0090-9556/05/3305-649–656$20.00
DRUG METABOLISM AND DISPOSITION
Vol. 33, No. 5
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2964/1198096
DMD 33:649–656, 2005
Printed in U.S.A.

MOLECULAR CLONING, BACULOVIRUS EXPRESSION, AND TISSUE DISTRIBUTION OF THE ZEBRAFISH ALDEHYDE DEHYDROGENASE 2

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Received November 10, 2004; accepted February 7, 2005

ABSTRACT:

Ethanol is metabolized to acetaldehyde mainly by the alcohol dehydrogenase pathway and, to a lesser extent, through microsomal oxidation (CYP2E1) and the catalase-H_2O_2 system. Acetaldehyde, which is responsible for some of the deleterious effects of ethanol, is further oxidized to acetic acid by aldehyde dehydrogenases (ALDHs), of which mitochondrial ALDH2 is the most efficient. The aim of this study was to evaluate zebrafish (*Danio rerio*) as a model for ethanol metabolism by cloning, expressing, and characterizing the zebrafish ALDH2. The zebrafish ALDH2 cDNA was cloned and found to be 1892 bp in length and encoding a protein of 516 amino acids (M_r = 56,562), approximately 75% identical to mammalian ALDH2 proteins. Recombinant zebrafish ALDH2 protein was expressed using the baculovirus expression system and purified to homogeneity by affinity chromatography. We found that zebrafish ALDH2 is catalytically active and efficiently oxidizes acetaldehyde (K_m = 11.5 μM) and propionaldehyde (K_m = 6.1 μM). Similar kinetic properties were observed with the recombinant human ALDH2 protein, which was expressed and purified using comparable experimental conditions. Western blot analysis revealed that ALDH2 is highly expressed in the heart, skeletal muscle, and brain with moderate expression in liver, eye, and swim bladder of the zebrafish. These results are the first reported on the cloning, expression, and characterization of a zebrafish ALDH, and indicate that zebrafish is a suitable model for studying ethanol metabolism and, therefore, toxicity.

Three enzyme systems are responsible for ethanol metabolism in the human liver (Lieber, 1999). The cytosolic hepatic alcohol dehydrogenase (ADH) pathway accounts for approximately 90% of ethanol oxidation, whereas the remaining 10% is metabolized by two accessory pathways: the ethanol-inducible cytochrome P450 (CYP2E1) in the smooth endoplasmic reticulum (Lieber, 1999), and the catalase pathway, which oxidizes alcohol in the presence of hydrogen peroxide within the peroxisomes (Oshino et al., 1973). Each pathway leads to the production of acetaldehyde, a potentially toxic ethanol metabolite. Excessive and/or chronic alcohol consumption may lead to liver damage due, in part, to the accumulation of acetaldehyde (Lieber, 1999), which can covalently bind to cellular nucleophiles, including proteins and nucleic acids, and form adducts that may interfere with the biological function of these macromolecules (Esterbauer et al., 1991). In addition, acetaldehyde promotes the peroxidation of cellular membrane lipids (Esterbauer et al., 1991), leading to the production of more reactive aldehyde species. Lipid peroxidation is associated with antigen generation and subsequent antibody production, increased collagen synthesis, glutathione depletion, and the development of alcohol liver disease (Lieber, 1999). Therefore, acetaldehyde metabolism is of primary importance for the survival of cells exposed to ethanol.

The major pathway of acetaldehyde detoxification is the oxidation by aldehyde dehydrogenases (ALDHs) (Vasiliou and Pappa, 2000). Human mitochondrial ALDH2 exhibits the highest affinity for acetaldehyde (K_m <5 μM) and constitutes the determinant enzyme in acetaldehyde clearance (Yoshida, 1992). The significance of ALDH2 in the detoxification of acetaldehyde becomes apparent in humans with a deficiency in ALDH2 enzymatic activity (Yoshida et al., 1984). This deficiency is due to a point mutation (G/C→A/T) in exon 12 of the ALDH2 gene, resulting in a Glu487Lys substitution (ALDH2*2 allele) (Yoshida et al., 1984). This mutation is dominant with regard to enzymatic activity, and heterozygous individuals have no or negligible activity in their livers (Crabb et al., 1989). Human ALDH2 deficiency results in blood acetaldehyde accumulation, causing an alcohol-induced flush reaction (Yoshida, 1992; Goedde et al., 1992). The latter is characterized by cutaneous vasodilation, tachycardia,

This work was supported in part by National Institutes of Health Grants AA11885 and EY14390 (V.V.), AA12783 (R.L.T.), A T32 AA 07464 (A.P.), and a FPE Pre-doctoral Fellowship (T.E.).

N.L. and T.E. equally contributed to this work.

The GenBank accession numbers for the zebrafish and human ALDH2 cDNA are AAM19352 and AY621070, respectively.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.

doi:10.1124/dmd.104.002964.

ABBREVIATIONS: ADH, alcohol dehydrogenase; ALDH2, aldehyde dehydrogenase 2; FAS, fetal alcohol syndrome; 2-ME, 2-mercaptoethanol; PVDF, polyvinylidene difluoride; Sf9, *Spodoptera frugiperda*; RACE, rapid amplification of cDNA ends; BCA, bicinchoninic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

nausea, and even cardiovascular collapse (Goedde et al., 1992), thus making drinking an unpleasant experience. These subjects, most commonly Asian, are generally protected against alcohol abuse (Yoshida, 1992).

Rodent models are used to study alcohol liver disease due to similarities between human and rodent ALDH liver isozymes (Klyosov et al., 1996). Zebrafish, however, have proven to be an excellent vertebrate model for the study of embryogenesis, gene functions, human disease, and toxicity. Although they are fish, zebrafish share many cellular and physiological characteristics with higher vertebrates, are economically maintained in large numbers, and have relatively short generation times (Westerfield, 1995). These properties make them well suited for genome saturation mutagenesis studies. Forward genetic screens have been successful for the identification of hundreds of previously unknown genes required for normal vertebrate development (Haffter et al., 1996). With the recent completion of the zebrafish genome sequencing project (Vogel, 2000), numerous reverse genetic approaches are now routine in zebrafish in which gene functions can be rapidly evaluated (Sumanas and Larson, 2002; North and Zon, 2003). Zebrafish was also the first animal model used to study human congenital sideroblastic anemia, which results from a mutation in the aminolevulinic acid synthase 2 gene (Brownlie et al., 1998). Mutations in the zebrafish aminolevulinic acid synthase 2 gene cause a phenotype similar to that observed in the human disease. Recently, the zebrafish has also been investigated as a model for studying alcoholism and fetal alcohol syndrome (FAS), a birth defect characterized by craniofacial malformations as well as physical and mental retardation (Gerlai et al., 2000; Reimers et al., 2004a). It has been documented that zebrafish embryos exposed to alcohol during development produced FAS-like abnormalities of the face, eye, ear, and heart that are comparable to those observed in mammals (Blader and Strahle, 1998; Reimers et al., 2004a). It has recently been shown that the zebrafish expresses functional ADH enzymes capable of oxidizing ethanol to acetaldehyde (Reimers et al., 2004b). The metabolism of acetaldehyde, however, by ALDH in zebrafish has not yet been investigated. In this paper, we cloned, expressed, and characterized the zebrafish ALDH2 to validate the use of zebrafish as a model for ethanol metabolism and toxicity. Our results demonstrate significant similarities between zebrafish and human ALDH2 protein sequences and metabolic properties, which confirms the ability of the zebrafish to detoxify acetaldehyde. In addition to expression in the liver, ALDH2 was also found at high levels in zebrafish heart, muscle, and brain. Overall, this work supports the use of the zebrafish for ethanol-related studies.

Materials and Methods

Materials. The 5′-3′ RACE Kit was purchased from BD Biosciences Clontech (Palo Alto, CA), the pGEM-T EASY was obtained from Promega (Madison, WI), and the baculovirus expression vector, pBlueBac 4.5, was purchased from Invitrogen (Carlsbad, CA). Restriction endonucleases were obtained from either Invitrogen or New England Biolabs (Beverly, MA), and 5′-AMP-Sepharose 4B was obtained from Amersham Biosciences Inc. (Piscataway, NJ). The bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical (Rockford, IL) and used according to the instructions provided by the manufacturer. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Corporation (Billerica, MA), horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody was obtained from Calbiochem (San Diego, CA), and reagents for chemiluminescence were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). Unless otherwise specified, all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Zebrafish ALDH2 cDNA Identification, Cloning, and Sequence Analysis. A 552-base pair expressed sequence tag sequence was identified in GenBank (accession number AI722643) that was similar to the mammalian ALDH2. Complete sequencing of the expressed sequence tag (fc29g10) revealed that it did not contain the complete open reading frame and lacked the predicted translation initiation codon, AUG. Therefore, a PCR-based approach was used to obtain the 5′ end of the zebrafish ALDH2 using the 5′-3′ RACE Kit according to the manufacturer's protocol. Poly(A^+) RNA was isolated from whole adult zebrafish and reverse-transcribed using avian myeloblastosis virus reverse transcriptase, and the Marathon cDNA synthesis primer was followed by second strand synthesis. After universal adaptor primer ligation to the cDNA ends, the adapted cDNA was used as a template for 5′ RACE, using ALDH2-specific reverse primer 2 (5′-GGTTGAAGAACAGGGCA-GAATGG-3′) with the supplied adapter-specific primer, under the following conditions: 30 s at 94°C and 4 min at 72°C. The original PCR reactions (5 μl) were diluted 1:200 and subsequently reamplified under the same conditions as above, using nested ALDH2-specific reverse primer 3 (5′-CTGCTCCACT-GCTTCTTCCATG-3′) and adaptor primers. Amplified products were resolved on 1.9% agarose gels and visualized by ethidium bromide staining and UV illumination. They were then subcloned into pGEM-T EASY. A single 800-base pair product was amplified, sequenced, and found to contain the 5′ end of the ALDH2 open reading frame. The entire open reading frame was created by cutting the PCR-amplified 5′ RACE clone with XhoI, a site within the ALDH2 sequence, and the vector-specific EcoRI. The 600-base pair fragment was ligated into the incomplete zebrafish pSport1 ALDH2 fragment, previously excised with XhoI and EcoRI. Both strands of the resulting construct were sequenced before GenBank submission (as accession number AF260121).

Gene Mapping. Genetic mapping was completed using the LN54 RH panel, a hybrid between zebrafish and mouse cells, which was obtained from Dr. Marc Ekker (Laboratory of Health Research Institute, Ottawa, ON, Canada). This panel of 94 DNAs was used to map the chromosomal location of zebrafish ALDH2 (according to previously described methods) (Hukriede et al., 1999). PCRs contained 100 ng of hybrid-cell DNA from each of the parental cell lines, a 0.25 μM concentration of each oligonucleotide primer, 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, and 1 U of TaqDNA polymerase in a total volume of 20 μl. PCR was performed for 32 cycles according to the following conditions: 60 s at 94°C, 90 s at 62°C, and 90 s at 72°C, using the ALDH2 forward 1 (5′-CTGATGTGGATAAAGCGGTG-3′) and ALDH2 reverse 1 (5′-CTA-AATAGGCAGCATCTCTC-3′) primers. PCR products were separated on 1.9% agarose gels and visualized by ethidium bromide staining and UV illumination. All PCR assays were performed in duplicate before linkage group assignment, using Dr. Igor Dawid's online mapping program (National Institute of Child Health and Human Development, National Institutes of Health, http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi).

Generation of ALDH2 Specific Antibodies. To generate specific antibodies for ALDH2, which would detect the zebrafish and human ALDH2 proteins, the predicted amino acid sequences were aligned and compared. The terminal 16 residues were 94% identical, with only a single amino acid difference at position 509, a Val to Ile substitution. Mammalian ALDH2 antisera had previously been raised against this region (Weiner et al., 1987). A synthetic peptide was produced (YTEVKTVTVKVPQKNS), which was designed to exactly match the human sequence. Following keyhole limpet hemocyanin conjugation, a standard two-rabbit antibody production was initiated (Alpha Diagnostics International, San Antonio, TX).

Construction of Recombinant Baculoviruses and Expression of Zebrafish and Human ALDH2. A human ALDH2 cDNA cloned in pT7T3 18U vector (clone number C45672) was purchased from Stratagene (La Jolla, CA), expanded, and sequenced before GenBank submission (GenBank accession number AY621070). The full-length human ALDH2 cDNA was removed from the pT7T3 18U plasmid by digestion with SstI and HindIII at the 5′ and 3′ ends, respectively, and subsequently ligated into the analogous sites of the pBlueBac 4.5 baculovirus vector. To generate recombinant zebrafish ALDH2, the open reading frame of zebrafish ALDH2 was subcloned into the corresponding KpnI and HindIII sites in the pBlueBac 4.5 vector. The expression constructs were verified by DNA sequencing prior to generation and coinfection of recombinant baculoviruses into *Spodoptera frugiperda* (Sf9) insect cells as described previously (Manzer et al., 2003; Pappa et al., 2003). Ten isolated recombinant clones were further amplified and examined for the

expression of each recombinant protein by Western blot analysis. For Western blot analysis, 30 μg of protein derived from crude cell lysates of Sf9 cells were separated by SDS-PAGE (12% gels) and transferred to PVDF membranes, which were subsequently incubated overnight in blocking buffer (50 mM Tris/150 mM sodium chloride, pH 7.5, containing 0.1% Tween 20 and 5% nonfat milk). Then, they were probed with the primary antibody generated against human ALDH2 (1:1000 dilution in blocking buffer) and a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000 dilution in blocking buffer). Labeled proteins were detected by enhanced chemiluminescence. Finally, clones with the highest ALDH2 expression were selected for consecutive infection of Sf9 insect cells at a multiplicity of infection of 1. Cells were harvested 48 h postinfection and cell lysates were used for ALDH2 purification.

Purification of Recombinant Zebrafish and Human ALDH2. Zebrafish and human ALDH2 were purified by a well established affinity chromatography method with slight variations (Pappa et al., 2003). Briefly, a 500-ml culture for each clone (containing approximately 600 $\times$ 10^6 cells) was harvested at 48 h postinfection. Cells were pelleted and resuspended in 12 ml of homogenization buffer, containing 100 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM 2-ME, and 0.02% Triton X-100 and the following protease inhibitors: 0.5 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 μg/ml aprotonin, and 100 μg/ml phenylmethanesulfonyl fluoride. Cells were lysed by sonication on ice for 30 s followed by a 30-s period of rest to prevent overheating. This was repeated for a total of six sonication cycles. The crude lysate was then centrifuged at 100,000g for 70 min at 4°C to separate the soluble fraction. The supernatant was collected and applied to a 1.6 $\times$ 6 cm 5′-AMP-Sepharose 4B affinity column equilibrated with binding buffer (100 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM 2-ME, and 0.01% Triton X-100) at 4°C. The human or zebrafish ALDH2 proteins were eluted from the column by applying a gradient of 0.25 mM NAD^+ dissolved in the binding buffer. The concentration range of the elution gradient was 0 to 0.25 mM NAD^+, with a linear increase of 0.005 mM NAD^+ per minute. Elution fractions (5 ml) were collected and fractions containing either human or zebrafish ALDH2 were determined by SDS-PAGE. The fractions containing ALDH2 were pooled and concentrated utilizing an Amicon device equipped with a 30-kDa membrane (Millipore) under N_2 gas. The purified proteins were dialyzed against a large volume of 100 mM potassium phosphate (pH 7.4) over 24 h at 4°C, using 10-kDa Slide-A-Lyzer dialysis cassettes (Pierce Chemical). The protein concentration was estimated using the BCA assay. The absorbance at 280 nm (ϵ = 62,070 M^{-1} cm^{-1} and 58,162 M^{-1} cm^{-1} for human and zebrafish, respectively) was also used to confirm the protein concentration in the final purified preparations. To verify the identity of the proteins, 30 μg of total protein from Sf9 cells or 0.05 μg and 0.2 μg of purified recombinant human and zebrafish ALDH2 proteins, respectively, were processed for Western blot analysis, as described above.

Determination of Kinetic Constants of Zebrafish and Human ALDH2. The enzymatic activity of zebrafish and human ALDH2 proteins was determined by monitoring the formation of NADH (340 nm) during the oxidation of aldehyde substrates at 25°C. Enzyme reaction mixtures contained 0.1 M sodium pyrophosphate, pH 8.0, 1 mM NAD^+, 50 mM 2-ME, 1 mM pyrazole (to inhibit alcohol dehydrogenase), and 10 μg of purified protein, and were incubated for 30 min under aluminum foil prior to enzyme assays. Aldehyde substrate stocks were prepared in aqueous solution and added to the reaction mixture to initiate oxidation. Apparent kinetic constants (V_{max} and K_m) were determined using a minimum of 8 to 10 substrate concentrations, three to five of which were below the apparent K_m and three to five above the apparent K_m. The resulting rates were then normalized for protein concentration and plotted by means of the Michaelis-Menten model using Sigma Plot Enzyme Kinetics software (version 7.0, 2001; SPSS Inc., Chicago, IL). The enzymatic activity was monitored in fresh protein to preserve its catalytic function. Enzyme activities are expressed as nmol of NADH/min/mg protein.

Zebrafish ALDH2 Tissue Distribution. Adult male zebrafish were euthanized by waterborne exposure to tricaine (ethyl 3-aminobenzoate, methanesulfonic acid; MS222) prior to individual tissue isolation. Individual organs were isolated and homogenized in 300 μl of extraction buffer containing 25 mM MOPS, pH 7.5, 1 mM EDTA, 5 mM EGTA, 0.02% NaN_3, 20 mM Na_2MoO_4, 10% (v/v) glycerol, 1 mM dithiothreitol, leupeptin (5 μg/ml), aprotonin (1 μg/ml), and pepstatin (5 μg/ml). The homogenate was sonicated in a 1.5-ml centrifuge tube three times on ice and homogenized using a Dounce homogenizer. Cellular debris was pelleted by centrifugation at 22,000g for 30 min. Protein concentrations of the supernatant were determined by the BCA method, using bovine serum albumin as a standard. To evaluate the zebrafish ALDH2 protein expression in the supernatant, 20 μg of each organ lysate was resolved on a 12% SDS-PAGE gel and transferred to a PVDF membrane, and immunochemical detection of ALDH2 was carried out by Western blotting as described above.

Results and Discussion

Zebrafish ALDH2 cDNA and Deduced Amino Acid Sequence. The isolated zebrafish ALDH2 cDNA was found to be 1892 bp in length, with 5′ and 3′ noncoding regions of 5 bp and 335 bp, respectively (Fig. 1; GenBank accession number AAM19352). The only putative polyadenylation signal (aataaa) was found 10 nucleotides upstream from the poly(A^+) tract. The open reading frame of the zebrafish ALDH2 cDNA encodes a protein of 516 amino acids (M_r = 56,562) that clusters with the mammalian ALDH2 proteins (a_dre-ALDH2 in Fig. 2). The zebrafish ALDH2 protein was determined to be 73 to 76% identical to horse, hamster, human, bovine, rat, and mouse ALDH2 proteins, 64 to 69% identical to human ALDH1 proteins, and 54 to 60% identical to plant ALDH2 proteins (Fig. 2). The clustering of the zebrafish ALDH2 with all the mammalian ALDH2 proteins is the first indication that the ALDH2 cDNA reported here is an ortholog of the mammalian ALDH2. It also indicates that ALDH2 proteins are highly conserved throughout evolution. The zebrafish ALDH2-deduced amino acid sequence contains critical residues that are conserved in mammalian ALDH2 proteins, further supporting the notion that the zebrafish ALDH2 is the ortholog of the mammalian ALDH2. Zebrafish ALDH2 also contains the mitochondrial targeting sequence, which is later cleaved to generate the mature protein (Fig. 3). This peptide leader typically contains 17 to 35 amino acids rich in positive charges and located at the N terminus of the precursor protein (Roise et al., 1988). The mitochondrial peptide leader sequence of the predicted zebrafish ALDH2 protein (16 amino acids) is rich in arginine, alanine, and serine residues, similar to the mitochondrial targeting sequences of human (17 amino acids) (Guan and Weiner, 1990), rat (19 amino acids) (Hammen et al., 1996), and mouse (19 amino acids) (Chang and Yoshida, 1994) (Fig. 3). The mitochondrial targeting sequences found within plant ALDH2 proteins are typically longer (13–85 amino acids) than those found within mammalian ALDH2 genes (Glaser et al., 1998).

The zebrafish ALDH2 protein sequence contains the catalytically essential amino acids Cys-302 and Glu-268 (numbering is based on the mature human ALDH2 protein) (Abriola et al., 1987), as well as Lys-192, Gly-245, Gly-250, Glu-399, and Phe-401, which are essential to the Rossmann fold (GxGxxG) required for coenzyme binding (Ni et al., 1997). Gly-299, a residue strictly conserved in the alignment of 145 ALDHs (Perozich et al., 1999), is also present in zebrafish ALDH2 (Fig. 3). The polymorphism in the human ALDH2 gene, leading to the ALDH2*2 allele that encodes an inactive form of ALDH2, is due to a single nucleotide mutation at position 487. This results in Lys substitution for Glu, causing substantial loss in the catalytic function of ALDH2 (Steinmetz et al., 1997). It has been suggested that the gain of a positive charge from the substituted Lys residue is responsible for the decreased affinity for NAD^+ (Steinmetz et al., 1997).

The chromosomal location of the zebrafish ALDH2 gene was mapped to chromosome 5, using the LN54 radiation hybrid panel, a PCR-based approach (data not shown). The human ALDH2 gene resides on chromosome 12 (Hsu et al., 1986), a chromosome of low

30 60 90
agaaa**atg**ctgcgcgccgtttttcccgaactttccctcaagtttttcgcatctcctcatgtcaacattcaacgattcctgcaccgaacgtt
M L R A V F S R T F P Q V F R I S S C Q H S T I P A P N V
120 150 180
cagcctgatgtgcattataacaagattttcattaataatgagtggcatgatgctgtaagcaagaagacttttccaaccatcaatcctgct
Q P D V H Y N K I F I N N E W H D A V S K K T F P T I N P A
210 240 270
actgctgaagtgatctgccacgtcgcggaggggataaggctgatgtggataaagcggtgaaggctgccagagatgcctttaagctgggc
T A E V I C H V A E G D K A D V D K A V K A A R D A F K L G
300 330 360
tctccctggcgccgcatggatgcgtctcagcgtgggctgctgctgaacagattagcagactgcattgagagagatgctgcctatttagct
S P W R R M D A S Q R G L L L N R L A D C I E R D A A Y L A
390 420 450
gagctagaaactcttgataatggaaagccatataccctctccttctgtgtggacttgcccatggtggtcaaatgcttgaggtattatgct
E L E T L D N G K P Y T L S F C V D L P M V V K C L R Y Y A
480 510 540
ggctgggcagacaaatgggagggtaaaaccatcccgattgatggcaattacttctgctacacaagacatgagcccattggagtctgtggg
G W A D K W E G K T I P I D G N Y F C Y T R H E P I G V C G
570 600 630
caaattattccttggaatttccctctattgatgcaggcgttaaagctgggtccggctctggccactggaaacacagtggtgatgaaggtg
Q I I P W N F P L L M Q A L K L G P A L A T G N T V V M K V
660 690 720
gcggagcagacgccactcactgcattgtatatcgccagtttgatcaaagaggttggttttcctgctggtgttgtcaacattgtcccaggg
A E Q T P L T A L Y I A S L I K E V G F P A G V V N I V P G
750 780 810
tttggtcccacagcaggagcagccattgcttctcatatggatgtggacaaagtagctttcaccggctccactgatgtcggtcaccttatc
F G P T A G A A I A S H M D V D K V A F T G S T D V G H L I
840 870 900
cagcaagcctcgagtgccagtaatctgaaaaatgtctcactggagctcggaggaaagagtccgaacatcatcctgtcagatgctaacatg
Q Q A S S A S N L K N V S L E L G G K S P N I I L S D A N M
930 960 990
gaagaagcagtggagcaggcccattctgccctgttcttcaaccaaggccagtgctgttgcgcaggcactcgcacttttgtacaggagagc
E E A V E Q A H S A L F F N Q G Q C C C A G T R T F V Q E S
1020 1050 1080
atctacgatgagtttgtggagcgaagtgtggagagagccaagaataggattgtgggagatccctttgacttaaacacggagcagggcccg
I Y D E F V E R S V E R A K N R I V G D P F D L N T E Q G P
1110 1140 1170
caggtggatgaggatcagttcaagaaggttctgggctacatcagcagcgggaagcgtgaaggagccaagctgatgtgtggaggagctcca
Q V D E D Q F K K V L G Y I S S G K R E G A K L M C G G A P
1200 1230 1260
gccgcagagcgcggatacttcatccagcccacagtctttggagacgtaaaggacgacatgaaaatcgctcgcgaggagatcttcgggcct
A A E R G Y F I Q P T V F G D V K D D M K I A R E E I F G P
1290 1320 1350
gttatgcaaattctgaagttcaaatctttggaggaagtgattgagagagccaacgacagcaaatatggcctcgcggctgctgtttttacc
V M Q I L K F K S L E E V I E R A N D S K Y G L A A A V F T
1380 1410 1440
cagaacattgacaaggccaattacatttcacatggcctgcgtgctgggactgtgtggattaactgctataacgtgttcggagttcaggct
Q N I D K A N Y I S H G L R A G T V W I N C Y N V F G V Q A
1470 1500 1530
ccgtttggaggctacaaagcgtcaggaattggtcgtgagttgggcgagtatggactggacatctacacg**gag**gtcaaaacggttacaatt
P F G G Y K A S G I G R E L G E Y G L D I Y T E V K T V T I
1560 1590 1620
aaggttcctcagaaaaattca**taa**atggagcaattaaagaagatgctgtttagttacgaattgcaacgttaagaattgaaaatattgtgg
K V P Q K N S *
taatgtgtagtaaaaaataggttttgtactgtaggtaaactccttaaaagtaaaggtctgccagagcttacttataaaattgatttgatt
ctgtttaattcagacttcatgtgaacagcataattgtttgaacgtatgagctgatttatgtattacatgtggttttgtttcattttttatt
tcatttatttttaatgcatgtcaatgcatgaccttttctgatgatcatttgtaataatatgcattagaaaataa**aataaa**atccaatatt
(a).

FIG. 1. The complete nucleotide and deduced amino acid sequence of the zebrafish cDNA encoding the 56-kDa mitochondrial ALDH2 protein. Bolded and underlined letters include the initiation (atg) and termination (taa) codons, the putative polyadenylation signal (aataaa), and the Glu487 codon (gag).

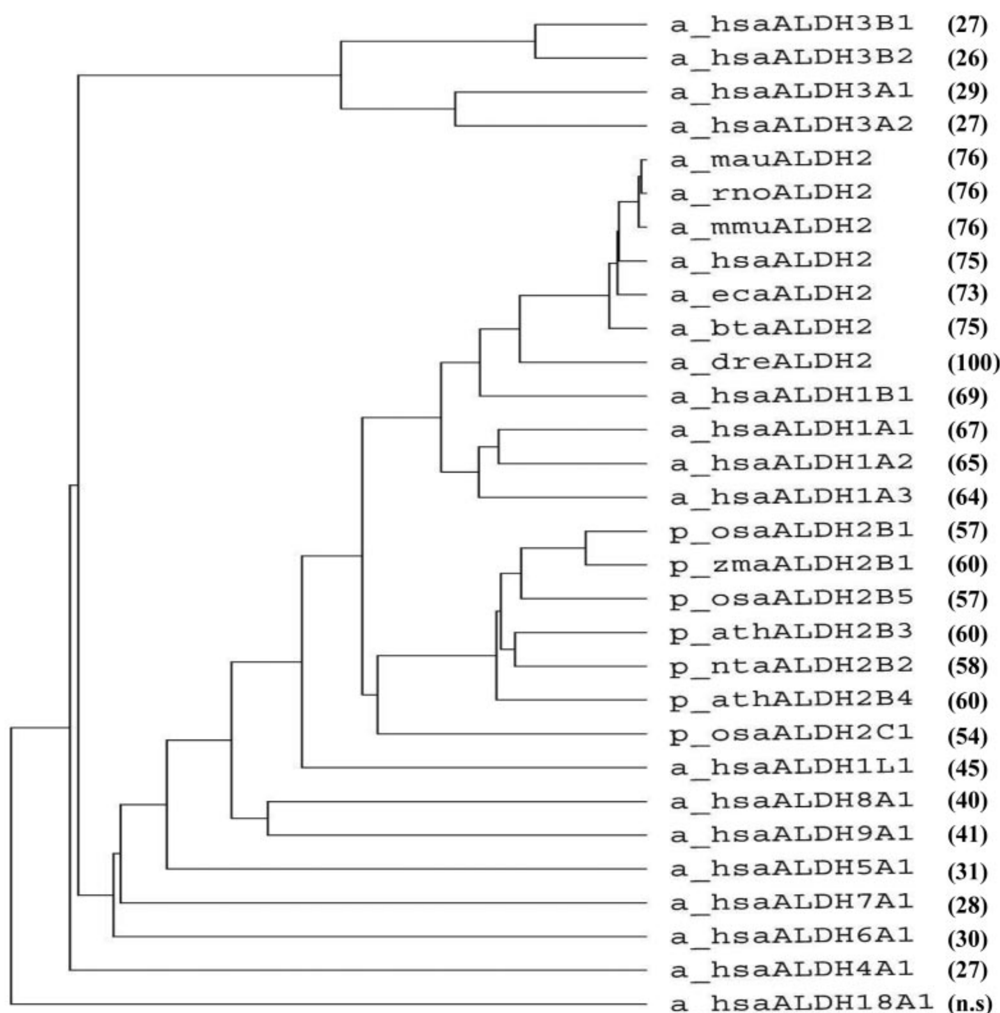

FIG. 2. Clustering algorithm, showing the evolutionary relationships between proteins encoded by the 17 human ALDH genes, 6 animal ALDH2 genes, and 7 plant ALDH2B and ALDH2C genes. The ALDH sequences have been abbreviated according to the following format: **a_bcdGENENAME**, where "**a**" stands for animal, "**b**" is the first character of the genus, and "**cd**" are the first two characters of the species, followed by the gene name. The same format was applied to the plants, where "**p**" denotes plant. Individual species abbreviations include: ath, *Arabidopsis thaliana* (thale cress); bta, *Bos taurus* (cow); dre, *Danio rerio* (zebrafish); eca, *Equus caballus* (horse); hsa, *Homo sapiens* (human); mau, *Mesocricetus auratus* (golden hamster); mmu, *Mus musculus* (house mouse); nta, *Nicotiana tabacum* (common tobacco); osa, *Oryza sativa* (rice); rno, *Rattus norvegicus* (Norway rat); and zma, *Zea mays* (maize). The numbers in parentheses on the right denote the percentage identity relative to the zebrafish ALDH2 protein. GenBank and SwissProt accession numbers of these sequences are available on the Internet (URL: http://www.aldh.org).

syntenic relationship to the zebrafish chromosome 5. Because most of the zebrafish genome contains duplicated segments, they often have two copies of a gene that is present as a single copy in mammals (Woods et al., 2000). It is important to note the possibility that the zebrafish genome may contain another ALDH2 gene.

Expression and Purification of Zebrafish and Human ALDH2. To generate substantial amounts ALDH2 for biochemical analysis, both zebrafish and human proteins were expressed in Sf9 insect cells by baculovirus infection. Successful expression of the two enzymes was determined by detecting ALDH2 protein using Western blot analysis of crude extracts of the Sf9 cells infected with human or zebrafish ALDH2 cDNAs. A single band with an apparent molecular weight of 56 kDa, which reacted with the human ALDH2 antibody, was observed in crude lysates from cells infected with zebrafish or human ALDH2. No reactive bands were detected in the extracts derived from Sf9 cells infected with the pBlueBac virus alone (data not shown). The Sf9 cell culture with the highest expression of zebrafish and human ALDH2 was selected for purification purposes. ALDH2 proteins were purified by the affinity chromatography method using 5′-AMP-Sepharose 4B. Analysis of the purified proteins by SDS-PAGE (Fig. 4) revealed a single protein band at approximately 56 kDa and at least 95% in purity after Coomassie Blue staining. When transferred, these bands reacted with the ALDH2 antibody during Western blotting, verifying the identity of the proteins as ALDH2 (Fig. 4). From a 500-ml culture of Sf9 cells, we were able to purify 2 and 4 mg of active zebrafish and human ALDH2 protein, respectively, representing a yield of 1 to 2% for each protein. Purification of human and zebrafish ALDH2 produced an approximate 37- and 51-fold increase in ALDH2 specific activity, respectively, as determined with acetaldehyde (data not shown).

Biochemical Characterization of Recombinant Zebrafish and Human ALDH2. During our study we observed that the purified zebrafish and human ALDH2 enzymes were prone to inactivation. Since it is common for proteins to be inactivated by oxidation of their sulfhydryl residues, we sought to measure enzymatic activity in the presence of a reducing agent, such as 2-ME. As anticipated, ALDH2 enzymatic activity was recovered with 2-ME, and this reversibility of inactivation indicated the oxidation of sulfhydryl groups present in both zebrafish and human ALDH2 proteins. To determine the optimal concentration of 2-ME, a titration curve was performed with increas-

```
Zebrafish  .......... .......... .......... ...MLRAVFS RTFPQVFRIS SCQHSTIPAP NVQPDVHYNK IFINNEWHDA   47
Human      .......... .......... .......... MLRAAARFGP RLG..RRLLS AAATQAVPAP NQQPEVFCNQ IFINNEWHDA   48
Mouse      .......... .......... .......... MLRAALTTVR RGPRLSRLLS AAATSAVPAP NHQPEVFCNQ IFINNEWHDA   50
Maize      MARRAASSLV SRCLLARAPA GAPPAAPSAP RRTVPADGMH RLLPGVLQRF STAAAVEEPI TPSVHVNYTK LLINGNFVDS   80

Zebrafish  VSKKTFPTIN PATAEVICHV AEGDKADVDK AVKAARDAFK LGSPWRRMDA SQRGLLLNRL ADCIERDAAY LAELETLDNG  127
Human      VSRKTFPTVN PSTGEVICQV AEGDKEDVDK AVKAARAAFQ LGSPWRRMDA SHRGRLLNRL ADLIERDRTY LAALETLDNG  128
Mouse      VSRKTFPTVN PSTGEVICQV AEGNKEDVDK AVKAARAAFQ LGSPWRRMDA SDRGRLLYRL ADLIERDRTY LAALETLDNG  130
Maize      ASGKTFPTLD PRTGEVIAHV AEGDAEDINR AVAAARKAFD EG.PWPKMTA YERSRILLRF ADLIEKHNDE LAALETWDNG  159

Zebrafish  KPYTLSFCVD LPMVVKCLRY YAGWADKWEG KTIPIDGNYF CYTRHEPIGV CGQIIPWNFP LLMQALKLGP ALATGNTVVM  207
Human      KPYVISYLVD LDMVLKCLRY YAGWADKYHG KTIPIDGDFF SYTRHEPVGV CGQIIPWNFP LLMQAWKLGP ALATGNVVVM  208
Mouse      KPYVISYLVD LDMVLKCLRY YAGWADKYHG KTIPIDGDFF SYTRHEPVGV CGQIIPWNFP LLMQAWKLGP ALATGNVVVM  210
Maize      KPYEQAAQIE VPMVARLMRY YAGWADKIHG LIVPADGPHH VQILHEPIGV AGQIIPWNFP LLMYAWKVGP ALACGNTLVL  239

           192                                                 245  250                268
Zebrafish  KVAEQTPLTA LYIASLIKEV GFPAGVVNIV PGFGPTAGAA IASHMDVDKV AFTGSTDVGH LIQQASSASN LKNVSLELGG  287
Human      KVAEQTPLTA LYVANLIKEA GFPPGVVNIV PGFGPTAGAA IASHEDVDKV AFTGSTEIGR VIQVAAGSSN LKRVTLELGG  288
Mouse      KVAEQTPLTA LYVANLIKEA GFPPGVVNIV PGFGPTAGAA IASHEGVDKV AFTGSTEVGH LIQVAAGSSN LKRVTLELGG  290
Maize      KTAEQTPLSA LYISKLLHEA GLPEGVVNVV SGFGPTAGAA LASHMDVDKI AFTGSTDTGK IILELAAKSN LKTVTLELGG  319

                                  299 302
Zebrafish  KSPNIILSDA NMEEAVEQAH SALFFNQGQC CCAGTRTFVQ ESIYDEFVER SVERAKNRIV GDPFDLNTEQ GPQVDEDQFK  367
Human      KSPNIIMSDA DMDWAVEQAH FALFFNQGQC CCAGSRTFVQ EDIYDEFVER SVARAKSRVV GNPFDSKTEQ GPQVDETQFK  368
Mouse      KSPNIIMSDA DMDWAVEQAH FALFFNQGQC CCAGSRTFVQ ENVYDEFVER SVARAKSRVV GNPFDSRTEQ GPQVDETQFK  370
Maize      KSPFIIMDDA DVDHAVELAH FALFFNQGQC CCAGSRTFVH ERVYDEFVEK AKARALKRVV GDPFRKGVEQ GPQIDDEQFN  399

                                                         399 401
Zebrafish  KVLGYISSGK REGAKLMCGG APAAERGYFI QPTVFGDVKD DMKIAREEIF GPVMQILKFK SLEEVIERAN DSKYGLAAAV  447
Human      KILGYINTGK QEGAKLLCGG GIAADRGYFI QPTVFGDVQD GMTIAKEEIF GPVMQILKFK TIEEVVGRAN NSTYGLAAAV  448
Mouse      KILGYIKSGQ QEGAKLLCGG GAAADRGYFI QPTVFGDVKD GMTIAKEEIF GPVMQILKFK TIEEVVGRAN DSKYGLAAAV  450
Maize      KILRYIRYGV DGGATLVTGG DRLGDKGFYI QPTIFSDVQD GMKIAQEEIF GPVQSILKFK DLNEVIKRAN ASQYGLAAGV  479

                                                                487
Zebrafish  FTQNIDKANY ISHGLRAGTV WINCYNVFGV QAPFGGYKAS GIGRELGEYG LDIYTEVKTV TIKVPQKNS.  516
Human      FTKDLDKANY LSQALQAGTV WVNCYDVFGA QSPFGGYKMS GSGRELGEYG LQAYTEVKTV TVKVPQKNS.  517
Mouse      FTKDLDKANY LSQALQAGTV WINCYDVFGA QSPFGGYKMS GSGRELGEYG LQAYTEVKTV TVKVPQKNS.  519
Maize      FTNSLDTANT LTRALRAGTV WVNCFDVFDA AIPFGGYKMS GIGREKGVDS LKNYLQVKAV VTPIKNAAWL  549
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FIG. 3. Alignment of predicted amino acid sequences of mitochondrial ALDH2 proteins from zebrafish, human, mouse, and maize. The open boxes enclose the mitochondrial leader sequence. Gray boxes indicate conserved amino acids (numbering is based on that of the mature human ALDH2) in the four species, which are discussed in the text. Underline indicates the peptide used to generate ALDH2-specific antibodies.

FIG. 4. Expression and purification of recombinant zebrafish and human ALDH2 proteins. Purified human and zebrafish ALDH2 proteins were subjected to SDS-PAGE on a 12% acrylamide gel. ALDH2 was detected by either Coomassie Blue staining (CB; 2 μg loading of each protein) or Western blotting (WB; 0.05 μg and 0.2 μg loading for human and zebrafish proteins, respectively). Protein molecular mass (kDa) markers are given on the left-hand side.

ing concentrations of 2-ME. Optimal activity recovery was obtained with 50 mM 2-ME (data not shown) and, therefore, this concentration was used for the rest of the study.

The kinetic constants for the recombinant zebrafish and human ALDH2 proteins for acetaldehyde and propionaldehyde were studied and found to be comparable. Both enzymes followed traditional Michaelis-Menten saturation kinetics for acetaldehyde (Fig. 5A) and propionaldehyde (Fig. 5B). The K_m for acetaldehyde was 3.2 and 11.5 μM for human and zebrafish ALDH2, respectively (Table 1). Propionaldehyde was also oxidized with a high apparent affinity by both human ($K_m = 2.4\ \mu$M) and zebrafish ($K_m = 6.1\ \mu$M) proteins (Table 1). Furthermore, the maximum velocity (V_{max}) values for acetaldehyde and propionaldehyde were similar for the human and zebrafish ALDH2 proteins (Table 1). Interestingly, the efficiency constants (V_{max}/K_m) appeared to be higher for human ALDH2 than for zebrafish ALDH2 (Table 1). Although this may indicate that human ALDH2 metabolizes acetaldehyde more efficiently than zebrafish ALDH2, the latter is still capable of metabolizing acetaldehyde with a substantially high affinity ($K_m = 11.5\ \mu$M). The *Zea mays* (maize) and *Oryza*

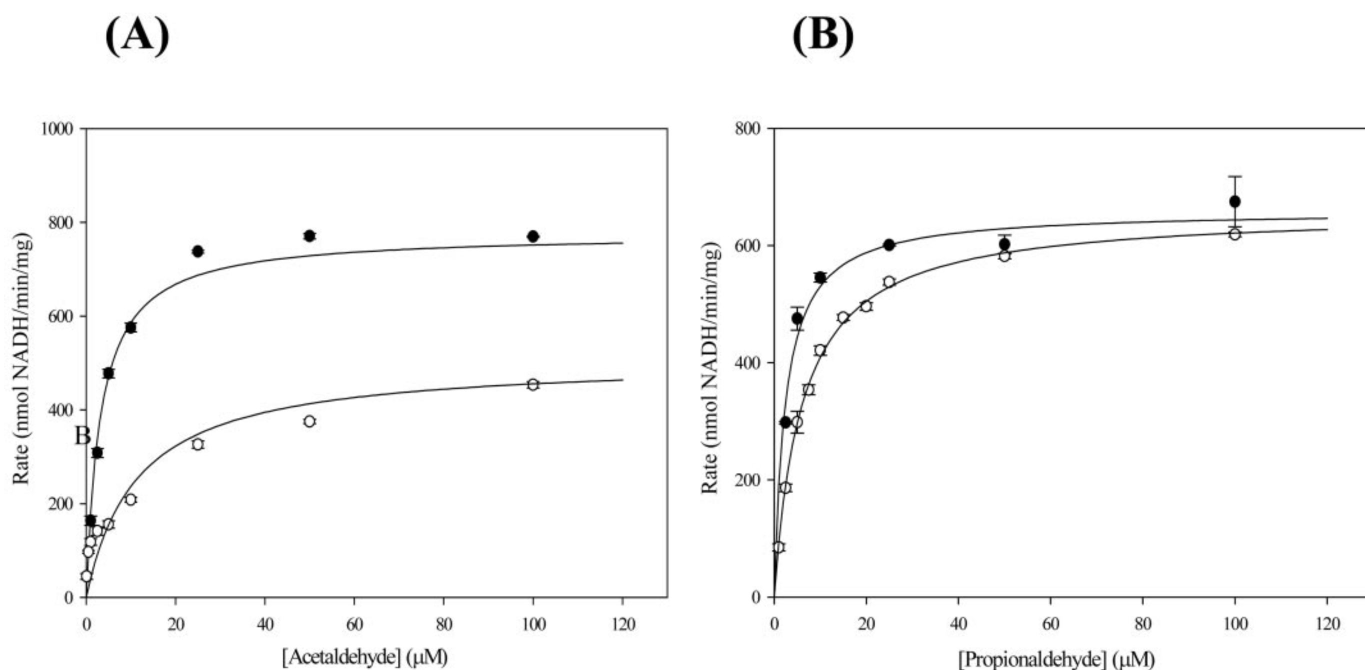

FIG. 5. Michaelis-Menten kinetic curves for zebrafish and human ALDH2 with acetaldehyde and propionaldehyde. Ten micrograms of purified human (closed circles) and zebrafish (open circles) ALDH2 proteins were incubated with increasing concentrations of acetaldehyde (A) or propionaldehyde (B) and 0.1 M sodium pyrophosphate buffer (pH 8.0), 1 mM NAD^+, 1 mM pyrazole, 50 mM 2-ME at 25°C. Values represent the mean ± standard error ($n = 3$) of at least three different experiments. Data were fit by Sigma Plot Enzyme Kinetics software (2001).

TABLE 1

Kinetic properties of recombinant zebrafish and human ALDH2

Apparent K_m and V_{max} values were determined by fitting the data to the Michaelis-Menten equation using Sigma Plot. Data represent mean ± S.E. from three separate experiments.

Substrate	V_{max}	K_m	V_{max}/K_m [a]
	nmol NADH/min/mg	*μM*	
Human ALDH2			
Acetaldehyde	775.9 ± 8.9	3.2 ± 0.2	239.5
Propionaldehyde	659.4 ± 9.1	2.4 ± 0.2	270.2
Zebrafish ALDH2			
Acetaldehyde	507.7 ± 2.0	11.5 ± 1.9	44.3
Propionaldehyde	659.1 ± 6.9	6.1 ± 0.2	107.6

[a] Aldehyde oxidizing capacity is expressed as V_{max}/K_m, in nmol NADH produced/min/mg protein/nmol aldehyde/ml.

sativa (rice) ALDH2 proteins also show a strong catalytic efficiency for acetaldehyde (low micromolar K_m values) (Nakazono et al., 2000; Liu and Schnable, 2002), in addition to *Saccharomyces cerevisiae* (baker's yeast) ALDH2 (Wang et al., 1997). As such, it would appear that ALDH2 protein function is highly conserved throughout evolution. These findings indicate that the zebrafish ALDH2 gene encodes a metabolically active enzyme that can efficiently oxidize acetaldehyde.

Adult Organ Distribution of ALDH2 in Zebrafish. Western blot analysis was used to detect ALDH2 in various zebrafish tissues (Fig. 6). In the upper panel, ALDH2 polyclonal antibodies were used to probe for ALDH2 protein, with 50 ng of recombinant zebrafish ALDH2 loaded as a positive control. ALDH2 was found to be highly expressed in the heart, skeletal muscle, and brain, and, to a lesser extent, in the eye, liver, and swim bladder. ALDH2 was not found in

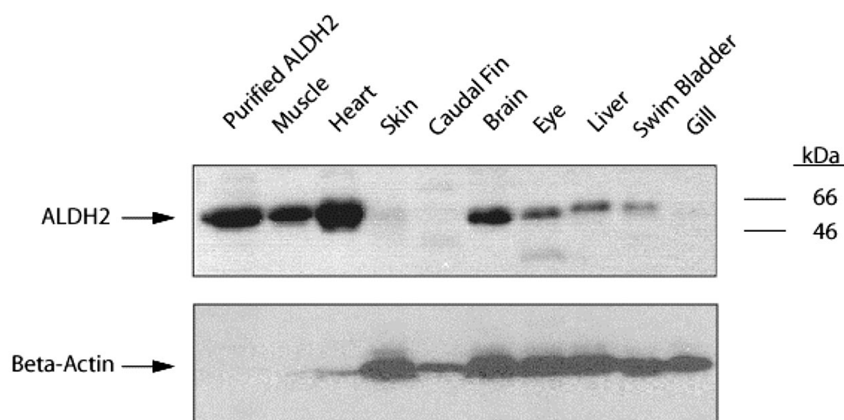

FIG. 6. Zebrafish ALDH2 tissue distribution. Individual organs were prepared for Western blot analysis of ALDH2 expression from adult male zebrafish (as described under *Materials and Methods*). Fifty nanograms of recombinant zebrafish ALDH2 protein was loaded into lane 1 (left lane) and 20 μg of protein from soluble extract, obtained from a variety of tissues, was loaded into each additional lane. The same membrane was reprobed for β-actin (bottom panel) for loading correction.

the skin, caudal fin, or gill (Fig. 6). The PVDF membrane was stripped and reprobed with anti-β-actin-specific antibody (Fig. 6, bottom panel), followed by ECL detection, to establish the relative loading of the samples. In the case of heart and skeletal muscle, which do not express β-actin, Ponseau-*S* staining was used to verify equal loading (data not shown). The tissue distribution of zebrafish ALDH2 appears to be similar but not identical to the distribution pattern of human ALDH2. Human ALDH2 is constitutively expressed at the highest levels in the liver and stomach, but is also widely distributed in other tissues including kidney, skeletal muscle, heart, and brain (Stewart et al., 1996). Considering the results of the kinetic studies, it is possible that ALDH2 may function in a different capacity in zebrafish than in humans. However, an active ALDH2, which is capable of oxidizing acetaldehyde, is present in the zebrafish liver and supports the use of this species as a model for human ethanol metabolism and toxicity.

The zebrafish has attracted interest as an animal model for investigating human diseases since it offers many advantages over traditional rodent models (Shin and Fishman, 2002; North and Zon, 2003). The zebrafish has recently been used to study both human alcoholism and FAS, even though the similarity between these species in relation to biochemical processing of ethanol has not been thoroughly examined (Gerlai et al., 2000; Reimers et al., 2004a). Our studies reveal that zebrafish ALDH2 shares a high sequence homology with human ALDH2 and is also expressed in zebrafish liver. We have shown that zebrafish ALDH2 efficiently metabolizes acetaldehyde with a micromolar apparent affinity constant. It can therefore be concluded that the dominant pathway of acetaldehyde metabolism attributed to mitochondrial ALDH2 is comparable for zebrafish and human ALDH2. These findings, taken together with the recent observation that zebrafish expresses an ADH that metabolizes ethanol (Reimers et al., 2004b), indicate that the zebrafish is an appropriate model for the study of human ethanol metabolism and toxicity.

Acknowledgments. We thank David Carbone for assistance with the genetic mapping studies, and the University of Colorado Comprehensive Cancer Center Tissue Culture/Monoclonal Antibody for assistance with the baculovirus-mediated expression of the recombinant ALDH2 proteins. We thank our colleagues, especially Drs. David Thompson and Richard A. Deitrich, for valuable discussions and critical reading of the manuscript.

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