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The Negative Regulation of the Rat Aldehyde Dehydrogenase 3 Gene by Glucocorticoids: Involvement of a Single Imperfect Palindromic Glucocorticoid Responsive Element

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ABSTRACT

Glucocorticoids repressed the polycyclic aromatic hydrocarbon-dependent induction of Class 3 aldehyde dehydrogenase (ALDH3) enzyme activity and mRNA levels in isolated rat hepatocytes by more than 50 to 80%, with a concentration-dependence consistent with the involvement of the glucocorticoid receptor (GR). No consistent effect on the low basal transcription rate was observed. This effect of glucocorticoids (GC) on polycyclic aromatic hydrocarbon induction was effectively antagonized at the mRNA and protein level by the GR antagonist RU38486. The response was cycloheximide-sensitive, because the protein synthesis inhibitor caused a GC-dependent superinduction of ALDH3 mRNA levels. This suggests that the effects of GC on this gene are complex and both positive and negative gene regulation is possible. The GC-response was recapitulated in HepG2 cells using transient transfection experiments with CAT reporter constructs containing 3.5 kb of 5′-flanking region from *ALDH3*. This ligand-dependent response was also observed when a chimeric GR (GR DNA-binding domain and peroxisome proliferator-activated receptor ligand-binding domain) was used in place of GR in the presence of the peroxisome proliferator, nafenopin. A putative palindromic glucocorticoid-responsive element exists between −930 and −910 base pairs relative to the transcription start site. If this element was either deleted or mutated, the negative GC-response was completely lost, which suggests that this sequence is responsible, in part, for the negative regulation of the gene. Electrophoretic mobility shift analysis demonstrated that this palindromic glucocorticoid-responsive element is capable of forming a specific DNA-protein complex with human glucocorticoid receptor. In conclusion, the negative regulation of *ALDH3* in rat liver is probably mediated through direct GR binding to its canonical responsive element.

The aldehyde dehydrogenases are a family of homodimeric enzymes, the function of which is to catalyze the oxidation of a wide variety of aliphatic and aromatic aldehydes to their corresponding carboxylic acids. They are classified according to their sequence similarity (Lindahl, 1994). In the rat, one family member, aldehyde dehydrogenase 3 (ALDH3), is induced by polycyclic aromatic hydrocarbons (PAH) or chlorinated compounds, such as TCDD (Dunn et al., 1988). These compounds are ligands for the aryl hydrocarbon (*Ah)* receptor, a transcription factor that binds a canonical consensus sequence [TNGCGTG; *Ah* responsive element (*Ah*RE)] to facilitate gene transactivation (Whitlock et al., 1996). These compounds represent a major class of environmental pollutants that produce a wide range of toxic and carcinogenic effects. Rat genes that are activated by this *Ah* receptor mechanism include *CYP1* family members, *ALDH3*, *GSTA2*, *QOR*, and *UGT1A6*. Multiple copies of the *Ah*RE sequence have been found in *CYP1A1*, although single copies have been found in the 5′ flanking regions of the *GSTA2* (Rushmore et al., 1990), *UGT1A6* (Emi et al., 1996) and *QOR* (Favreau and Pickett, 1991) genes. Two sequences that have significant similarity with the *Ah*RE core have been observed near positions −3500 and −687 in the 5′-flanking region of the rat *ALDH3* gene (Takimoto et al., 1994; Xie et al., 1996).

Previous studies in our laboratory (Xiao et al., 1995; Prough et al., 1996) have shown that several genes regulated by the *Ah* receptor (namely, *GSTA2*, *QOR*, and *ALDH3*) are negatively regulated by physiological levels of glucocorticoids

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ABBREVIATIONS: PAH, polycyclic aromatic hydrocarbon; *Ah*RE, aryl hydrocarbon receptor response element; ALDH, aldehyde dehydrogenase; AP-1, activator protein-1; BA, 1,2-benzanthracene; bp, base pair(s); CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; CMV, cytomegalovirus; DEX, dexamethasone; DMSO, dimethyl sulfoxide; GC, glucocorticoid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; P-450, cytochrome P-450; PCR, polymerase chain reaction; pGRE, palindromic glucocorticoid response element; NF-1, Nuclear Factor 1; PPAR, peroxisome proliferator-activated receptor; QOR, NAD(P)H:quinone acceptor oxidoreductase, SDS, sodium dodecyl sulfate; SSC, standard saline citrate; UGT, UDP-glycosyltransferase

(GCs) in primary cultures of rat hepatocytes and *GSTA2* and *QOR* in adrenalectomized rats (Sherratt et al., 1989) or neonatal rats whose circulating levels of GCs are negligible (Linder and Prough, 1993). In contrast, other members of the *Ah* gene family whose PAH-dependent induction are positively regulated by GCs, include *CYP1A1* and *UGT1A6*. We have shown that in the case of *GSTA2*, the negative regulation involves the GC receptor (GR) binding to a palindromic, canonical binding sequence (TGTYCT) found in the 5′-flanking sequence of that gene (Falkner et al.1998). The hypothesis of the current study is that the regulation of *ALDH3* by GCs occurs at the transcriptional level and that it involves the binding of the GR to its canonical consensus element in the 5′ flanking region of this gene. In this article, we describe the effects of GCs on *ALDH3* expression in primary rat hepatocyte cultures and test our hypothesis using transient transfection techniques in cultured HepG2 cells.

Experimental Procedures

Materials. Collagenase (type H), chlorophenol red-β-D-galactopyranoside and random primed DNA-labeling kits were purchased from Boehringer Mannheim (Indianapolis, IN). Benzaldehyde, 1,2-benzanthracene (BA), bicinchoninic acid solution, chloramphenicol, dexamethasone (DEX), Hanks' modified balanced salts, and insulin-transferrin-sodium selenite medium supplement were obtained from the Sigma Chemical Co. (St. Louis, MO). Restriction endonucleases and T4 ligase were purchased from either Promega (Madison, WI) or New England Biolabs (Beverly, MA). pCR II cloning kits were obtained from InVitrogen (San Diego, CA). Nafenopin was obtained from Ciba Gigy Chemical Co. (Ardsley, NY) and RU38486 was obtained from Roussel Uclaf (Romainville, France). *n*-Butyryl CoA and poly dI-dC were obtained from Pharmacia (Piscataway, NJ). [^{3}H]Chloramphenicol and [^{32}P]dCTP were obtained from Du Pont New England Nuclear (Boston, MA). Oligonucleotides were purchased from National Bioscience Inc. (Plymouth, MN). The polyclonal antihuman GR antibodies (PA1–511) were obtained from Affinity Bioreagents, Inc. (Golden, CO). Dulbecco's modified Eagle's medium (DME/High modified) was purchased from JRH BioSciences (Lexena, KS). Premium grade fetal bovine serum was obtained from Harlan Bioproducts for Science (Indianapolis, IN). Plasmid purification was performed using kits from Qiagen (Chatworth, CA). Polymerase chain reaction (PCR) reagents were purchased from Perkin Elmer Cetus (Norwalk, CT). Antibiotic-antimycotic solution, arginine, arginine-free Eagle's medium, and Luria broth base were purchased from Life Technologies, Inc.(Gaithersburg, MD). Matrigel was purchased from Collaborative Research Inc. (Bedford, MA). All other reagents were of either American Chemical Society or molecular biology grade.

Primary Rat Hepatocyte Cultures. Hepatocytes were routinely prepared from male adult Sprague-Dawley rats (180 to 250 g, Hsd: Sprague-Dawley) from Harlen Sprague-Dawley (Indianapolis, IN) by in situ liver collagenase perfusion and cultured with a matrigel overlay (Schuetz et al., 1988) with modifications (Xiao et al., 1995). After 24 h in culture, inducing agents were added after fresh medium was provided to the cells. Control cells received solvent alone. After 48 to 72 h in culture, the medium was removed by aspiration, the cells were washed with phosphate-buffered saline, harvested by scraping, and the protein/mRNA was stored at −80°C. Protein concentrations were determined using the bicinchoninic acid method with bovine serum albumin as the standard (Smith et al., 1985). Enzyme activity was stable in hepatocyte samples stored for up to 1 month and ALDH3 protein was stable for more than 6 months at −80°C when analyzed by Western blot analysis (data not shown).

Hepatoma Cell Culture. The human hepatoblastoma cell line HepG2 was obtained from the American Type Culture Collection tissue-culture cell collection (Rockville, MD). HepG2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with antibiotic-antimycotic solution, nonessential amino acids, and 10% fetal bovine serum. The cells were incubated at 37°C in a humidified, 5% carbon dioxide environment and were subcultured every 2 to 3 days.

Enzyme Assays. Aldehyde dehydrogenase activity was determined by monitoring the increase in absorbance at 340 nm caused by NADPH production during the oxidation of benzaldehyde as substrate (Lindahl, 1977). 7-Ethoxyresorufin *O*-deethylase activity was determined by the fluorometric assay described previously (Prough et al., 1978).

Northern Analysis. Total RNA was isolated as described by Chomczynski and Sacchi (1987) with minor modifications as described previously (Xiao et al., 1995). Northern blot experiments were performed after size fractionation of denatured RNA (25 μg) on formaldehyde-containing 1% agarose gels (FMC Bioproducts, Rockland, ME) and transfer of RNAs to Zetaprobe membranes (Bio-Rad Laboratories, Hercules, CA) by diffusion. Hybridization was carried out overnight at 43°C in 0.25 M sodium phosphate buffer, pH 7.2, containing 0.25 M NaCl, 7% sodium dodecyl sulfate (SDS), 0.5× standard saline citrate (SSC), 1 mM EDTA, and ^{32}P-labeled cDNA probe. The hybridized membranes were washed sequentially for 15 min with 2× SSC and 0.1% SDS, 0.5× SSC and 0.1% SDS, or 0.1× SSC and 0.1% SDS. Autoradiography was performed and the intensity of relevant bands quantitated using a Bio-Rad model 620 video densitometer.

Probes for mRNA Analysis. ALDH3 mRNA was measured using a *Eco*RI/*Hin*dIII cDNA fragment of the plasmid pSelALDHX, the *ALDH3*-specific clone (Dunn et al., 1988). Cytochrome P-450 (P-450) 1A1 mRNA was measured using a *Pst*I fragment from the plasmid pA8 provided by R. N. Hines, Department of Pharmacology and Toxicology, Wayne State University (Hines et al., 1985). As a control, β-actin mRNA levels were measured using a mouse cytoskeletal β-actin (Xiao et al., 1997). These nucleic acid probes were labeled with [^{32}P]dCTP using the random primer labeling procedure.

Generation of PCR Products. To create reporter plasmids in which the palindromic GRE in the 5′-flanking region of pALDH3.5CAT was mutated, a wild-type upstream primer OKF11 (5′-GGAGGACAAAGTGTTGCTATG-3′) and mutant primer OKF12 (5′-GGACGGCAAAGTGTTGCTATG-3′) complimentary to base pairs (bp) −930 to −910 were used in conjunction with the downstream primer OKF13 (5′-AGCTGCTGTTCTCTGAGTCC-3′) to produce a 605-bp product. The primers OKF11 and OKF12 differ in the putative palindromic GRE, causing a AGGACA-to-ACGGCA mutation in the perfect half site. PCR was performed in a Barnstead Thermolyne Amplitron II thermal cycler (Dubuque, IA) with a Mg^{2+} concentration of 2 mM. The PCR products were produced through 20 cycles of annealing temperature of 55°C for 1 min, elongation at 70°C for 1 min, and denaturing at 90°C for 30 s.

Plasmid Constructs. The 5′ flanking region construct of rat *ALDH3* (pALDH3.5CAT) has been described previously (Takimoto et al., 1994). pCMVβ was obtained from Clonetech (PaloAlto, CA). The chimeric GR containing the ligand-binding domain for PPARα was generously provided by Thomas H. Rushmore, Merck Research Laboratories, West Point, PA (Boie et al., 1993). pRSVGR, the expression vector for the human GR, was a kind gift from Michael Mathis (Louisiana State University Medical School, Shreveport, LA). *E. coli* DH5α were routinely transformed with plasmids of interest for preparation of transfection-quality plasmids. pCRII derived plasmids were grown in the *Escherichia coli* strain provided (InVitrogen, San Diego, CA.). The pΔ(−1057/−392)CAT construct was created by digesting pALDH3.5CAT with *Pst*I and religating the parent fragments; this created a deletion between position −1054 and −392 in the 5′-flanking sequence. The pΔ(−1057/−930)CAT construct was generated by initially subcloning the 605-bp PCR product produced using OKF 11 and OKF 13 into the pCR II vector. Digestion of the resulting plasmid with *Pst*I resulted in the liberation of a 568 bp

fragment which was then subcloned in a vector made from *Pst*I digestion of pΔ(−1057/-392)CAT. The normal orientation of the fragment was confirmed with *Eco*RI digestion of the resulting plasmid pΔ(−1057/−930)CAT. The pΔ(−1057/−930)MUTCAT construct was produced in an identical fashion to pΔ(−1057/−930)CAT except OKF12 and OKF 13 were used to generate the mutated pGRE PCR product.

Transfection of HepG2 Cells. Cells were transfected, treated, and harvested using methods described previously (Rushmore et al., 1990; Xiao et al., 1997). The cells were approximately 40% confluent when transfected. All cells were cotransfected with 2 μg of pCMVβ as a transfection control. Routinely, 2 μg of cotransfected plasmids (pGRPPAR or pRSVGR) and 4 μg of pALDH3.5CAT or related constructs were added per flask. The inducing agents BA, DEX, nafenopin, and/or RU38486 were added as 500× concentrated stocks in dimethyl sulfoxide (DMSO). Controls received DMSO alone.

Assays for β-Galactosidase and Chloramphenicol Acetyltransferase Activity. The chloramphenicol acetyltransferase assay employed in this study was a variant of that described by Gorman et al. (1982) and has been described previously (Xiao et al., 1997). For the β-galatosidase assays, cell extracts (30 μg of protein) were incubated at 37°C for 1 h. Activity was determined spectrophotometrically in the presence of chlorophenol red β-galactopyranoside by measuring absorbance formed at 595 nm on a Titretek Uniskan II plate reader (Flow Laboratories, McLean, VA).

Electrophoretic Mobility Shift Assay Protocol. Nuclear extracts were prepared from either HepG2 or HepG2-GR4 cells by methods described previously (Dignam et al., 1983), and were then aliquoted and stored at −80°C until used. Cells from the HepG2-GR4 cell line are stably transfected with an expression vector for the human GR and have been described previously (Falkner et al. 1998). Nuclear extracts were incubated at 30°C for 30 min with either competitor ds-oligonucleotides followed by incubation with radiolabeled probe or with radiolabeled probe before addition and incubation with polyclonal-human anti-GR antibody before resolution on a polyacrylamide gels using 0.5× Tris/borate/EDTA buffer. The gels were dried and analyzed by exposure to a Phosphor Screen in a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical Analysis. All data was analyzed using a Student's *t* test.

Results

Concentration-Dependent Effects of DEX on BA-Dependent Induction of ALDH3 Activity in Primary Rat Hepatocytes. Previous experiments in our laboratory have shown that GCs regulate the expression of many genes that require the action of the Ah receptor (Xiao et al., 1995; Prough et al., 1996). To examine whether the negative regulation of *ALDH3* expression was mediated by GR, we initially tested the concentration-dependence of the repression phenomenon (Fig. 1A). As anticipated, aldehyde dehydrogenase activity was induced 4.6-fold by inclusion of 50 μM BA in the culture media. This result is consistent with the selective induction of this activity via an *Ah* receptor mechanism (Dunn et al., 1988). Addition of the synthetic GC DEX caused a simple monotonic decline in PAH-inducible ALDH3 activity in the concentration range between 10^{-6} and 10^{-12} M, with significant decreases (<50%) in activity being observed with concentrations of 10^{-8} M or greater. No significant effect was observed on the very low level of basal ALDH activity of rat hepatocytes, which may be caused by other constitutive ALDH isoenzymes that also display low levels of catalytic activity toward benzaldehyde. We observed a 2- to 3-fold increase in PAH-inducible, P-450 1A1-dependent 7-ethoxyresorufin *O*-deethylation activity with addition of increasing amounts of DEX (Fig. 1B), concomitant with the decrease in ALDH3 activity, similar to our past studies (Xiao et al., 1995; Prough et al., 1996). Similar results at the mRNA and protein levels were obtained from Northern and Western analysis, respectively (data not shown). These results demonstrate that GCs suppress the levels of ALDH3 enzyme activity and specific mRNA levels in cultured rat hepatocytes exposed to PAH with a concentration-dependence consistent with the involvement of GR.

Effects of GR Antagonist RU38486 on the DEX-Dependent Repression of BA-Induced ALDH3 mRNA Levels in Primary Rat Hepatocytes. To further characterize the involvement of the GR in the DEX-dependent suppression of ALDH3 mRNA levels and activities, we examined the effects of RU38486 to effectively antagonize the suppression by GCs. As shown in Fig. 2, treatment of the hepatocytes with BA caused a 14-fold induction in the mRNA level for this gene. Administration of DEX suppressed BA-dependent induction of this gene by 75%; the basal level of expression was

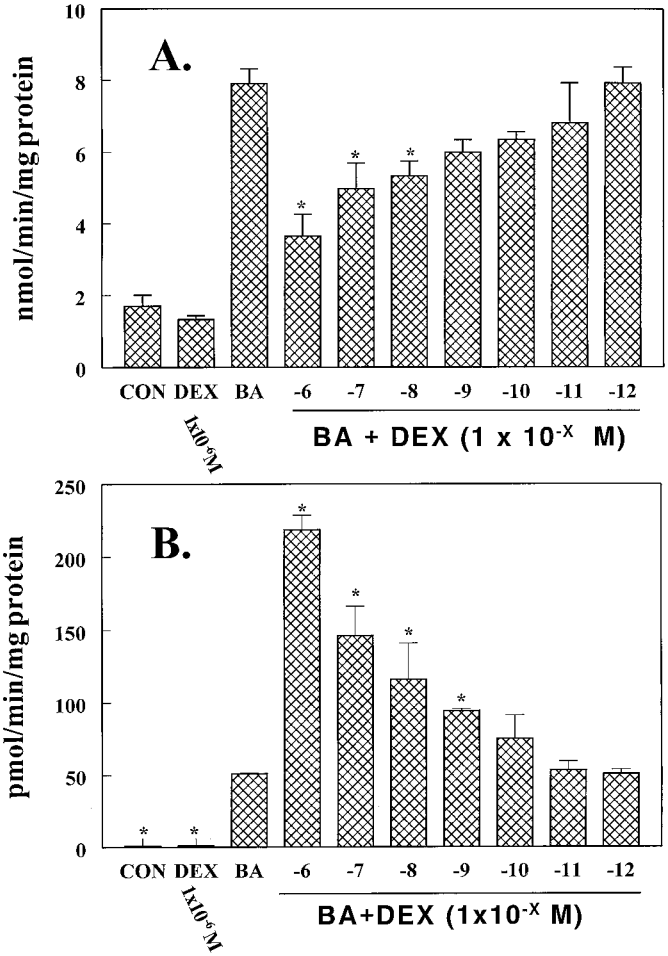

Fig. 1. Effect of DEX on ALDH3 and P-450 1A1 enzyme activity in cultured adult rat hepatocytes. Primary rat hepatocytes were prepared as described under *Experimental Procedures* and cultured with a Matrigel overlay. Twenty-four hours after plating, the medium was changed and cells were treated with either DMSO or with 50 μM BA in DMSO, 1 μM DEX in DMSO, or combinations of the two agents for 72 h. Cells were harvested by scraping and enzyme activity was determined. A, ALDH3 enzyme activity using benzaldehyde as substrate. B, P45 1A1 enzyme activity using 7-ethoxyresorufin as substrate. *, statistically different from control ($p < .01$).

sufficiently low that no statistically significant effect of RU38486 could be observed. RU38486 seemed to slightly decrease the BA-dependent induction (11-fold compared with 14-fold for BA alone), but significantly reversed the DEX-dependent repression of BA-induced expression of this gene.

Although the progesterone receptor also binds RU38486, R5020, an agonist of the progesterone receptor, had no observed effect on regulation of this gene in HepG2 cells co-transfected with human GR (data not shown). Thus, RU38486 was an effective antagonist of the GC-mediated negative regulation of this PAH-inducible gene. Likewise, the effect of GCs potentiating the BA-dependent induction of P-450 1A1 mRNA levels was also effectively antagonized by RU38486, as reported previously (Xiao et al., 1995). In concert, these results are consistent with the involvement of GR and support the conclusion that the effects of GC on *ALDH3* expression occur at the pretranslation level.

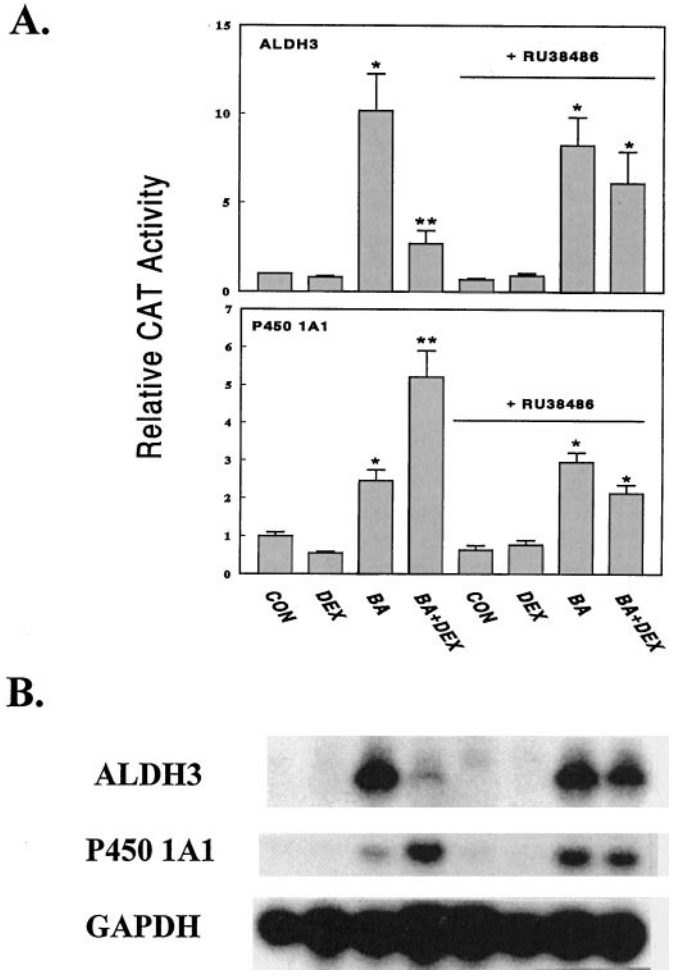

Fig. 2. Effect of the GR antagonist RU38486 on ALDH3 and P-450 1A1 mRNA levels. Primary rat hepatocytes were cultured as described under *Experimental Procedures*. Twenty-four hours after plating, the medium was changed and cells were treated with either DMSO, 50 μM BA in DMSO, 1 μM DEX in DMSO, 10 μM RU38486 or combinations of the three agents for 72 h. mRNA was harvested and Northern blotting performed as described under *Experimental Procedures*, using ALDH3, CYP1A1, and GADPH cDNA probes. The hybridization complexes were recorded using X-ray film and quantified using a Bio-Rad model 620 video densitometer and normalized to the level of GAPDH message. A, data for ALDH3 and P-450 1A1 mRNA levels from three independent experiments. B, data for representative experiment. *, statistically different from control cells ($p < .01$); **, statistically different from BA-treated cells ($p < .04$).

Effect of the Protein Synthesis Inhibitor Cycloheximide (CHX) on the DEX-Mediated Repression of BA-Induced ALDH-3 mRNA Levels in Rat Hepatocytes. Unlike other GR-regulated genes, the effects of GC on *ALDH3* expression is negative. To test the hypothesis that this effect might occur either through de novo synthesis of a second transcription factor or interaction with an intrinsically labile protein, we tested the CHX sensitivity of the DEX-dependent repression of *ALDH3* expression. As shown in Fig. 3A, administration of CHX (5 μg/ml for 24 h) completely blunted the repressive effect of GC and actually resulted in a GC-dependent superinduction of *ALDH3* compared with control hepatocytes. In addition, CHX caused an increase in the level of P-450 1A1 mRNA induction by BA (Fig. 3B). This superinduction phenomenon with *CYP1A1* expression has been observed previously (Lusska et al., 1992). Superinduction by CHX in the presence of DEX suggests that the negative regulation of *ALDH3* by GC most likely involves a second, labile (i.e., CHX-sensitive) transcription factor, because we would expect the level of expression observed if de novo synthesis were involved to be the same as that of cells treated with CHX and BA alone. Because the

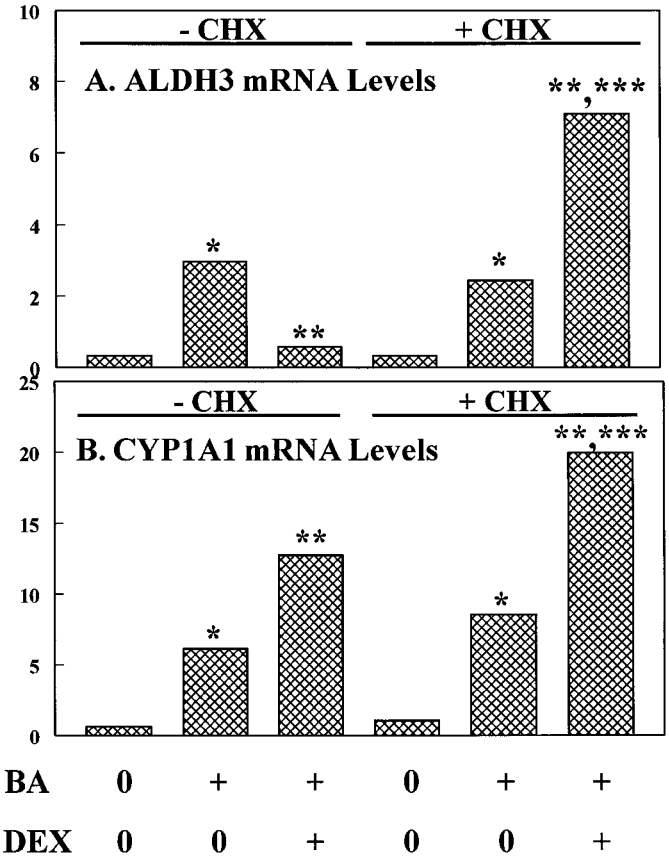

Fig. 3. Effect of the protein synthesis inhibitor CHX on GC repression of ALDH3 mRNA levels. Primary rat hepatocytes were cultured as described under *Experimental Procedures*. Twenty-four hours after plating, the medium was changed and cells were treated with either DMSO, 50 μM BA in DMSO, 1 μM DEX in DMSO, 0.5 μg/ml CHX or combinations of the three agents. As shown in Fig. 2, the data shown for ALDH3 and P-450 1A1 mRNA levels was normalized to the level of GADPH message. A, ALDH3 message levels. B, P-450 1A1 message levels. *, statistically different than level of message in untreated cells ($p < .01$); **, statistically different from level of message induced by BA alone ($p < .08$); ***, statistically different from message in cells not treated with CHX ($p < .01$).

positive regulatory effect of DEX on *CYP1A1* expression was unaffected, it is unlikely that the loss of negative regulation is caused by a shortage of functional GR. The superinduction observed is intriguing and indicates that the interaction of ligand-activated GR with *ALDH3* is complex, possibly allowing for both positive and negative regulation depending on the transcription factors present in a given cell type. Several constitutive negative regulatory regions have been described and are implicated in the regulation of this gene (Xiao et al., 1995; Xie et al., 1996).

Effects of BA, DEX, and RU38486 on pALDH 3.5CAT Expression. As anticipated, BA induced expression of pALDH 3.5CAT approximately 20-fold when this reporter construct was transiently transfected in HepG2 cells (Fig. 4). Interestingly, we did not observe appreciable BA-induced CAT activity when a plasmid containing a 5.5-kb portion of the *ALDH3* 5′-flanking region (pALDH5.5CAT) was transfected (results not shown). This suggests that the distal negative response element of *ALDH3*, reported to reside between −5.5 and −3.5 kb, acts more effectively in HepG2 cells than in HII4E cells used in studies reported by Takimoto et al. (1994). Therefore, we used pALDH3.5CAT to elucidate the role of GR in regulation of this gene. DEX suppressed the BA-dependent induction of pALDH3.5CAT approximately by 40 to 50% in HepG2 cells transiently cotransfected with both pALDH3.5CAT and a functional GR-expression plasmid, pRSVGR (Fig. 4). This is in good agreement with data observed from both mRNA and enzyme activities in primary rat hepatocyte cultures, supporting our hypothesis that GCs suppress PAH-dependent induction of *ALDH3* gene at the transcriptional level. In primary hepatocytes and transiently transfected HepG2 cells, the basal *ALDH3* transcription rate of the native and reporter gene is low and no consistent effect of DEX was noted on the low basal expression.

When HepG2 cells were cotreated with the GR antagonist RU38486 (Fig. 4), no effect was observed on either the basal or BA-induced levels of pALDH3.5CAT expression. In fact, the levels of pALDH3.5CAT expression in HepG2 cells treated with either BA or BA and DEX were identical in the presence of the GR antagonist. RU38486 was an effective antagonist of the DEX-dependent suppression of pALDH3.5CAT expression, implying that the response involves GR.

Deletion Analysis of the Palindromic GRE Located between bp −1057 and −380 in the 5′ Flanking Region. From sequence analysis of the 5′-flanking sequence of *ALDH3*, we identified a imperfect pGRE sequence at approximately −920 bp relative to the transcription start site. Deletion of a *Pst*I fragment (−1057 to −392) from the 5′-flanking region of pALDH3.5CAT to form pΔ(−1057/−392)CAT had dramatic effects on both the PAH-dependent induction of CAT activity and the DEX-dependent suppression of induction (Fig. 5). With pΔ(−1057/−392)CAT, BA caused only a 4-fold increase in CAT activity relative to the 20-fold induction observed with the parent construct, whereas basal activity was nearly identical between the two constructs. A sequence related to the canonical *Ah*RE consensus is located within the *Pst*I fragment (between bp −690 and −685 at the 5′ end of the transcription start site). Our results are consistent with the active involvement of this AhRE sequence in determining the magnitude of the PAH induction of *ALDH3* expression in HepG2 cells. Cotreatment of the cells with DEX had no effect on the reduced BA-dependent induction of this deletion construct, which suggests that sequences critical for the GC effect may be located between bp −1057 and −390.

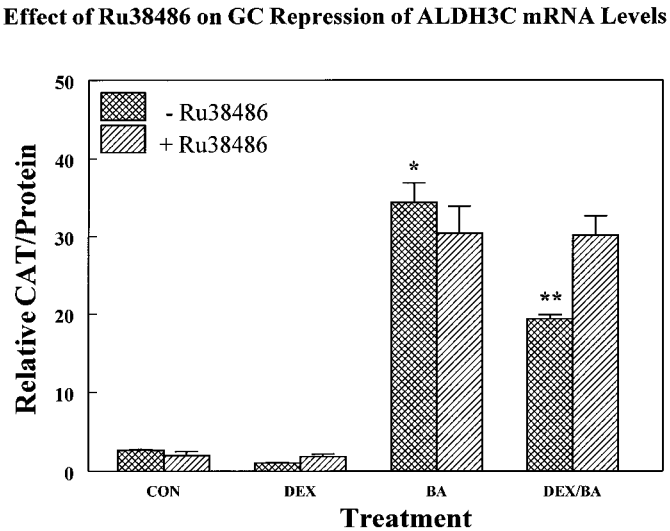

Fig. 4. Effects of RU38486 on CAT activity of HepG2 Cells transiently transfected with p3.5ALDHCAT and treated with either BA, DEX, and/or RU38486. CAT and β-galactosidase assays were performed on lysates from HepG2 cells that had been transiently transfected with p3.5ALDHCAT and the expression plasmids for pCMVβ and pRSVGR and then treated with either 50 μM BA, 1 μM DEX, 10 μM RU38486, or combinations of these compounds for 24 h as described under *Experimental Procedures*. The normalized CAT activity is the percentage conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean of three flasks ± standard deviation (S.D.). *, statistically different from CAT activity of control cells ($p < .001$); **, statistically different from CAT Activity of BA-treated cells ($p < .005$).

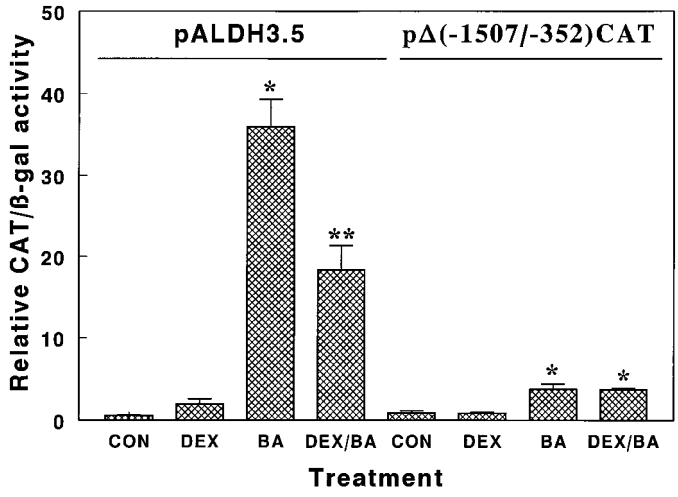

Fig. 5. Effects of an *ALDH3* 5′-flanking region deletion construct on CAT activity of transiently transfected HepG2 cells. CAT and β-galactosidase assays were performed on lysates from HepG2 cells that had been transiently transfected with either p3.5ALDHCAT or pΔ(−1057/−392)CAT (deletion of sequences between position −1057 to −390) and the expression plasmids for pCMVβ and pRSVGR and then treated with either 50 μM BA, 1 μM DEX, or combinations of these compounds for 24 h as described under *Experimental Procedures*. The normalized CAT activity is expressed as the percentage conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean of three flasks ± S.D. *, statistically different from CAT activity of control cells ($p < .002$); **, statistically different from CAT activity of BA-treated cells ($p < .003$).

This is consistent with the involvement of the pGRE located −920 bp in the GC regulation of this gene.

Role of the Palindromic GRE Sequence in ALDH3 Expression. To test whether GCs act through the putative pGRE, we used a PCR-based strategy to generate variant pALDH3.5CAT constructs that contained either a mutated (AGGACA to ACGGCA) or wild-type sequence of the pGRE. The PCR products were synthesized to span the region between −930 and −374 in the 5′-region of ALDH3. The rationale was to produce plasmids that had full BA-dependent inducibility containing the AhRE sequences at −690 to −685 and the distal element at approximately −3.5 kb, but with mutations in the −920 pGRE site. Because of the location of restriction sites in the 5′-flanking region when PCR products were reintroduced into pΔ(−1057/−392)CAT, the resulting plasmids lack 127 bp of sequence located at −1057 to −930 from the transcription start site and immediately 5′ of the putative pGRE. We reported previously that this deletion resulted in a 5-fold increase in basal expression, but had no affect on the PAH induction of this reporter construct (Xiao et al., 1997). As shown in Fig. 6A, constructs pΔ(−1057/−930)CAT and pΔ(−1057/−930)MUTCAT possessed all other sequences from −3.5 kb through the transcription start site. Both plasmids tested (wild-type and mutant GRE) were induced approximately 20-fold by BA, nearly identical with the induction observed with pALDH3.5CAT. When both BA and DEX were coadministered, only pΔ(−1057/−930)CAT, which contains the wild-type GRE, was repressed by approximately 50 to 60%. DEX failed to significantly repress expression of pΔ(−1057/−930)MUT CAT activity with a construct which contains a 2-bp mutation in the GRE. We have subsequently performed transient transfection experiments with CAT reporter constructs containing either a standard xenobiotic response element or multiple copies of a GRE enhancer. We examined the effects of either 1 μM DEX, 50 μM BA, or combinations of both chemicals. BA had no effect under any condition on the GRE enhancers and DEX had no effect on either basal or PAH-induced expression through the *Ah*RE enhancers (data not shown). These results indicate that there can not be a simple interaction between GR and *Ah*R working through their respective canonical responsive elements. In conclusion, the palindromic GRE is functionally involved in the negative regulation of the *ALDH3* gene and neither GC nor GR affect the function of *Ah*R.

Effect of a Chimeric GR on Negative Regulation of pALDH3.5CAT. In our previous studies on the rat glutathione *S*-transferase *A2* gene, we demonstrated that a chimeric GR [GR DNA-binding domain and peroxisome proliferator-activated receptor-α (PPARα) ligand-binding domain] does not regulate that gene, which suggests that a second transcription factor must be required for the coregulation of that gene (Falkner et al., 1998). Because the *GSTA2* and *ALDH3* genes may have conserved mechanisms for the negative regulation by GR, we used the chimeric GR-PPAR in transient transfection experiments with pALDH3.5CAT. As seen in Fig. 7, cotransfection of this chimeric receptor plasmid resulted in a nafenopin-induced decrease in reporter expression, although nafenopin had no effect on the pALDH3.5CAT expression when cotransfected with a plasmid that expresses human GR. This result demonstrates that the binding of GR to the canonical GRE of *ALDH3* is required for negative regulation of this gene, independent of the ligand-binding domain, which can interact directly with other transcription factors, such as the CCAAT/enhancer-binding protein (Nishio et al., 1993). Although there was some suppression of BA-induced activity by DEX in the presence of cotransfected

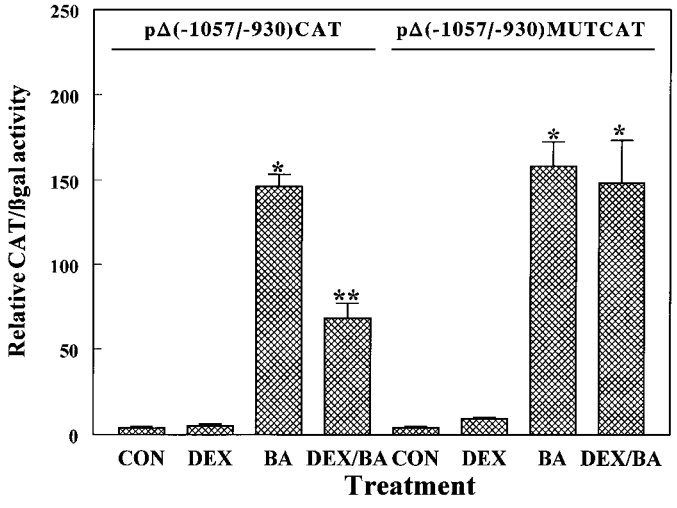

Fig. 6. Effects of mutation of the palindromic GRE in the 5′-flanking region of p3.5ALDHCAT on CAT activity of transiently transfected HepG2 cells treated with BA and DEX. CAT and β-galactosidase assays were performed on lysates from HepG2 cells that had been transiently transfected with p3.5ALDHCAT constructs containing either a native core GRE (pΔ(−1057/−930)CAT) or a mutated core GRE (pΔ(−1057/−930)MUTCAT) and the expression plasmids for pCMVβ and pRSVGR and then treated with either 50 μM BA, 1 μM DEX, or combinations of these compounds for 24 h as described under *Experimental Procedures*. The normalized CAT activity is expressed as the percentage conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean of three flasks ± S.D. *, statistically different from CAT activity of control cells ($p < .001$); **, statistically different from CAT activity of BA-treated cells ($p < .0003$).

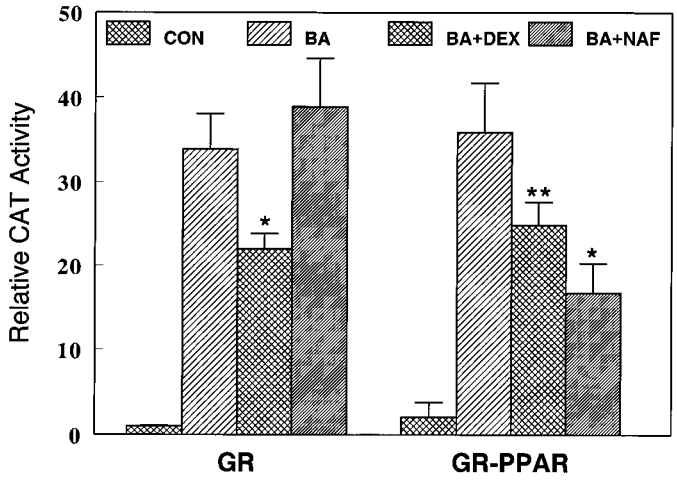

Fig. 7. Effects of a chimeric GR on pALDH3.5CAT Activity of transiently transfected HepG2 cells treated with BA and either DEX or nafenopin. CAT and β-galactosidase assays were performed on lysates from HepG2 cells that had been transiently transfected with p3.5ALDHCAT and cotransfected with pCMVβ and plasmids expressing either human pRSVGR or the chimeric GR-PPAR. The HepG2 cells were treated with either 50 μM BA, 1 μM DEX, 50 μM nafenopin, or combinations of these compounds for 24 h as described under *Experimental Procedures*. The normalized CAT activity is expressed as the percentage conversion of chloramphenicol to its acetyl derivative relative to β-galactosidase activity and is the mean of three flasks ± S.D. *, statistically different from CAT activity of BA-treated cells ($p < .01$). **, statistically different from CAT activity of BA-treated cells transfected with GR ($p = .04$).

GR-PPAR, this effect was not as large or reproducible as the effect seen with cotransfected GR, which is consistent with our observations that HepG2 cells express low and variable levels of GR (Falkner et al., 1998). Because the GR-PPAR functions to negatively regulate *ALDH3* reporter gene expression, GR must regulate this gene's expression, but in a manner different than its action in *GSTA2* expression.

Electrophoretic Mobility Shift Assay of the Palindromic GRE. To establish that the putative GRE sequence was capable of binding human GR, we performed electrophoretic mobility shift experiments with the ds-oligonucleotides shown in Fig. 8A. Extracts from HepG2-GR4 cells, which are stably transfected to express human GR, formed a quantitatively larger amount of DNA-protein complex with a ds-oligonucleotide with sequences identical with the *ALDH3* pGRE than do extracts from HepG2 cells (Fig. 8B). Formation of the complex could be prevented by inclusion of either excess unlabeled oligonucleotide or antibodies specific for GR. The antibody was used under conditions identical with those we have used previously to supershift a DNA-protein complex formed with the pGRE derived from *GSTA2* (Falkner et al., 1998). The diminished formation of the specific complex is consistent with our hypothesis that GR binds to this pGRE sequence of *ALDH3* (Fig. 8B). To further characterize the complex, we tested the ability of other ds-oligonucleotides to prevent DNA-protein complex formation. Increasing concentrations of ds-oligonucleotides derived from sequences either identical with the *ALDH3* pGRE or a consensus pGRE identical with that from the mouse mammary tumor virus long terminal repeat caused clear, graded declines in complex formation consistent with their being specific competitors (Fig. 8C). However, no clear, graded declines in specific protein-DNA complexes were observed with ds-oligonucleotides containing either a mutated pGRE or the unrelated oligonucleotide (such as the *Ah*RE from *CYP1A1*). Thus, complex formation could be prevented with unlabeled ds-oligonucleotides with sequence identity to the canonical GR binding sequence, but not unrelated sequences. In concert, these results are consistent with GR binding directly to the putative GRE.

Discussion

Our results demonstrate that the negative regulation of the *ALDH3* gene by GC occurs via a GR-dependent process. The negative regulation was observed in both primary rat hepatocytes and in transient transfection assays with *ALDH3* 5′-flanking region reporter gene constructs. The expression of *ALDH3* is known to be under multiple regulatory processes. *Ah*RE sequences allow regulation of this gene by xenobiotic compounds that are ligands for the *Ah* receptor. Additionally, at least two other negative regulatory regions have been described in the 5′-flanking region of *ALDH3* (Takimoto et al., 1994; Xie et al., 1996). These regions, in part, may explain the marked differences in constitutive Class 3 ALDH protein content; almost no constitutive expression is observed in liver, but high levels of expression are seen in corneal epithelium, stomach, and heart. The presence of the most distal negative regulatory region (Takimoto et al., 1994) located between −5.5 and − 3.5 kb in the 5′ region

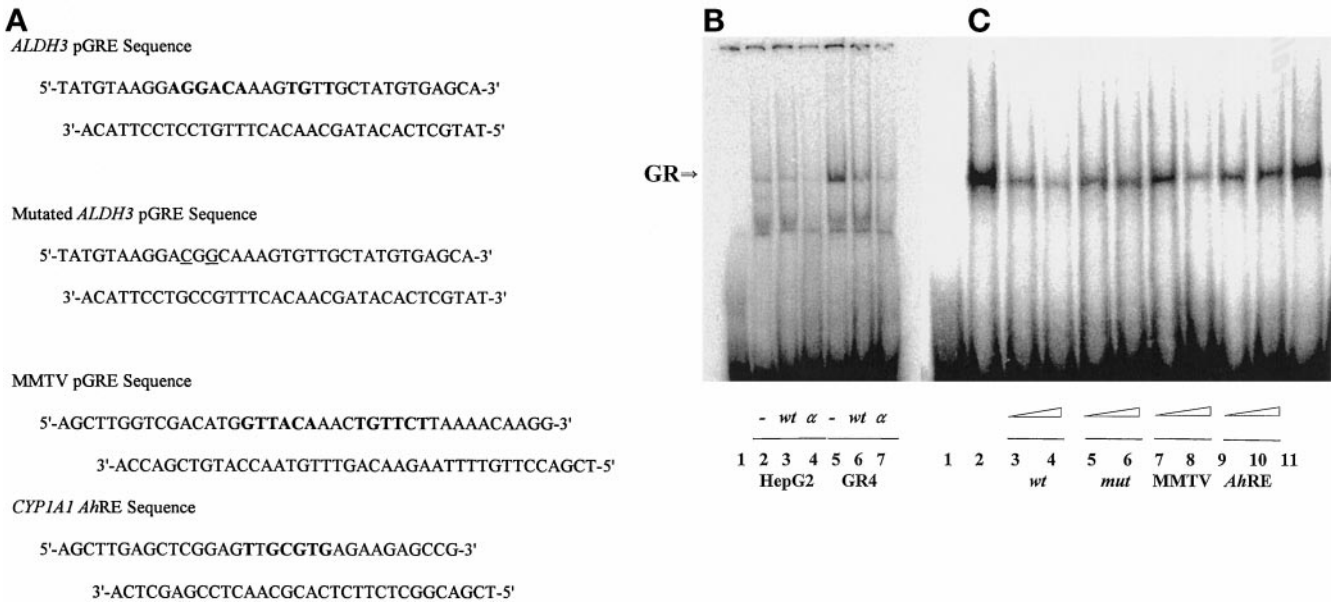

Fig. 8. DNA-protein interactions at the palindromic GRE located at −920 bp in the 5′-flanking region of *ALDH3*. A, ds-stranded oligonucleotides used for the ^{32}P-labeled *ALDH3* pGRE probe and for competition are shown. Consensus nucleotides to the GRE palindrome (30) are marked in bold, and mutated bases are underlined in bold. B, nuclear extracts from HepG2 or GR4 cells treated with 1×10^{-6} M DEX were incubated with the radiolabeled *ALDH3* pGRE probe and resolved using a low-ionic-strength polyacrylamide gel electrophoresis. The DNA-protein complexes were measured using a Molecular Dynamics PhosphorImager. *Lane 1*, probe alone; *lane 2*, probe and extract from DEX-treated HepG2 cells; *lane 3*, probe and extract from DEX-treated HepG2 cells with a 100-fold excess of unlabeled *ALDH3* pGRE; *lane 4*, probe and extract from DEX-treated HepG2 cells with 6 μg of anti-GR globulin; *lane 5*, probe and extract from DEX-treated GR4 cells; *lane 6*, probe and extract from DEX-treated GR4 cells with a 100-fold excess of unlabeled *ALDH3* pGRE; *lane 7*. Probe and extract from DEX-treated GR4 cells with 6 μg anti-GR globulin. C, *lane 1*, probe alone; *lanes 2* and *11*, probe and extract from DEX-treated GR4 cells; *lanes 3* and *4*, probe and extract from DEX-treated GR4 cells with a 50- and 100-fold excess of *ALDH3* pGRE, respectively; *lanes 5* and *6*, probe and extract from DEX-treated GR4 cells with a 50- and 100-fold excess of mutated *ALDH3* pGRE, respectively; *lanes 7* and *8*, probe and extract from DEX-treated GR4 cells with a 50- and 100-fold excess of MMTV pGRE, respectively; *lanes 9* and *10*, probe and extract from DEX-treated GR4 cells with a 50- and 100-fold excess of the unrelated oligonucleotide *Ah*RE, respectively.

results in loss of both basal and BA-inducible expression of reporter constructs transfected into HepG2 cells. The more proximal negative regulatory region (Takimoto et al., 1994; Xie et al., 1996) spans bp −1057 to −390, where multiple transcription factor binding sites have been suggested. A negative-acting cAMP responsive element is located between bp −1057 and −991 (Xiao et al., 1997). Binding sites for the Nuclear Factor 1(NF-1)-like transcription factors, the consensus sequences of which lie at positions −899 to −886 and −844 to −831, are also implicated in the negative regulation of this gene. The data presented here indicates that the pGRE located at −910 to −930 bp is responsible for GC-mediated suppression of PAH-induction of *ALDH3*.

Although the negative regulation by GC is CHX-sensitive, the pGRE must be involved in at least one mode of negative regulation. GC-dependent superinduction of mRNA levels for *ALDH3* in hepatocytes grown in defined GC-free media cannot be explained merely by the loss of a GC-inducible repressor protein. The effects of CHX or CHX plus GC on the level of gene expression would be identical if that were the case. The positive effects are most likely the result of GR interacting with other transcription factors by a non-CHX-dependent mechanism. This positive interaction must be suppressed under normal conditions, possibly by switching GR interaction from one with a non-CHX-dependent transcription factor that elicits a positive response to one with a CHX-sensitive repressor protein resulting in negative regulation.

In both isolated hepatocytes and in transient transfection studies, no significant regulation by GCs was observed in the basal expression of *ALDH3*, which suggests that the mechanism of negative GC regulation of PAH-induction of ALDH3 protein requires direct involvement with events/processes mediated by *Ah*R. In hepatocytes, basal expression of *ALDH3* is low and the inability to observe a negative effect may be caused in part by the closeness of the activities to the limits of detection for the assays employed. As potentiation of PAH-induction via GR-GRE interactions in genes such as *CYP1A1* was shown in this article, it is unlikely that a simple inhibition of *Ah* receptor action or decreases in *Ah*R levels is involved in repression of *ALDH3* expression in the presence of PAH. The role of the ligand-activated *Ah* receptor in induction of *CYP1A1* expression may involve chromatin remodeling (Whitlock et al., 1996) and an alteration in the manner in which constitutive transcription factors interact. Hines et al. (1988) suggested that PAH-dependent induction of P-450 1A1 involves a de-repression event in which the actions of negative regulatory elements are ameliorated, as well as the active recruitment of transcription factors required for gene activation. Likewise, the mode of negative regulation observed for *ALDH3*, which apparently involves a CHX-sensitive transcription factor, may not be observed until the gene is induced. This suggests a complex interplay between the various transcription factors, including the GR and *Ah*R, regulates the expression of *ALDH3*.

Several modes of negative regulation have been described that involve GR (Starr et al., 1996). The simplest involves squelching caused by the removal of essential factors from the nucleus before receptor binding. This mode of negative regulation (Mukaida et al., 1994) does not require a GRE and RU38486 often acts as an agonist rather than an antagonist in such situations. This mechanism is thus clearly different from the mode of GC repression of expression observed with the *ALDH3* gene. Other modes of repression mediated by GR involve competition for binding sites, as seen with the osteocalcin gene (Stromstedt et al., 1991), in which a palindromic GRE overlaps the TATA box. In *ALDH3*, the area in which the pGRE resides has not been implicated in the positive regulation of this gene, because basal activity does not change when this region is mutated and there do not seem to be any other significant DNA-protein complexes formed in the electrophoretic mobility shift experiments.

More complex modes of GR regulation involve interaction with other transcription factors, presumably either through protein-protein interaction or by remodeling of chromatin structure in areas immediately adjacent to a second transcription factor. The best example of complex interaction of GR with other transcription factor is at the composite activator protein-1 (AP-1)/GRE found in the proliferin gene (Miner and Yamamoto, 1992), in which both positive and negative GC-responses may be observed depending on the nature of the AP-1 dimers (e.g., jun-jun or fos-jun) that bind to the site. Other examples of this composite mechanism include the phosphoenopyruvate kinase gene (Imai et al., 1993) in which transcription factors are clustered to elicit a maximal positive GC response. Several groups have described CHX-sensitivity of GC responsiveness in genes, such as the α1-acid glycoprotein gene (Klein et al., 1988), in which protein-protein interaction via CCAAT/enhancer-binding proteins and other unidentified transcription factors are thought to play a role in forming GC-responsive elements. Because the action of many ligand-activated receptors is modulated by other transcription factors through protein-protein interaction, we used an expression plasmid for a chimeric GR (GRPPAR) in which the GR DNA-binding domain is present, but contains the ligand-binding domain of murine PPARα. Our results with GRE mutation and chimeric receptor offers solid proof that the negative regulation of *ALDH3* is a GR-dependent process involving DNA binding to the 5′-flanking region of this gene. In *ALDH3*, the effect of GC is complex, but the negative regulation involves GC acting as ligand for its receptor which then binds a canonical pGRE consensus sequences. In the 5′-flanking region of *ALDH3*, several other putative negative element sequences exist either immediately 5′ or 3′ of the pGRE, including putative cAMP responsive element and NF1 sites. Because plasmids constructs pΔ(−1057/−930)CAT and pΔ(−1057/−930)MUTCAT lack the cAMP-responsive element, but pΔ(−1057/−930)CAT retains GC-sensitivity nearly identical with the intact gene, the negative cAMP responsive element does not seem to be obligatorily involved with the GC regulation of *ALDH3*. Immediately 3′ of the pGRE are binding sites for NF-1 or NF-1-like factors (Xie et al., 1996), which have also been implicated as negative regulators of *ALDH3*. Furthermore, this negative regulation is distinct from that seen with glutathione *S*-transferase *A2* for which the chimeric GR-PPAR was unable to regulate the 5′-flanking reporter construct (Falkner et al., 1998). Our results are consistent with a hypothesis that GR works in coordination with other transcription factors, which will be the focus for further study.

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