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NEGATIVE REGULATION OF THE MURINE CYTOSOLIC ALDEHYDE DEHYDROGENASE-3 (*Aldh-3c*) GENE BY FUNCTIONAL CYP1A1 AND CYP1A2 PROTEINS

Vasilis Vasiliou, Alvaro Puga, and Daniel W. Nebert*

Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, Ohio 45267-0056

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We have examined enzyme activities and mRNA levels corresponding to aldehyde dehydrogenase-3 genes encoding cytosolic (ALDH3c) and microsomal (ALDH3m) forms. In contrast to negligible activities in the intact mouse liver, both ALDH3c and ALDH3m enzyme activities are inducible by benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mouse hepatoma Hepa-1c1c7 cell cultures. Constitutive mRNA levels of ALDH3c are virtually absent, whereas those of ALDH3m are substantial; using Hepa-1 mutant lines, we show that both ALDH3c and ALDH3m are TCDD-inducible by an Ah receptor-dependent mechanism. Basal mRNA levels of ALDH3c, but not those of ALDH3m, are strikingly elevated in untreated mutant cells lacking a functional CYP1A1 enzyme; low ALDH3c basal mRNA levels can be restored by introduction of a functional murine CYP1A1 or human CYP1A2 enzyme into these mutant cells. These data suggest that the TCDD induction process is distinct from the CYP1A1/CYP1A2 metabolism-dependent repression of constitutive gene expression; we suggest that this latter property classifies the ***Aldh-3c*** gene, but not the ***Aldh-3m*** gene, as a member of the murine [***Ah***] battery.

Aldehyde dehydrogenases [aldehyde:NAD(P)$^+$ oxidoreductase, EC 1.2.1.3 and 1.2.1.5] represent a group of enzymes that catalyze the oxidation of various aliphatic and aromatic aldehydes to the corresponding carboxylic acids (1, 2). These enzymes are involved in the metabolism of acetaldehyde (3), corticosteroids (4), biogenic amines (5) and other neurotransmitters (6), retinoic acid (7), and in lipid peroxidation (8). Following the cloning and sequencing of more than a dozen of the aldehyde dehydrogenase cDNAs (the sources ranging from bacteria and plants to fungi, yeast and mammals), a superfamily comprising three gene families (***ALDH1***, ***ALDH2*** and ***ALDH3***) has been proposed on the basis of divergent evolution (1). Within the ***ALDH3*** family, cloning and sequencing has been reported for the rat 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin; TCDD)-inducible ***ALDH3c*** (9-11). Cloning and sequencing of the human homologue, which is highly expressed in stomach, has also been reported (12); the rat and human gene encode a cytosolic enzyme (ALDH3c). Another cDNA, corresponding to a rat liver microsomal aldehyde dehydrogenase, was recently cloned and proposed to be named Class 4 ALDH (13); however, the amino acid sequence is 65.5% identical to ALDH3c. Based on the evolutionary scheme of Lindahl and Hempel (1), therefore, this gene should more appropriately be called a member of a new subfamily within the ***ALDH3*** family, the gene and enzyme hereafter referred to as ***ALDH3m*** and ALDH3m, respectively.

*To whom correspondence should be addressed.

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Aldehyde dehydrogenases are considered to be "drug-metabolizing enzymes." A large number of drugs, foods and other environmental substances are metabolized by enzymes that have classically been defined as "Phase I" and "Phase II" (14). An evolutionary argument has recently been put forth, suggesting that particular subsets of drug-metabolizing enzymes are coordinately regulated in response to high concentrations of various foreign chemicals that mimic endogenous ligands effecting growth, differentiation, homeostasis, and neuroendocrine functions (15). Distinctly different subsets of these enzymes are induced, for example, by steroids, ethanol, peroxisome proliferators, phenobarbital, and TCDD (16).

The best studied example of coordinately regulated genes encoding drug-metabolizing enzymes is the murine [*Ah*] gene battery (16, 17). This battery comprises at least six genes that are coordinately induced by TCDD and polycyclic aromatic hydrocarbons such as benzo[a]pyrene; this induction process requires a functional aromatic hydrocarbon-responsive (Ah) receptor. In addition to two Phase I cytochrome P450 genes--*Cyp1a-1* and *Cyp1a-2*--this laboratory has proposed (17) that the four Phase II genes include: NAD(P)H:menadione oxidoreductase (*Nmo-1*); a tumor-specific aldehyde dehydrogenase (*Aldh-3*); a UDP glucuronosyltransferase having 4-methylumbelliferone as substrate (*Ugt1*06*); and a glutathione transferase having 2,4-dinitro-1-chlorobenzene as substrate (GST Ya, *Gst1a-1*). Interestingly, whereas the Ah receptor-mediated coordinate induction involves all six [*Ah*] battery genes, a gene on chromosome 7 is necessary for the up-regulation of the four Phase II [*Ah*] battery genes and this appears to occur via a mechanism quite independent of *Cyp1a-1* and *Cyp1a-2* induction (16, 17).

Many conclusions about the interactions of the [*Ah*] battery genes would not have been possible without the development of several Hepa-1 benzo[a]pyrene-resistant (BP^r) mutant lines (18): *c37*, CYP1A1 metabolism-deficient (P_1^-); *c4*, chromatin binding-defective (*cb*$^-$), also called nuclear translocation-impaired (*nt*$^-$); and *c2*, Ah receptorless (r^-) containing <10% of normal functional receptor levels. For example, expression of the murine *Cyp1a-1* gene is transcriptionally derepressed in Hepa-1 mutant lines that contain missense mutations in the *Cyp1a-1* structural gene (19), and this derepression appears to include *Nmo-1* (20, 21) and possibly other genes in the [*Ah*] battery (16, 20). The *Cyp1a-1* gene product seems to be necessary in a mechanism of feedback regulation, not only of the *Cyp1a-1* gene (22) but also of the NMO1 mRNA levels (21). By introducing expression plasmids carrying the appropriate cDNA into the mutant *c37* P_1^- cells, RayChaudhuri and coworkers showed that expression of a functional, exogenous murine CYP1A1 protein, or a human CYP1A2 protein, is sufficient to restore the repression of the endogenous *Cyp1a-1* gene, as well as to restore the repression of the NMO1 mRNA levels, and that this effect for *Cyp1a-1* takes place primarily at the level of transcription (21).

Using the rat *ALDH3c* cDNA as a probe, this laboratory has recently cloned and sequenced the murine microsomal aldehyde dehydrogenase-3 (*Aldh-3m*) cDNA; the gene is located on mouse chromosome 11, and alignment with the rat ALDH3m amino acid sequence shows 95% identity [V. Vasiliou, C.A. Kozak, R. Lindahl and D.W. Nebert, manuscript in preparation]. Using the Hepa-1 *wt* and its three mutant lines described above, plus the *c37* stable transfectants containing the murine *Cyp1a-1* cDNA or the human *CYP1A2* cDNA, we show in this report that the murine *Aldh-3c*, but not *Aldh-3m*, can be classified as a *bona fide* member of the [*Ah*] battery.

MATERIALS AND METHODS

Cell Culture Conditions. The *wt* mouse hepatoma cell line Hepa-1c1c7 and its three mutant lines--the CYP1A1 metabolism-deficient derivative *c37*, the chromatin binding-defective *c4*, and the receptorless *c2* (18)--were generous gifts of O. Hankinson (UCLA, Los Angeles, CA). We used the following four *c37* stable transfectants: **CX4**, in which the aromatic hydrocarbon- responsive domain (AhRD, coordinates -1100 to -896) in its native orientation, ligated in the *cis* configuration to the SV40 promoter, drives the murine *Cyp1a-1* cDNA; **CE1**, in which the AhRD in its opposite orientation, ligated in the *cis* configuration to the SV40 promoter, drives the murine *Cyp1a-1* cDNA; **CP47**, in which the AhRD is on a separate plasmid (*trans* configuration) to that containing the SV40 promoter and murine *Cyp1a-1* cDNA; and **CA3.4**, in which the AhRD in its native orientation, ligated in the *cis* configuration to the SV40 promoter, drives the human *CYP1A2* cDNA (21). All cell lines were routinely grown in modified Eagle's α-medium containing 10% fetal calf serum. When required, TCDD treatment (20 nM, first dissolved in *p*-dioxane) was carried out for 12-24 h, or benzo[a]pyrene treatment (10 μM, first dissolved in dimethylsulfoxide) was carried out for 6-48 h.

ALDH3c and ALDH3m Enzyme Assays. After being rinsed twice with ice-cold phosphate-buffered saline, the cells were scraped from the tissue culture flasks. The harvested suspension was centrifuged at 1,500 x g for 5 min, and the cell pellet was resuspended in the homogenization buffer [sodium pyrophosphate (0.1 M, pH 8.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM mercaptoethanol]. Typically, one culture flask of cells was resuspended in 1 ml of buffer. The cell suspensions were sonicated in ice, with 3 periods of 10 s interrupted by two intervals of the same duration, in order to avoid overheating (23). The cell-free suspension was centrifuged at 3,000 x g for 10 min, and the supernatant fraction was centrifuged at 105,000 x g for 1 h. The soluble fraction was used for the ALDH3c assays, and the microsomal pellet was resuspended in 0.3 ml of homogenization buffer and used for the ALDH3m assays. Determinations of ALDH activity were carried out with a Beckman spectrophotometer (Model DU-70), by monitoring NAD(P)H production at 340 nm and 37 °C. To measure the NAD^+-dependent oxidation of propionaldehyde (P/NAD activity), the assay mixture contained sodium pyrophosphate buffer (75 mM, pH 8.0), 1 mM pyrazole (to inhibit alcohol dehydrogenase), 1 mM NAD^+, and 5 mM propionaldehyde. To measure the $NADP^+$-dependent oxidation of benzaldehyde (B/NADP activity), assay conditions were the same except benzaldehyde (5 mM originally in 20% methanol) was substituted for propionaldehyde, and the coenzyme $NADP^+$ (2.5 mM) was used instead of NAD^+. In either enzyme assay, the reaction was started by adding the substrate subsequent to a 5-min preincubation, and a blank was run without the substrate (24). Protein was measured by the bicinchoninic acid method (Pierce Chemical Company, Rockford IL), according to details supplied by the manufacturer. Units denote nmoles of NAD(P)H formed per min; specific activities are expressed in units/mg protein. Statistical analyses of the results were performed by Student's two-tailed t-test.

RNA Extraction and Northern Blots. RNA was extracted by the acid guanidinium thiocyanate method (25). Total RNA (10 μg) was separated in formaldehyde-agarose gels and transferred to Nytran. Transfers were carried out for 2 to 4 h with the use of a semi-dry blotting apparatus (JKA BioTech, Copenhagen, Denmark) at ≤0.8 mA per cm^2 of gel surface. Prehybridizations and hybridizations were carried out at 42 °C in a solution containing 50% deionized formamide, 6X SSC (SSC = 0.9 M NaCl and 0.09 M sodium citrate, pH 7.0), 2.5X Denhardt's solution, 0.5% sodium dodecylsulfate (SDS), and denatured salmon sperm DNA (0.1 mg/ml). Radioactively labeled probes were prepared by nick translation (26) or by random priming (27), using [α-^{32}P]dCTP (3000 Ci/mmole, New England Nuclear/DuPont) as the labeled precursor, and were added to the hybridization solutions at 5 x 10^6 to 10 x 10^6 cpm/ml. After hybridization for 16-20 h, the filters were washed twice in 2X SSC and 0.1% SDS for 10 min at room temperature and then twice in 0.1X SSC and 0.1% SDS for 30 min at 50 °C. The filters were then exposed for 48 h to Kodak XAR-5 film at -70 °C with intensifying screens. Probes included the rat *ALDH3c* full-length cDNA (9), the murine *Aldh-3m* full-length cDNA [V. Vasiliou, C.A. Kozak, R. Lindahl and D.W. Nebert, manuscript in preparation], and chicken β-actin-1 cDNA from plasmid pBA1 (28).

RESULTS AND DISCUSSION

ALDH3c and ALDH3m enzyme activities induced in murine Hepa-1 cells. Expression and induction of hepatic ALDH3 activity in the intact animal is known to be different between rats and mice. Whereas ALDH3c is present and inducible by benzo[a]pyrene and TCDD in rats, this is not the

TABLE 1. ALDH3c induction by benzo[a]pyrene in Hepa-1 *wt* cells

Time (hours)	B/NADP	P/NAD	B/NADP to P/NAD Ratio
0	2.2 ± 0.1	5.3 ± 0.3	0.42
12	10.8 ± 0.3	4.2 ± 0.9	2.6
24	18.3 ± 1.7	9.6 ± 0.5	1.9
48	23.0 ± 0.9	10.4 ± 0.9	2.2

Cells were treated with benzo[a]pyrene (10 μM), and the cytosolic fraction was prepared. Specific activities are expressed as units/mg protein in mean ± standard deviation, from duplicates in three separate experiments. B/NADP, $NADP^+$-dependent benzaldehyde oxidation. P/NAD, NAD^+-dependent propionaldehyde oxidation. A ratio of B/NADP to P/NAD of >1.00 is generally believed to reflect dioxin-inducible ALDH3c activity (30, 31).

case in mice (29). To our surprise, ALDH3c enzyme activity was detectable and inducible by benzo[a]pyrene in murine Hepa-1 cell cultures (Table 1). We have also found similar results in other mouse strains and cell culture lines derived from these mouse strains [V. Vasiliou and D.W. Nebert, in preparation]. These data thus suggest that, in mouse liver tissue culture cells or in hepatoma cell culture, either an inhibitor of ***Aldh-3c*** gene expression is extinguished or an activator of ***Aldh-3c*** gene expression is activated, as compared with hepatic ALDH3c activity in the intact mouse.

To see if this induction process by benzo[a]pyrene would also occur after TCDD treatment, and to see if this process might include ALDH3m as well as ALDH3c, we compared cytosolic and microsomal ALDH3 enzyme activities in control and TCDD-treated Hepa-1 cell cultures (Table 2). Increases in the B/NADP to P/NAD ratio in cytosolic fractions, from 1.0 to 3.4, confirmed that the cytosolic ALDH3c is inducible by TCDD in these mouse cell cultures. For the microsomal ALDH3m activity, however, the increase in B/NADP to P/NAD ratio was from 0.27 to 1.0. Since a ratio of B/NADP to P/NAD of >1.00 is generally believed to reflect dioxin-inducible ALDH3 activity (30, 31), the Table 2 observation of microsomal ALDH3 activity is inconclusive. One possibility is that the ALDH3m, like the ALDH3c, is inducible by TCDD; an alternative possibility is that the TCDD-induced microsomal ALDH3 activity represents contamination of microsomes with the highly

TABLE 2. Effect of TCDD on ALDH3c and ALDH3m enzyme activities in Hepa-1 *wt* cells

	B/NADP	P/NAD	B/NADP to P/NAD Ratio
Cytosol:			
Control	1.3 ± 0.4	1.3 ± 0.2	1.0
TCDD	16.3 ± 2.1	4.8 ± 0.6	3.4
Microsomes:			
Control	0.4 ± 0.1	1.5 ± 0.2	0.27
TCDD	3.1 ± 0.6	3.1 ± 0.7	1.0

Cells were treated with TCDD (20 nM) for 24 h. Specific activities and abbreviations are the same as those described in Table 1.

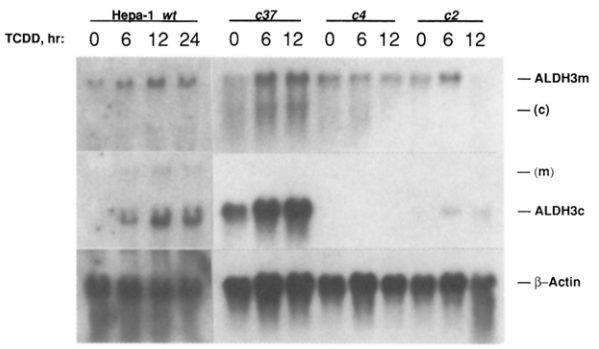

FIG. 1. Northern hybridization analysis of ALDH3m and ALDH3c mRNAs in the Hepa-1 *wt* wild-parent cell line and the three mutant lines: CYP1A1 metabolism-deficient *c37*, chromatin binding-defective *c4*, and "receptorless" *c2*. The times of TCDD treatment (20 nM) are indicated. The β-actin probe was used as a control to ensure that the amount of RNA per lane is relatively constant. **(c)**, the 1.9-kb ALDH3c mRNA that appears to cross-hybridize weakly with the ***Aldh-3m*** cDNA probe. **(m)**, the 2.8-kb ALDH3m mRNA that appears to cross-hybridize weakly with the ***ALDH3c*** cDNA probe.

inducible cytosolic ALDH3c form. To distinguish between these two possibilities, therefore, we compared ALDH3c and ALDH3m mRNA levels.

ALDH3c and ALDH3m mRNA levels in *wt* and mutant cell lines. In the Hepa-1 wild-type parent line, the ALDH3c mRNA basal levels were negligible and the mRNA was highly induced by TCDD; the ALDH3m mRNA basal levels were high and also significantly induced by TCDD (Fig. 1). In addition to hybridizing to the ALDH3m 2.8-kb mRNA, the murine ***Aldh-3m*** cDNA probe cross-hybridized weakly with the ALDH3c 1.9-kb mRNA (Fig. 1, especially ***6th & 7th lanes***). In addition to hybridizing to the murine ALDH3c 1.9-kb mRNA, the rat ***ALDH3c*** cDNA probe cross-hybridized weakly with the murine ALDH3m 2.8-kb mRNA (Fig. 1, especially ***lanes 2-4***). In a recent study in which the rat ALDH3c cDNA probe was used on Northerns from rat hepatoma H4IIE and HTC cell cultures, a faint, higher-molecular-weight RNA band was detected and believed to be a precursor of the mature ALDH3c mRNA (32); however, based on our results, we find it more likely that this faint band might represent the rat ALDH3m mRNA.

In the absence of endogenous ***Cyp1a-1*** expression in the *c37* mutant cells (Fig. 1), ALDH3c mRNA basal levels were markedly elevated, and the mRNA levels were further induced after TCDD treatment. In contrast, ALDH3m constitutive mRNA levels in *c37* cells were not increased over those in *wt* cells, although induction by TCDD was observed.

In the *c4* mutant line that lacks the ARNT protein responsible for nuclear translocation of the Ah receptor (33), ALDH3c mRNA basal levels were nil and no induction occurred with TCDD; on the other hand, ALDH3m mRNA basal levels were elevated but not further increased by TCDD. In the *c2* mutant line in which a small amount of functional Ah receptor exists, ALDH3c mRNA basal levels were negligible but induced slightly by TCDD as expected; in contrast, ALDH3m mRNA basal levels

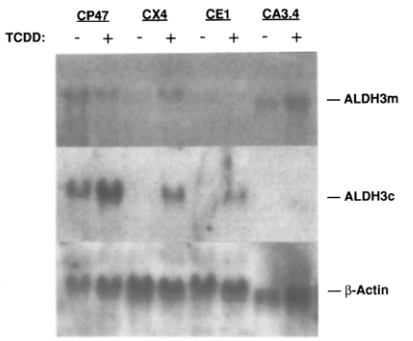

FIG. 2. Northern hybridization analysis of ALDH3m and ALDH3c mRNAs in *c37* stable tranfectants containing exogenously expressed mouse *Cyp1a-1* or human *CYP1A2* cDNA. The three probes are the same as those in Figure 1. TCDD treatment (20 nM) was for 12 h. **CP47** contains the aromatic hydrocarbon-responsive domain (AhRD) on a separate plasmid (*trans* configuration) to that containing the SV40 promoter and murine *Cyp1a-1* cDNA. **CX4** contains the AhRD in its native orientation, ligated in *cis* to the SV40 promoter and the murine *Cyp1a-1* cDNA. **CE1** contains the AhRD in its opposite orientation, ligated in *cis* to the SV40 promoter and the murine *Cyp1a-1* cDNA. **CA3.4** contains the AhRD in its native orientation, ligated in *cis* to the SV40 promoter and the human *CYP1A2* cDNA.

were elevated and induction by TCDD, if any, was difficult to discern. These results therefore indicate that TCDD-induced accumulation of either ALDH3c or ALDH3m mRNA requires a functional Ah receptor, whereas ALDH3m basal mRNA expression does not.

ALDH3c and ALDH3m mRNA levels in *c37* stable transfectants. The **CX4** and **CE1** cell lines are derivatives of *c37* cells into which the murine *Cyp1a-1* cDNA expressing a functional murine CYP1A1 protein is stably expressed (21). The ALDH3c mRNA basal levels--that are dramatically elevated in *c37* cells (Fig. 1)--are decreased to negligible levels by the exogenously expressed CYP1A1 enzyme in the **CX4** and **CE1** cell lines (Fig. 2, *lanes 3 & 5*), and the ALDH3c mRNA is clearly elevated by TCDD treatment (Fig. 2, *lanes 4 & 6*). In contrast, the ALDH3m mRNA basal levels were not decreased in the **CX4** and **CE1** lines, and only slight increases by TCDD were observed (Fig. 2, *lanes 3-6*). The same effects were seen in the **CA3.4** line, into which a functional human *CYP1A2* cDNA is stably transfected (Fig. 2, *last 2 lanes*). We have found that either low or high expression of the CYP1A1 or CYP1A2 functional enzyme in these *c37* stable transfectants is capable of repressing the endogenous CYP1A1 and NMO1 mRNA levels (21, 22); the same results are seen with ALDH3c, but not ALDH3m, mRNA levels in Fig. 2.

Lastly, the **CP47** cell line contains the AhRD enhancer in *trans* to the SV40 promoter and murine *Cyp1a-1* cDNA and has no functional CYP1A1 enzyme (21). The expression of ALDH3c and ALDH3m mRNA levels was indistinguishable from that seen in the *c37* line from which **CP47** had been derived (Fig. 2, *first 2 lanes*).

In conclusion, TCDD inducibility *per se* is not sufficient to classify a gene as a member of the [*Ah*] battery. What determines whether a gene is a member of the [*Ah*] battery is its intricate inter-relationship and control by a functional *Cyp1a-1* gene and CYP1A1 enzyme (or *CYP1A2* gene and

CYP1A2 enzyme) (34). In addition to ***Nmo-1*** (21), and now shown here for ***Aldh-3c***, we have also found this intricate interrelationship to be true for the ***Ugt1*06*** and ***Gst1a-1*** genes [V. Vasiliou, S.F. Reuter and D.W. Nebert, unpublished]. These results would suggest that a critical regulatory region of all four of these [***Ah***] battery Phase II genes might have a common DNA recognition sequence for similar, or the identical, ***trans***-acting control protein(s). How, or if, the Ah receptor is involved in this intriguing CYP1A1/CYP1A2 metabolism-dependent process, will require further study.

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