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Three human alcohol dehydrogenase subunits: cDNA structure and molecular and evolutionary divergence

(cDNA cloning/oligonucleotide probe/molecular evolution)

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ABSTRACT **Class I human alcohol dehydrogenase (ADH; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) consists of several homo- and heterodimers of α, β, and γ subunits that are governed by the *ADH1*, *ADH2*, and *ADH3* loci. We previously cloned a full length of cDNA for the β subunit, and the complete sequence of 374 amino acid residues was established. cDNAs for the α and γ subunits were cloned and characterized. A human liver cDNA library, constructed in phage λgt11, was screened by using a synthetic oligonucleotide probe that was matched to the γ but not to the β sequence. Clone pUCADHγ21 and clone pUCADHα15L differed from β cDNA with respect to restriction sites and hybridization with the nucleotide probe. Clone pUCADHγ21 contained an insertion of 1.5 kilobase pairs (kbp) and encodes 374 amino acid residues compatible with the reported amino acid sequence of the γ subunit. Clone pUCADHα15L contained an insertion of 2.4 kbp and included nucleotide sequences that encode 374 amino acid residues for another subunit, the α subunit. In addition, this clone contained the sequences that encode the COOH-terminal part of the β subunit at its extended 5′ region. The amino acid sequences and coding regions of the cDNAs of the three subunits are very similar (≈93–95% identity). A high degree of resemblance is observed also in their 3′ noncoding regions. However, distinctive differences exist in the vicinity of the Zn-binding cysteine residue at position 46—i.e., Cys-Gly-Thr in the α, Cys-Arg-Thr in the wild-type β_1, Cys-His-Thr in the Oriental-type β_2, and Cys-Arg-Ser in the γ, reflecting the differences in their kinetic properties. Based on the cDNA sequences and the deduced amino acid sequences of the three subunits, their structural and evolutionary relationships are discussed.**

The human liver and other tissues contain several cytosolic and microsomal alcohol dehydrogenase isozymes (ADH; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1). Among these isozymes, "class I" ADH, which exhibits high activity for ethanol oxidation, is considered to play a major role in ethanol catabolism. Class I ADH contains several homo- and heterodimers, formed by the association of three types of subunits, α, β, and γ, which are governed by the three separate structural loci, *ADH1*, *ADH2*, and *ADH3*, respectively (1–3). The ADH2 and ADH3 are strongly expressed in adult livers, whereas the ADH1 is predominant in fetal and infant livers (4).

The *ADH2* and *ADH3* loci are polymorphic, whereas the *ADH1* locus appears to be monomorphic (1–3). The *ADH2*^{1} producing β_1 subunit is common in Caucasians and the *ADH2*^{2} producing β_2 subunit is prevalent in Orientals (5). Two types of *ADH3*—i.e., *ADH3*^{1} for γ_1 subunit and *ADH3*^{2} for γ_2 subunit—are commonly found in various populations (1).

The amino acid sequences of β and γ subunits were proposed, partly relying on a presumed homology between the horse and human ADH (6, 7). A full length of cDNA for the β subunit was cloned and characterized, and the complete amino acid sequence of the β subunit was deduced from the cDNA sequence without ambiguity (8). The structure of the fetal form subunit, the α subunit, is totally unknown.

This paper reports molecular cloning of full-length cDNAs for the α and γ subunits and the complete amino acid sequences deduced from the nucleotide sequences. The molecular and evolutionary divergence of these subunits is discussed.

MATERIALS AND METHODS

Cloning of cDNAs for α and γ Subunits. The adult human liver cDNA library constructed in phage λgt11 was provided by Savio L. C. Woo (Howard Hughes Medical Institute, Houston, TX). The library was screened by the plaque hybridization method (9) with a synthetic oligonucleotide probe whose sequence is matched to the known amino acid sequence of the γ subunit but not to the β subunit (Fig. 1). The nucleotide was labeled at the 5′ OH end with [γ-^{32}P]ATP (>7000 Ci/mmol; 1 Ci = 37 GBq; INC Radiochemicals) by T4 polynucleotide kinase (Bethesda Research Laboratories) to give a specific activity of $>2 \times 10^8$ cpm/μg (10).

Subcloning of Phage Inserts and Preparation of Cloned DNA. Recombinant phage DNA was prepared from a large-scale liquid culture and purified by gradient centrifugation in cesium chloride (11). The inserted cDNA was separated from *Eco*RI-digested λ DNA by gel electrophoresis on a 3.5% polyacrylamide gel (12) and ligated to the *Eco*RI-digested pUC13 vector (13). Competent *Escherichia coli* TB1 cells were transformed with the ligated DNA, and the plasmid DNA was prepared from the cells grown in a liquid culture by the alkali/NaDodSO$_4$ method (11).

Restriction Maps and DNA Sequence Analysis. The location of restriction endonuclease cleavage sites in the inserted DNA was determined by digesting the DNA with several restriction enzymes under the conditions recommended by the manufacturer. The DNA fragments to be sequenced were ligated into M13 mp18 and/or mp19 vectors (14). *E. coli* JM 107 or JM 103 was transformed with the ligated vectors by calcium chloride treatment (15). The single-stranded template DNA was prepared from white plaques and subjected to DNA sequencing analysis by the dideoxynucleotide chain-termination method of Sanger *et al.* (16). The universal M13 primes (17-mer) were used for the sequencing. DNA sequences were established by analysis of both strands and analysis of the fragments across the restriction sites.

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Abbreviations: ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; kbp, kilobase pair(s).

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β subunit

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                        14  15  16  17  18  19
Amino Acid Sequence:    Leu-Trp-Glu-Val-Lys-Lys

mRNA:              5' CUA·UGG·GAG·GUA·AAG·AAA  3'

cDNA:              3' GAT·ACC·CTC·CAT·TTC·TTT  5'
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γ subunit

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                        14  15  16  17  18  19
Amino Acid Sequence:    Leu-Trp-Glu-Leu-Lys-Lys

mRNA:              5' CUA·UGG·GAG·U/CUA·AAG·AAA  3'

cDNA:              3' GAT·ACC·CTC·A/GAT·TTC·TTT  5'
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FIG. 1. Synthetic oligonucleotide probe used for cDNA screening. The amino acid and cDNA sequences of the β subunit are from the previous report (8). The amino acid sequence of the γ subunit is based on the proposed sequence of γ_1 (7).

RESULTS AND DISCUSSION

Cloning of cDNAs for α and γ Subunits. Approximately 10 × 10^4 recombinant λgt11 phage plaques were screened by hybridization with the oligonucleotide probe for the γ subunit (Fig. 1), and 44 positive plaques were obtained. In the subsequent screening, 8 single plaques, which were positive to the γ cDNA probe, were cloned. Insert sizes of these recombinant phage clones, estimated by *Eco*RI digestion, ranged from 1.3 to 2.4 kilobase pairs (kbp).

Digestion of these eight cDNAs by endonucleases revealed that all have *Kpn* I and *Pst* I sites. Three cDNA clones were digested by *Hpa* I, whereas the other five cDNA clones lacked the *Hpa* I site. The coding sequence of cDNA for the β subunit contains a *Kpn* I site, three *Pst* I sites, and an *Hpa* I site (8). The 185th position of the γ subunit was reported to be lysine (7), whereas this position of the β subunit is asparagine (codon AAC) (8). Therefore, the coding sequence of the γ cDNA might not bear an *Hpa* I site. It was further confirmed, by Southern hybridization analysis, that the three *Hpa* I-positive cDNA clones were hybridized with the synthetic oligonucleotide probe for the β subunit and that five *Hpa* I-negative cDNA clones were strongly hybridized with the γ probe. These five cDNA clones could be either for the α or for the γ subunit of human ADH.

Restriction mapping of these five cDNA clones revealed that one clone, designated pUCADHα15L, exhibits a unique restriction map—i.e., it contains three *Kpn* I sites, whereas the other clones have only one *Kpn* I site. A clone, designated pUCADHγ21, which carried an insert of about 1.5 kbp, and pUCADHα15L, which contained an insert of 2.4 kbp, were subjected to nucleotide sequence determination.

Nucleotide Sequences and Deduced Amino Acid Sequences. Restriction endonuclease cleavage maps of pUCADHγ21 and pUCADHα15L are shown in Fig. 2. For comparison, a map of one of the β cDNA clones, pUCADHβ14, which was cloned in our previous study, is also shown in Fig. 2.

The nucleotide sequences and deduced amino acid sequences of these three cDNA clones are summarized in Fig. 3. The deduced amino acid sequence of pUCADHγ21 is identical to the proposed amino acid sequence of the human γ_1 subunit, except that position 276 was valine in γ_1 and methionine in pUCADHγ21L. The *ADH3* locus is polymorphic—i.e., *ADH3*^{1} for γ_1 and *ADH3*^{2} for γ_2 are almost equally common in various human populations (1). It is most likely that the discrepancy is due to the polymorphism not to sequence errors. The corresponding position of the β and α subunits is methionine. Therefore, γ_2 is considered to represent a prototype, and γ_1 is a diverse type.

The nucleotide and deduced amino acid sequences of pUCADHα15L differ from those of β and γ, although they are very similar. Human class I ADH isozymes are governed by the three homologous gene loci—i.e., *ADH1* for α, *ADH2* for β, and *ADH3* for γ subunit (1–3). Other human ADH isozymes, class II (π) and class III (χ), are distinctive from the class I isozymes with respect to their enzymatic properties, amino acid compositions, structure, and immunological characteristics (17, 18). Therefore, pUCADHα15L should not be a cDNA for class II (π) or class III (χ) isozymes. One can conclude that pUCADHα15L is a cDNA for the α subunit.

All three clones have a chain-initiation signal and a chain-termination signal and encode 375 amino acid residues, including methionine at the NH_2 terminus, which is eventually removed in the completed subunit chains. A high degree of resemblance exists not only in their coding sequence but also in their 3′ noncoding region. The poly(A) signal, AATAAA, is located at the same position of the α and the γ

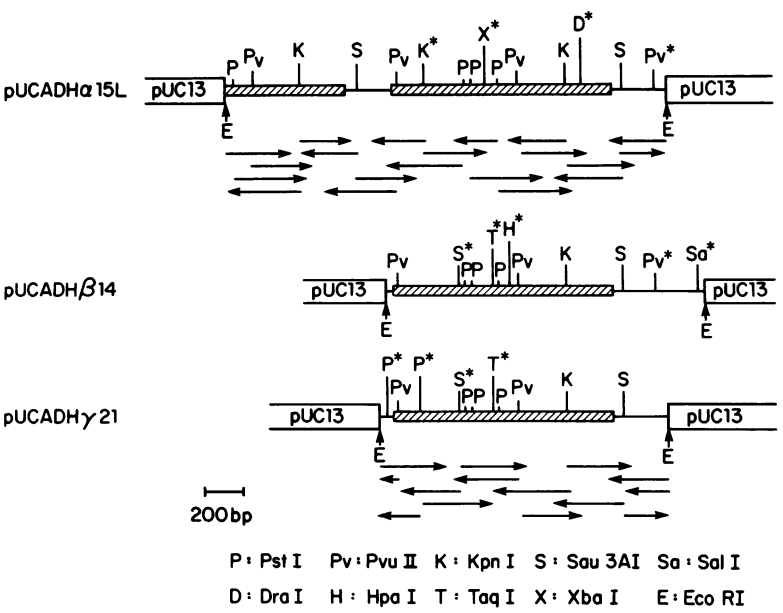

FIG. 2. Restriction maps of cDNA inserts of the three clones and the sequence determination strategy. Horizontal arrows indicate the direction and extent of sequencing. Restriction endonuclease cleavage sites are indicated by vertical lines. Asterisks indicate the restriction sites that are not common in the three clones. bp, Base pairs.

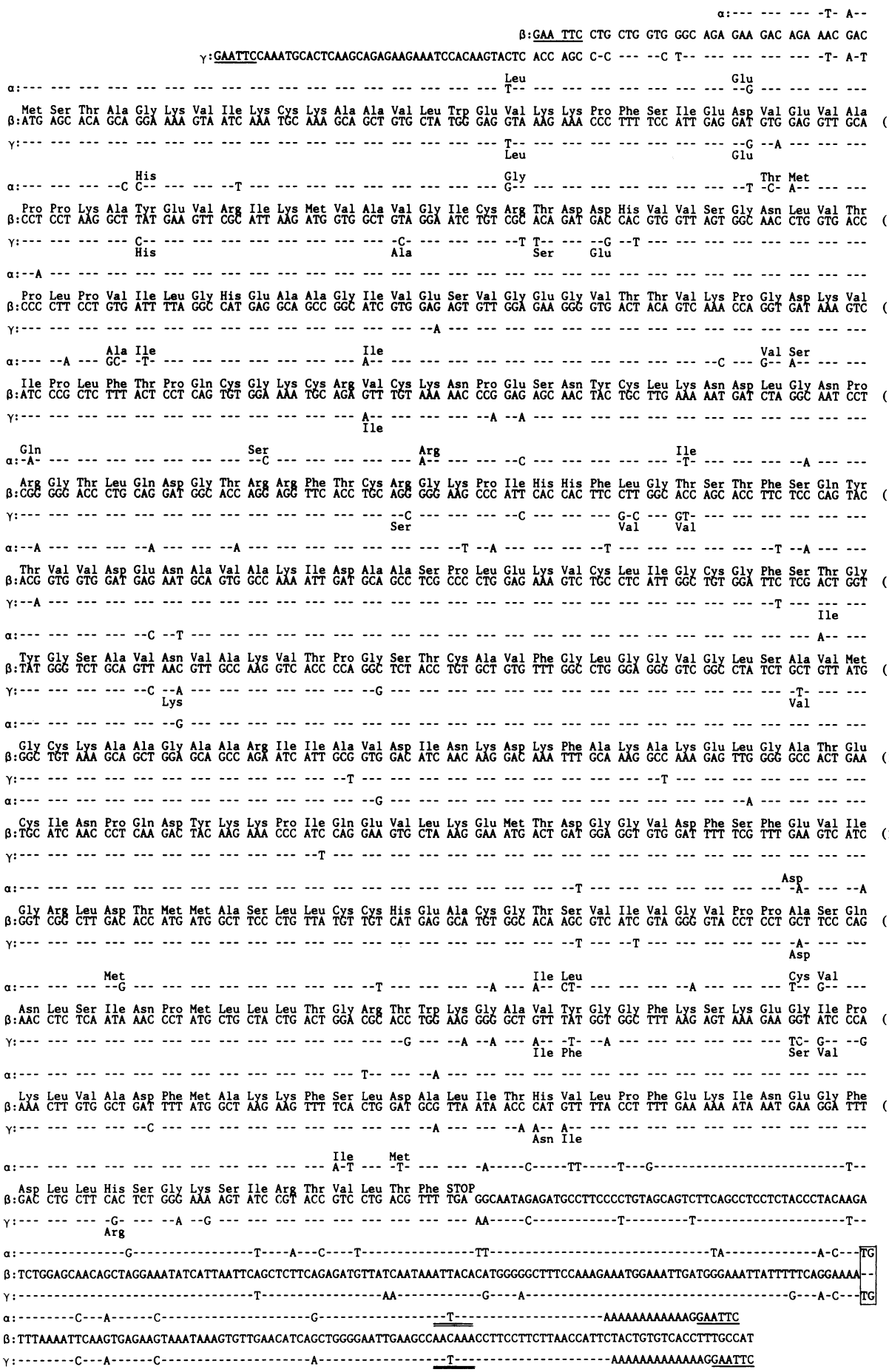

FIG. 3. (*Legend appears on the opposite page.*)

cDNA clones, and the poly(A) chain is also closely located in both cDNA clones. It was observed that the poly(A) chain is located further downstream of a β cDNA clone that contains a longer 3′ noncoding region (unpublished observation). Two short base gaps exist in the β cDNA (Fig. 3).

pUCADHα15L contained the coding sequence of the COOH-terminal region of the β subunit, in addition to the coding sequence for the full-length α subunit, at its extended 5′ region. The origin and characterization of this unique region of pUCADHα15L will be reported elsewhere.

Structural Diversion of the Three Human ADH Subnits. The three subunits are very similar—i.e., the degrees of amino acid identity are α–β_1 = 94%, β_1–γ_2 = 94.6%, and γ_2–α = 93.0%; the degrees of identity in their coding nucleotide sequences are α–β_1 = 95.1%, β–γ_2 = 95.6%, and γ_2–α = 94.0%. A total of 53% of the nucleotide changes involve "silent mutations" that cause no amino acid changes. A high degree of similarity is observed also in their 3′ noncoding regions of 258 base pairs—i.e., α–β = 91.1%, β_1–γ_2 = 92.6%, and γ_2–α = 93.0%. In the case of mouse metallothionein I and II, their coding regions had higher similarity (75.4%) than their 3′ noncoding region (31.8%) (19). These facts would imply that the *ADH* locus was recently duplicated and diverged in the hominoid.

The three-dimensional structure of horse ADH has been proposed from x-ray crystallographic analysis, and the active site pocket, the NAD-binding domain, and the catalytic domain are defined (20). Enzymatic properties of the human isozymes consisting of the α, β, and γ subunits differ widely. In comparison to the isozymes consisting of α and β subunits and horse ADH-E, the ethanol oxidizing activity of $\beta_1\beta_1$ isozyme is very low (17, 21). However, the variant $\beta_2\beta_2$ isozyme, which is prevalent in Orientals, exhibits nearly 100 times the activity of that of the $\beta_1\beta_1$ isozyme (21). The only structural difference between the β_1 and β_2 subunits is a single amino acid substitution—i.e., arginine in β_1 and histidine in β_2 at position 47 (8, 21, 22). Cysteine at position 46, histidine at position 67, and cysteine at position 174 ligate the catalytic Zn atom, and the functional importance of arginine at position 47 and serine at position 48 of the horse ADH has been stressed—i.e., the positively charged arginine was implicated in binding to the pyrophosphate group of NAD and serine to the substrate (20). The structural differences exist in the region adjacent to Cys-46—i.e., Cys-Gly-Thr in α, Cys-Arg-Thr in β_1, Cys-His-Thr in β_2 and yeast ADH, and Cys-Arg-Ser in γ and horse ADH. The substitution of serine at position 48 by threonine in the β_1 subunit would restrict the active center space, resulting in drastic reduction of the catalytic activity in $\beta_1\beta_1$ isozyme. The substitution of arginine at position 47 by histidine in β_2 would restore the active center space and its catalytic activity. The striking amino acid change is the replacement of arginine by glycine in the α subunit, in view of the previously emphasized functional importance of the positively charged arginine at position 47 (20, 23). Since the activity of the $\alpha\alpha$ isozyme is not retarded, the previous model for ADH activity needs to be modified. It should be mentioned that sheep sorbitol dehydrogenase has Cys-Gly-Ser sequence at the corresponding region (24).

γ_1 and γ_2 have the same sequence, Cys-Arg-Ser, at their positions 46–48. As described in the previous section, the only structural difference between the two subunits is valine in the γ_1 and methionine in the γ_2 at position 276. The two isozymes, $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$, exhibit similar enzymatic properties (17).

FIG. 3. Nucleotide sequences and deduced amino acid sequences of human α, β, and γ subunits. Dotted horizontal lines indicate parts that are identical to the β subunit. *Eco*RI sites are singly underlined and the poly(A) signals, AATAAA, are doubly underlined. Numbers in parentheses are positions of amino acid residues from the NH_2-terminal serine. The gap in β cDNA is boxed. The amino acid sequence and the cDNA nucleotide sequence of the β subunits are from the previous publication (8).

Table 1. Amino acid changes in the human α, β, and γ subunits and in the human, horse, and mouse ADH

	Human ADH				Horse	Mouse
	Consensus	α	β	γ	ADH-E	ADH
Consensus	0	15	10	12	43	58
α	15	0	24	27	47	63
β	10	24	0	20	48	62
γ	12	27	20	0	44	56
Horse	43	47	48	44	0	58
Mouse	58	63	62	56	58	0

The consensus human cDNA sequence was deduced from the consensus nucleotide sequence of α, β, and γ cDNAs. The amino acid sequences of horse and mouse ADHs are from previous reports (25, 26).

Evolution of the ADH Isozymes. To clarify the evolutionary relationships of the α, β, and γ subunits, the consensus cDNA sequence (i.e., the most common codon at a given nucleotide position) was deduced from their cDNA sequences. The consensus amino acid sequence—i.e., structure of presumed archetype human ADH subunit—was derived from the consensus cDNA sequence. The amino acid and nucleotide differences in the α, β, and γ subunits and in the human consensus sequence, horse ADH-E, and mouse ADH are shown in Table 1. It is evident that the β–γ group and α diverged first, and β and γ diverged later (Fig. 4). β_2 diverged from β_1 and γ_1 from γ_2, in one-step base substitutions quite recently. Since great apes have multiple class I isozymes (D. Goldman and R. Cotton, personal communication), the first gene duplication in the hominoid probably occurred about 10 million years ago. The estimated rate of mutation acceptance of ADH in hominoids is about five changes per 100 links per 10 million years, and it is among the highest in various proteins.

The amino acid substitution sites of various subunits in these molecular regions are illustrated in Fig. 5. It is readily noticeable that the NAD-binding domain (residue from 176 to 318) is well conserved, and there are only six amino acid changes in this region of the α, β, and γ subunits. There is only one amino acid change in the six parallel β strands (A, B, C, D, E, and F), which commonly exist in nucleotide-binding enzymes (27). No amino acid change exists in a region from 209 to 296. The catalytic domain, from 1 to 175 and from 319 to 374, is more variable than the NAD-binding domain in the human subunits. In comparison to the amino acid changes within the human subunits, interspecies amino acid changes are observed in whole molecular regions (Fig. 5). The most distinctive difference between intrahominoid

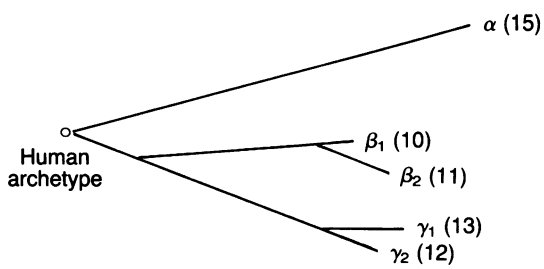

FIG. 4. Proposed evolutionary tree of the human α, β, and γ subunits. Numbers in parentheses are amino acid differences between the present subunits and the consensus subunit, which would represent an archetypal human subunit.

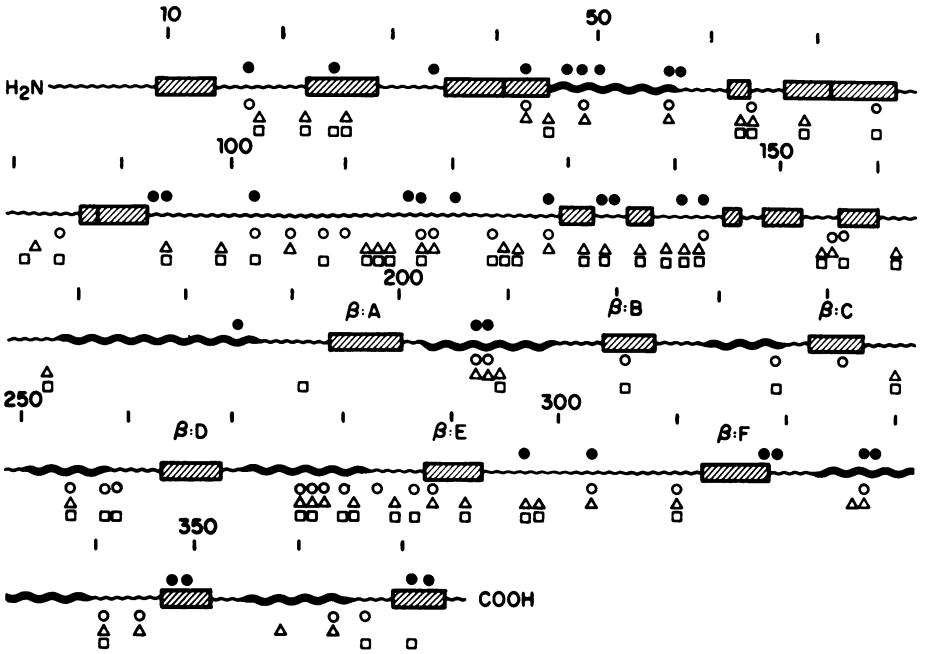

FIG. 5. Schematic diagram of the amino acid substitution sites in human, horse, and mouse ADH subunits. The secondary structure is based on the three-dimensional model of horse ADH proposed by Eklund *et al* (20). The amino acid sequences of horse ADH-E and mouse ADH are from previous reports (25, 26). Shadowed box, β sheets; large, bold wavy line, α-helices; small wavy line, random coils. Amino acid substitution sites are between human consensus and either one of α, β, and γ subunits (●); between human consensus and horse (○); between human consensus and mouse (△); between horse and mouse (□).

and interanimal species is that there is no amino acid change in αE–βE region within the human subunits, but the region is highly variable in interspecies. These facts indicate that different selective pressures operated at the *ADH* locus in the process of intrahominoid evolution and intermammalian evolution.

Remarkable racial differences of alcohol-metabolizing enzymes exist between Caucasians and Orientals. Most Caucasians have the usual $ADH2^1$, which produces catalytically less active β_1 subunit, whereas about 90% of Orientals have the "atypical" $ADH2^2$, which produces superactive β_2 subunit (5). The gene frequency of $ADH2^2$ is >70% in Orientals and <10% in Caucasians. Virtually all Caucasians have two major aldehyde dehydrogenase isozymes (ALDH; aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3)—i.e., cytosolic ALDH1 and mitochrondrial ALDH2. By contrast, ≈50% of Orientals are homozygous atypical $ALDH2^2/ALDH2^2$ and lack the active ALDH2 isozyme (28, 29). The atypical Orientals have a catalytically inactive variant form of the enzyme $ALDH2^2$ (30). The structural difference between the active $ALDH2^1$ and the inactive (or far less active) $ALDH2^2$ is a single amino acid substitution Glu → Lys at the 14th position from the COOH terminus (31, 32). Since all mammals thus far examined have active cytosolic and mitochondrial ALDH isozymes, the inactive $ALDH2^2$ was a late variant that spread in Orientals. The simultaneous development of the two variant genes on different loci—i.e., $ADH2^2$ from $ADH2^1$ and $ALDH2^2$ from $ALDH2^1$—would not be mere coincidence. Rather, the two variant genes were coadapted to an environment, such as diet, to which Orientals were exposed, since ADH and ALDH are complementary in the metabolic pathway of various alcohols.

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