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Evolution

Cloning and sequencing of cDNA encoding baboon liver alcohol dehydrogenase: Evidence for a common ancestral lineage with the human alcohol dehydrogenase β subunit and for class I *ADH* gene duplications predating primate radiation

(alcohol dehydrogenase/nucleotide sequence/deduced amino acid sequence/structural comparisons/enzyme evolution)

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ABSTRACT **The baboon has at least five alcohol dehydrogenases (ADH; alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) and has distinct liver and kidney class I isozymes. A rat liver class I ADH partial cDNA was used to screen a baboon liver cDNA library. A cDNA clone was isolated and sequenced and found to contain the entire coding region for baboon liver ADH, 12 nucleotides of the 5′ noncoding region, and 256 nucleotides of the 3′ noncoding region. The amino acid sequence deduced from this cDNA most closely resembles that of human liver ADH β subunit (ADH-β): 363 of 374 residues were identical. This suggested that baboon liver class I ADH is of the same ancestral lineage as the human ADH-β. In contrast to human liver, only a single ADH-β transcript is observed in baboon liver. A comparison of human and baboon *ADH* 3′ noncoding regions suggests that a single nucleotide change in a polyadenylylation signal consensus sequence may, in part, be responsible for the generation of ADH-β transcripts with variable-length 3′ ends in human liver. A nucleotide substitution rate of 0.5×10^{-9} substitutions per site per year for primate class I *ADH* genes was deduced from the data, which suggests that the α-βγ separation of human *ADH* genes occurred about 60 million years ago, and that primate class I *ADH* gene duplications predated primate radiation.**

Alcohol dehydrogenase (ADH; alcohol:NAD$^+$ oxidoreductase; EC 1.1.1.1) is the major enzyme of alcohol metabolism in the body and exists as a family of enzymes separated into three distinct classes based upon differential substrate and inhibitor specificities and on comparative amino acid and cDNA sequences (1–6). Human class I ADH isozymes exist as homo- and heterodimers of the α, β, and γ subunits (ADH-α, -β, and -γ; ref. 7), for which structures are known at the protein and cDNA levels (3, 8–16). These class I subunits share positional identity at >90% of all positions. The corresponding human genes (*ADH1*, *ADH2*, and *ADH3*) are closely linked on the long arm of human chromosome 4 (17), indicating that they have recently evolved from a common ancestor via tandem gene duplication. Class II and III ADH cDNA sequences showed 60–65% positional identity with each other and with the class I isozymes, confirming the separation of human ADHs into, at least, three discrete classes (3, 18).

The baboon has been used as a model for studying alcoholic liver injury and the effects of alcohol consumption on the rate of alcohol metabolism in the body (19, 20). More recent studies have examined the biochemical properties of baboon ADH isozymes, providing evidence for five major forms of this enzyme (21, 22). The major baboon liver isozyme (designated ADH2) has been purified to homogeneity and biochemically characterized, showing properties consistent with other mammalian class I ADHs (53). Two other baboon liver ADHs have also been reported, ADH4 and ADH5, with properties resembling those of class II and class III human liver ADHs, respectively. Recent studies have examined the effects of chronic alcohol consumption upon liver ADH isozyme phenotype in the baboon (23). Dramatic decreases in class II liver ADH activity (ADH4) and a shift in liver class I isozymes were observed, perhaps reflecting adaptations in the liver to high alcohol levels in the body. The major baboon kidney ADH (designated ADH1) exhibited class I properties, whereas the major corneal and stomach ADH (designated ADH3) showed class II kinetic properties, similar to that previously reported for mouse gastric ADH (2).

We are interested in the regulation of *ADH* gene expression in the baboon, the genetic and biochemical basis of multiplicity for this enzyme, and its evolutionary relationships with other mammalian ADHs. Here we report the nucleotide sequence† of the cDNA encoding the entire baboon liver ADH and the complete amino acid sequence deduced from it. The results provide evidence for a common ancestral lineage of the baboon liver ADH2 subunit with the human ADH-β subunit (also called ADH2) and for gene duplications for primate class I *ADH* genes that predate the separation of human and baboon species during evolution.

MATERIALS AND METHODS

Preparation of RNA and RNA Blot (Northern) Analysis. Freshly dissected or frozen tissue was homogenized by using 30-sec bursts of an Ultra Turrax homogenizer in 8 volumes of a 1:1 mixture of buffer (20 mM Tris·HCl, pH 8.6/10 mM NaCl)-saturated phenol and the same buffer containing 3 mM magnesium acetate and 5% sucrose (24, 25). The homogenate was extracted twice with buffer-saturated phenol and once with buffer-saturated phenol/chloroform/isoamyl alcohol, 25:24:1 (vol/vol), and the nucleic acids were precipitated with ethanol. The RNA was then precipitated with LiCl (26), extracted with phenol again, and finally precipitated with ethanol. The total RNA yield was 2 mg per g of tissue and had an A_{260}/A_{280} ratio of 1.9–2.0. Poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Collaborative Research type 3) (27). Northern blot analysis of baboon poly(A)$^+$ RNA was carried out essentially as described by Seed (28).

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Abbreviations: ADH, alcohol dehydrogenase; ADH-α, ADH-β, and ADH-γ, α, β, and γ subunits of ADH.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25035).

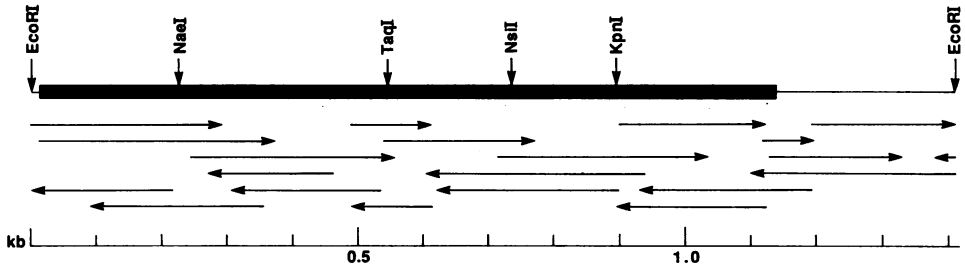

FIG. 1. Restriction map of the baboon liver ADH cDNA clone and the sequence-determination strategy. Horizontal arrows indicate the direction and extent of sequencing. Restriction endonuclease cleavage sites are indicated by vertical arrows. The solid region indicates the ADH coding sequence.

Construction and Screening of cDNA Libraries. cDNA was synthesized from baboon poly(A)$^+$ RNA by the method of Gubler and Hoffman (29) with commercially supplied reagents (Amersham). *Eco*RI linkers were ligated to the cDNA, which was then ligated into the *Eco*RI site of phage λgt10, packaged *in vitro*, and transduced into *Escherichia coli* NM514 (30). A rat liver cDNA library in λgt11 was kindly provided by G. Howlett (University of Melbourne). This was screened with two oligonucleotide probes: one was a mixture of 16 different 14-mers corresponding to amino acid residues 225–229 of rat liver alcohol dehydrogenase (31), and the second was a unique 33-mer corresponding to the human ADH-β cDNA sequence (amino acid residues 329–339; see ref. 11). Oligonucleotides were end-labeled with [γ-^{32}P]ATP using phage T4 polynucleotide kinase (32). Plaques which hybridized with both probes were identified. The largest cDNA insert was 0.65 kilobase (kb); sequence analysis revealed that this cDNA encompassed the region from the polyadenylylation site to a position corresponding to amino acid 202 (33). This partial rat liver cDNA was inserted into a "Riboprobe" vector (pGEM-3Z blue; Promega) and ^{32}P-labeled RNA transcripts (see ref. 34) used to screen the baboon liver cDNA library. Recombinant phage λ plaques from both the rat and baboon liver cDNA libraries were transferred to nitrocellulose, amplified, and hybridized with ^{32}P-labeled probes as described by Woo (35).

Subcloning and DNA Sequencing. Small-scale phage λ DNA preparations were made from positive plaques (36, 37). The cDNA insert was isolated from agarose after electrophoresis of *Eco*RI-digested λ DNA and ligated into the *Eco*RI site of pUC118. Recombinant plasmids were prepared by standard procedures (32) and used as a source of DNA for sequencing by the dideoxy chain-termination method (38, 39) with modified phage T7 DNA polymerase (40) obtained commercially (United States Biochemicals).

RESULTS AND DISCUSSION

Cloning of cDNA for Baboon Liver ADH2 (ADH-β Subunit). From 160 ng of cDNA, an unamplified library of 10^6 recombinant phage λ plaques was obtained. Of these, 5000 were screened, of which 9 hybridized with the rat ADH cDNA probe. Of these, the 2 largest cDNA inserts were 1.4 and 1.1 kb. These cDNA inserts were subcloned to give pBL14 and pBL11, respectively. The nucleotide sequence of the cDNA insert of pBL14 was determined according to the strategy in Fig. 1. The sequence (Fig. 2) contains the entire coding region, 12 nucleotides of the 5′ noncoding region, and 256 nucleotides of the 3′ noncoding region.

Deduced Amino Acid Sequence and Comparison with Human Class I ADH Amino Acid Sequences. A comparison of the deduced amino acid sequence for baboon liver ADH with those of human class I ADH-α, -β, and -γ previously reported (8, 9, 15, 16) reveals a high degree of similarity with all three human subunits, confirming the baboon enzyme as a mammalian class I ADH (Table 1). A higher degree of sequence identity was observed, however, with human ADH-β (11 residues different) as compared with the ADH-α (29 differences) and ADH-γ (25 differences) subunits; hence, we refer to the baboon enzyme as ADH-β. In terms of deduced protein structure and enzyme properties, the baboon ADH-β has retained all zinc-liganding residues as well as other amino acids with defined functions (41). The baboon enzyme also retains the human ADH-β^1 residue (arginine) at position 47, as compared with the high-activity Oriental (ADH-β^2) variant (histidine at position 47) (42). Nearly all of the amino acid substitutions are conservative, with the exception of a valine/threonine substitution at residue 190 in a nonessential region of the molecule (43).

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                                                                      12
                                                                GACAGAAACAAC

                  27                  42                  57                  72
ATG AGC ACA GCA GGA AAA GTC ATC AAA TGC AAA GCA GCT GTG CTA TGG GAG GTA AAG AAA
MET Ser Thr Ala Gly Lys Val Ile Lys Cys Lys Ala Ala Val Leu Trp Glu Val Lys Lys

                  87                 102                 117                 132
CCC TTT TCC ATT GAG GAT GTG GAG GTT GCA CCT CCT AAG GCT TAT GAA GTT CGC ATT AAG
Pro Phe Ser Ile Glu Asp Val Glu Val Ala Pro Pro Lys Ala Tyr Glu Val Arg Ile Lys

                 147                 162                 177                 192
ATG GTG GCT GTA GGA ATC TGT CGC ACA GAT GAC CAC GTG GTT AGT GGC AAC CTG GTG AGC
MET Val Ala Val Gly Ile Cys Arg Thr Asp Asp His Val Val Ser Gly Asn Leu Val Ser

                 207                 222                 237                 252
CCC CTT CCT GCG ATT TTA GGC CAT GAG GCA GCC GGC ATC GTG GAA AGT GTT GGA GAA GGG
Pro Leu Pro Ala Ile Leu Gly His Glu Ala Ala Gly Ile Val Glu Ser Val Gly Glu Gly

                 267                 282                 297                 312
GTG ACT ACA GTC AAA CCA GGT GAT AAA GTC ATC CCG CTC TTT ACT CCT CAG TGT GGA AAA
Val Thr Thr Val Lys Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro Gln Cys Gly Lys

                 327                 342                 357                 372
TGC AGA GTT TGT AAA AGC CCA GAA GGC AAC TAC TGT GTG AAA AAT GAT CTG AGC AAT CCT
Cys Arg Val Cys Lys Ser Pro Glu Gly Asn Tyr Cys Val Lys Asn Asp Leu Ser Asn Pro

                 387                 402                 417                 432
CGG GGG ACC CTG CAG GAT GGC ACC AGG AGA TTC ACC TGC AGG GGG AAG CCC ATT CAC CAC
Arg Gly Thr Leu Gln Asp Gly Thr Arg Arg Phe Thr Cys Arg Gly Lys Pro Ile His His

                 447                 462                 477                 492
TTC GTT GGC ACC AGC ACC TTC TCC CAG TAC ACG GTG GTG GAT GAG AAT GCA GTG GCC AAA
Phe Val Gly Thr Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Asn Ala Val Ala Lys

                 507                 522                 537                 552
ATT GAT GCA GCC TCG CCC CTG GAG AAA GTC TGC CTC ATT GGC TGT GGA TTT TCG ACT GGT
Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly Phe Ser Thr Gly

                 567                 582                 597                 612
TAT GGG TCT GCA GTT AAT GTT GCC AAG GTC ACC CCA GGC TCT GTC TGT GCT GTG TTT GGC
Tyr Gly Ser Ala Val Asn Val Ala Lys Val Thr Pro Gly Ser Val Cys Ala Val Phe Gly

                 627                 642                 657                 672
CTG GGA GGA GTC GGC CTC TCT GCT GTT ATG GGC TGT AAA GCA GCT GGA GCA GCC AGA ATC
Leu Gly Gly Val Gly Leu Ser Ala Val MET Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile

                 687                 702                 717                 732
ATT GCG GTG GAC ATC AAC AAG GAC AAA TTT GCA AAG GCC AAA GAG TTG GGT GCC ACT GAA
Ile Ala Val Asp Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Leu Gly Ala Thr Glu

                 747                 762                 777                 792
TGC ATC AAC CCT CAA GAC TAC AAG AAA CCC ATC CAG GAG GTG CTA AAG GAA ATG ACT GAT
Cys Ile Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Lys Glu MET Thr Asp

                 807                 822                 837                 852
GGA GGT GTG GAT TTT TCG TTT GAA GTC ATC GGT CGG CTT GAT ACC ATG ATG GCT TCC CTG
Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg Leu Asp Thr MET MET Ala Ser Leu

                 867                 882                 897                 912
TTA TGT TGT CAT GAG GCA TGT GGC ACA AGC GTC ATC GTA GGG GTA CCT CCT GAT TCC CAG
Leu Cys Cys His Glu Ala Cys Gly Thr Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln

                 927                 942                 957                 972
AAC CTC TCA ATA AAC CCT ATG CTG CTA CTG ACT GGA CGC ACC TGG AAG GGG GCT GTT TAT
Asn Leu Ser Ile Asn Pro MET Leu Leu Leu Thr Gly Arg Thr Trp Lys Gly Ala Val Tyr

                 987                1002                1017                1032
GGT GGC TTT AAG AGT AGA GAA GGT ATC CCA AAA CTT GTG GCT GAT TTT ATG GCT AAG AAG
Gly Gly Phe Lys Ser Arg Glu Gly Ile Pro Lys Leu Val Ala Asp Phe MET Ala Lys Lys

                1047                1062                1077                1092
TTT TCA CTG GAC GCA TTA ATA ACC CAT GTT TTA CCT TTT GAA AAA ATA AAT GAA GGC TTT
Phe Ser Leu Asp Ala Leu Ile Thr His Val Leu Pro Phe Glu Lys Ile Asn Glu Gly Phe

                1107                1122                1137                1155
GAC CTG CTT CGC TCT GGG AAA AGT ATC CGT ACC GTC CTG ACG TTT TGA GGCAATACAGATGCC
Asp Leu Leu Arg Ser Gly Lys Ser Ile Arg Thr Val Leu Thr Phe

           1170                1185                1200                1215                1230
TTCCCTTGTAGCAGT CTTCATCCTCCTCTA CTCTACATGATCTGG AGCAACAGCTGGGAA ACATCATAATTCTGC

           1245                1260                1275                1290                1305
TCTTCAGAGATGTAT TCAATAAATTACACA AAGGGGCTTTCTAAA GAAATGGAAATTGAT GGGAAATTATTTTTC

           1320                1335                1350                1365                1380
AAGCAAAAATTTAAA ATTCAAGTGAGAACT AAATAAAGTGTTGAA CATCAGCTGGGAAAT TGAAGCCAATAAACC

           1395                1410
ATCCGTCTTAACCAT TAAAAAAAAAAAAA AAA
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FIG. 2. Nucleotide sequence of baboon liver ADH cDNA and the deduced amino acid sequence of baboon liver ADH-β.

Table 1. Amino acid sequence differences (and nucleotide sequence differences in parentheses) between baboon ADH-β and human ADH-α, ADH-β^1, and ADH-γ^1 (and between nucleotide sequences of class I ADH-encoding genes)

		Sequence differences			
		Human ADH subunits			
ADH	Subunit	α	β^1	γ^1	Baboon ADH-β
Human	α	0 (0)	23 (55)	25 (69)	30 (73)
	β^1	23 (55)	0 (0)	20 (49)	11 (28)
	γ^1	25 (69)	20 (49)	0 (0)	25 (61)
Baboon	β	30 (73)	11 (28)	25 (61)	0 (0)

The amino acid and nucleotide sequences of human ADH subunits (α, β^1, and γ^1) are from previous reports (3, 8–16).

Nucleotide Sequence. The sequence immediately preceding the AUG initiator codon conforms well to the "−1 to −5" consensus sequence reported by Kozak (44), with matches at three of five positions, including the highly conserved adenosine residue at −3. The 3′ noncoding regions of both pBL14 and pBL11 are identical, and each contains 256 nucleotides. Comparison of these with the 3′ noncoding regions of human ADH-α, -β, and -γ cDNA sequences (11–16), reveals 88.3%, 91%, and 88.7% sequence identity, respectively. The polyadenylylation site is identical in baboon ADH-β and in human ADH-α and -γ^2 cDNA sequences; the polyadenylylation site in the case of ADH-γ^1 differs by one or three nucleotides (13, 16). The human *ADH* gene encoding the β subunit (designated *ADH2*) gives rise to variably sized mRNA molecules, differing in the length of their 3′ noncoding sequences (14). Three size classes of 3′ region are evident: short (213 nucleotides), intermediate (590 nucleotides), and long (1330 nucleotides). The former seems to be the most abundant and is closest in length to the baboon ADH-β 3′ noncoding region reported here (256 nucleotides). There is no evidence from Northern blot experiments that different-size ADH transcripts occur in baboon liver, since only a single mRNA of 1.6 kb is observed using the baboon ADH-β cDNA as a probe (Fig. 3). There are three polyadenylylation signal consensus sequences (AATAAA; ref. 45), which are at nucleotide positions 1248–1253, 1337–1342, and 1373–1378 (Fig. 4). The latter is 18 nucleotides from the polyadenylylation site and hence is presumably involved in polyadenylylation. Significantly, the equivalent polyadenylylation signal in the human gene encoding ADH-β has a T→C change, which presumably accounts for the use of alternative AATAAA sequences (see ref. 14). Furthermore, whereas in the baboon *ADH* DNA sequence, the consensus sequence CAYTG (see ref. 46) is matched at four of five positions (nucleotides 1393–1397 in Fig. 4) immediately adjacent and 5′ to the polyadenylylation site (as in "class I" messenger RNA sequences; ref. 46), this consensus sequence is lacking at the 3′ termini of the human ADH-β mRNA sequences (14). Thus, these differences between human and baboon ADH-β cDNA sequences may, at least in part, be related to the formation of multiple mRNA sequences, differing in the length of their 3′ ends, in human liver.

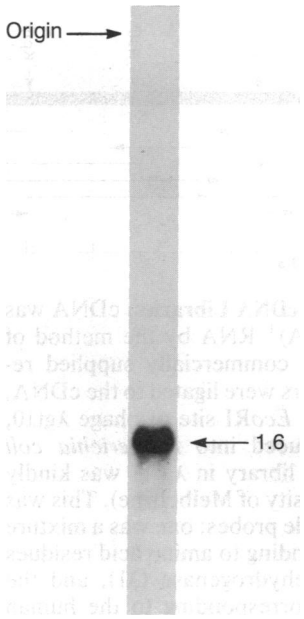

FIG. 3. Northern blot of baboon liver mRNA, probed with ^{32}P-labeled baboon ADH-β cDNA, indicating the presence of a single species ≈1600 bases long (1.6 kb). Molecular weight standards (not shown) were eukaryotic and prokaryotic ribosomal RNAs.

Remarkably, a sequence of 10 nucleotides in the 3′ untranslated region (positions 1151–1160; Fig. 4) is identical in

```
                1150      1160      1170      1180      1190      1200
Baboon β  GGCAATACAGATGCCTTCCCTTGTAGCAGTCTTCATCCTCCTCTACTCTACATGATCTGG
Human  α  .A..........TT.........G.........G.........C.............
       β1 .......G...........C.............G.........C....GA.......
       γ1 AA..............................T....G.........C.............

                1210      1220      1230      1240      1250      1260
AGCAACAGCTGGGAAACATCAT AATTCTGCTCTTCAGAGATGTATTCAATAAATTACACA
...............T.....T..........A...C....T.TA.............TT
..........A.....T.....T.....A..............TA..............
..........A.....T.....T.....................TAAA............G

                1270      1280      1290      1300      1310      1320
AAGGGGCTTTCTAAAGAAATGGAAATTGATGGGAAATTATTTTTCAAGCAAAAA  TTTAAA
TG.........C...................TA..............  C...TG......
TG.........C.....................................G.  ....  ......
TG..A......C.....................................G.....  C...TG......

                1330      1340      1350      1360      1370      1380
ATTCAAGTGAGAAACTAAATAAAGTGTTGAACATCAGCTGGGAAATTGAAGCCAATAAACC
..C...A.....................................G.....................
.............G.....................................G...........C.....
..C...A.....................................A.....G.....................

                1390      1400      1410
ATCCGTCTTAACCATTAAAAAAAAAAAAAAAAA
T...T..........
T...T..........
T...T..........
```

FIG. 4. Comparative nucleotide sequences for the 3′ regions of baboon ADH-β, and human ADH-α, ADH-β^1, and ADH-γ^1 cDNAs. Sequences for the human cDNAs are from refs. 15, 14, and 16, respectively. Note in particular the underlined region for three polyadenylylation signal consensus sequences (AATAAA) at nucleotide positions 1248–1253, 1337–1342, and 1373–1378, and the T → C base substitution for the human sequence of ADH-β at nucleotide 1375.

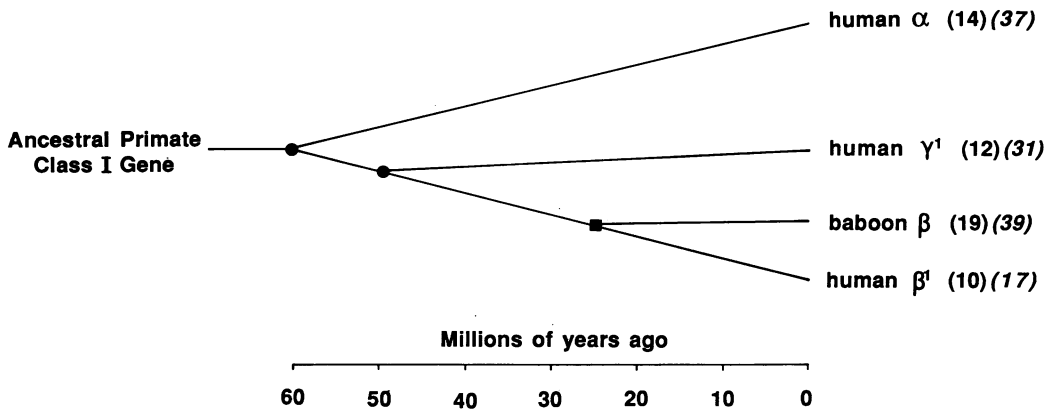

FIG. 5. Proposed evolutionary tree of genes for human ADH-α, ADH-β^1, and ADH-γ^1 (based on ref. 13) and baboon ADH-β. Numbers in parentheses are amino acid differences of the products (first set of parentheses) and nucleotide differences (second set of parentheses) between the genes for the human and baboon subunits and the consensus human subunit. The time scale is calibrated from a date for the Catarrhini branch point in primate evolution (most recent common ancestor of human and baboon) at approximately 25 million years ago (48), and a corresponding nucleotide substitution rate of 0.5×10^{-9} substitutions per site per year for all primate class I ADH genes. A different gene divergence topology to that suggested here, and by Ikuta *et al.* (13), has been proposed (49) and is based upon different amino acid substitution rates for separate class I ADHs. ●, Gene duplication of primate class I ADH genes; ■, species separation of humanoid and baboon ADH genes.

the sequences of human and baboon ADH-β, human ADH-γ, and (with the exception of two changes) human ADH-α and also is completely conserved in the ADH-β sequences of rat and mouse (see refs. 33 and 47). This sequence is 10 nucleotides after the stop codon in all cases.

Evolution of Primate Class I ADHs. A comparison of the cDNA nucleotide sequences for the coding regions of ADH-α, -β, and -γ (see ref. 13) with that of the baboon ADH-β (Table 1) reveals a high degree of sequence identity (>93%) between these structures. Moreover, the human ADH-β and baboon ADH-β cDNA sequences show the highest level of sequence identity (97.6%). The results suggest that these ADHs have arisen from a recent common ancestral mammalian class I *ADH* gene. Fig. 5 illustrates a phylogenetic tree for primate class I *ADH* genes, based upon the amino acid and nucleotide sequences. Molecular genetic evidence obtained from primate pseudo-η-globin DNA sequences have, in association with palaeontological time points, provided a date for the Catarrhini branchpoint in primate evolution (most recent common ancestor of human and baboon) at 22.2–28.2 million years ago (48). A comparison of the human ADH-β and baboon ADH-β cDNA coding sequences (1125 base pairs) was used to establish a nucleotide substitution rate of $\approx 0.5 \times 10^{-9}$ $[(28/1125)/(2 \times 25.2 \times 10^6)]$ substitutions per site per year. This substitution rate may be compared with the neutral rate of 1.3×10^{-9} substitutions per site per year in descent from the Catarrhini branchpoint, reported by Koop *et al.* (48) for the pseudo-η-globin sequences. By using the ADH-β nucleotide substitution rate as an evolutionary clock for all primate class I *ADH* genes, it is now possible to date an α-$\beta\gamma$ separation at $\approx$60 million years ago (see Fig. 5). This corresponds to the appearance of protoprimates in the fossil record, prior to primate radiation (50). The data also suggest that the separation of the genes encoding ADH-β and ADH-γ occurred later, $\approx$50 million years ago. Evidence from molecular and biochemical genetic studies of murine class I *ADH* are consistent with a single gene occurring in this species (51, 52), as compared with the multiple class I *ADH* genes in humans, supporting the model for class I *ADH* gene duplications occurring in the protoprimate ancestor rather than in an earlier common ancestor for eutherian mammals.

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