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THE MITOCHONDRIAL ALDEHYDE DEHYDROGENASE GENE RESIDES IN AN HTF ISLAND BUT IS EXPRESSED IN A TISSUE-SPECIFIC MANNER

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The tissue distribution of mitochondrial aldehyde dehydrogenase (ALDH2) in rats was analyzed by activity assays, and by Western and Northern blotting. ALDH2 was expressed at highest levels in liver. The mRNA levels were intermediate in the kidney and lung, while lower levels were found in spleen and heart. The transcript was undetectable in other tissues tested. The human *ALDH2* 5' flanking region (-200 to +60) contains similar numbers of CpG and GpC dinucleotides and the rat *ALDH2* gene was undermethylated in liver, kidney, and spleen. This suggests that the *ALDH2* promoter resides in a Hpa II tiny fragment (HTF) island, unlike most genes expressed in a tissue-specific manner.

The isozymes of aldehyde dehydrogenase (ALDH, E.C. 1.2.1.3) are grouped into three classes based on sequence similarity. Class 1 consists of cytosolic ALDHs (e.g., ALDH1) that have low K_ms for small aliphatic aldehydes such as acetaldehyde (10-100 μM) (1). The class 2 ALDHs are the mitochondrial ALDH isoenzymes (ALDH2 and probably $ALDH_x$ (2)) with very low K_m for acetaldehyde (1 μM), in the range that occurs in liver during ethanol consumption. Class 3 ALDHs have a much broader substrate specificity and very high K_ms for acetaldehyde (1 mM). This class includes mammalian ALDHs from stomach, cornea, lung, and urinary bladder as well as liver microsomes and certain tumors (3).

ALDH tissue distribution has been studied by assaying total aldehyde oxidizing activity of tissue extracts or activity staining of electrophoretic gels. Most tissues expressed both ALDH1 and ALDH2, but the relative levels of the various isoenzymes vary. The liver and kidney have the highest levels of total ALDHs in humans and rats (4-7). However, activity staining of gels is not quantitative, and ALDH2 mRNA levels in various tissues have not been determined to date.

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Abbreviations:
ALDH, aldehyde dehydrogenase; HTF, Hpa II tiny fragment; SDS, sodium dodecylsulfate.

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In this study, we examined the tissue distribution of ALDH2 by measuring low K_m ALDH activity, determining immunoreactive protein levels by Western blotting, and mRNA levels. We then examined *ALDH2* 5' flanking sequences to explain the tissue-distribution of ALDH2. This region is very rich in G+C residues and suggested that *ALDH2* resides in an Hpa II tiny fragment (HTF) island.

MATERIALS AND METHODS

Preparation of tissue extracts

One gram of tissue was homogenized in 1 ml of homogenization buffer (30 mM $NaPO_4$, pH 6.0, 1 mM EDTA, 1% Triton X100, and 0.1% ß-mercaptoethanol) with a Polytron homogenizer (Ultra-Turrax SDT, Tekmar) at 50% speed for 30 s. For liver, 0.5 g were homogenized in 4 ml of homogenization buffer. Extracts were centrifuged (30 min at 120,000xg) and the supernatants were assayed for ALDH activity and total protein and DNA content (8,9).

ALDH activity assay

ALDH activity was assayed spectrophotometrically in 40 mM KCl, 90 mM KH_2PO_4, 1 mM $MgCl_2$, 0.01 mM 4-methylpyrazole, and 2.6 mM NAD^+. The low K_m and total ALDH activity were determined using 25 μM and 10 mM propionaldehyde, respectively. One unit of activity (U) was defined as the amount of enzyme catalyzing the conversion of 1 μmol/min NAD^+ to NADH. High K_m ALDH activity was determined by subtracting the low K_m from the total ALDH activity.

SDS-PAGE and Western blot analysis

Ten μg of protein extract was denatured in 2% sodium dodecylsulfate (SDS) and 5% ß-mercaptoethanol and fractionated on a SDS-polyacrylamide gel (10). The proteins were electrophoretically transferred to nitrocellulose, blocked with 5% casein in phosphate buffered saline with 0.05% v/v Tween 20 for 30 min at 40°C, and then incubated with an antibody raised against purified human ALDH2 (11) as previously described (12). The primary antibody was detected by incubation with 5 μCi of [^{125}I] protein A. The membranes were exposed to X-ray film for 24 h with one intensifying screen.

Isolation and Northern blot analysis of RNA

Total cellular RNA was prepared by a single extraction with guanidinium thiocyanate (13). One hundred milligrams of tissue were homogenized in 1 ml of extraction buffer with a Polytron homogenizer (Ultra-Turrax SDT, Tekmar) at 50% maximum speed for 30 seconds. The RNA was extracted once with phenol:chloroform and precipitated twice with isopropanol. Northern blotting was performed as described (12). The filter was hybridized with the rat ALDH2 (14) and 28S rRNA (15) cDNAs labelled by random hexamer priming (16). The filter was washed 3 times in 0.1X SSC/0.5% SDS at 55°C for 20 minutes and then exposed to X-ray film with two intensifying screens at -70°C for 8 days for the ALDH2 probe and 1 day for the 28S rRNA probe.

Isolation and Southern blot analysis of genomic DNA

Liver, kidney, and spleen were excised, minced, and quickly frozen in liquid nitrogen. Genomic DNA was extracted as described (17). The DNA purity and concentration were determined from the optical density at 260 and 280 nm. Ten μg of genomic DNA was digested with MspI or HpaII (at 37°C, overnight) and then electrophoresed on a 1.2% agarose gel. The DNA was exposed to ultraviolet light for 15 min, denatured, and transferred to nitrocellulose

(18). 600 bp of the human *ALDH2* 5' flanking sequence was cloned using the polymerase chain reaction (19). This was radioactively labelled by random hexamer labelling (16). After hybridization, the filter was washed three times in 1X SSC, 0.1% SDS at room temperature and once stringently (0.5X SSC and 0.05% SDS at 55°C). The filter was exposed to X-ray film for three days with one intensifying screen at -70°C.

RESULTS AND DISCUSSION

Based upon the results of activity staining of electrophoretic gels, it has often been stated that ALDH2 is expressed ubiquitously. In order to interpret the results of other studies in our laboratory on the ALDH2 promoter, it was necessary to re-examine the tissue-specific expression of this gene. The liver had considerably higher low and high K_m ALDH activity than other tissues (Table 1), whether the activity was expressed as U/mg protein or U/mg DNA. Spleen and kidney had intermediate, while lung, heart, intestine, and stomach had low levels of low K_m ALDH activity. Kidney, intestine, and stomach had intermediate levels of high K_m ALDH activity, while spleen, heart, and lung had lower high K_m activity. There is considerable high K_m ALDH3 activity in stomach and gut that probably accounts for the high K_m ALDH activity observed in those tissues (20-22). Class 1, but not class 3, ALDHs would be partially active at the concentration of substrate used for the low K_m ALDH assay (23,24). Thus, the activity

Table 1. ALDH ACTIVITY IN VARIOUS RAT TISSUES

	U/tissue		U/g tissue		U/mg protein		U/mg DNA	
	Low Km	High Km	Low Km	High Km	Low Km	High Km	Low Km	High Km
Tissue								
Liver	13.3	35.8	0.84	2.3	4.0	10.7	0.33	0.88
Kidney	0.12	0.71	0.08	0.52	0.64	3.9	0.11	0.66
Spleen	0.12	0.19	0.12	0.19	0.49	0.74	0.14	0.22
Stomach	0.10	0.79	0.05	0.36	0.75	5.6	0.05	0.41
Lung	0.11	0.25	0.06	0.13	0.31	0.71	0.06	0.14
Heart	0.06	0.11	0.04	0.08	0.55	1.0	0.06	0.10
Intestine	0.42	4.2	0.04	0.42	0.61	6.1	0.06	0.59

The tissues were homogenized and centrifuged, and the supernatant was analyzed for ALDH activity as described in Materials and Methods. The high K_m activity was determined by subtracting the low K_m from the total ALDH activity. Values shown are the average of determinations made using extracts of organs from two male rats.

assay would tend to overestimate the contribution of ALDH2. We therefore examined the tissue distribution of immunoreactive ALDH2 and the ALDH2 mRNA.

The highest levels of ALDH2 immunoreactivity were found in the liver (Figure 1). We were surprised to find the next highest levels in intestine and stomach, followed by heart and kidney. Spleen and lung had even lower levels of ALDH2 immunoreactivity. The faint higher molecular weight band seen with the liver extract may be the ALDH2 precursor containing the 19 amino acid mitochondrial signal peptide (14) or a cross-reacting protein. The high molecular weight bands seen in the stomach and intestine lanes were consistently seen, but their nature is unknown. The high levels of immunoreactive ALDH in the stomach and intestine compared with their minimal low K_m ALDH2 activity raised the possibility that the antibody cross-reacts with other ALDHs. Class 3 ALDHs have 24% identity to ALDH2 (14,25,26) and the majority of the identical amino acids occur in stretches of 5 to 10 consecutive amino acids. Allowing for conservative substitutions, the degree of similarity is 35%. The class 1 ALDHs are even more similar to ALDH2, with amino acid identities of about 68% (11). The antiserum used for Western blot analysis was generated against purified human ALDH2 and was shown not to cross react with ALDH1 when tested using non-denaturing electrophoresis and immunoblotting (11). It seems likely that the denaturing electrophoresis technique used here exposed additional epitopes in ALDH1 and ALDH3 that cross reacted with the antiserum.

To clarify the results obtained by activity assay and Western blots, Northern blots were performed using a rat ALDH2 cDNA probe (14), since at the nucleotide level the various ALDH isozymes are no more than approximately 66% identical. The highest levels of ALDH2 mRNA

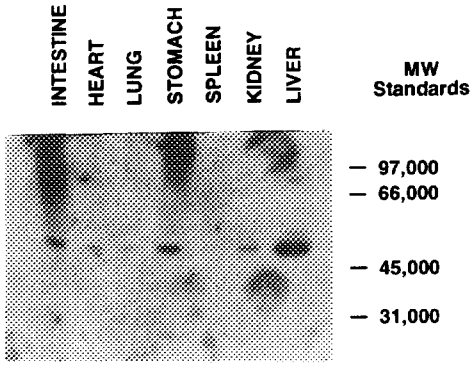

Figure 1. Western Blot Analysis of ALDH2 Distribution in Rat Tissues

Western blot analysis of proteins extracted from various rat tissues. Ten μg of protein was electrophoresed in an SDS-PAGE gel and blotted to nitrocellulose. Immunoreactive ALDH was identified with an antiserum raised against purified human ALDH2 (11) and the antibody-enzyme complex was detected with ^{125}I protein A.

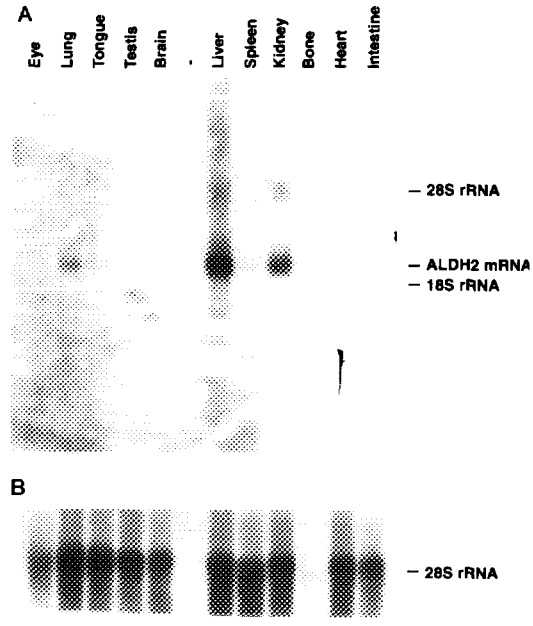

Figure 2. Distribution of ALDH2 mRNA in Rat Tissues
Twenty μg of total RNA from various rat tissues was subjected to formaldehyde-agarose gel electrophoresis and Northern blotted. The filter was hybridized to a radiolabelled rat ALDH2 cDNA (Panel A) or to a cDNA for the rat 28S rRNA (Panel B).

were in the liver, followed by kidney, and then lung (Figure 2). A faint band was seen in heart and spleen RNA, while ALDH2 mRNA was almost undetectable in intestine and other tissues examined, including stomach (data not shown). A more prominent band was seen in blots of heart RNA in other experiments.

Given the high degree of liver-specific expression of ALDH2, we analyzed the 5' flanking region of the human *ALDH2* gene (19) (the rat gene has not been cloned) in search of recognition sites for hepatocyte-specific nuclear factors. However, from -200 to +60, the G+C content was 76% and the CpG/GpC ratio was 0.80 (Figure 3), characteristics of Hpa II tiny

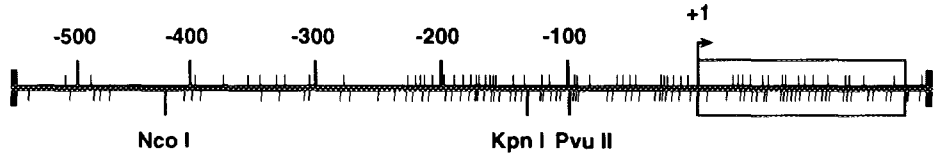

Figure 3. Structure of the Human ALDH2 5' Upstream Sequences
The transcription start site (arrow) was 60 bp 5' to the ATG translation start site, corresponding to the putative bovine ALDH2 transcription start site (33). Vertical lines above the promoter indicate CpG dinucleotides while those below indicate GpC dinucleotides. The open box indicates the location of the first exon.

fragment (HTF) islands (27). HTF islands are also undermethylated. When rat genomic DNA (from liver, kidney, and spleen) was digested with the isoschizomers HpaII or MspI, a similar pattern was observed, indicating undermethylation of the DNA (Figure 4). Therefore, the *ALDH2* flanking region from -200 to +60 probably resides in an HTF island. Interestingly, part of this region (from -18 to -55 bp) is bound by nuclear proteins found in all tissues examined, while sequences further 5' are bound by liver-specific nuclear factors (28). This suggests that the region from -18 to -55 bp, which is part of the CpG island, may be important for low levels of expression seen in kidney, heart, spleen, and lung.

HTF islands are usually associated with the 5' flanking sequences and first exons of genes that are constitutively expressed. There are, however a few examples of genes that are expressed in a tissue-specific manner that occur in HTF islands (including human carbonic anhydrase III, α-globin, aldolase A, class II MHC ß subunit, retinol binding protein, and chicken skeletal muscle actin and collagen α2(I) collagen genes) (27,29). *ALDH2* may belong to this small number of tissue-specific genes residing in an HTF island. Alternatively, this HTF island may be important for expression of a neighbouring gene (27,30). ALDH2 has been mapped to the long arm of chromosome 12 (q24.2) (31). Of genes localized to this region, only

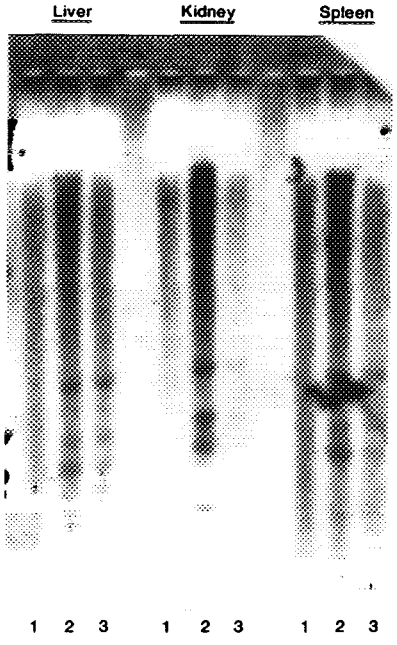

Figure 4. Southern Blot Analysis of the Methylation Pattern of ALDH2 in Rat Tissues
Southern blot analysis was performed with genomic DNA from rat liver, kidney, or spleen. The DNA was mock digested (lane 1), digested with MspI (lane 2) or with HpaII (lane 3), electrophoresed in an agarose gel and blotted, then hybridized with the 600 bp human *ALDH2* promoter shown in Figure 3 (-540 to +60 bp).

that for acyl-coenzyme A dehydrogenase is clearly a housekeeping gene ((32) and references within).

This study demonstrates quantitatively that ALDH2 mRNA is expressed primarily in the liver, with lower levels expressed in kidney and even lower amounts in lung, heart, and spleen. Northern blots clarified the confusion that has existed secondary to the inability of enzyme assays or polyclonal antisera to distinguish the multiple isozymes of ALDH present in many tissues. Unexpectedly, the ALDH2 5' flanking region was found to reside in an HTF island. The fact that the HTF island corresponds to a region that is bound by nuclear proteins in all tissues examined while further upstream regions outside of the HTF island are bound by liver-specific proteins defines interesting areas for further investigation into the mechanism for the liver-specific expression of ALDH2.

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