We investigated the inhibitory effects of intracellular cyclic adenosine monophosphate (cAMP) levels in regulating class 3 aldehyde dehydrogenase (aldh3) gene expression using cultures of primary rat hepatocytes and transient transfection experiments with HepG2 cells. In addition to regulation by an Ah receptor-dependent mechanism, expression of many members of the Ah gene battery have been shown to be negatively regulated. As was seen for the cytochrome P450 (cyp1A1) gene, aldh3 is transcriptionally inducible by polycyclic aromatic hydrocarbons (PAH), and this induction involving function of the arylhydrocarbon (Ah) receptor is inhibited by the protein kinase C (PKC) inhibitors, 1-(5-isoquinolinesulfonfonyl)-2-methylpiperazine di-HCl (H7) and staurosporine. However, PAH induction of ALDH-3 activity, protein, and mRNA was potentiated 2–4-fold by addition of the protein kinase A (PKA) inhibitors, N-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide di-HCl (H8) and N-(2-guanidinoethyl)-5-isoquinolinesulfonamide HCl (HA1004). These PKA inhibitors had no effect on the PAH induction of the cyp1A1. Protein kinase A activity of cultured hepatocytes was specifically inhibited by H8 and HA1004 in a concentration-dependent manner, but not by H7, and there was an inverse correlation observed between potentiation of PAH-induced aldh3 gene expression and inhibition of specific PKA activity by the PKA inhibitors. The cAMP analog dibutyryl cAMP, the adenylyl cyclase activator forskolin, and the protein phosphatase inhibitor okadaic acid dramatically inhibited both PAH induction and H8 potentiation of PAH induction of aldh3 expression but had no effect on induction of cyp1A1 expression in cultured hepatocytes.

Both basal and PAH-dependent expression of a chloramphenicol acetyltransferase expression plasmid containing approximately 3.5 kilobase pairs of the 5′-flanking region of aldh3 (pALDH3.5CAT) were enhanced 3–4-fold by the PKA inhibitor H8 but not by the PKC inhibitor H7 (>20 μM). cAMP analogs, activators of PKA activity, or protein phosphatase inhibitors diminished expression of the reporter gene in a manner identical to the native gene in cultured rat hepatocytes. Using deletion analysis of the pALDH3.5CAT construct, we demonstrated the existence of a negative regulatory region in the 5′-flanking region between −1057 and −991 base pairs which appears to be responsible for the cAMP-dependent regulation of this gene under both basal and PAH-induced conditions. At least two apparently independent mechanisms which involve protein phosphorylation regulate aldh3 expression. One involves function of the Ah receptor which requires PKC protein phosphorylation to positively regulate both aldh3 and cyp1A1 gene expression and the other a cAMP-responsive process which allows PKA activity to negatively regulate expression of aldh3 under either basal or inducible conditions.

The aldehyde dehydrogenases (ALDH,1 aldheyde NAD(P)+ oxidoreductase EC 1.2.1.3) are a family of NAD(P)+-dependent enzymes that oxidize a broad class of aldehydes to their carboxyl acids. The family is divided into at least three classes based on sequence similarity, and the class 3 aldehyde dehydrogenase gene is expressed in a tissue-specific manner in microsomal and cytosolic fractions (1, 2). The highest level of basal expression of aldh32 is seen in corneal epithelium, stomach, and heart, whereas PAH-induced expression is seen principally in liver, lung, bladder, colon, spleen, and thymus of rodents. This gene is also expressed at high levels in neoplastic tissue and some cell lines. Takimoto et al. (5) and Xie et al. (6) have characterized the 5′-flanking region of the aldh3 gene and demonstrated that it contains at least three major functional domains: a strong promoter proximal to the transcription start site, an inhibitory region just upstream of the promoter, and a PAH-responsive enhancer region. The transcription of the aldh3 gene appears to be controlled by cooperation of at least these three functional domains.

Expression of this gene in liver is mediated by the arylhydrocarbon (AhR) receptor, a cytosolic protein capable of binding PAH as ligands (7). After ligand binding, the AhR-ligand complex is translocated into the nucleus, forms a heterodimeric complex with the arylhydrocarbon nuclear transporter (ARNT), and they interact with specific DNA sequences, designated xenobiotic responsive elements (XREs), to alter the transcription of specific genes. The AhR mediates induction of the aryl hydrocarbon (Ah) receptor; ARNT, arylhydrocarbon receptor nuclear transporter; BA, 1,2-benzanthracene; CAT, chloramphenicol acetyltransferase; CYP1A1, cytochrome P4501A1; EROD, 7-ethoxyresorufin O-deethylase; H7, 1-(5-isoquinolinesulfonfonyl)-2-methylpiperazine di-HCl; H8, N-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide di-HCl; HA1004, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide HCl; PAH, polycyclic aromatic hydrocarbons; PKA, protein kinase A; PKC, protein kinase C; XRE, xenobiotic responsive element; AH, kilobase pair(s); bp, base pair(s).

1 The abbreviations used are: ALDH-3, aldehyde dehydrogenase class 3; AhR, arylhydrocarbon receptor; ARNT, arylhydrocarbon receptor nuclear transporter; BA, 1,2-benzanthracene; CAT, chloramphenicol acetyltransferase; CYP1A1, cytochrome P4501A1; EROD, 7-ethoxyresorufin O-deethylase; H7, 1-(5-isoquinolinesulfonfonyl)-2-methylpiperazine di-HCl; H8, N-(2-(methylamino)ethyl)-5-isoquinolinesulfonamide di-HCl; HA1004, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide HCl; PAH, polycyclic aromatic hydrocarbons; PKA, protein kinase A; PKC, protein kinase C; XRE, xenobiotic responsive element; AH, kilobase pair(s); bp, base pair(s).

2 The gene/isoform designations used for cytochrome P450 and class 3 aldehyde dehydrogenase adhere to the systematic nomenclature recommended by Nelson et al. (3) and Hempel et al. (4), respectively.
a number of xenobiotic metabolizing enzymes (termed the Ah gene battery), including cytochrome P4501A1, cytochrome P4501A2, glutathione S-transferase Ya1 (GST1), NAD(P)/H:quinone oxidoreductase, (QOR), UDP-glucuronosyltransferase 1.6 (UGT1A6), and class 3 aldehyde dehydrogenase (8). The first gene whose activation was shown to be directly mediated by the AhR was cyp1A1 (7, 9); multiple XREs have been identified in the 5'-upstream regulatory region of the cyp1A1 gene. Although there are sequences of the aldh3 PAH-responsive enhancer nearly identical to the XREs in the cyp1A1 flanking sequences, one functional aldh3 XRE is located much farther upstream than those seen in either cyp1A1, glutathione S-transferase Ya1, or NADPH-quinone oxidoreductase genes (5, 10, 11). A second feature of genes in the Ah gene battery is the low levels of constitutive expression that appears to be due to negative control mechanisms (12). Several different mechanisms have been described for the negative regulation of cyp1A1, including putative regulatory genes on mouse chromosome 7 (12), regulation by nucleosome structure (13), and specific negative regulatory transcription factors (14).

Several studies have suggested that protein kinase C (PKC) plays an important role in the regulation of cyp1A1 gene expression. Carrier et al. (15) and Reiners et al. (16) have provided evidence that some step in transactivation by the AhR is dependent upon phosphorylation by PKC. Our studies (17) with cultured rat hepatocytes demonstrated that the PKC inhibitors H7 and staurosporine concomitantly inhibited PAH induction of all five genes we tested, including aldh3, cyp1A1, gst1, qor, and ugt1.6 (ugt1A6). This result suggests that PKC-dependent phosphorylation of the AhR is required for PAH induction of all five xenobiotic metabolizing enzymes. The protein kinase A inhibitors H8 and HA1004 were without effect on all of these genes at equivalent concentrations, except PAH induction of ALDH-3 mRNA and protein which was stimulated 2–4-fold (17). In this study, we sought to characterize the synergistic effects of the PAH, 1,2-benzanthracene, and protein kinase A inhibitors, H8 and HA1004, on aldh3 gene expression and the mechanism whereby PAH regulates levels of the ALDH-3 enzyme in cultured adult rat hepatocytes. Utilizing reporter genes containing the 5'-flanking region of the aldh3 gene transfected into HepG2 tumor cells, we demonstrate the existence of a CAMP-dependent negative cis-acting element.

**EXPERIMENTAL PROCEDURES**

**Materials**

Collagenase (type I) and chlorophenol red-β-galactopyranoside were obtained from Boehringer Mannheim. Benzaldehyde, 1,2-benzanthracene, chloramphenicol, cytochrome c, dibutylryl cAMP, forskolin, Hank's modified balanced salt, insulin-transferrin-sodium selenite medium supplement, NADP+ NADPH, l-tartaric acid were purchased from Sigma and arginine free Eagle's modified medium (high modified) were purchased from JRH BioSciences (Lexena, KS). Fetal bovine serum and fungizone were obtained from Harlan Bioproducts for Science (Indianapolis, IN). n-Butyryl-CoA was obtained from Pharmacia Biotech Inc., and plasma purification kits were purchased from Qiagen (Chatsworth, CA). [3H]Chloramphenicol and [32P]-nucleotide triphosphates were obtained from Du Pont NEN.

**Methods**

**Primary Hepatocyte Cell Culture—**Hepatocytes were routinely prepared from male adult Sprague-Dawley rats (180–250 g, Harlan Sprague-Dawley SD, Harlan Sprague-Dawley, Indianapolis, IN) by in situ liver collagenase perfusion (18, 19) with modification (17). At 24 h in culture, inducing agents were added to the fresh media. H7, H8, HA1004, dibutyryl cAMP, forskolin, and okadaic acid were added 1 h prior to addition of BA. Equivalent amounts (±1% v/v) of solvent were added to control wells. At the desired times, the media were removed from the dishes by aspiration, the cells were washed with Dulbecco's phosphate-buffered saline (2 × 1 ml), and cells were harvested (17). Protein concentrations were determined utilizing bichononic acid with bovine serum albumin as a protein standard (20). The samples were stored at −70 °C and analyzed within 2–4 days for enzyme activity. The proteins were stable for over 6 months at −70 °C when analyzed by Western immunoblot analyses.

**Enzyme Assays—**Cytochrome P4501A1 activity was determined using the specific substrate 7-ethoxyresorufin (21). Aldehyde dehydrogenase 3 activity was measured by monitoring the increase in absorbance at 340 nm caused by NADPH production during the oxidation of benzaldehyde as a substrate (22). Protein kinase A activity was measured as described by using the protein kinase A assay system obtained from Life Technologies, Inc.

**Northern Analysis—**Total RNA was isolated by modification of the method of Chomczynski and Sacchi (23) as described previously (17). Northern blot experiments were performed after size-fractionation of the denatured RNA (25 µg) on formaldehyde-containing 1% agarose gels. The RNA was transferred on to Zetaprobe membranes by capillary blotting (24). Hybridization was carried out at 45 °C overnight in 0.25 M sodium phosphate buffer, pH 7.2, containing 0.25 M sodium chloride, 50% formamide, 7% SDS, 1 mM EDTA, and [32P]-labeled cDNA probe. The hybridized membranes were washed three times 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 at room temperature. The washed membrane was used to expose x-ray film at −70 °C with an intensifying screen (Du Pont NEN). The exposed x-ray film was developed, and the optical density of the relevant bands was quantitated by densitometry using a Bio-Rad Model 620 video densitometer (La Jolla, CA).

**Probes Used for mRNA Analysis—**Cytochrome P4501A1 mRNA was measured using a 635-bp PsI fragment of the plasmid pA6 for cyp1A1 provided by R. N. Hines, Department of Pharmacology and Toxicology, Wayne State University (25). Aldehyde dehydrogenase 3 mRNA was measured using a EcoRI/HindIII cDNA fragment of the plasmid pALDHX, the aldh3-specific clone (1). As a control, β-actin mRNA levels were measured using the cDNA plasmid encoding the mouse cytoskeletal β-actin (26). These nuclei acid probes were labeled with [32P]dCTP using the random primer labeling procedure (27).

**Preparation of CAT Constructs—**A CAT construct (p3239CAT, containing approximately 3.5 kb of 5'-flanking region of the aldh3 gene containing functional XREs (5) was used to test for the effects of PAH and PKA inhibitors/activators. A series of deletion mutants of the proximal 5'-flanking region of aldh3 were prepared by polymerase chain reaction as described previously (6), namely pI-3, pI-4, pI-5, pI-6, and pI-8. Construct pI-1054/392ALDH was produced by treating pALDH 3 with the restriction enzyme PsI at a point 1054 bp upstream of the aldh3 gene. This construct was selected for study because the 5'-flanking region from −1057 bp to −392 bp relative to the transcription start site; the resulting plasmid was gel purified and religated to yield pI-1057/392ALDH. Construct pI-1057/930 was prepared using polymerase chain reaction to generate a fragment from −374 bp to −930 bp (upstream primer 5'GGAGGCAAAAGTTGCTATTG-3' and downstream primer, 5'-AGGCTGCTGTCGTTGTCG-3'). The poly-}

reactions were performed in 100-μl reaction mixtures containing the cell extract (approximately 120 μg of protein) in 0.25 M Tris-HCl buffer, pH 7.5, containing 3.7 mM chloramphenicol (25 nCi), and 5 μg n-butylryl-CoA. The samples were extracted with 300 μl of xylene and back-extracted with 100 μl of Tris-HCl buffer. Aliquots of the xylene phases and the original reaction mixture were assayed for tritium content. β-Galactosidase activity was measured spectrophotometrically using chlorophenol red β-galactopyranoside as substrate by measuring the absorbance formed after 1 h at 595 nm on a Titrbytek Uniskan II plate reader (Flow Laboratories, McLean, VA). Protein concentrations were determined using the method of Smith et al. (20). All transient transfection experiments were performed three times in triplicate. Although a single experiment is shown in the data presented, the results seen in three separate transfection experiments were similar. Statistical Analysis—All data were analyzed using a Student’s t test.

RESULTS

Effects of Protein Kinase Inhibitors on the Induction of P4501A1 and ALDH-3 by PAH—Previous studies have demonstrated that protein kinase C-mediated protein phosphorylation may be a determinant in the regulation of PAH-dependent gene expression of aldh3, cyp1A1, gst1, qor, and ugt1.6 (10, 11, 17). As we have previously shown (17), the PKC inhibitor H7 (50 μM) potently inhibited BA induction of both cyp1A1 and aldh3 mRNA by over 80% in cultured rat hepatocytes (Fig. 1). In contrast, the PKA inhibitors H8 (50 μM) and HA1004 (50 μM) had little or no effect on PAH induction of mRNA levels for cyp1A1; in fact, they served as negative controls for inhibitor studies on PKC activity since they only inhibit this activity at very high concentrations (<200 μM). Surprisingly, the PKA inhibitors H8 and HA1004 strikingly potentiated PAH induction of mRNA for aldh3 by over 2-fold (Fig. 1). These results were also seen in the levels of ALDH-3 and P4501A1 proteins (data not shown), demonstrating that the changes seen are caused by the regulation of pretranslational processes.

The time courses for induction of ALDH-3 and EROD (7-ethoxyresorufin O-deethylase activity for P4501A1) activity in cultured rat hepatocytes in the presence of 50 μM BA or BA plus 50 μM H8 were compared (Fig. 2). Maximal induction of EROD activity by BA occurred at day 3, and H8 had no observable effects on either BA-inducible or basal EROD activity. The time courses of induction of ALDH-3 activity by BA or BA plus H8 were very similar. Maximal induction occurred at 4 days, and H8 potentiated PAH induction by 2–3-fold as early as 1 day and as late as 6 days in culture. Basal ALDH-3 activity (6.2 nmol/min/mg protein) was observed on day 0 but declined to 0.5 nmol/min/mg protein by day 3 in culture. H8 treatment alone at any concentration did not appreciably affect the basal level of ALDH-3 enzyme activity. H8 potentiated PAH induction of ALDH-3 activity over 3-fold in a concentration-dependent manner (Fig. 3) but had no effect on basal activity at any concentration tested.

PKA activity was measured using a specific peptide substrate labeled with a fluorescent dye, and an appropriate PKA standard with a known activity was used to confirm the specificity of PKA activity (29). Specific PKA enzyme activity in rat hepatocytes was inhibited (>80%) in a concordant but opposite manner than was observed with the H8 and HA1004 potentiation of PAH induction of ALDH-3 enzyme activity (Fig. 4). As a control, the same concentrations of a structurally similar PKC inhibitor, H7, were observed to have little or no effect on PKA activity. Since higher concentrations of H8 and HA1004 (>100 μM) also inhibit PKC activity (17), we routinely used 50 μM concentrations of PKA inhibitors at which hepatocyte PKC activity was unaffected.

Effect of Elevated Intracellular cAMP Levels on PAH Induction and H8 Potentiation of PAH-mediated Induction of aldh3 Expression—The level of intracellular cAMP, and therefore the activation of cAMP-dependent protein kinase, was modulated in primary rat hepatocytes by addition of the cAMP analog, dibutyryl cAMP, or the PKA activator, forskolin. After a period of 24 h in culture and immediately prior to initiating PAH induction, hepatocytes were exposed for 60 min to either dibutyryl cAMP or forskolin. PAH-mediated induction of aldh3 mRNA and H8 potentiation of PAH-mediated induction of aldh3 mRNA levels were inhibited more than 60 and 70%, respectively, by either dibutyryl cAMP or forskolin (Fig. 5). This treatment had no effect on the PAH-mediated induction of levels of cyp1A1 mRNA. Furthermore, the changes in levels of aldh3 mRNAs were concordant with the changes observed for ALDH-3 enzyme activity (Fig. 6) and protein measured by Western immunoblot analysis (data not shown). These results demonstrate that cAMP analogs and activators of adenyl cyclase led to repression of both PAH induction and H8 potentiation of PAH induction of aldh3 gene expression. This effect is specific for the aldh3 gene, since these agents had little or no effect on expression of cyp1A1.

Effect of Protein Phosphatase Inhibitors on the PAH Induc-
Okadaic acid is a potent and specific inhibitor of protein phosphatases 1 and 2A and appears to exert its effects by preventing dephosphorylation of specific transcription factor proteins in cells. Okadaic acid decreased both PAH induction and H8 potentiation of PAH induction of ALDH-3 activity by over 50 and 80%, respectively, in a concentration-dependent manner as expected (Fig. 7). Interestingly, H8 potentiation of PAH induction of ALDH-3 enzyme activity was more sensitive to okadaic acid than was PAH induction of ALDH-3 activity. For example, okadaic acid (15–20 nM) resulted in 50% inhibition of ALDH-3 activity induced by BA alone, whereas 5–10 nM okadaic acid resulted in 50% inhibition of ALDH-3 activity induced by treatment with BA plus H8 (Fig. 7).

To assess whether these effects of okadaic acid involve a pretranslational mechanism of action, we performed Northern analysis of mRNA isolated from cells treated with 50 μM H8, 50 μM BA, 20 nM okadaic acid, or combinations of these agents (Fig. 7). These results document that the decrease in aldh3 mRNA levels and enzyme activity by okadaic acid are apparently caused by changes in pretranslational processes specific for aldh3 expression. Addition of okadaic acid to cultured rat hepatocytes treated with BA or BA plus H8 had no effect on PAH induction of cytochrome P4501A1 and levels of its mRNA (data not shown).
Effect of PKA Inhibitor on Basal and PAH-inducible Expression of pALDH3.5 CAT—Lindahl and co-workers (1, 5, 6, 30) have developed a highly inducible 5′-flanking construct containing 3.5 kb of upstream sequence of the rat aldh3 gene in a chloramphenicol acetyltransferase (CAT) reporter gene. Basal level expression of this gene is cell type-specific, and liver displays very low basal expression (1, 2). Unlike the native gene in rat primary hepatocyte cultures, there is measurable basal expression of the pALDH3.5 plasmid construct in transient transfection into HepG2 cells. We observed a 2–4-fold increase in pALDH3.5CAT expression in the presence of the PKA inhibitor H8 in the absence (Fig. 9A) or presence of 1,2-benzanthracene (Fig. 9B). In these experiments, we noted that H7 and H8 also stimulated the expression of the transfection control plasmid, pCMV-βgal. Therefore, we have expressed the data relative to total cellular protein, since our routine transfection efficiency with pCMV-βgal normally yields β-galactosidase activity which varied less than ±20% within a single transfection experiment. The PKC inhibitor, H7, used as a control for the structurally related H8 compound, had no effect on basal expression at the concentrations used, suggesting that the mechanism of regulation by PKA activators and inhibitors is independent of the action of the AhR (ligand- or phosphorylation-dependent) or PKC activation. Inhibition was seen at higher concentrations of H7 and H8 (50 μM), but this concentration of either agent was toxic to HepG2 cells in our hands. Subsequent transfection experiments demonstrated that forskolin, dibutyryl cAMP, and okadaic acid inhibited the transient expression of this reporter gene by 60% in the presence of BA and by 75% in the presence of BA plus H8, similar to the manner they affect expression of the native gene in cultured rat hepatocytes (Fig. 10). In addition, both basal and PAH-induced reporter gene activity was inhibited in a similar manner to the PKC inhibitor H7 (Fig. 9), suggesting that the regulation of the aldh3 gene by PKA activity functions independently of the AhR action.

Deletion Analysis to Define cis-Acting Elements Associated
with Regulation by PKA—To map the region of the 5'-flanking region of the aldh3 gene responsible for a putative PKA-responsive element, we tested a series of 5'-flanking CAT constructs derived from the rat gene (5, 6). Of these constructs (Fig. 11), only two CAT constructs were positively regulated by the PKA inhibitor H8, namely pALDH3.5 and pI-8. The shorter deletion CAT constructs pl-3, pl-4, pl-5, and pl-6 displayed higher basal level expression than that observed with pALDH3.5CAT and pl-8CAT, suggesting that a cis-acting negative regulatory element exists located between nucleotides −1125 and −991 of the 5'-flanking region of aldh3. Due to the effects of PKA activators and inhibitors seen in experiments utilizing pALDH3.5, this element appears to be regulated by a PKA-dependent phosphorylation event.

Since the expression of the pl-8 construct, but not pl-5, was affected by H8, we prepared an internal deletion in the 3.5-kb construct around the PstI site at −1057 bp. Specifically, the construct, pΔ(−1057/−930)ALDH, was prepared from pALDH3.5 which had an internal deletion between nucleotides −1057 and −930 of the flanking region (Fig. 11). This construct had higher levels of basal expression like the pl-5 or shorter constructs and was not regulated by the PKA inhibitors, demonstrating loss of the PKA-regulated element from the reporter gene. Given the results seen with the deletion analyses, we propose that the PKA-responsive elements associated with regulation by PKA lies within a 66-bp region from nucleotides −1057 and −991.

**DISCUSSION**

A number of extracellular signals regulate various intracellular processes via a complex cascade of receptors, transducers, and second messengers (31, 32). These second messengers, cyclic nucleotides including cAMP and cGMP, are well characterized biochemical systems (33, 34). The resulting activation of cyclic nucleotide-dependent protein kinases is the basis for many effects of cyclic nucleotides on cellular function. Recently, a number of in vivo and in vitro studies have been focused on the effects of protein kinase activation on the specific signal transduction pathways in the control of cyp1A1 gene expression. Notably, protein kinase C-mediated phosphorylation (15, 16) appears to be a determinant in the regulation of PAH-inducible cyp1A1 gene expression. Our past experiments suggested that PKC-dependent phosphorylation of AhR appears to be required for PAH induction of all of the xenobiotic metabolizing enzymes in the Ah gene battery, not just for cyp1A1 (17).

Other studies have reported that P450-mediated biotransformation activities associated with phenobarbital-inducible isozymes, including cyp2B1, cyp2B2, and cyp3A1, were depressed by agents that elevated cAMP levels in rat liver and in rat hepatocytes (35), indicating a negative regulatory role for cAMP-dependent signal transduction pathway on gene induction by this barbiturate. This is not the case for cyp1A1, the control gene used in the studies reported herein.

In the current study, we used rat hepatocytes and transient transfection of reporter genes in HepG2 cells to investigate the regulatory control exerted by intracellular CAMP on basal and PAH-induced aldh3 gene expression. H8 and HA1004 are effective inhibitors of cyclic nucleotide-dependent protein kinases (29) and are especially effective as PKA and PKG inhibitors. Our results indicate that these inhibitors potentiate PAH induction of ALDH-3 activity in a concentration-dependent manner, but neither PKA inhibitor had any effect on PAH induction of P4501A1. Protein kinase A activity was shown to be effectively inhibited by H8 and HA1004 but not by H7 in cultured hepatocytes, demonstrating an inverse correlation between potentiation of PAH-dependent regulation of aldh3 gene expression and inhibition of specific PKA activity.

As H8 is also a potent protein kinase G inhibitor, we were concerned that the inhibition of this protein kinase (PKG) by H8 might be responsible for the H8 potentiation effect. However, elevation of intracellular cAMP levels, achieved through the use of a membrane-permeable CAMP analog, dibutyryl cAMP, also resulted in inhibition of PAH-mediated induction and H8 potentiation of PAH-mediated induction of aldh3 gene expression. Striking inhibition of BA induction and H8 potentiation of BA induction of aldh3 gene expression also was noted when intracellular cAMP levels were modulated via adenylate cyclase activation in hepatocytes with 25 μM forskolin. In recent studies by Björnsson et al. (36) and Sidhu and Omiecinski (35), treatment of primary rat hepatocytes with forskolin results in dramatic stimulation of intracellular CAMP levels and decrease in expression of cyp2B. Finally, okadaic acid is a potent, selective inhibitor of protein phosphatase type 1 and 2A and apparently exerts its effects by inhibiting protein dephosphorylation in cells. Okadaic acid decreased PAH induction and H8 potentiation of PAH induction of ALDH-3 activity and mRNA levels in a concentration-dependent manner as expected. Dibutyryl CAMP, forskolin, and okadaic acid had no
effect on the PAH induction of cyp1A1 gene expression under any condition. Since PKC inhibitors had pronounced effect on PAH induction of P4501A1, but okadaic acid did not, PKC-dependent phosphorylation of the AhR must be near maximal in cultured primary rat hepatocytes (17).

Transient transfection experiments utilizing a CAT construct containing 3.5 kb of the 5′-flanking regulatory region of the aldh3 gene and were tested by transient transfection into HepG2 cells. The cells were treated with either dimethyl sulfoxide or H8 (20 μM) in dimethyl sulfoxide 24 h after transfection, and the cellular protein was harvested at 48 h after transfection. CAT activity and cell protein were measured as described under “Methods.” The CAT activity was normalized to unity relative to cell protein, and the standard deviation (n = 3) was less than 10% of the average normalized CAT activity. *, statistically different from basal activity of the given construct (p < 0.005); **, statistically different from basal activity of pALDH3.5 or pI-8 constructs (p < 0.005).

Fig. 11. Deletion analysis of the 5′-flanking region of the aldh3 gene. A series of CAT constructs were prepared with deletions in the 3.5-kb 5′-flanking regulatory region of the aldh3 gene and were tested by transient transfection into HepG2 cells. The cells were treated with either dimethyl sulfoxide or H8 (20 μM) in dimethyl sulfoxide 24 h after transfection, and the cellular protein was harvested at 48 h after transfection. CAT activity and cell protein were measured as described under “Methods.” The CAT activity was normalized to unity relative to cell protein, and the standard deviation (n = 3) was less than 10% of the average normalized CAT activity. *, statistically different from basal activity of the given construct (p < 0.005); **, statistically different from basal activity of pALDH3.5 or pI-8 constructs (p < 0.005).

Using deletion analysis of the regulatory 5′-flanking region of this gene, we have identified a region that likely contains a negative regulatory element. The 66 bp region (−1057 to −991) of the rat aldh3 gene (Fig. 11) and that of its immediate flanking regions contain no unique sequences signal transduction pathway in basal and PAH-inducible aldh3 gene expression.

Using deletion analysis of the regulatory 5′-flanking region of this gene, we have identified a region that likely contains a negative regulatory element. The mechanism for this decreased expression has not been fully established. In Saccharomyces cerevisiae, the aldehyde dehydrogenase 2 (ALD2) gene (37) has been shown to be negatively regulated by protein kinase A, and its 5′-flanking region contains two classical cAMP-responsive elements. The 66-bp region (−1057 to −991) of the rat aldh3 gene (Fig. 11) and that of its immediate flanking regions contain no unique sequences...
with high consensus to other canonical cAMP-responsive element binding protein binding elements (classical or nonclassical, Ref. 38) or other elements suggesting that the rat gene may be uniquely regulated by a novel PKA-regulated transcription factor.

Our studies demonstrate that there are apparently two protein phosphorylation mechanisms involved in the regulation of aldh3 gene expression in hepatocytes (Fig. 12). One involves the AhR, which requires PKC phosphorylation to positively regulate aldh3 gene in the presence of PAH ligands. The second appears to involve a cAMP-responsive process, which may require PKA phosphorylation to negatively regulate aldh3 gene expression (Fig. 12). Future studies will characterize the cis- and trans-acting elements involved in the negative regulation of the class 3 aldehyde dehydrogenase gene of rat.

Acknowledgments—We express our thanks to Jaydev Dholakia and Michael Waterman for their useful input to this project and to Mary Pendleton for technical assistance.

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