Molecular Basis of the Alcohol Dehydrogenase-negative Deer Mouse

EVIDENCE FOR DELETION OF THE GENE FOR CLASS I ENZYME AND IDENTIFICATION OF A POSSIBLE NEW ENZYME CLASS*

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The molecular basis of the alcohol dehydrogenase (ADH)-negative deer mouse (Peromyscus maniculatus) has been investigated. Several classes of mammalian ADHs have been recognized based upon biochemical and structural properties. ADH cDNA clones identified by hybridization to a mouse class I ADH cDNA clone were obtained from a deer mouse ADH-positive liver cDNA library. This cDNA has been identified as being a class I sequence and represents the deer mouse Adh-1 gene. An additional cDNA sequence identified in both the ADH-positive and -negative deer mouse cDNA libraries was identified by weak cross-hybridization to the mouse cDNA. This cDNA encodes an amino acid sequence representing a new class of mammalian ADH, and the deer mouse gene for this ADH is named Adh-2. ADH-negative deer mice do not produce mRNA, that is detected by the Adh-1 cDNA probe. However, both stocks of deer mice produce high levels of Adh-2 mRNA in liver. Southern analysis using an essentially full-length Adh-1 cDNA probe has shown that the Adh-1 gene is deleted in the ADH-negative mice. Biochemical analysis of enzyme activity suggests at least three ADH polypeptides are expressed in different tissues and have somewhat different substrate specificities, as in the mouse.

The alcohol dehydrogenase (E.C.1.1.1.1) (ADH)-deficient (ADH-negative) and ADH-positive deer mouse (Peromyscus maniculatus) stocks (1, 2) have been widely used to study the contributions of various pathways of ethanol metabolism in the liver. ADH-negative animals metabolize ethanol in vivo at rates approximately one-half that found for ADH-positive animals. Both stocks contain an ethanol-inducible microsomal enzyme-oxidizing system, although the activity is elevated about 2-fold in ADH-negative deer mice compared with ADH-positive deer mice in both control and ethanol-fed animals (3, 4).

The relative contributions of the non-ADH pathways (namely, catalase and cytochrome P-450-mediated microsomal ethanol-oxidizing activity) have been studied in this animal model with differing interpretations. Using different aminotriazole treatment protocols to inhibit catalase activity in ADH-negative deer mice, investigators have concluded that insignificant (5) or substantial (6) ethanol metabolism via the peroxidatic activity of catalase occurs in the liver. Reports relying upon isotope discrimination effects on the different pathways to assess the role of non-ADH-mediated ethanol metabolism have suggested a predominant cytochrome P-450 role (7) or, alternatively, a significant catalase contribution (8) in ADH-negative animals. Deuterium exchange experiments have suggested dehydrogenase contributions in the ADH-negative deer mice (8), whereas this was not noted in other studies (7).

Because this is a widely used model to study the role of various pathways in ethanol metabolism, a more detailed molecular and biochemical study of the ADHs in the deer mouse has been undertaken. Previous studies have shown that a highly basic ADH is detected in the deer mouse liver using physiological concentrations of substrate ethanol. This isoyme is encoded by a single gene with three identified alleles (1). Two alleles encode electrophoretic variants of the enzyme, and the third allele encodes the enzyme deficiency. ADH-negative mice also lack cross-reacting antigenic material (2).

Attempts to identify other ADHs in the deer mouse have not been systematically explored. The ADH isoymes in mammals, including humans, were originally grouped into class I, II, or III based upon electrophoretic, kinetic, and immunological properties (9). Structural similarity of nearly 95% in amino acid sequence is found between members of a class even between distant species, while sequence similarity of about 60% exists between different classes of isoyme found within a species (10). More recent structural studies have suggested that the rat stomach ADH represents a new class IV isoyme (11), and a recently characterized genomic and cDNA sequence reported from human is a structurally distinct class (12). Thus, the mammalian ADHs known to exist as a minimum of five structurally distinct classes. Here, the molecular nature of the deer mouse ADH-negative phenotype is examined. A cDNA clone obtained from an ADH-positive liver cDNA library was determined to encode a class I ADH. ADH-negative deer mice do not contain liver mRNA detectable by hybridization to this clone, and analysis of genomic DNA suggests that the gene for class I ADH is deleted in ADH-negative mice. However, a cDNA clone isolated and characterized from the ADH-negative liver cDNA library was found to encode a new structural class of mammalian ADH. Of the tissues examined, the mRNA for this class of ADH is expressed at high level only in liver and in both strains of deer mice. Biochemical analysis has suggested at least three forms of the ADH in the deer mouse with similar electropho-
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EXPERIMENTAL PROCEDURES

Animals—ADH-positive (Adh^+ /Adh^+ genotype) and ADH-negative (Adh^−/Adh^+) deer mice were obtained from a breeding colony in the Peromyscus Stock Center at the University of South Carolina. The animals were fed chow diet and water ad libitum while being housed under 18 h of light and 8 h of dark conditions.

Cloning and Characterization of Deer Mouse Adh-1 cDNA Clones—Approximately 1 × 10^6 recombinant λgt10 plaques from both the ADH-positive and -negative liver cDNA libraries were screened by hybridization as previously described (16). The ADH class I cDNA insert from the mouse pADHFn1 plasmid (17) was labeled with [32P]dCTP by nick translation (18) and used as probe. Hybridization conditions were the same as used for Southern blotting. Plaques were purified by three subsequent rounds of screening. Phage DNA was isolated (19), and inserts flanked by EcoRI restriction sites were subcloned into EcoRI-digested pEM72(+) (+). A deletion series of clones were prepared by exonuclease III digestion (20) of some clones to aid in sequencing. Other subclones were obtained using convenient restriction sites to further aid in sequencing. One oligonucleotide primer was made for sequencing one part of one strand on the Adh-2 cDNA clone.

Double-stranded plasmids were prepared, and both strands were sequenced by the dideoxyxynucleotide chain termination method (21) using [α-^32P]dATP as the radiolabeled nucleotide. Sequence 2.0 was used following the supplier’s protocols for the reactions (U.S. Biochemical Corp.). The sequencing gels were 8 μm urea and 8% acrylamide. DNA sequences were analyzed on a VAX computer using the University of Wisconsin Genetics Computer Group Program and compared with Genbank and EMBL sequence banks (22).

RNA and DNA Isolation—High molecular weight genomic DNA was isolated from liver tissue after mice were starved overnight. The procedure used was a modification of other methods. Liver from a single animal was homogenized in 10 ml of 0.1 M EDTA, 1% SDS, 10 mM Tris-HCl (pH 8.0) by 2–3 short bursts of a polytron homogenizer. The homogenate was treated overnight with 100 μg/ml Proteinase K at 56 °C with rotary shaking. Two phenol/chloroform, two chloroform, and four ether extractions followed. The aqueous sample was then incubated at 37 °C for 4 h in the presence of 100 μg/ml of RNase A and 100 units/ml of RNase T1 followed by 100 μg/ml of promerase for 4–12 h. After one phenol/chloroform and one chloroform extraction, the DNA was precipitated twice with ethanol and dissolved in TE (10 mM Tris-HCl, pH 7.0, 1 mM EDTA).

Total cellular RNA was isolated (14) and analyzed for integrity by agarose gel electrophoresis in 1 X TBE (0.089 M Tris-borate, 0.089 M boric acid, 2 mM EDTA). RNA purity was determined by the A260/A280 ratio, and concentration was estimated by A260.

Northern and Southern Analyses—Electrophoresis of RNA in formaldehyde denaturing agarose gels, blotting, and hybridization under stringent conditions were done to sequence the cDNA inserts. A polyvinylpyrrolidone, 0.08% Ficoll, and 5 mg/ml glycerine; for 30 min in 3 X SSPE, 1% SDS; and twice for 30 min in 0.3 X SSPE, 1% SDS, all at 65 °C. The filter was subjected to autoradiography at −70 °C with intensifying screens.

Electrophoretic Analysis of Tissue Alcohol Dehydrogenase—Twenty percent tissue extracts were prepared in 50 mM Tris-HCl (pH 7.0) containing 1 mM diethiothreitol at 4 °C using a polytron homogenizer on medium setting for 20 s. Supernatants were prepared by centrifugation for 20 min at 27,000 × g. The supernatant was added (90 μl) to fill the slots in 12% starch gels made with 8 mM Tris/3 mM citric acid (adjusted to pH 7.2 with NaOH). The gel was connected to electrode-containing buffer chambers containing the same buffer 27.5 times more concentrated, and electrophoresis was conducted for 14 h at 7 V/cm at 4 °C. The gels were 18 × 31 × approximately 0.5 cm. Enzyme activity in the horizontal gel slices was visualized using various alcohols in the histochemical staining method described by Holmes et al. (24).

RESULTS

Cloning and Sequencing of Adh-1 cDNA—About 1 × 10^6 recombinant λgt10 phage from the ADH-positive liver cDNA library were screened with a mouse class I ADH cDNA probe. Nearly 100 positive plaques were initially picked from this library and two were initially chosen for sequencing and further study after subcloning the cDNA inserts into pGEM plasmids. Initially, the complete sequence of the 0.9-kb insert from pADHF72 was obtained. Since the cDNA in pADHF72 was not full-length, the library was rescreened with a 5'-fragment obtained from a full-length mouse cDNA clone called pCK1.2 Additional clones were obtained using this probe, and AADH12−3 was further analyzed and found to contain a larger cDNA insert of about 1.4 kb. The insert was subcloned into pGEM and subsequently called pADH12−3 from which the additional 5'-end sequence was obtained. The complete sequence of the deer mouse class I ADH cDNA representing the Adh-1 gene was determined from these two clones and is presented in Fig. 1. Both strands were sequenced, as were all overlaps. The encoded amino acid sequence is 374 amino acid residues and has a relatively short 3' untranslated region. A polyadenylation signal (AATAAA) is located upstream of the poly(A) tract. The 5'-untranslated region is 47 nucleotides, but this cDNA may not represent the full transcript.

Cloning and Sequencing of Adh-2 cDNA—An additional clone, AADHn1, was identified by a faint hybridization signal in the ADH-negative cDNA liver library. Further comparison identified a related clone, AADHF65, which had been isolated from the ADH-positive library. Insert from both of these cDNA clones were subcloned into pGEM plasmids. The sequence of the cDNA insert in pADHN1 is shown in Fig. 2. This clone was sequenced on both strands, and all overlaps were determined. An open reading frame encoding a 374-amino acid sequence is found within this sequence. The sequence has 135 nucleotides of 5'-untranslated sequence and a long 612-nucleotide 3'-untranslated sequence. A polyadenylation signal (AATAAA) is located upstream of the polyadenylation site.

Comparison of Deduced Amino Acid Sequences—The deduced amino acid sequence of the deer mouse Adh-1 cDNA is 94% identical to the mouse Adh-1 (16, 25) deduced sequence; therefore, the deer mouse Adh-1 gene clearly encodes a class I enzyme. This is further confirmed by comparison with the deduced amino acid sequence encoded by the six human genes (Table I). The human ADH1, ADH2, and ADH3 genes all encode structurally closely related class I enzymes, and the deer mouse Adh-1-encoded sequence exhibits greatest similarity to these sequences. The encoded amino acid sequence

C. K. Boyle and M. R. Felder, unpublished results.
Fig. 1. Nucleotide sequence of the Adh-1 cDNA. Nucleotides 1–520 were obtained from the insert in pADH12–3, and nucleotides 440–1304 were obtained from pADHF72. The overlapping sequence was identical in the two clones, and the insert in pADH12–3 is essentially full-length based upon insert size. The asterisk indicates the stop codon. Nucleotides are numbered in the left column, and amino acids are numbered in the right column.

Fig. 2. The Adh-2 cDNA nucleotide sequence. The complete sequence of the insert in pADH1 is presented. Numbering is as in Fig. 1. The asterisk indicates the stop codon. The (0) denotes those 9 amino acid residues conserved in 47 members of the zinc-containing ADH family, and the (1) indicates those additional conserved residues when \(\text{wcrystallin is excluded}\).
Adh-1 and Adh-2 genes encode classes of the ADH-positive and -negative deer mice. Three classes of ADHs have been identified: Adh-1, Adh-2, and Adh-3. The Adh-1 and Adh-2 genes are expressed in ADH-positive and ADH-negative deer mice, respectively. The Adh-3 gene is recently identified as a distinct class that is equally similar to the other two classes in terms of size and expression patterns.

The Adh-3 gene is expressed in approximately equal amounts in the liver of ADH-positive and ADH-negative deer mice. The Adh-3 gene is also expressed in the kidney, heart, and brain of these animals. The Adh-3 gene is smaller in size compared to the other two classes and is expressed at a lower level in the liver compared to the other tissues.

The Adh-2 gene is expressed at a high level only in liver with a faintly detectable signal seen in kidney RNA. Expression was not detectable in the other tissues examined (Fig. 4B).

**Southern Analysis of the Adh-1 and Adh-2 Genes in ADH-positive and -negative Deer Mice—** Southern blot analysis was used to examine the nature of the Adh-1 gene in ADH-negative and -positive animals. When DNA from ADH-positive deer mice is digested with restriction enzymes and the separated fragments are probed with Adh-1-specific sequences from the nearly full-length insert in pADH12, a single copy sequence appears to be detected. However, faintly detectable bands are also seen in the autoradiograph (Fig. 5B). The strongly hybridizing bands detected in ADH-positive DNA with the Adh-1 probe are not seen in DNA from ADH-negative animals. Only very faintly hybridizing DNA restriction fragments are seen in the ADH-negative DNA, and these correspond closely to those weak signals seen in the ADH-positive DNA. This suggests that the ADH-negative mice are due to a deletion of all or most of the Adh-1 gene.

Both ADH-positive and -negative deer mice contain the Adh-2 gene (Fig. 5B). This provides a good control, since the same DNA restriction digests were analyzed separately with the Adh-1 and Adh-2 probes. Some restriction fragments that faintly hybridize with the Adh-1-specific probe hybridize strongly with the Adh-2-specific probe. As examples, these include the 4.0-kb PstI fragments found in both genetic stocks, the approximately 4.9-kb EcoRI fragments, and the 6.6-kb PvuII fragments. One clear example is the HindIII polymorphic restriction fragment, which is 1 kb in ADH-positive mice and 0.8 kb in ADH-negative mice. This is easily detectable with the Adh-2 gene probe but is only faintly detectable with the Adh-1 gene probe. Another HindIII restriction site polymorphism is detected only with the Adh-2 probe. This polymorphism is the approximately 2-kb fragment in the ADH-positive sample and the nearly 5-kb fragment in the ADH-negative sample. It is not surprising to find such DNA polymorphisms in the ADH-positive animals, but it is surprising that they are not inbred lines or congenic with one another.

**Biochemical Detection of Tissue Alcohol Dehydrogenases—** The enzyme activities in various deer mouse tissues were analyzed by starch gel electrophoresis and histochemical detection. Electrophoretic mobility, tissue specificity, and substrate utilization of the ADHs detected in the deer mouse are similar to ADHs in the mouse, and the dimeric subunit structure of the isozymes is designated A2, B2, and C2 as in the mouse system (24, 30, 31). With 20 mM ethanol as substrate, the cathodally migrating class I ADH-A2 is apparent in liver and kidney of ADH-positive mice (Fig. 6A, lanes 1 and 2) but is deficient in ADH-negative mice (lanes 7 and 8). This isozyme is the product of the Adh-1 gene. Anodally migrating enzyme activity, designated B2, is present mostly in liver of both stocks of animals, and a small amount of activity is also observed in the lung. The B2 designation on the gel is actually resolved into two bands of activity.

At higher ethanol concentrations (250 mM), the stomach C2 enzyme is detectable (Fig. 6B), and this form is also detectable using benzyl alcohol and trans-2-hexene-1-ol as substrates (Fig. 6, C and D). The B2 isozyme seems to have broad substrate specificity using benzyl alcohol and trans-2-hexene-1-ol efficiently as substrates. The A2 form is detectable with these substrates but stains less intensely than with ethanol.

The liver has the highest level of expression of the activity designated ADH-B2, and the tissue specificity is similar to the expression of the Adh-2 gene as measured by Northern analy-
The restriction endonucleases used were as follows: enzymes, and equal amounts of the digestion products were loaded with Adh-I-specific (insert from pADH12-3) and Adh-2-specific (insert from pADHnl) cDNA sequences as indicated on the figure. The RNA sample from each tissue was denatured, divided, and analyzed separately with the two probes.

**FIG. 4.** Northern analysis of Adh-1 and Adh-2 expression in various tissues of ADH-positive mice. The lanes contained total RNA isolated from liver (lane 1), kidney (lane 2), seminal vesicle (lane 3), lung (lane 4), stomach (lane 5), testis (lane 6), and adrenal gland (lane 7). All lanes contained 5 μg of RNA except lane 7, which contained only 1 μg. The blots were probed with the Adh-1 cDNA insert in pADHFT2 (A) and the Adh-2 cDNA insert in pADHnl1 (B). The RNA sample from each tissue was denatured, divided, and analyzed separately with the two probes.

**DISCUSSION**

In this report, the molecular basis of the ADHs in the deer mouse has been investigated. The ADH-positive and -negative stocks have been widely used in studies on ethanol metabolism, and the molecular basis of the ADH-negative variant is now better understood. Two different cDNAs for ADH have been obtained and sequenced from the deer mouse. One complete cDNA sequence was obtained from two overlapping clones isolated from the ADH-positive liver cDNA library. This cDNA sequence contained a 374-amino acid open reading frame, which shared 94% sequence identity with the mouse Adh-1-encoded class I ADH amino acid sequence (16, 25). This deer mouse class I ADH sequence is designated as being encoded by the Adh-1 gene. Further support that the deer mouse Adh-1 gene encodes a class I ADH is the greater than 80% sequence identity at the amino acid level with the three class I human ADHs (26, 27). Most importantly, the ADH-negative mice do not produce an mRNA in liver, which is detectable by hybridization to the Adh-1 cDNA probe. Furthermore, Southern analysis has shown that the Adh-1 gene is substantially or entirely deleted in the ADH-negative deer mice.

The other cDNA sequence was detected by faint hybridization signals found when the ADH-negative cDNA library was screened with a mouse Adh-1 cDNA. The cDNA sequence in the clone obtained from the ADH-negative library was also found to encode a 374-amino acid polypeptide. This amino acid sequence was found to be only 57% identical to the amino acid sequence encoded by the deer mouse Adh-1 gene. This cDNA is designated as representing the mRNA of the deer mouse Adh-2 gene. When the Adh-2-encoded amino acid sequence is compared with the sequences encoded by the six known human alcohol dehydrogenase genes, the sequence was only 51–58% identical to five of the sequences representing class I, II, and III ADHs. The deer mouse Adh-2-encoded sequence is 67% identical to the sequence encoded by the human ADH6 gene, which represents the recently identified additional human class of ADH (12). Furthermore, an identity of only 50% was found between the deer mouse Adh-2-encoded sequence and the partial rat stomach ADH sequence (173 available amino acid residues), which is the only additional known mammalian class of ADH (11). This suggests that the deer mouse Adh-2 gene encodes a new enzyme class not represented by any of the six known human genes representing four classes and the rat stomach ADH representing a fifth class of mammalian ADH. The Adh-2-encoded protein has the highest sequence identity with the protein product of the ADH6 gene, but the human ADH6 gene is expressed in liver and stomach, whereas the deer mouse Adh-2 gene is not expressed in stomach.

The protein encoded by the deer mouse Adh-2 gene contains all 13 residues (Fig. 2) conserved in 47 members of the zinc-containing ADH family including β-crystallin (32). Nine residues are conserved when β-crystallin is included, and 8 of these are glycine, suggesting a side chain in these locations would disrupt a structure required for enzymatic function. One cluster of strictly conserved residues located in the substrate-binding domain has glycine at positions 66, 71, 77, and
of the substitutions involve amino acid replacements with partial similarity.

Expression of the \textit{Adh-1} and \textit{Adh-2} genes in tissues of the deer mouse is substantially different. The \textit{ADH-1} mRNA is found at high levels in liver, kidney, and adrenal gland in \textit{ADH}-positive mice with a much lower level being found in seminal vesicle tissue. In contrast, \textit{ADH-2} mRNA is detected only at high level in liver and at very low level in kidney. While \textit{ADH-1} mRNA is not detectable in liver of \textit{ADH}-negative mice, \textit{ADH-2} mRNA is found in liver of both stocks of animals. The \textit{ADH-2} mRNA is about 400 nucleotides longer than the \textit{ADH-1} mRNA, as measured by Northern analysis, and this is seemingly due to the substantially longer \textit{3'}-untranslated region in the \textit{ADH-2} mRNA as determined from the cDNA sequence.

An effort was made to correlate molecular expression of the \textit{Adh-1} and \textit{Adh-2} genes with \textit{ADH} enzyme activities in various tissues. \textit{ADH}-positive liver and kidney supernatants possess a basic \textit{ADH} protein (\textit{ADH-A}_2) with activity at low ethanol substrate concentrations. This activity is deficient in the \textit{ADH}-negative deer mice correlating with the expression of the \textit{Adh-1} gene. Both stocks of mice have an acidic \textit{ADH} activity (\textit{ADH-B}_2) (Fig. 6), which is detectable with ethanol at low substrate concentrations and with other alcohols. This \textit{ADH} isozyme seems to actually be resolved into two forms in the gel electrophoresis system used. The liver-specific expression of \textit{ADH-B}_2 correlates with the molecular expression of the \textit{Adh-2} gene. However, the mouse \textit{ADH-B}_2 cDNA has recently been sequenced and is 87% identical at the nucleotide level to the human class \textit{II} \textit{ADH} (34). The mouse \textit{ADH-B}_2 cDNA nucleotide sequence is only 62.9% identical to the deer mouse \textit{ADh-2} cDNA sequence, suggesting these are not orthologous genes. That the gel system employed here resolves the \textit{ADH-B}_2 region into two zones of activity makes it a possibility that this region of activity may be the result of expression of two genes, one of which could be \textit{Adh-2}. An effort was made to identify an electrophoretic variant for the \textit{ADH-B}_2 region between \textit{Peromyscus polionotus} and \textit{P. maniculatus}, but none was found. This could have determined whether more than one gene encodes the two bands of activity in this region. The multiple bands in the \textit{ADH-A}_2 form are due to differential binding of NAD cofactor (1) and are the product of a single gene. The role the \textit{ADH-B}_2 activity and the product of the \textit{Adh-2} gene may play in ethanol metabolism remains unclear. Part of the residual ethanol metabolism found in the \textit{ADH}-negative mice may be due to these \textit{ADH}s, and some may be due to the cytochrome P450-mediated microsomal ethanol-oxidizing system.

Not only do both \textit{ADH}-positive and -negative deer mice express \textit{ADH-B}_2 in liver tissue, but both stocks express \textit{ADH-C}_2 in stomach tissue, as does the mouse (24, 30, 31). The isozyme is detectable with high ethanol substrate concentrations and with benzyl alcohol and trans-2-hexene-1-ol as is the mouse isozyme (24, 31).

Not only are the \textit{ADH}-negative deer mice reduced in their ability to metabolize ethanol, but they are also greatly reduced in their ability to synthesize retinoic acid from retinol (35), although the mice appear to possess the ability to produce retinoic acid at physiologically required levels. In the liver and kidney, \textit{ADH}-negative deer mice retain about one-eighth the ability to metabolize retinol as found in \textit{ADH}-positive deer mice. These results would suggest that the protein product of the \textit{Adh-2} gene is not involved in this residual metabolism, since the \textit{Adh-2} gene is expressed at near negligible level in kidney.

The \textit{ADH}-negative deer mice are here clearly shown to be
due to a deletion of the Adh-1 gene, which encodes a class I ADH. A number of protein deficiencies are known in mammalian systems, but not a large number are due to substantial or entire gene deletions. A multiexon deletion of about 9 kb in the procollagen III gene in humans is known to cause a mild disorder (36). The entire gene for arylamine N-acetyltransferase is deleted in a genetically identified group of rabbits deficient in the ability to metabolize certain drugs (37). The deletion of the β-hexosaminidase α-chain in some French Canadians with Tay-Sachs disease appears to be associated with Alu sequences flanking the deleted sequences (38). A deletion of a large portion of the gene for the low density lipoprotein receptor is the molecular basis of familial hypercholesterolemia. This deletion is also flanked by Alu sequences flanking the deleted sequences (39). The possible molecular mechanism by which the Adh-1 gene became deleted in the ADH-negative deer mouse is currently unknown.

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