. All ligation products were used to transform SURE Epicurian coli cells (Strategene) by electroporation. Deletion mutagenesis of XRE3 and XRE4 was performed using the GeneEditor™ mutagenesis kit (Promega). The five base pair core sequence of XRE4 (gcgtg) was deleted using the oligo XREn6dmuta, (5'-TCGAGTGTGCCCTGACTTTGTGGTACCTATG-3'). Fifteen base pairs encompassing the XRE3 cluster (ggtgcgtgtgtgcgc) were deleted using oligo XREn6pmuta, (5-TGGCCTACTGCTAGTGGGAAGATGTGCAACACACACAAGG TGAAGGGGCTTAGTTGTTGAC-3').

Reporter assays. EC3 cells were transfected in 35 mm plates with 1.75 μg pALDH reporters and 0.25 μg gWiz-beta-Gal (Gene Therapy Systems) using GenePorter transfection reagent (Gene Therapy Systems). Corneal epithelial cells were transfected with 7.0 μg pALDHluc8.0 and 1.0 μg gWiz-beta-Gal. *ALDH3A1*- luciferase reporter activity was determined using LucLite assay reagent (Packard Bioscience) supplemented with 100 mM each $MgCl_2$ and $CaCl_2$ on a TopCount-NXT luminescence counter (Packard Bioscience). Beta-

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galactosidase activity was determined using Galacton-Plus kit (Tropix). Luciferase values were normalized to beta-galactosidase as a control for transfection efficiency.

Northern blot analysis. RNA was extracted using TRI REAGENT (Sigma). Five μg total RNA from corneal epithelial cells was separated in a 1% formaldehyde-agarose gel and transferred to a Hybond-N nylon membrane (Amersham) by upward capillary transfer. *ALDH3A1* mRNA was detected using a 1.2 kb EcoRI/BglII fragment from pTALDH cDNA (Lin et al., 1988) labeled with [α-^{32}P]dCTP by random priming using RadPrime (Gibco). Autoradiography was performed and fold induction was determined by densitometry using ethidium bromide-stained 18S ribosomal RNA for standardization.

Western blot analysis. Protein lysates from H4-II-EC3 and corneal epithelial cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Bio-Rad). Membranes were probed with a goat polyclonal antibody against rat AhR (Novus) diluted 1:1000 in nonfat dry milk (NFDM). AhR-primary antibody complexes were detected with a mouse anti-goat secondary antibody conjugated to horseradish peroxidase diluted 1:10,000 in NFDM and developed by chemiluminescence using ECL reagent (Amersham).

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Results

The first generation of *ALDH3A1* reporter gene constructs were derived from a lambda Charon 35 library clone (Takimoto et al., 1994). The most striking features of the expression pattern of the pALDHCAT deletion reporters are 1) the strong activity produced by pALDHCAT-3.2 which contains a functional XRE located near -2.0, and 2) the fact that reporters containing segments of 5' flanking region longer than ~4.0 kb exhibit sharply reduced 3-MC-induced and constitutive reporter gene expression (Takimoto et al., 1994, figure 2; Boesch et al., 1999, figure 1) (figure 1). This presented the possibility that a negative regulatory region exists upstream of -3.2, and that other positive regulatory regions may exist further upstream. In order to study the 5' flanking region in a more complete context, we subcloned an 8,017 bp fragment from a lambda DASH rat genomic clone, UTR-1 (Asman et al., 1999). This 8.0 kb fragment consists of the same 5.0 kb region represented in the ALDH3A1 CAT reporters plus 3,013 bp of additional 5' flanking region previously unexamined (figure 2 A). Each *ALDH3A1* reporter construct includes 238 bp of the first non-coding exon and partial first intron (Takimoto et al., 1994). The 8.0 kb fragment was inserted into pGL3 basic to create pALDHluc8.0. pALDHluc8.0 was then used to create a series of luciferase *ALDH3A1* deletion constructs that emulate the *ALDH3A1*-CAT reporters (figure 2 B). The entire 8.0 kb fragment was sequenced in reverse and forward directions. The sequence has been deposited in Genbank (accession number EF015593).

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Analysis of luciferase-based *ALDH3A1* reporter constructs. Two cell culture systems were used for these studies. For xenobiotic-inducible expression we used a rat hepatoma line, H4-II-EC3. These cells express *ALDH3A1* only after xenobiotic induction, and thus serve as a model system for hepatic *ALDH3A1* expression (Lin et al., 1988). Rat primary corneal epithelial cells were used to study constitutive expression. These cells exhibit very high *ALDH3A1* expression in the absence of xenobiotic induction (Boesch et al., 1996). The luciferase *ALDH3A1* reporter constructs were used to transfect H4-II-EC3 and corneal epithelial cells and extracts were tested for luciferase activity. Consistent with previous *ALDH3A1* CAT reporter data, pALDHluc3.2, which contains the XRE near -2.0 kb, drives strong reporter expression relative to pALDHluc0.6 which contains only the basal promoter (figure 3). In contrast to *ALDH3A1* CAT reporter results, we did not observe reduced reporter activity in constructs containing greater than 4.0 kb (figure 3). Sequence analysis of the *ALDH3A1* CAT reporter sequence and the *ALDH3A1* luciferase version did not reveal any sequence variation within the region between -3.0 and -5.0 that could explain this discrepancy (not shown). The new full length *ALDH3A1* reporter, pALDHluc8.0, drove *ALDH3A1* reporter expression at much higher levels compared to any other reporter construct, indicating that positive regulatory elements exist between -5.0 and -8.0 (figure 3 A and B).

Analysis of XRE-containing regions. Previous studies demonstrated that the XRE located near -2.0 kb (designated XRE2 for the current study) mediates both

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constitutive and xenobiotic-inducible *ALDH3A1* expression (Boesch et al., 1999). We therefore asked whether other XREs in the 5' flanking region also mediate *ALDH3A1* expression, and what their relative contribution is in the absence of XRE2. The core XRE sequence is GCGTG, and typically occurs as TNGCGTG, where N represents 0 to 2 nucleotides (Neuhold et al., 1999; Shen et al., 1992; Yao et al., 1992). In addition to the XRE near -2.0 kb, previous sequence analysis of the *ALDH3A1* 5' flanking region revealed a XRE consensus sequence at positions -387 to -382 (Asman et al., 1999). For the present study we have designated this consensus sequence XRE1 (figure 4 A). Our analysis revealed at least two other XRE consensus sequences. Within the new sequence upstream of -5.0 kb we identified a cluster of XRE consensus sequences between -5852 to -5837, which we designated XRE3 (figure 4 A). This cluster contains four overlapping core XRE sequences on both strands, in both forward and reverse orientation. In addition, a single XRE consensus sequence, designated XRE4, lies between -6107 to -6101 (figure 4 A).

To test regions containing XRE consensus sequences we generated additional *ALDH3A1* 5' flanking region reporters (figure 4 B). pALDHluc0.6 was extending by 180 bp to generate pALDHluc0.8, resulting in the addition of a fragment containing XRE1. Compared to pALDHluc0.6, pALDH0.8 produces 3.5 fold higher constitutive *ALDH3A1* reporter activity in corneal epithelial cells than pALDHluc0.6 (figure 5 A). In H4-II-EC3 cells treated with 3-MC, this reporter displays 11 fold induction compared to 2 fold induction for pALDHluc0.6 (figure 5

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B). These results support the idea that XRE1 mediates both modes of reporter expression. To assess the ability of regions containing XRE3 and XRE4 to drive *ALDH3A1* reporter expression in the absence of XRE2, we generated a series of deletion mutants (figure 4 B). When fused to 600 bp of the proximal promoter (reporter pALDHXRE3/4), a 700 bp fragment containing both XRE3 and XRE4 strongly enhances constitutive reporter activity in both H4-II-EC3 and corneal epithelial cells (figure 5). In addition, 3-MC induction in H4-II-EC3 cells was increased from 2 fold (pALDHluc0.6) to 3.85 fold (figure 5 B). 5' truncation of this 700 bp fragment, which resulted in removal of XRE4 (reporter pALDHXRE3), sharply reduces positive effects of the 700 bp XRE fragment on constitutive corneal reporter expression, and virtually eliminates 3-MC induction in H4-II-EC3 cells. Deletion of a 2.4 kb BglI fragment, including XRE2, from the 8.0 kb *ALDH3A1* fragment sharply increases both constitutive and 3-MC-induced expression in (figure 5 A and B). These results indicate that the region containing XRE2 is dispensable for *ALDH3A1* reporter expression, and may exert negative effects. Therefore to further investigate this issue, we took advantage of an artifact created during subcloning of the 8.0kb *ALDH3A1* fragment. This artifact resulted in deletion of a 150 bp fragment immediately surrounding and including XRE2. Effects of the 150 bp deletion on 3-MC-induced reporter expression were measured in the context of pALDH8.0 (pALDH8.0ΔXRE2) and pALDH3 (pALDH3.2ΔXRE2). Interestingly, in both cases the deletion results in enhanced 3-MC-induced hepatic reporter expression relative to the wild type reporters (figure 6).

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Mutagenesis of XRE consensus sequences. To directly test the role of XREs 3 and 4 in mediating *ALDH3A1* reporter expression we performed deletional mutagenesis. Fifteen base pairs encompassing XRE3, and the 5 base pair core sequence of XRE4 were deleted from the 700 base pair XRE fragment in the reporter pALDHXRE3/4. In corneal epithelial cells deletion of either XRE3 or XRE4 significantly reduced constitutive reporter gene expression (figure 7 A). Deletion of XRE3 did not effect 3-MC-induced reporter expression in H4-II-EC3 cells. However, deletion of XRE3 or both XRE3 and XRE4 eliminated induction of reporter activity by 3-MC (figure 7 B). Interestingly, the double deletion mutant produces a slight but reproducible increase in expression compared to both single deletion mutants in corneal cells, and compared to the XRE4 deletion mutant in H4-II-EC3 cells.

Xenobiotic induction of *ALDH3A1* expression in corneal epithelial cells. Studies of *ALDH3A1* expression have been conducted under the premise that in specific tissues, expression occurs as either constitutive or xenobiotic-inducible, but not both. In the course of our studies we reexamined this issue, and asked whether we could induce *ALDH3A1* expression above high constitutive levels in corneal epithelial cells with 3-MC. Cells were treated with 3-MC for 24 hours and tested for effects on *ALDH3A1* reporter gene expression and *ALDH3A1* mRNA. 3-MC treatment results in a 3 fold induction of *ALDH3A1* reporter expression (figure 8 A). Northern blot analysis confirms strong upregulation of *ALDH3A1*

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message. We previously showed that hypoxia downregulates inducible and constitutive *ALDH3A1* expression in H4-II-EC3 and corneal epithelial cells respectively (Reisdorph and Lindahl, 1998). We therefore asked what effects concurrent treatment of hypoxia and 3-MC would have on xenobiotic-induced *ALDH3A1* expression in corneal cells. As shown in figure 8 B, 1% oxygen downregulates both constitituve and 3-MC-induced *ALDH3A1* expression in corneal epithelial cells.

Previously it was reported that AhR is not detectable in corneal epithelial extracts by Western blot analysis (Boesch et al., 1999). However, our observation that 3-MC induces *ALDH3A1* expression in corneal clearly indicates that AhR or a similar protein must mediate this response. Therefore we revisited this question and probed corneal extracts with an antibody against AhR. Figure 9 clearly shows that corneal extracts possess AhR polypeptide at levels comparable to those in H4-II-EC3 extracts.

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Discussion

Our results indicate that *ALDH3A1* expression is influenced by at least four XRE elements distributed throughout approximately 6.1 kb of 5' flanking region sequence. These XRE elements appear to act in concert to drive both xenobiotic-induced and constitutive *ALDH3A1* expression. Of particular interest in the current study are the XREs located between -5.8 and -6.1 kb which exert cell type-specific effects on *ALDH3A1* reporter expression. Our data suggest that whereas the distal most XRE (XRE4) mediates both 3-MC-induced and constitutive expression, the cluster of overlapping XREs (XRE3) primarily mediates constitutive expression. In addition, XRE3 and XRE4 appear to both be required for constitutive expression, as deletion of either eliminates positive effects on constitutive reporter activity. One unexpected result was that deletion of a 150 bp region surrounding and including XRE2 did not reduce reporter expression, but rather resulted in an increase in 3-MC-induced expression. This is in contrast to previous mutagenesis work which confirmed XRE2 mediates both modes of *ALDH3A1* expression. The results of these two studies need not be contradictory. It is likely that the region immediately surrounding XRE2 actually represses *ALDH3A1* expression, and that XRE2 serves as a means of relieving this repression. This could be explained by the recruitment of transcriptional co-activators by AhR/ARNT. Co-activator proteins including creb binding protein (CBP)/p300 (Kallio et al., 1998), Gcn5 (for general control nonrepressed) (Brownell et al., 1996), the Spt-Ada-Gcn5-acetyltransferase complex (SAGA) (Hampsey et al., 1999), and the NCoA/SRC-1/p160 family of

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transcriptional coactivators, either possess or are associated with histone acetyl transferase (HAT) activity. Acetylation of lysine residues in amino termini of histones H3 and H4 reduced the stability of histone/DNA interactions, thus promoting unfolding of chromatin, allowing better access to general transcription factors (Grunstein et al, 1997). Both ARNT and AhR have been demonstrated to associate with NCoA/SRC-1/p160 family coactivators in a TCDD-dependent manner (Beischlag et al., 2002). Also, recently it was reported that AhR recruits the co-activator GAC63 to XREs (Chen et al., 2006). Another mechanism of chromatin remodeling involves the SWI/SNF complex. The SWI/SNF complex utilizes ATPase activity to remodel chromatin conformation in enhancer regions thereby stimulating gene expression. AhR has been shown to interact with Brm/SWI-2 related gene (BRG-1) a subunit of the SWI/SNF chromatin remodeling complex. Further, forced expression of BRG-1 in a BRG-1-minus background restored endogenous P4501A1 (*CYP1A1*), expression, and enhanced XRE-driven reporter gene expression (Song Wang and Hankinson 2002). Given the established relationship between AhR/ARNT dimers and chromatin remodeling factors, it is plausible that the region surrounding XRE2 exists in a tightly packaged conformation and that binding by AhR/ARNT dimers, and recruitment of co-activator activity, induces a more relaxed conformation, allowing *ALDH3A1* expression to occur (figure 10). While it remains to be proven, such a scenario could provide a mechanism that contributes to tissue-specific regulation of *ALDH3A1* expression.

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Based on previous *ALDH3A1* CAT reporter analyses, we anticipated that the luciferase reporter pALDH4.6 would generate sharply reduced luciferase expression compared to pALDH3.2. However, reporter activity of pALDH4.6 was virtually identical to pALDH3.2, indicating that negative regulatory elements do not exist upstream of -3.0. Sequence analysis did not reveal variations between the lambda Charon 35 clone used to generate the CAT reporters and the lambda DASH clone used for the luciferase reporters. The most dramatic negative effects on ALDH3A1 CAT reporter expression were observed with the longest reporter which included approximately 5.0 kb of the ALDH3A1 5' flanking region. It is important to note that the ALDH3A1 luciferase reporter most similar to this CAT reporter is pALDH4.6, which includes 380 fewer base pairs at the 5' end. Thus it remains a possibility that negative regulatory elements exist in this region. However, in CAT reporter gene analyses, inclusion of 4.0 kb of 5' flanking region was sufficient to sharply reduce reporter activity, which we did not observe with pALDH4.6. Therefore it is possible that the negative effects on reporter activity observed in the CAT studies were caused by an artifactual context created by fusing *ALDH3A1* sequence with sequence from the pBLCAT reporter. In any case, our results show that additional positive regulatory elements exist upstream of -5.0, indicating that the results of early studies were influenced by an incomplete genomic context.

As reported here, XRE2 is located between -2,057 and -2,062. In earlier work utilizing the *ALDH3A1* CAT reporters, XRE2 was reported to be near -3.0 relative

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to the transcriptional start site. The length of the original deletion fragments was estimated by electrophoretic mobility rather than direct sequencing. Further, direct sequencing that resulted in the identification of XRE2 was performed from the 5' end of pALDHCAT-3.2, and did not extend significantly downstream of XRE2. For the current study we sequenced the entire 8.0kb 5' flanking region fragment on both strands; thus the positioning of the elements of interest has been precisely established.

Finally, we have demonstrated that *ALDH3A1* expression is inducible by 3-MC in corneal epithelial cells. This is significant because *ALDH3A1* expression has previously been thought to occur either constitutively, or by xenobiotic induction, but not by both mechanisms in the same tissue. Our results clearly show that AhR is present and functional in corneal epithelial cells. To our knowledge this is the first demonstration of 3-MC-inducibility of *ALDH3A1* expression in rat corneal epithelial cells. Given that *ALDH3A1* expression in corneal epithelial cells is constitutively quite high, the physiologic relevance of xenobiotic *ALDH3A1* induction is not immediately clear. The proposed roles for ALDH3A1 in the cornea include[RR1] structural[RR2] support (as a corneal crystallin), oxidation of toxic lipid aldehydes, absorption of UV radiation, and replenishment of reduced NAD(P) cofactors (Kinoshita et al., 2001; Piatigorsky, 1988). While ALDH3A1 likely functions to a degree in all of these roles, the physiologic importance of each are not clear. Nor is it clear if cellular ALDH3A1 is functionally partitioned, or if the entire pool is available for multiple physiologic tasks. There is

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considerable evidence that ALDH3A1 enzyme activity plays an important role in protecting cells from oxidative damage, particularly damage inflicted by reactive lipid aldehydes. Lipid peroxidation-derived aldehydes, such as 4-hydroxy-2-nonenal (HNE), exert deleterious effects including inhibition of DNA and RNA synthesis, inhibition of mitochondrial respiration, disturbances of calcium homeostasis, growth inhibition and eventually apoptosis. In cell models, *ALDH3A1* expression protects against HNE-induced protein adduct formation and growth inhibition, and enhances cell survival (Muzio et al, 2001; Townsend et al, 2001). This protection may be especially important in corneal epithelium. Under normal circumstances, the cornea is subjected to wide and frequent fluctuations in oxygen tension, ranging from levels essentially equal to that of the atmosphere (a condition hyperoxic relative to perfused tissues), to near anoxia (Lubbers and Baumgartl, 1997; Baum, 1997). The potential oxidative damage associated with these fluctuations must present a significant challenge to cellular survival and tissue function. In this scenario, the ALDH3A1 enzyme is well suited to play a protective role, as the protein half-life has been established to be 96 hours (Huang and Lindahl, 1990). Therefore, although *ALDH3A1* expression is inhibited during periods of hypoxia, the longevity of the ALDH3A1 enzyme ensures that a sufficient level of functional protein remains when cellular oxygen levels spike upward and protection is critical. Thus ALDH3A1 appears to be a component of an adaptive strategy that allows cells to temporarily decrease production of a vital part of their defensive machinery, yet still be able to rely on the function of that machinery when it is needed most. Under conditions in which

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cells are subjected to multiple environmental insults, e.g. xenobiotic and hypoxic exposure, xenobiotic induction would provide additional ALDH3A1 that may be required for survival.

Our results support the idea that, like xenobiotic-inducible expression, constitutive *ALDH3A1* expression is mediated by an AhR/ARNT-dependent mechanism. The possibility that constitutive expression is regulated by the classical AhR/ARNT mechanism is intriguing because it would suggest that AhR is activated by an endogenous ligand in tissues wherein *ALDH3A1* occurs constitutively. There is significant evidence to support this possibility. For example, several structurally diverse compounds have been shown to bind to and modulate AhR activity including dietary indoles, flavonoids and tryptophan derivatives (Seidel et al., 1992; ,Denison and Nagy, 2003). The compound 6-formylindolo[3,2-b]carbazole (FICZ) is a tryptophan photoproduct that binds to AhR with high affinity and activates its transcriptional activity (Wei et al., 1998). 2-(1_*H*-indole-3_-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) is also a potent agonist of AhR (Henry et al., 1996). In addition, studies of effects of AhR and ARNT overexpression on transcriptional activation in the absence of endogenous ligands demonstrate a requirement for the AhR ligand binding domain, indicating the presence of an endogenous agonist (Chang and Puga, 1998). There is a great deal to be discovered about physiologically relevant endogenous AhR ligands. Models for constitutive *ALDH3A1* expression such as

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our corneal system will likely provide important details about specific endogenous ligands and their roles in regulating AhR/ARNT transcriptional activity.

In summary, we show that a previously-unexamined region of the *ALDH3A1* 5' flanking region located between -4.6 kb and -7.8 kb drives xenobiotic-inducible and constitutive *ALDH3A1* reporter gene expression. Our results indicate that *ALDH3A1* expression is driven in part by a series of XRE response elements distributed throughout approximately 6.1 kb of 5' flanking region sequence. We also demonstrate 3-MC-inducibility of *ALDH3A1* expression in a constitutive corneal expression model system.

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Footnotes

Submitted by R. R. in partial fulfillment of the requirements of the PhD degree in the Graduate School, University of South Dakota School of Medicine.

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Figure legends

Figure 1. *ALDH3A1* CAT reporter expression profiles. Corneal epithelial cells were transfected with *ALDH3A1* CAT reporter constructs and extracts were assayed for CAT activity 48 hours post-transfection.

Figure 2. Schematic of ALDH3A1 5' flanking region and ALDH3A1 luciferase reporters. A.UTR-1 clone; the unshaded region represents ALDH3 gene sequence downstream of translational start site. Grey shaded region depicts 5.0 kb region studied previously in pALDHCAT reporters. The black region depicts additional sequence added for current studies. B. The 8.0 kb SacI/BglII fragment shown in A was subcloned into pGL3basic to generate pALDHluc8.0 which was then used to create deletion reporters. The numerical value of each construct signifies the approximate length (kb) of 5' flanking region present in each construct plus 238 bp of non-coding exon one and part of the first intron. The black vertical bar represents the previously-characterized XRE2 near -2.0 kb. The checkered vertical bars represent XREs examined in the current study.

Figure 3. *ALDH3A1* luciferase reporter expression profiles. Corneal epithelial cells (A) and H4-II-EC3 cells (B) were transfected with *ALDH3A1* luciferase reporter constructs and extracts were assayed 48 hours post-transfection. H4-II-EC3 cells were treated with 3-MC for 24 hours.

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Figure 4. Schematic of *ALDH3* 5' flanking region and reporter gene constructs. A. *ALDH3A1* 5' flanking region. XRE consensus sequences are depicted by vertical bars and their positions are indicated. The sequence immediately surrounding targeted XREs is listed and specific XRE core sequences are encased in boxes. XRE3 contains a cluster of overlapping XRE consensus sequences in forward and reverse orientation, and each is encased in a box with a distinct border. The bases deleted for mutagenesis studies are indicated in lower case font. XRE2 was characterized in previous studies. B. *ALDH3A1* deletion constructs. XREs targeted in the current study are depicted by checkered vertical bars. Previously characterized XRE2 is indicated by a black vertical bar. The grey region adjacent to the luciferase gene (in black) indicates the non-coding first exon and a portion of the first intron. See Materials and Methods section for greater detail of each construct.

Figure 5. Expression profile of *ALDH3* deletion reporters. Deletion reporters were tested for corneal constitutive (A) and H4-II-EC3 xenobiotic-inducible (B) expression. Specific reporters are indicated on X axis (see figure 4 for detail). Fold induction for 3-MC treated H4-II-EC3 cells is indicated. The extremely high value for the reporter pALDH8.0ΔBglI in H4-II-EC3 cells was omitted to preserve scale and for clarity.

Figure 6. Deletion of the XRE2 region enhances 3-MC-induced *ALDHA1* reporter activity. H4-II-EC3 cells were transfected with pALDH3.1, pALDH8.0,

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or versions lacking 150 bp surrounding and including XRE2 (designated Δ150). 24 hours post-transfection, cells were treated with 1 mM 3-MC for 24 hours and extracts were assayed for luciferase activity.

Figure 7. Effects of deletional mutagenesis of XREs on *ALDH3A1* reporter activity. pALDHXRE3/4 deletion mutants were generated that lack either XRE3, XRE4, or both . XREs in each reporter are indicated by vertical bars. Solid black bars indicate wild type, white bars indicate deleted XREs. Corneal epithelial (A) and H4-II-EC3 cells (B) were transfected with wild type pALDHXRE3/4 or indicated mutant reporters and extracts were assayed for luciferase activity 48 hours post transfection. H4-II-EC3 cells were treated with 1 mM 3-MC for 24 hours.

Figure 8. Northern blot analysis of the effects of 3-MC and hypoxia on *ALDH3A1* expression in corneal epithelial cells. A. Corneal epithelial cells were treated with 1 mM 3-MC for 24 hours. 5 μg total RNA was probed with radiolabeled ALDH3A1 cDNA and autoradiography was performed (inset). Radioactive counts per lane were normalized against 18S ribosomal RNA. Experiments were performed three times triplicate. B. Corneal epithelial cells were treated with 1%O_2, 1 mM 3-MC, or both for 24 hours. Northern blot analysis was performed as for A.

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Figure 9. Western blot analysis of AhR expression in hepatoma and corneal epithelial cell lysates. Protein extracts from corneal epithelial cells and H4-II-EC3 cells were resolved by SDS-PAGE, transferred to PVDF membrane and probed with antibodies against AhR.

Figure 10. Proposed model for transcriptional regulation of *ALDH3A1*. ARNT serves as a dimerization partner for both AhR and Hif1a, and thus mediates transcriptional activation of both xenobiotic- and hypoxia-responsive gene expression. In some model systems functional ARNT appears to be limited, resulting in suppressed xenobiotic-induced gene expression during concurrent hypoxia exposure. The negative transcriptional effects exerted by the region encompassing XRE2 may be due to tight chromatin packaging. Recruitment of transcriptional coactivators by AhR/ARNT could result in modification of histones and subsequent relaxation of chromatin structure, allowing access to additional AhR/ARNT dimers and other general transcription factors. In corneal epithelial cells, an unidentified mechanism, which may include an endogenous ligand, mediates constitutive expression. Exogenous ligands such as 3-MC further induce ALDH3A1 expression in corneal epithelial cells. The ALDH3A1 enzyme oxidizes lipid peroxides generated by oxygen radicals and thus likely serves an important role in cell survival during physiologic fluctuations in the partial pressure of oxygen.

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Figure 1

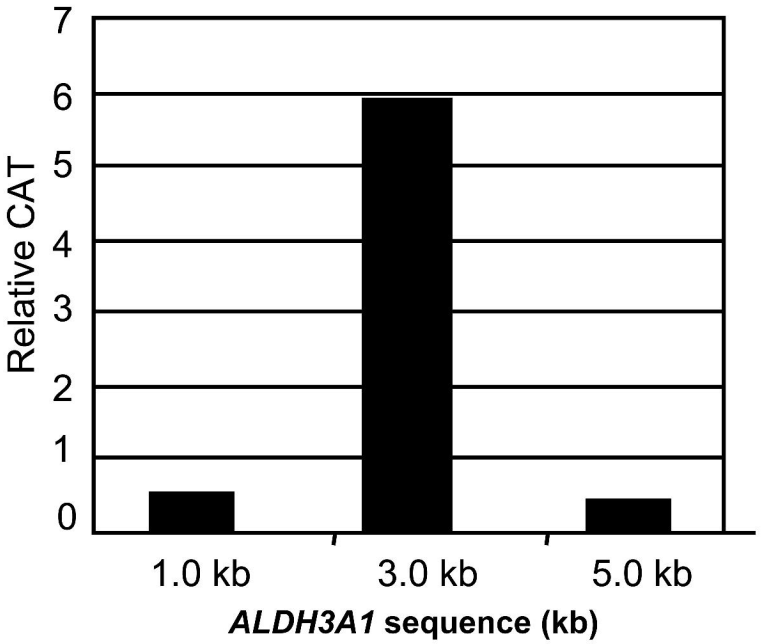
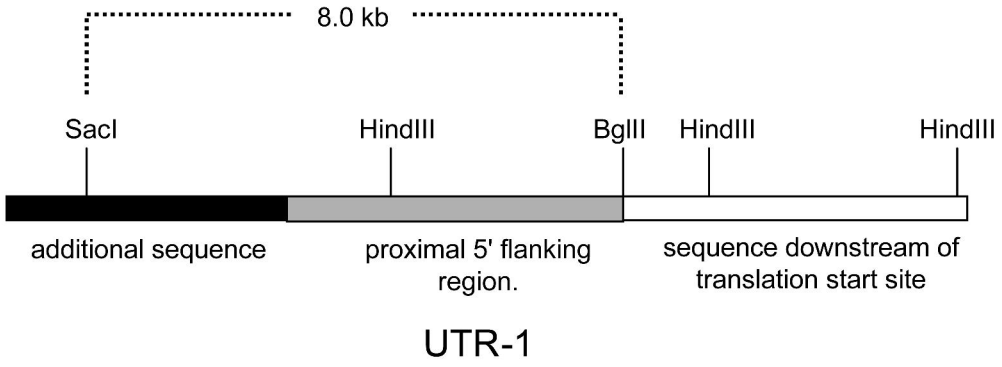


Figure 2

A



UTR-1

B

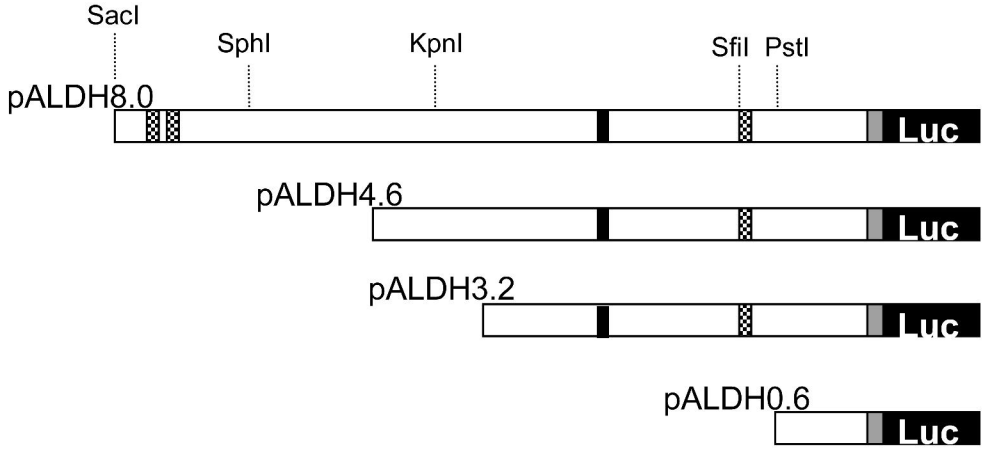
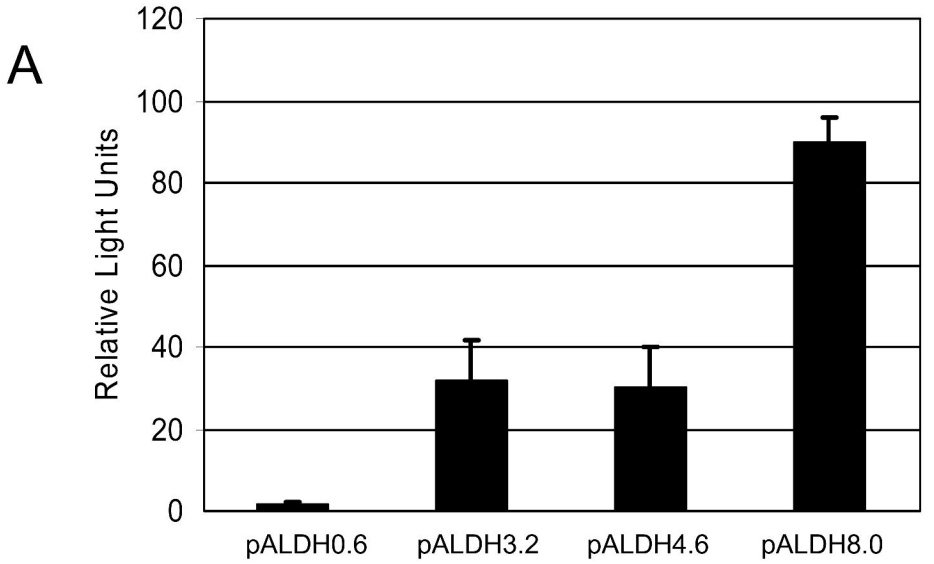


Figure 3


A
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40
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Relative Light Units
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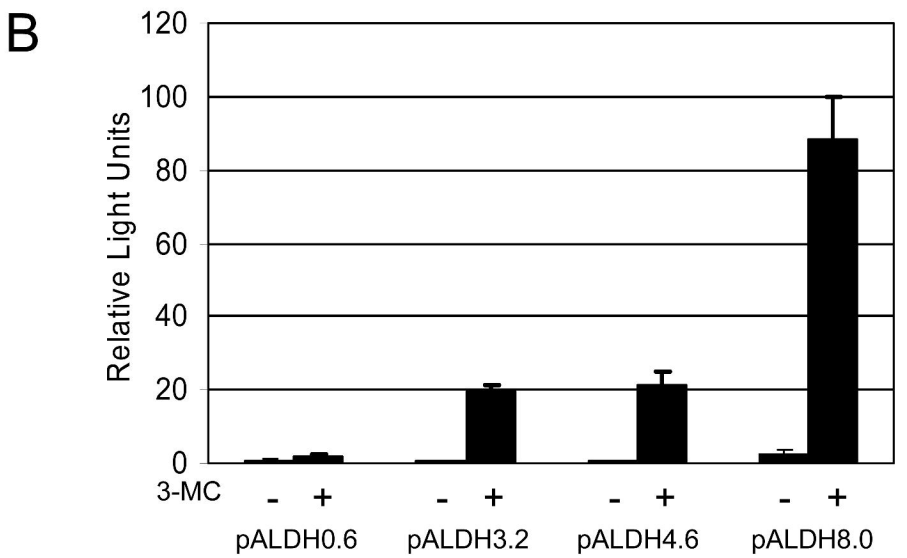
pALDH3.2
pALDH4.6
pALDH8.0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Relative Light Units
3-MC
- + - + - + - +
pALDH0.6
pALDH3.2
pALDH4.6
pALDH8.0

Figure 4


A
XRE4
(-6107 to -6101)
XRE3
-5852 to -5837)
XRE2
(-2062 to -2056)
XRE1
(-387 to -382)
--7,777
+1
ALDH3 5' flanking region
700XRE
fragment
XRE4
XRE3
...GCCCTGgcgtgACTTT...
...ATGTGCAGgtgcgtggtgtgcgcac...
...CGCGCCGCCTGCGTG
ACTGCAGCTT...

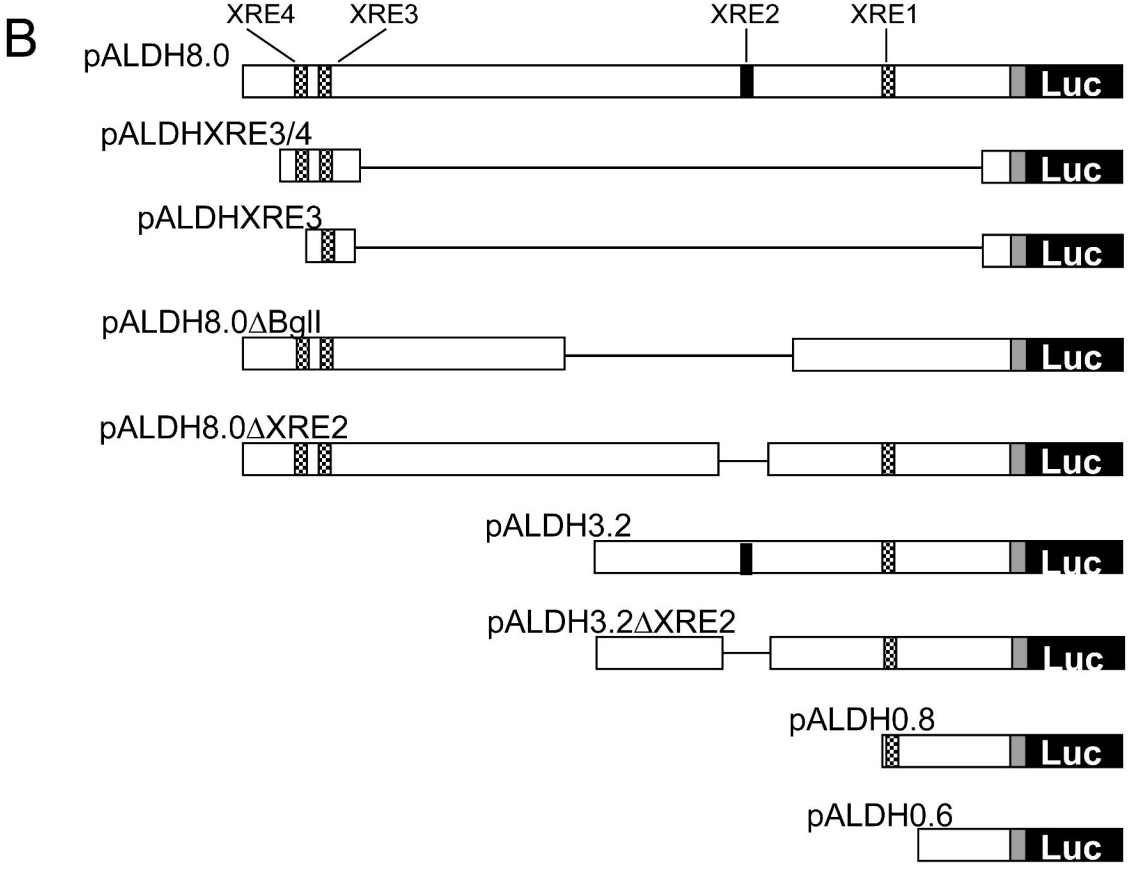
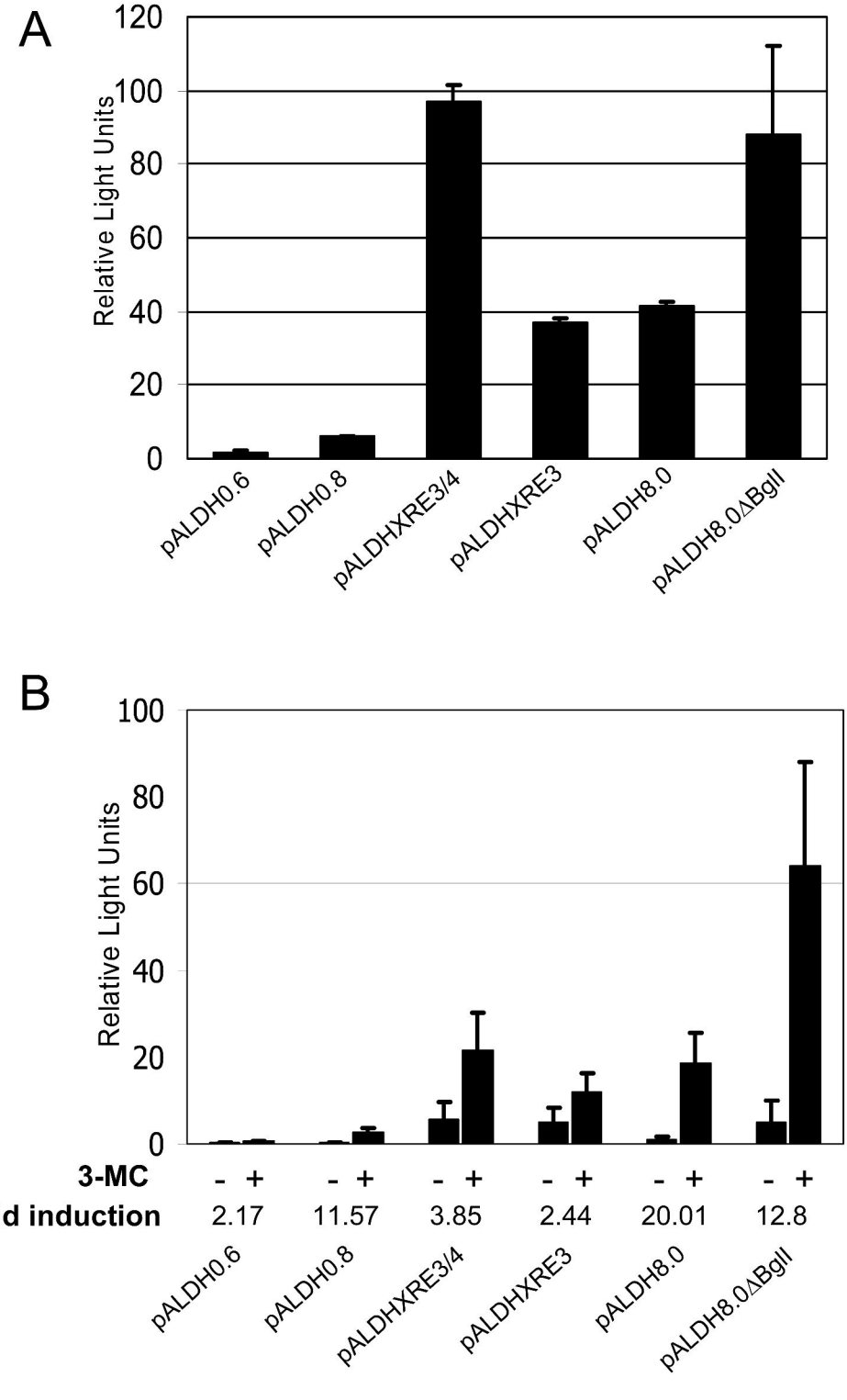

B
XRE4
XRE3
XRE2
XRE1
pALDH8.0
Luc
pALDHXRE3/4
Luc
pALDHXRE3
Luc
pALDH8.0ΔBglI
Luc
pALDH8.0ΔXRE2
Luc
pALDH3.2
Luc
pALDH3.2ΔXRE2
Luc
pALDH0.8
Luc
pALDH0.6
Luc

Figure 5


A
120
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Relative Light Units
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pALDH0.8
pALDHXRE3/4
pALDHXRE3
pALDH8.0
pALDH8.0ΔBglI
B
100
80
60
40
20
0
Relative Light Units
3-MC
- + - + - + - + - + - +
Fold induction
2.17
11.57
3.85
2.44
20.01
12.8
pALDH0.6
pALDH0.8
pALDHXRE3/4
pALDHXRE3
pALDH8.0
pALDH8.0ΔBglI

Figure 6

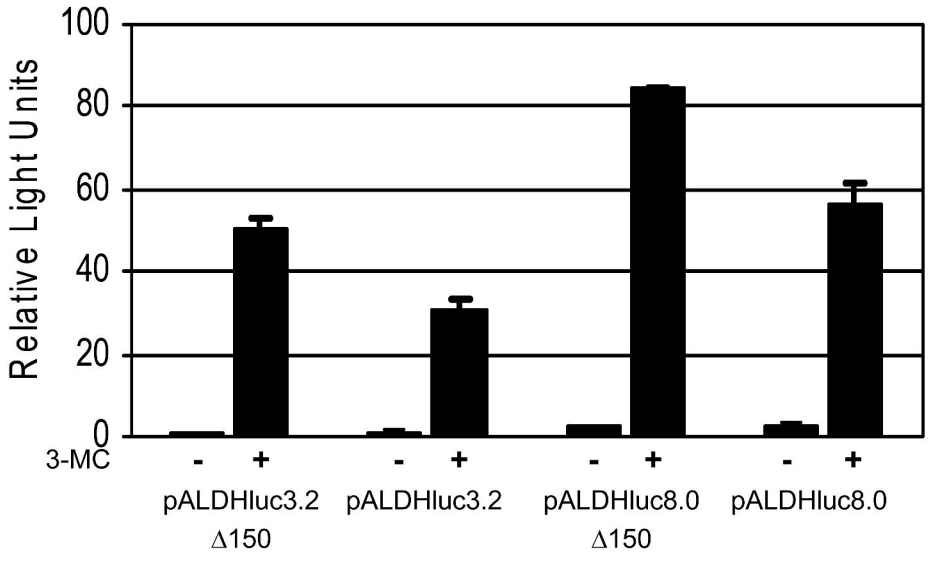

Relative Light Units
100
80
60
40
20
0
3-MC
- + - + - + - +
pALDHluc3.2 Δ150
pALDHluc3.2
pALDHluc8.0 Δ150
pALDHluc8.0

Figure 7

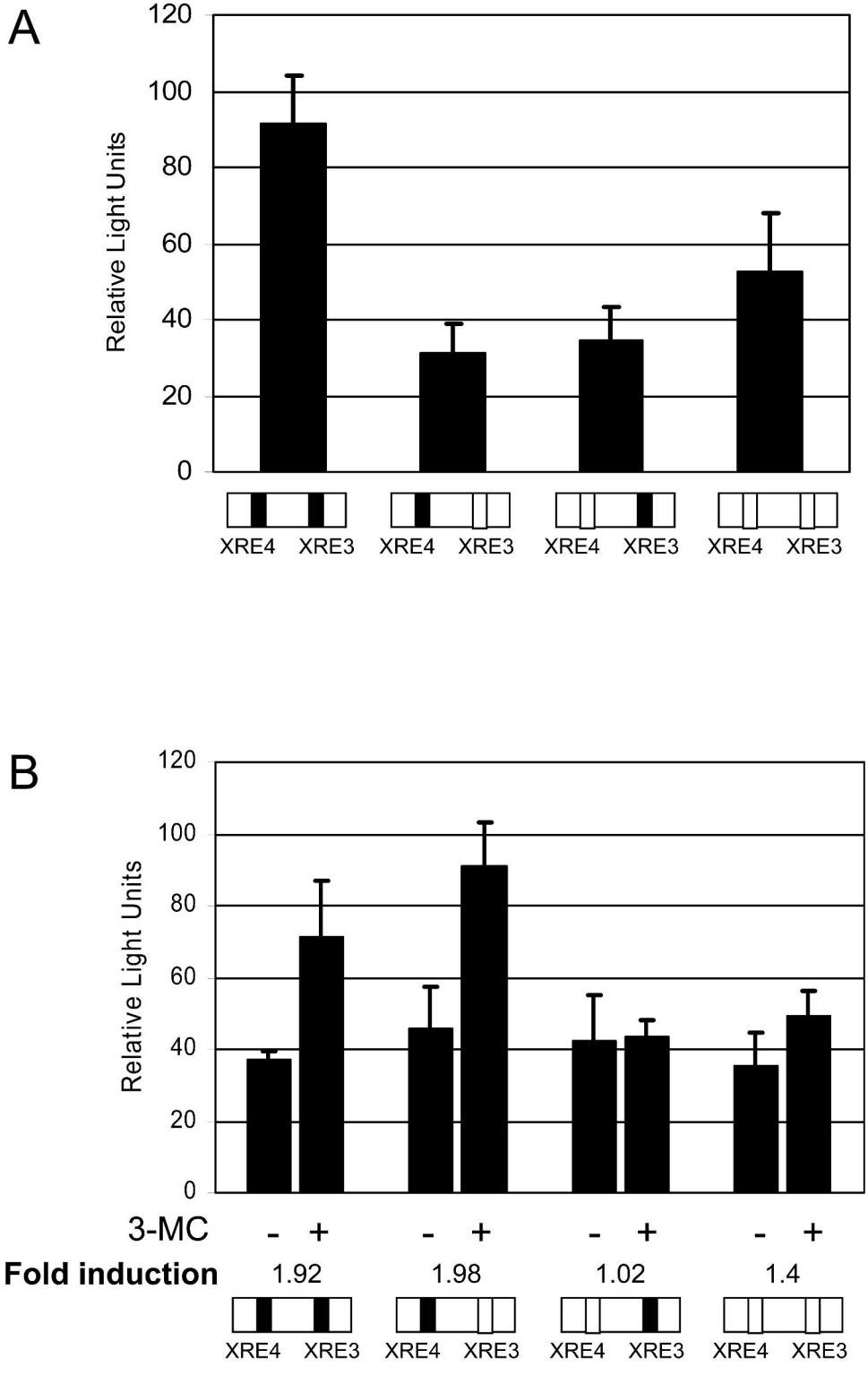
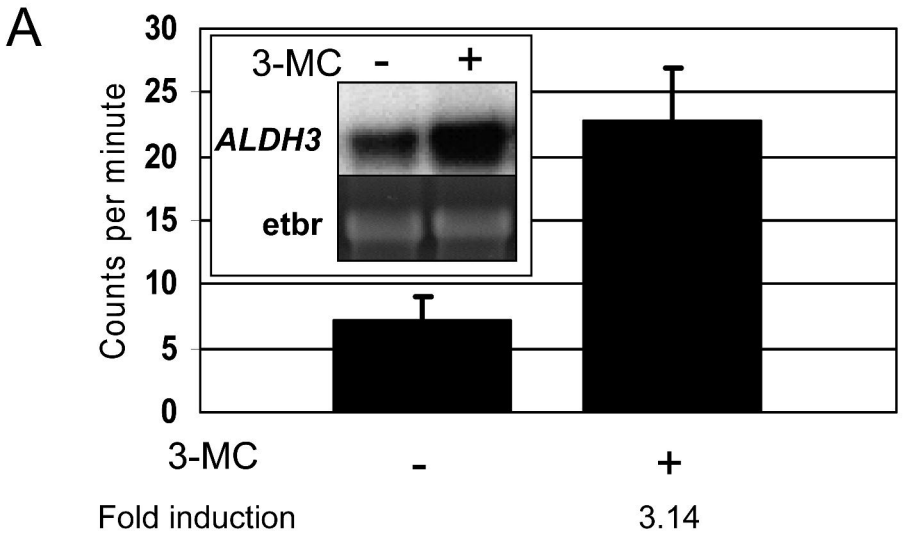

A
120
100
80
60
40
20
0
Relative Light Units
XRE4 XRE3
XRE4 XRE3
XRE4 XRE3
XRE4 XRE3
B
120
100
80
60
40
20
0
Relative Light Units
3-MC - + - + - + - +
Fold induction 1.92 1.98 1.02 1.4
XRE4 XRE3
XRE4 XRE3
XRE4 XRE3
XRE4 XRE3

Figure 8


A
30
25
20
15
10
5
0
Counts per minute
3-MC - +
ALDH3
etbr
3-MC - +
Fold induction 3.14

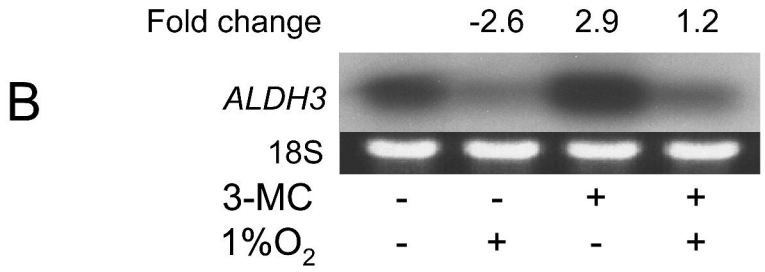

B
Fold change -2.6 2.9 1.2
ALDH3
18S
3-MC - - + +
1%O2 - + - +

Figure 9

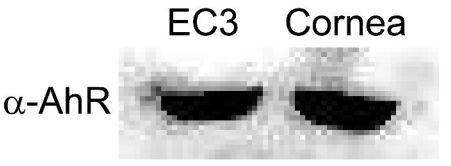


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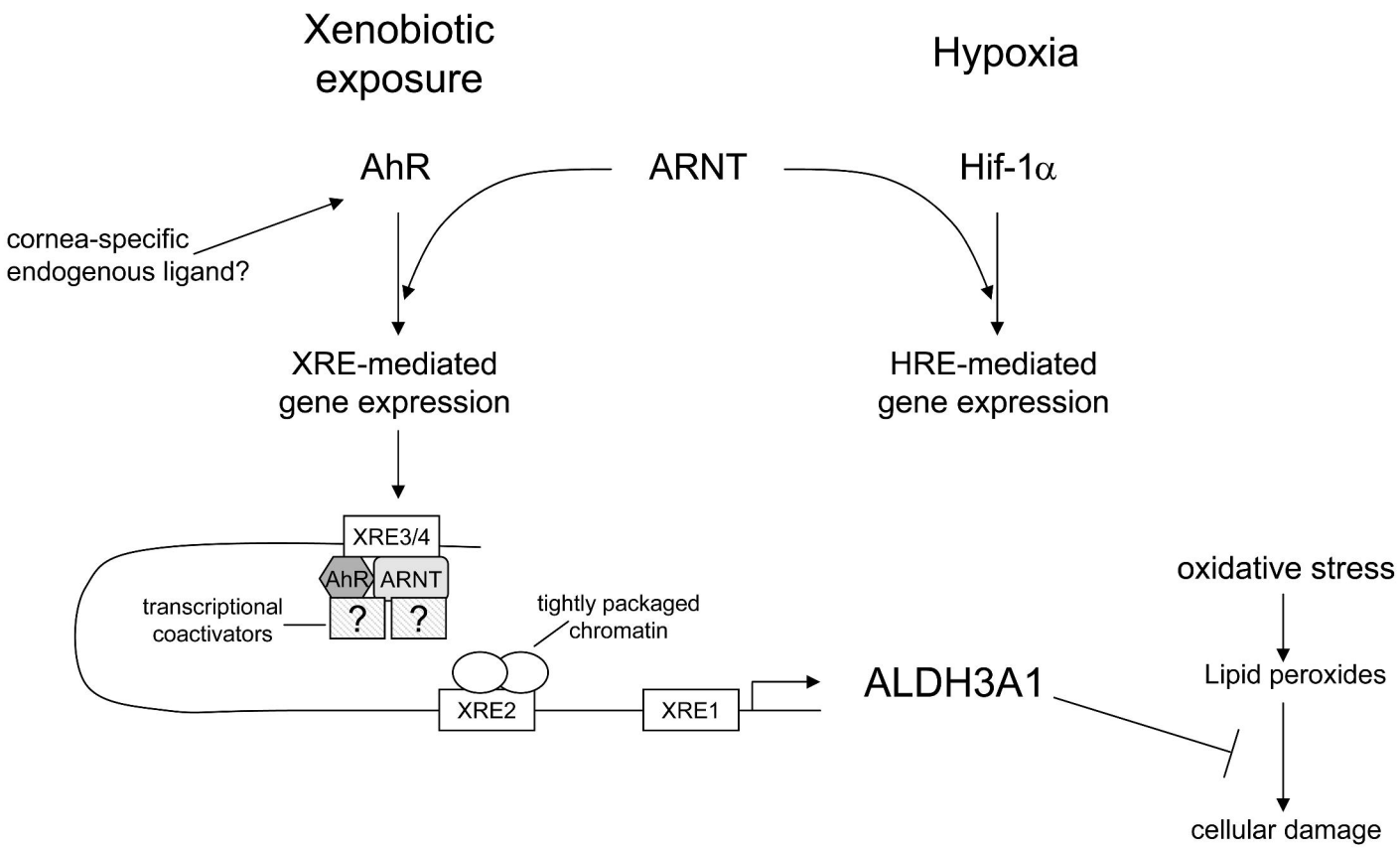
Figure 10



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