Constitutive and 3-Methylcholanthrene-Induced Rat ALDH3A1 Expression Is Mediated by Multiple Xenobiotic Response Elements

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ABSTRACT:
The rat class 3 aldehyde dehydrogenase gene (ALDH3A1) is expressed constitutively or by xenobiotic induction depending on the tissue in which it occurs. Although 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-kilobase (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 noncoding first exon that drives strong ALDH3A1 reporter activity. This region contains xenobiotic response element 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-methylcholanthrene induces ALDH3A1 expression above high constitutive background in corneal epithelial cells.

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 depletion, protein damage, growth inhibition, and apoptosis (reviewed in Yu, 1994). 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 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 corneal epithelium (Boesch et al., 1996; Su et al., 2002). In the latter tissue, ALDH3A1 protein may account for up to 40% of cytosolic protein in some species (Piatigorsky, 1988; Kinoshita et al., 2001). 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, and 3-methylcholanthrene (3-MC) (Dunn et al., 1988). With the exception of hepatoma cell lines, xenobiotic 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 (XREs). 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 pALDH3A1CAT reporter containing the largest segment of ALDH3A1 5′ flanking region (~5.0 kb) displays sharply reduced

ABBREVIATIONS: HNE, 4-hydroxy-2-nonenal; ALDH3A1, rat class 3 aldehyde dehydrogenase; 3-MC, 3-methylcholanthrene; ARNT, aryl hydrocarbon nuclear translocator; AhR, aryl hydrocarbon receptor; XRE, xenobiotic response element; CAT, chloramphenicol acetyl transferase; kb, kilobase(s); UTR, untranslated region; bp, base pair(s).
constitutive and xenobiotic-induced reporter activity compared with 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 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 down-regulated by hypoxia (Reisdorph and Lindahl, 1998). This phenomenon has been demonstrated for 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 down-regulated by hypoxia. Although a definitive mechanism by which down-regulation occurs has not been described, it may involve competition for limiting transcription factors, since ARNT is also a dimerization partner for hypoxia-inducible factor-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 down-regulation is more complex than simple sequestration of a single transcription factor (Pollenz et al., 1999). Regardless of the specific mechanism of hypoxia-induced down-regulation 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.

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 10:47,365,155 to 47,374,873. The 8.0-kb fragment includes the 5.0 kb used in previous ARNT reporter expression studies. 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, to our knowledge, that 3-MC strongly induces ALDH3A1 expression above constitutive levels, and that hypoxia down-regulates 3-MC-induced expression in corneal epithelial cells.

**Materials and 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 t-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml amphotericin B (all from Sigma-Aldrich, St. Louis, MO). 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 × 15 mm culture plate (Falcon; BD Biosciences Discovery Labware, Bedford, MA). Explants were fed for 4 days with serum-free Dulbecco’s modified Eagle’s medium-Ham’s F-12 supplemented with 2 mM t-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml amphotericin B, 0.5% dimethyl sulfoxide, 0.1 μg/ml cholaer toxin, and 10 ng/ml epidermal growth factor (all from Sigma-Aldrich). Insulin was omitted. After 4 days, cells were fed with medium described above, supplemented with 10% fetal bovine serum, until harvested between 10 and 18 days in culture (or at about 90% confluence). For xenobiotic induction, 3-MC (Sigma-Aldrich) in dimethyl sulfoxide 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, Inc., Del Mar, CA) that was flushed with a gas mix comprising 1% O2, 5% CO2, balance N2. The chamber was then placed in an incubator at 37°C 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 7651 bp of ALDH3A1 5′ flanking region, the entire 40-bp noncoding first exon, and 198 bp of the first intron were subcloned into pGL3basic (Promega, Madison, WI) to generate the ALDH3A1-luciferase reporter, pALDH8.0. Deletion constructs were then generated using the following restriction enzymes: SacI/Sphl (pALDH4.6), Kpol (pALDH3.2 and pALDH3.2ΔXRE2), SacI/SfII (pALDHuc0.8), and Kpol/PstI for pALDHuc0.6. pALDH0.2ΔBglII was generated by deletion of a 2.7-kb BglII fragment from pALDHubc8.0. The 730-bp Apal/HincII fragment containing XRE3 and XRE4 was subcloned into pALDH0.6 to create pALDHREX3/4. pALDHXRE3 was created by removing a Kpol fragment from the 5′ end of pALDHREX3/4. pALDHXRE4 was generated using the GeneEditor mutagenesis kit (Promega). The 5-bp core sequence of XRE4 (gattgt) was deleted using the oligo XREFnedm4ta (TGCAGTGTGCCCTGACTTTGTGGTACCTATG-3′). Fifteen base pairs encompassing the XRE3 cluster (gtggtggtggtggtggtggt) were deleted using oligo XREfndm3ta (TGGGCTTACTGTCTGTCGGCCAGTGGTCAACACACACAACAGG TGAAGGCGGTAGTTGGTGTAC-3′).

**Reporter Assays.** EC3 cells were transfected in 35-mm plates with 1.75 μg of pALDH reporters and 0.25 μg of gWiz-β-Gal (Gene Therapy Systems, Inc., San Diego, CA) using GenePorter transfection reagent (Gene Therapy Systems). Corneal epithelial cells were transfected with 7.0 μg of pALDHuc8.0 and 1.0 μg of gWiz-β-Gal. ALDH3A1-luciferase reporter activity was determined using LucLite assay reagent (PerkinElmer Life and Analytical Sciences, Boston, MA) supplemented with a 100 mM concentration of MgCl2 and CaCl2 on a TopCount-NXT luminescence counter (PerkinElmer Life and Analytical Sciences). β-Galactosidase activity was determined using a Galac-ton-Plus kit (Tropix, Bedford, MA). Luciferase values were normalized to β-galactosidase as a control for transfection efficiency.

**Northern Blot Analysis.** RNA was extracted using TRI Reagent (Sigma-Aldrich). Five micrograms of total RNA from corneal epithelial cells was separated in a 1% formaldehyde-agarose gel and transferred to a Hybrid-Bond nylon membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) by upward capillary transfer. Membranes were probed with a goat polyclonal antibody against rat AhR (Novus Biologicals, Inc., Littleton, CO) diluted 1:1000 in nonfat dry milk. AhR-primary antibody complexes were detected with a mouse anti-goat secondary antibody conjugated to horseradish peroxidase (Novus Biologicals, Inc., Littleton, CO) diluted 1:10,000 in nonfat dry milk. 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 polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). Membranes were probed with a goat polyclonal antibody against rat AhR (Novus Biologicals, Inc., Littleton, CO) diluted 1:1000 in nonfat dry milk. AhR-primary antibody complexes were detected with a mouse anti- goat secondary antibody conjugated to horseradish peroxidase diluted 1:10,000 in nonfat dry milk and developed with chemiluminescence using ECL reagent (GE Healthcare).

**Results**

The first generation of ALDH3A1 reporter gene constructs was 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, Fig. 2; Boesch et al., 1999, Fig. 1) (Fig. 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. To study the 5′ flanking region in a more complete context, we subcloned an 8017-bp fragment from a lambda DASH rat genomic clone, UTR-1 (Asman et al., 1993). This 8.0-kb fragment consists of the same 5.0-kb region represented in the ALDH3A1 CAT reporters plus 3013 bp of additional 5′ flanking region previously unexamined (Fig. 2A). Each ALDH3A1 reporter construct includes 238 bp of the first noncoding exon and partial first intron (Takimoto et al., 1994). The 8.0-kb fragment was inserted into pGL3 basic to generate pALDHLuc8.0. pALDHLuc8.0 was then used to create a series of luciferase ALDH3A1 deletion constructs that emulate the ALDH3A1-CAT reporters (Fig. 2B). The entire 8.0-kb fragment was sequenced in reverse and forward directions. The sequence has been deposited in GenBank (accession number EF015593).

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 (Fig. 3). In contrast to ALDH3A1 CAT reporter results, we did not observe reduced reporter activity in constructs containing greater than 4.0 kb (Fig. 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 with any other reporter construct, indicating that positive regulatory elements exist between -5.0 and -8.0 (Fig. 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 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 TNGGCGTG, where N represents 0 to 2 nucleotides (Neuhold et al., 1989; Shen and Whitlock, 1992; Yao and Denison, 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., 1993). For the present study
we have designated this consensus sequence XRE1 (Fig. 4A). 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 and −5837, which we designated XRE3 (Fig. 4A). 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 and −6101 (Fig. 4A).

To test regions containing XRE consensus sequences, we generated additional ALDH3A1 5′ flanking region reporters (Fig. 4B). pALDHuc0.6 was extending by 180 bp to generate pALDHuc0.8, resulting in the addition of a fragment containing XRE1. Compared with pALDHuc0.6, pALDH0.8 produces 3.5-fold higher constitutive ALDH3A1 reporter activity in corneal epithelial cells than pALDHuc0.6 (Fig. 5A). In H4-II-EC3 cells treated with 3-MC, this reporter displays 11-fold induction compared with 2-fold induction for pALDHuc0.6 (Fig. 5A). 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 BglII fragment, including XRE2, from the 8.0-kb ALDH3A1 fragment sharply increases both constitutive and 3-MC-induced expression in H4-II-EC3 cells (Fig. 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 8.0-kb 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 pALDH3ΔXRE2. Interestingly, in both cases the deletion results in enhanced 3-MC-induced hepatic reporter expression relative to the wild-type reporters (Fig. 6).

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 (Fig. 7A). Deletion of XRE3 did not affect 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 (Fig. 7B). Interestingly,
the double deletion mutant produces a slight but reproducible increase in expression compared with both single deletion mutants in corneal cells and compared with 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 h 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 (Fig. 8A). Northern blot analysis confirms strong up-regulation of the ALDH3A1 message. We previously showed that hypoxia down-regulates 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 Fig. 8B, 1% oxygen down-regulates both constitutive 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 extracts 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.

**Discussion**

Our results indicate that ALDH3A1 expression is influenced by at least four XREs distributed throughout approximately 6.1 kb of 5′ flanking region sequence. These XREs 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 both appear to be required for constitutive expression, since deletion of
either eliminates positive effects on constitutive reporter activity. One unexpected result was that deletion of a 150-bp region surrounding 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 that 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 coactivators by AhR/ARNT. Coactivator proteins, including cAMP response element-binding protein/p300 (Kallio et al., 1998), Gcn5 (for general control nonrepressed) (Brownell et al., 1996), the Spt-Ada-Gcn5-acetyltransferase complex (SAGA) (Hampsey, 1997), and the NCoA/SRC-1/p160 family of transcriptional coactivators, either possess or are associated with histone acetyl transferase 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, 1997). Both ARNT and AhR have been demonstrated to associate with NCoA/SRC-1/p160 family coactivators in a 2,3,7,8-tetrachlorodibenzop-dioxin-dependent manner (Beischlag et al., 2002). Also, recently, it was reported that AhR recruits the coactivator GAC63 to XREs (Chen et al., 2006). Another mechanism of chromatin remodeling involves the SWI/SNF complex. The SWI/SNF complex uses 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. Furthermore, forced expression of BRG-1 in a BRG-1-minus background restored endogenous CYPIA1 expression and enhanced XRE-driven reporter gene expression (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 coactivator activity, induces a more relaxed conformation, allowing ALDH3A1 expression to occur (Fig. 10). Although it remains to be proven, such a scenario could provide a mechanism that contributes to tissue-specific regulation of ALDH3A1 expression.

Based on previous ALDH3A1 CAT reporter analyses, we anticipated that the luciferase reporter pALDH4.6 would generate sharply reduced luciferase expression compared with 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

![Fig. 10. Proposed model for transcriptional regulation of ALDH3A1. ARNT serves as a dimerization partner for both AhR and hypoxia-inducible factor-1α, 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.](image-url)
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 \(-2057\) and \(-2062\). In earlier work using the \(ALDH3A1\) CAT reporters, XRE2 was reported to be near \(-3.0\) relative to the transcriptional start site. The length of the original deletion fragments was estimated by electrophoretic mobility rather than direct sequencing. Furthermore, direct sequencing that resulted in the identification of XRE2 was performed from the 5' end of \(P_{ALDH}CAT-3.2\) and did not extend significantly downstream of XRE2. For the current study, we sequenced the entire 8.0-kb 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 structural support (as a corneal crystallin), oxidation of toxic lipid aldehydes, absorption of UV radiation, and replenishment of reduced NAD(P)H cofactors (Piatigorsky, 1988; Kinoshita et al., 2001). Although \(ALDH3A1\) probably functions to a degree in all of these roles, the physiologic importance of each is not clear. Nor is it clear whether cellular \(ALDH3A1\) is functionally partitioned, or whether the entire pool is available for multiple physiologic tasks. There is 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 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 (Muzzio 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 that is hyperoxic relative to perfused tissues), to near anoxia (Baum, 1997; Lubbers and Baumgart, 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 h (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 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 trophtophan derivatives (Seidel et al., 2001; Denison and Nagy, 2003). The compound 6-formylindolo[3,2-b]carbazole 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 is also a potent agonist of AhR (Henry et al., 2006). 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 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 XREs 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.

References

Asman DC, Takimoto K, Pitot HC, Dunn TJ, and Lindahl R (1993) Organization and characteriza-
MR, Rosenfeld MG, and Hankinson O (2002) Recruitment of the NCoR/SMRT/p160 family of
transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nu-
Bosch J, Lee C, and Lindahl R (1996) Constitutive expression of class 3 aldehyde dehydro-
element is required for constitutive and inducible expression of the mammalian aldehyde dehydro-
Tetramethyl histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetyl-
transcriptional activation mediated by the aryl hydrocarbon receptor. J Biol Chem 281:
12422–7.
Denison MS and Nagy SR (2003) Activation of the aryl hydrocarbon receptor by structurally
tetrachlorodibenzo-p-dioxin (TCDD). Noncoordinate regulation of a TCDD-induced aldehyde
M, and Poellinger L (1996) Functional interference between hypoxia and dioxin signal
transduction pathways: competition for recruitment of the Arnt transcription factor. Mol
389:249–252.
429.
ligand for the aryl hydrocarbon receptor has potent agonist activity in vitro and in vivo. Arch
Biochem Biophys 277:296–300.
Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the
CBP/p300 coactivator by the hypoxia-inducible factor-1a. EMBO (Eur Mol Biol Organ) J
17:6573–6585.


Yao EF and Denison MS (1992) DNA sequence determinants for binding of transformed Ah receptor to a dioxin-responsive enhancer. *Biochemistry* **31:**5060–5067.

Yu BP (1994) Cellular defenses against damage from reactive oxygen species. *Physiol Rev* **74:**139–162.

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