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# Molecular cloning and characterization of a cDNA for the β subunit of human alcohol dehydrogenase

(synthetic DNA probe/M13 DNA sequence analysis/amino acid sequence/multigene family)

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ABSTRACT    Human alcohol dehydrogenase (ADH) is encoded by at least five genes that fall into three classes. The class I ADH genes encode the three closely related α, β, and γ polypeptides. Molecular genetic analysis of class I ADH genes has been initiated by isolating a cDNA clone from a human adult liver cDNA library. A synthetic oligonucleotide mixture encoding a portion of the β subunit of ADH was used as an in situ hybridization probe for the cDNA library. One positively hybridizing clone, pADH12, which contained an 1100-base-pair cDNA insert, was subjected to DNA sequence analysis. The sequence indicated that the cDNA encoded information for the carboxyl-terminal 91 amino acids of a class I ADH and a 3' untranslated region of 593 nucleotides. Comparisons with the carboxyl terminus of the human ADH β subunit indicated that the cDNA encoded the β polypeptide. This probe may facilitate genetic studies of various human alcohol-related syndromes, as well as enable basic molecular studies on human ADH gene expression.

Mammalian alcohol dehydrogenase (ADH; alcohol:NAD⁺ oxidoreductase, EC 1.1.1.1) is responsible for the oxidation of a wide variety of primary, secondary, and aromatic alcohols (1) but is most noted as being the enzyme primarily responsible for catalyzing the first reaction in the metabolism of ethanol. Horse liver ADH is the most well-studied form (2), but the enzyme from man was early shown to be closely related (3). Human ADH exists as a set of at least 20 different isoenzymes that exhibit a wide range of mobilities on starch gel electrophoresis (1, 4–6). All isoenzymes are similar in that they are dimeric, each monomer having a molecular weight of ≈40,000 (7) like those of the horse enzyme (2). Because of different substrate specificities, differential inhibition by 4-methylpyrazole, and different electrophoretic properties, the human isoenzymes have been placed in one of three classes (8). Class I contains a large group of isoenzymes possessing various combinations of α, β, and γ subunits, coded for by three genes designated ADH1, ADH2, and ADH3, respectively (1). Class II contains the π isoenzyme (6, 9) and class III contains the χ isoenzyme (5). Amino acid analysis (6) and peptide mapping data (8) suggest that the three classes of ADH differ significantly in primary structure, although they are similar in molecular weight. The differences within group I are also considerable. Thus, the β and γ chains have recently been shown to differ at 21 positions (unpublished results) out of a total of 373. The complete primary structure of the β chain has recently been determined (unpublished results), as has the nature of a mutational amino acid exchange that explains the properties of "atypical" β chains present in some Oriental (10) and Caucasian (unpublished results) lines.

Homo- and heterodimers of the α, β, and γ subunits have been observed (1, 11–13) and the distribution of those subunits in human tissue is variable. Adult liver has large amounts of α, β, and γ subunits; adult kidney, stomach, and intestine have more γ than β and no α subunits; adult lung has only β subunits (1, 12). Developmental regulation of ADH is also evident. In fetal liver, the α subunit is expressed during the first trimester and this is followed by expression of the β subunit during the second trimester and of the γ subunit 5 months after birth (1).

Genetic polymorphisms within each of the class I subunits have been detected (14). Most noted is the polymorphism at the ADH2 locus in which most Caucasians have the $\beta_1$ allele, giving "typical" $\beta_1$ chains. An atypical enzyme was early described (15) and is now known to be explained by subunits ($\beta_2$-Bern chains) from the $\beta_2$ allele. Similarly, an atypical enzyme with $\beta_2$-Oriental chains appears to be present in nearly 90% of Japanese and Chinese individuals (14, 16). Furthermore, another β variant has been ascribed to individuals of African origin with the β-Indianapolis enzyme subunit (4, 17). Different amino acid exchanges explaining the $\beta_2$ allelic variants were initially reported for the $\beta_2$-Bern (18) and $\beta_2$-Oriental (19) chains. However, these two types of atypical chains are now known to be identical and compatible with a single Arg–His exchange at position 47 (10) that explains all the differences in functional properties. Since at physiological pH the activity of the $\beta_2$ subunit is much higher than that of the $\beta_1$, it has been suggested that the high frequency of acute alcohol intoxication among Orientals may be related to the presence of the $\beta_2$-subunit allele and the resultant rapid accumulation of acetaldehyde after alcohol ingestion (16, 20). Polymorphism at the ADH locus is also suspected to have importance in determining the degree of susceptibility of certain individuals to fetal alcohol syndrome (21), alcoholism (22), and chlorpropamide-induced alcohol sensitivity (23).

Knowledge of the structure of the β subunit of ADH has facilitated the molecular genetic analysis of human ADH genes. Initially, a partial amino acid sequence of the human β subunit (18) enabled the selection and synthesis of an oligonucleotide probe complementary to β-subunit mRNA. This synthetic oligonucleotide was used to isolate a cDNA clone for human ADH. Later, the complete $\beta_1$-subunit amino acid sequence (unpublished results) and the $\beta_1/\gamma$ differences (unpublished results) provided conclusive evidence that the cDNA encoded a portion of the human β chain. This cDNA should serve as a useful probe for analysis of ADH gene expression and polymorphism in various human tissues.

## MATERIALS AND METHODS

**Colony Screening of the cDNA Library.** The adult human liver cDNA library used was provided by S. H. Orkin of

Abbreviation: ADH, alcohol dehydrogenase.

Harvard Medical School and has been described (24). Colonies were screened with a synthetic oligonucleotide purchased from Applied Biosystems (Foster City, CA) as a hybridization probe. The synthetic DNA probe was constructed using information from a previously published comparison of the partial human $\beta$-subunit and the complete horse E-chain sequences (18, 25). A mixture of 16 oligonucleotides (each 14 nucleotides long) encoding the portion of the human $\beta$ subunit between amino acids 332 and 336 [corresponding to positions 333–337 in the horse protein because there is a single amino acid deletion in the NH$_2$-terminal half of human $\beta$ subunits relative to those of horse E chain] was synthesized simultaneously by inserting all possible nucleotides at positions corresponding to amino acids specified by more than one codon (see Fig. 1). Oligonucleotides were labeled at the 5' end by transfer of $^{32}$P from [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Bethesda Research Laboratories) as described (26).

A total of 10,000 colonies was screened on ten 82-mm-diameter Whatman 541 filters (27) using the prehybridization and hybridization conditions of Wallace *et al.* (28). Filters were then washed with four changes of 0.30 M NaCl/0.030 M Na citrate, pH 7.0, at room temperature (10 min for each wash with continuous shaking) followed by two changes of the same citrate buffer at 42°C (30 min for each wash with continuous shaking). Filters were dried and autoradiography was carried out for 24 hr with DuPont Lightning Plus intensifying screens at −70°C.

**Isolation and Restriction Analysis of Plasmid DNA.** Plasmid DNA was isolated by using an alkaline lysis procedure (29) and further purified by CsCl/ethidium bromide density gradient centrifugation. Restriction endonuclease mapping was carried out by standard double-digestion procedures.

**DNA Sequence Analysis.** DNA sequencing was carried out by the dideoxynucleotide chain-termination method described by Sanger *et al.* (30). Appropriate restriction fragments were cloned in either M13mp8 or M13mp9 vectors (31). Single-stranded DNA isolated from recombinant phage served as a template for DNA sequence analysis, with the primer being either the mixture of 14-mers used for screening the cDNA library or the M13 universal primer (17-mer) purchased from Collaborative Research (Waltham, MA).

**Amino Acid Sequence Analysis.** $\beta_1\beta_1$ ADH was isolated from Caucasian human livers of typical phenotype, frozen 10–20 hr after death, and stored at −20°C. The enzyme was purified on CapGapp-Sepharose (ref. 32; unpublished results) followed by CM-cellulose chromatography (13) as described. The pure isoenzyme was carboxymethylated with iodo[2-$^{14}$C]acetate and different samples were treated with CNBr and trypsin. Peptides obtained were first fractionated by exclusion chromatography on Sephadex G-50 and then purified by reversed-phase high-performance liquid chromatography using acetonitrile for elution (33) and checked for composition. Manual sequence analysis was carried out by the dimethylaminoazobenzene method (34) using by-products for identification (35), and by liquid-phase sequencer degradations carried out in a Beckman 890D instrument using a 0.1 M peptide program in the presence of precycled Polybrene (33). The complete structural determination of $\beta_1\beta_1$ ADH was carried out in cooperation with Bert L. Vallee (Boston).

## RESULTS

**Identification of an ADH cDNA Clone.** A mixture of 16 oligonucleotides (Fig. 1), one of them perfectly complementary to ADH mRNA, was used as a hybridization probe for colony screening. Labeled oligonucleotides were hybridized *in situ* to filters containing lysed colonies of the human adult liver cDNA library. Ten thousand colonies were initially

| RESIDUE NUMBER: | 332 333 334 335 336 |
|---|---|

| AMINO ACID: | Ala Asp Phe Met Ala |
|---|---|

mRNA:    5'– GCN GA$_C^U$ UU$_C^U$ AUG GCN–3'

SYNTHETIC DNA:    3'– CGN' CT$_G^A$ AA$_G^A$ TAC CG –5'

FIG. 1.   ADH synthetic oligonucleotide sequence. The region of the ADH subunit corresponding to amino acids 332–336 was chosen for the synthesis of a mixture of oligonucleotides having the least amount of codon ambiguity. The mixture consisted of 16 distinct 14-mer oligonucleotides, one being perfectly complementary to ADH mRNA. N, all four possible nucleotides; N', the complementary nucleotides.

screened in an unordered fashion on 82-mm Petri dishes at a density of 1000 clones per dish. Positive clones were replated in an ordered fashion and rescreened. A 42°C wash of the filters after hybridization proved to be important in reducing the amount of nonspecific hybridization without severely reducing the specific signal. About 40 positively hybridizing clones were observed in the unordered screening procedure. After rescreening in an ordered fashion, one clone, designated pADH12, was selected for further characterization.

Plasmid DNA was purified and *Pst* I digestion indicated that pADH12 contained a cDNA insert of approximately 1100 base pairs. A restriction map was generated by further digestion of the isolated cDNA insert with various enzymes (Fig. 2). DNA sequencing was carried out to verify that pADH12 was in fact a cDNA for class I ADH.

**DNA Sequence Analysis of pADH12.** The DNA sequence of the ADH cDNA fragment was determined according to the strategy indicated in Fig. 2. Initially, the cDNA-containing *Pst* I fragment of pADH12 (Fig. 2) was cloned in both orientations into M13mp8 to enable sequencing of either strand of the cDNA. Using the mixture of 14-mers as a primer, an M13 clone containing the strand complementary to the 14-mer was subjected to sequence analysis by the method of Sanger *et al.* (30). This clone provided sufficient sequence information to confirm that pADH12 contained a cDNA for ADH. Sequencing of additional fragments cloned into either M13mp8 or M13mp9 established the DNA sequence of the entire cDNA fragment (Fig. 3).

The amino acid sequence predicted from the cDNA shares 84% homology with the carboxyl-terminal 91 amino acids of the published structure of the horse E chain (positions 284–374) (25). It is also in complete agreement with the amino acid sequence determination for the corresponding region of human ADH $\beta_1$ chains (below). Thus, in the region between residues 283 and 373, all 91 amino acids from the $\beta_1$ chain match the predicted amino acid sequence from the cDNA.

The $\beta$-chain cDNA sequence contains a long 3' untranslated region of 593 base pairs. Within this region, there are four copies of the sequence A-A-T-A-A-A (residues 385–390, 472–477, 727–732, and 843–848; Fig. 3), the last one being located 18 base pairs upstream of the poly(A) tract and presumably functioning as a polyadenylylation signal (36, 37).

**Amino Acid Sequence Analysis of the Human ADH $\beta$ Chain.** Since the amino acid sequence predicted from the ADH cDNA did not match the previous partial estimate of the human ADH $\beta$ chain (18), additional sequence analysis was required to establish which subunit the cDNA encoded. The ADH $\beta$ subunit was isolated from human liver, carboxymethylated, and cleavaged with trypsin and with CNBr, and then the peptides were purified and structurally analyzed, as described in *Materials and Methods*. The amino acid se-
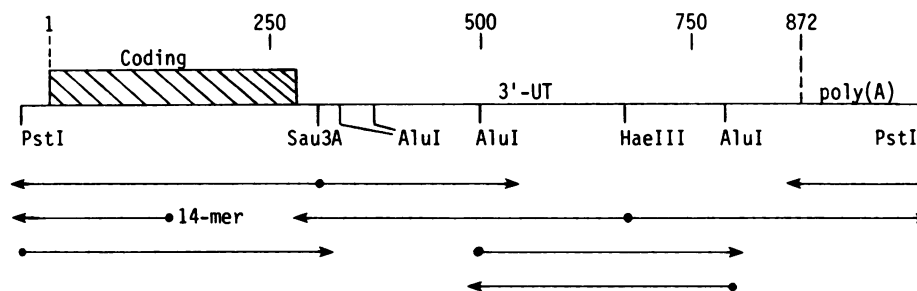
FIG. 2. Restriction map of the cDNA insert of pADH12 and strategy for DNA sequence analysis. The restriction sites that were mapped are indicated. The scale at the top is in base pairs. The sequence of the entire cDNA insert was determined according to the strategy shown at the bottom using M13mp8 and M13mp9 subclones of the cDNA insert. The arrow labeled 14-mer indicates sequence information obtained using the 14-mer oligonucleotide mixture as a primer.

quence obtained for the carboxyl-terminal 98 residues of the $\beta_1$ chain is shown in Fig. 4, as are the positions of all peptides analyzed. The amino acid sequence from residues 283–373 was in perfect agreement with the sequence predicted from the cDNA. These data confirmed that the cDNA that we isolated encoded the $\beta$ subunit of human ADH.

## DISCUSSION

The usefulness of synthetic oligonucleotide probes 14–17 nucleotides long for the detection of specific DNA sequences in bacterial colonies has been well documented (26, 28). Using this approach, we have isolated a cDNA clone for the $\beta$ subunit of human ADH. DNA sequence analysis and amino acid sequence analysis were both employed to verify that the clone indeed coded for the $\beta$ polypeptide. Because there were significant differences (at six positions) between the amino acid sequence predicted by the cDNA and that earlier estimated from compositions of peptides (18), it was necessary to examine the human ADH $\beta_1$ chain by complete amino acid sequence analysis to determine which ADH subunit

the cDNA encoded. These studies indicated that the cDNA corresponded exactly to the carboxyl-terminal 91 amino acids of the $\beta$ subunit. In this context, it may be noticed that the studies on ADH, exactly like those on aldehyde dehydrogenase (38), illustrate that total amino acid compositions and homology alignments give unreliable estimates of exact relationships. Obviously, multiple substitutions of related residues are concealed in ordering by composition only. This was already evident from peptide studies (38), but it is confirmed by the fact that the cDNA structure supports the complete ADH peptide analyses.

Because of the similarities between the human class I ADH subunits (1, 7, 8), the $\beta$-subunit cDNA described here should prove to be a useful probe for studying the molecular genetics of the $\alpha$ and $\gamma$ subunits also. In further support of this, the amino acid sequence of the carboxyl-terminal end of human $\gamma$ chains (unpublished results) indicates that there is a high degree of homology with $\beta$ chains. Amino acid sequence data for human $\alpha$ subunits are lacking. Based on the similarities between $\alpha$, $\beta$, and $\gamma$ subunits, it is reasonable to propose that the class I *ADH* genes have evolved as a multi-

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283                          290                                        300                                      310
Glu Ala Cys Gly Thr Ser Val Ile Val Gly Val Pro Pro Ala Ser Gln Asn Leu Ser Ile Asn Pro Met Leu Leu Leu Thr Gly Arg Thr
GAG GCA TGT GGC ACA AGC GTC ATC GTA GGG GTA CCT CCT GCT TCC CAG AAC CTC TCA ATA AAC CCT ATG CTG CTA CTG ACT GGA CGC ACC
                                                                                                                        90

                             320                                        330                                      340
Trp Lys Gly Ala Val Tyr Gly Gly Phe Lys Ser Lys Glu Gly Ile Pro Lys Leu Val Ala Asp Phe Met Ala Lys Lys Phe Ser Leu Asp
TGG AAG GGG GCT GTT TAT GGT GGC TTT AAG AGT AAA GAA GGT ATC CCA AAA CTT GTG GCT GAT TTT ATG GCT AAG AAG TTT TCA CTG GAT
                                                                                                                        180

                             350                                        360                                      370
Ala Leu Ile Thr His Val Leu Pro Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu His Ser Gly Lys Ser Ile Arg Thr Val Leu Thr
GCG TTA ATA ACC CAT GTT TTA CCT TTT GAA AAA ATA AAT GAA GGA TTT GAC CTG CTT CAC TCT GGG AAA AGT ATC CGT ACC GTC CTG ACG
373                                                                                                                     270
Phe Ter
TTT TGA GGCA ATAGAGATGC CTTCCCCTGT AGCAGTCTTC AGCCTCCTCT ACCCTACGAG ATCTGGAGCA ACAGCTAGGA AATATCATTA ATTCAGCTCT
                                                                                                                        370

  TCAGAGATGT TATCAATAAA TTACACATGG GGGCTTTCCA AAGAAATGGA AATTGATGGG AAATTATTTT TCAGGAAAAT TTAAAATTCA AGTGAGAAGT
                                                                                                                        470

 AAATAAAGTG TTGAACATCA GCTGGGGAAT TGAAGCCAAC AAACCTTCCT TCTTAACCAT TCTACTGTGT CACCTTTGCC ATTGAGGAAA AATATTCCTG
                                                                                                                        570

 TGACTTCTTG CATTTTTGGT ATCTTCATAA TCTTTAGTCA TCGAATCCCA GTGGAGGGGA CCCTTTTACT TGCCCTGAAC ATACACATGC TGGGCCATTG
                                                                                                                        670

 TGATTGAAGT CTTCTAACTC TGTCTCAGTT TTCACTGTCG ACATTTTCCT TTTTCTAATA AAAATGTACC AAATCCCTGG GGTAAAAGCT AGGGTAAGGT
                                                                                                                        770

 AAAGGATAGA CTCACATTTA CAAGTAGTGA AGGTCCAAGA GTTCTAAATA CAGGAAATTT CTTAGGAACT CAAATAAAAT GCCCACATTT TACTACAAAAAAA150
                                                                                                                        870
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FIG. 3. DNA sequence of the cDNA insert of pADH12. The cDNA insert of pADH12 was sequenced according to the dideoxynucleotide chain-termination method of Sanger *et al.* (30). The nucleotides are numbered 1–870 in the 5′ to 3′ direction. The predicted amino acid sequence of the carboxyl-terminal 91 amino acids of the $\beta$ subunit of ADH (residues 283–373) is shown above the nucleotide sequence, immediately followed by a UGA translation termination (Ter) codon. Four putative polyadenylylation signals in the 3′ untranslated region are underlined. There are approximately 150 adenylate residues in the poly(A) tract.
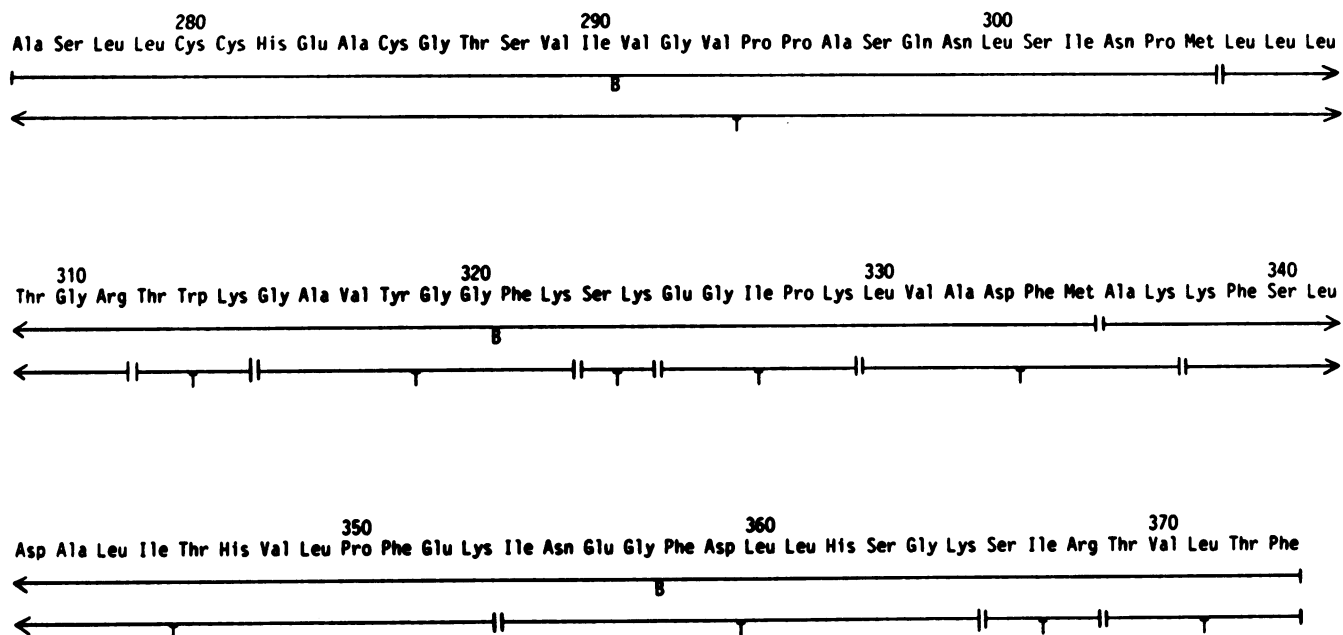
FIG. 4. Carboxyl-terminal amino acid sequence of the human ADH $\beta_1$ chain. The sequence was obtained by analysis of peptides derived from digestion with CNBr (B) and with trypsin (T). Regions covered by the peptides are shown below the sequence. The sequence from residue 276 to the carboxyl-terminal residue 373 is shown.

gene family. Since $\pi$ (class II) and $\chi$ (class III) subunits have been shown to differ significantly from the class I subunits biochemically (5, 6, 8) and immunologically (39), it is unlikely that the $\beta$-subunit cDNA will aid genetic analysis of either of these two ADH subunits.

The presence of four putative polyadenylylation signals (37) in the 3′ untranslated region of the $\beta$-subunit cDNA raises questions as to the sequence and/or secondary structural requirements for this signal. It is not clear what would prevent the three proximal signals from being used. Other genes with particularly long 3′ untranslated regions also display multiple polyadenylylation signals (40, 41). The porcine preproenkephalin B mRNA contains five such signals, the most distally located one being the only functional signal in the hypothalamus (40). On the other hand, the dihydrofolate reductase gene of Chinese hamster ovary cells has four poly-adenylylated mRNAs of distinct sizes, each presumably using a different polyadenylylation signal in the 3′ untranslated region (41).

In other systems, the cloning and molecular genetic analysis of ADH is well underway, notably in yeast (42–44), *Drosophila* (45), and maize (46). The availability of a cDNA for the human ADH $\beta$ subunit now makes it possible to study the ADH multigene family at the molecular level in humans. Chromosomal mapping of human *ADH* genes should be facilitated through the use of this cDNA probe. Also, since polymorphic variants of the *ADH1*, *ADH2*, and *ADH3* genes may be involved in the differences in ethanol metabolism observed in various population groups, one may be able to identify restriction-fragment-length polymorphisms correlating to each of these genes by using the cDNA probe (or genomic *ADH* probes derived from it) to screen genomic DNA samples from various individuals. In addition, analysis of cloned genomic fragments obtained using this probe should enable a detailed study of human $\alpha$-, $\beta$-, and $\gamma$-subunit *ADH* gene structure and expression.

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